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aquatic animal health subprogram

Aquatic Animal Health Subprogram. Vibrios of Aquatic Animals: Development of a National Standard Diagnostic Technology

Jeremy Carson, Melissa J. Higgins
Teresa K. Wilson, Nicholas Gudkovs,
***and* Trevor N. Bryant**

December 2006

FRDC Project No. 2001/628

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1 NON-TECHNICAL SUMMARY

01/628 Aquatic Animal Health Subprogram. Vibrios of Aquatic Animals: Development of a National Standard Diagnostic Technology

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OBJECTIVES:

- Undertake a definitive numerical taxonomic analysis to describe and characterise the phenotypes of aquatic animal Vibrios in Australia
- Develop practical, robust phenotypic identification systems for *Vibrio* species using computer assisted identification software
- Develop and implement PCR gene probes for the rapid identification of key bacterial pathogens
- Determine the range of *Vibrio* species associated with farmed fish, shellfish and crustacea in Australia
- Technology transfer workshop for state veterinary diagnostic microbiologists
- Develop an ANZSDP for the Identification of *Vibrio* species in aquatic animals

NON-TECHNICAL SUMMARY:

The *Vibrionaceae* is a large group of important marine bacteria, of which over half are the cause of disease in a diversity of aquatic animals, plants and man. Diseases due to *Vibrionaceae* are known to occur in most major aquatic animal groups from marine mammals to teleost fish, crustacea such as prawns and crabs, molluscan shellfish and octopus through to zooxanthellae, the symbiotic plant cells of coral. The risk of disease in farmed aquatic animals is increased because of captivity stress when significant losses can rapidly occur due to vibriosis caused by pathogenic species of *Vibrionaceae*. More insidious is non-specific disease due to opportunistic infection caused by any one of a diverse range of *Vibrionaceae*. This type of infection in particular is evident in juvenile animals occurring mostly during larval rearing stages. Conversely, several species appear beneficial and have, like *Vibrio alginolyticus*, been used to good effect as probiotics to control disease in various farmed aquatic animals.

Although this all-pervasive group of bacteria has far reaching effects for aquatic animal health, the methods available to identify members of the *Vibrionaceae* are at best rudimentary and piecemeal for other than the few species of significance for human health such as *V. cholerae*. The purpose of this project was to develop a robust and practical system for the identification of *Vibrionaceae* and to obtain an inventory of species associated with aquatic animals in Australia.

A library of 823 strains of *Vibrionaceae*, including 56 type strain species and biovars, was assembled from farmed and wild aquatic animals in Australia. The principle sources of

strains were from Western Australia, Queensland and Tasmania, representing tropical and temperate faunas. A numerical taxonomy of the library was undertaken using a panel of 107 tests, most in miniaturised form, to determine the phenotype (descriptive characteristics) of the assembled strains. Relationships based on similarities were assessed by cluster analysis so that common characteristics could be determined for groups of related strains. A total of 86 distinct types were recognised of which 56 groups, or clusters, represented the known species and biovars of the *Vibrionaceae* that formed the library of strains. The remaining groups represented distinct biovars of *Photobacterium damselae* ssp. *damselae*, *Vibrio fischeri*, *V. harveyi* and *V. ichthyoenteri* and 25 un-named protospecies of *Vibrionaceae*. The library of strains was also analysed by molecular taxonomy. A partial DNA sequence of the 16S rRNA gene was determined for each strain and an identification reached by matching sequences with strains in the GenBank data library. The homogeneity of cluster membership was assessed by comparing the group identity reached by numerical taxonomy with that determined by molecular taxonomy. Mostly there was good agreement between the two methods, confirmation that the clusters form homogeneous groups of strains. The agreement was less good for a few clusters indicating some heterogeneity in membership. Neither method of taxonomic assessment is absolute and the basis for disagreement was not always evident. In some instances it would appear that the sequence data was confounding, due probably to the use of partial sequences which did not have sufficient resolution to reliably determine a species identity.

Complete 16S rRNA gene sequences were obtained for representatives of the 25 protospecies as a preliminary step in determining their identity. Several of the clusters, defined as phenons, were designated as biovars of known species: *Ph. damselae* ssp. *damselae* biovar II, *V. fischeri* biovar II, *V. harveyi* biovar II and *V. ichthyoenteri* biovar II. Four of the phenons were tentatively designated as *Aeromonas sobria, sensu stricto*, *V. coralliilyticus, V. pomeroyi* and *V. xuii*; the remaining phenons appear to be potential new species that warrant further investigation to establish their true taxonomic status.

The phenotypic characteristics of the phenons defined by numerical and molecular taxonomy, were used for the development of a database for computer-assisted probabilistic identification. A software program, PIBWin was developed for this purpose. Using an implementation of Bayes theorem, the most likely identification of an unknown is obtained by reference to the phenotype of known species. The program not only computes the most likely identification but also provides additional measures to assess the likelihood of a correct identification. The program was designed for ease of use with a highly intuitive interface. Several tools for assessing the functionality of a probability matrix are included in the software package. A probability matrix, VibEx7 was developed for the identification of all 86 phenons using a standardised panel of 39 miniaturised and five antibiotic sensitivity tests. Using the V36 panel of tests an identification can be obtained within two days. Performance of the matrix was assessed by several means. Using an identification threshold of $P \geq 0.99$ and phenetic data from the numerical taxonomy, 93% of the library of strains were correctly identified to their parent cluster with only 0.3% incorrectly identified, an indication that the V36 identification panel and VibEx7 probability matrix accurately reflect the phenons defined by the numerical taxonomy. Using prospective data obtained from routine diagnostic samples, 79% of strains had an identification score of $P \geq 0.99$ and represents a significant improvement in identification rates compared to previous methods. The ability to accurately identify almost 80% of wild type strains is further evidence that the VibEx7 identification matrix is a good reflection of the diversity of *Vibrionaceae* in Australian aquatic animals and can be used with a degree of assurance to obtain a correct identification for unknown strains. A known limitation of the VibEx7 matrix is the absence of 35 species which were formally proposed while the project was nearing completion. Based on the 16S rDNA sequence identification, it seems that few if any of these new species were present in the library of strains from Australian

aquatic animals. It would be prudent nevertheless to ensure that these species are included in the VibEx7 identification matrix in the future.

Rapid identification using the DNA amplification technique of polymerase chain reaction (PCR) was evaluated as a means of either confirming the identity of species or determining the presence of specific pathogenicity factors. A range of PCR primers were evaluated for *Ph. damselae* ssp. *damselae*, *Ph. damselae* ssp. *piscicida, V. anguillarum, V. harveyi* and *V. vulnificus*. All the primer sets for these important pathogens of aquatic animals were implemented and evaluated for use with template DNA extracted from pure cultures. Most of the primer sets were directed not at conserved genes such as 16S or 23S rRNA, but at pathogenicity genes such as haemolysin or for regions of chromosomal DNA of unknown function. Targets such as these were considered to be of limited value as a means of species identification since not all strains may contain these more labile genes or may occur in other unrelated species.

Despite the extensive composition of the library of *Vibrionaceae* assembled for the survey, few unusual species were encountered. The most commonly occurring were *Ph. damselae* ssp. *damselae* and *V. harveyi* from a varied range of hosts. An unusual finding was the isolation of *V. penaeicida* from rock lobster phyllosoma and it was possible also to confirm the presence of *V. scophthalmi* and *V. ichthyoenteri* in both Atlantic salmon and barramundi. The library of strains provided good evidence that several species of *Vibrionaceae* remain exotic pathogens including *M. viscosa, V. ordalii, V. salmonicida* and *V. wodanis* in salmonids, and *V. tapetis* and *V. pectenicida* in shellfish. The cause of blister disease in farmed abalone was confirmed as a unique form of *V. harveyi* designated biovar II while several, as yet un-named protospecies, were associated exclusively with specific aquatic animals; most noticeable of these were Phenons 15 and 46 from abalone and Phenon 29 from larval striped trumpeter. The role of these protospecies as pathogens is as yet unknown.

A draft Australian New Zealand Standard Diagnostic Procedure has been developed for the identification of *Vibrionaceae* using the V36 panel of tests and the VibEx7 probability matrix. The standardised approach for the identification of *Vibrionaceae* associated with aquatic animals should enable diagnostic laboratories to identify isolates with greater assurance and obtain meaningful results of value to farmers, veterinarians and regulators.

The approach used for the identification of *Vibrionaceae* will enable additions and improvements to be made to the VibEx7 identification matrix and ensure that it stays relevant and useful into the future.

OUTCOME ACHIEVED: A standardised panel of phenotypic tests was developed for the identification of bacteria from the *Vibrionaceae* using computer assistance and the software package PIBWin developed for this purpose. The database for the *Vibrionaceae* was developed from a combined molecular and numerical taxonomic study of bacterial strains from tropical and temperate water aquatic animals in Australia. Phenotypic identification is supported by the use of PCR, a molecular DNA technique, for the rapid identification of key aquatic animal pathogens. These procedures form the basis of a uniform approach for the accurate identification of *Vibrionaceae* for use by diagnostic laboratories. The improved basis for the identification of *Vibrionaceae* will be of benefit to the aquaculture industry, microbiologists, veterinarians and regulators.

KEYWORDS: *Vibrionaceae*, *Grimontia, Moritella, Photobacterium, Vibrio,* numerical taxonomy, phenotyping, molecular taxonomy, 16S rRNA, sequencing, genotyping, probabilistic identification, computer assisted identification, software, PCR, aquatic animal disease

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3 BACKGROUND

Vibriosis is one of the most frequent causes of marine aquatic animal disease and affects a wide range of farmed species including finfish, crustacea and shellfish (Actis *et al.*, 1999). In Australia, catastrophic losses are regularly observed in larvae and juveniles in hatcheries as well as in adult farmed animals. Vibriosis is a national problem that affects aquaculture from the temperate regions of the south through to tropical northern Australia. The purpose of this project is to develop a practical and reliable laboratory based test system for the identification of Australian *Vibrionaceae*.

No single species is associated with the condition vibriosis. Currently 89 taxa in the genera *Enterovibrio, Grimontia, Moritella, Photobacterium* and *Vibrio* are recognised of which 32 cause disease in aquatic animals and a further 11 are known human pathogens. At least four species, *V. mediterranei*, *V. coralliilyticus*, *Ph. rosenbergii* and *En. coralii* are associated with, or are the cause of, coral bleaching arising from infection of zooxanthellae (Banin *et al.*, 2001; Ben-Haim *et al.*, 2003a,b). In Australia, outbreaks of vibriosis have occurred in a variety of farmed aquatic animals at levels that can be serious barriers to production, Table 1.1. The range of significant pathogens is surprisingly small given the diversity of aquatic animals that are farmed in Australia with

Table 1.1 *Vibrionaceae* as a cause of significant disease in farmed aquatic animals within Australia

Pathogen	Host
Photobacterium damselae ssp. *damselae*	Atlantic salmon, barramundi
Vibrio anguillarum	Atlantic salmon, rainbow trout, striped trumpeter
Vibrio harveyi	Barramundi, abalone, rock lobster
Vibrio splendidus biovar I	Snapper
Vibrio tubiashii	Pacific oyster

a climate that spans temperate to tropical regions and containing a multiplicity of habitat types. In other countries, serious epizootics are associated with a diversity of *Vibrionaceae* across a range of animal species, Table 1.2, an indication of the variety of potentially important pathogens that could emerge in Australia.

While specific species are associated with acute disease, significant cumulative production losses also occur on farms and in hatcheries as a result of opportunistic infection by *Vibrionaceae*. Often reported simply as non-specific vibriosis, most of the bacteria isolated from this common condition are not identified. Typically, diagnostic laboratories identify less than half the isolates recovered in culture. It could be argued that identification of a flora beyond the level of *Vibrionaceae* is unnecessary in that speciation will add little value to the understanding of a clinical situation. A narrow

Table 1.2 Significant pathogenic *Vibrionaceae* of aquatic animals outside Australia

Host	Pathogen
Clams	*Vibrio tapetis*
Eels	*Vibrio vulnificus* biovar II
Japanese flounder	*Vibrio ichthyoenteri*
Kuruma prawns	*Vibrio penaeicida*
Lobster	*Vibrio fluvialis*
Salmonids	*Moritella viscosa* *Vibrio ordalii* *Vibrio salmonicida* *Vibrio wodanis*
Scallops	*Vibrio pectenicida*
Sea bass Sea bream	*Photobacterium damselae* ssp. *piscicida*
Turbot	*Vibrio scophthalmi*

view such as this has long term risks that go beyond the immediate requirements of a particular investigation. Failure to determine the composition of a flora, whether comprised of a single species or many species, precludes either the possibility of obtaining any perspective on subsequent clinical events or the opportunity to differentiate between pathogens, opportunists and normal flora. Significantly, if there is no capacity or willingness to identify species other than those known to occur commonly, then there is a real risk that known pathogens newly emerging, or species not previously considered pathogenic, may go undetected. More important than the exigencies of clinical diagnosis, a failure in capacity and ability to identify *Vibrionaceae* means that gaining an understanding of the interaction between host and bacterial flora is compromised. Floras other than pathogens are of increasing interest, particularly those associated with larval rearing (Hansen & Olafsen, 1999), gut flora and nutrition (Ringø *et al.*, 1995; Oxley *et al.*, 2002) and probiotics as disease control agents, some of which are species of *Vibrio* (Verschuere *et al.*, 2000). A capacity to identity components of these floras is a prerequisite to assessing their importance.

Identification methods for *Vibrionaceae* vary enormously in their complexity, utility and accuracy. Molecular methods arising from phylogenetic taxonomic studies of the *Vibrionaceae* would appear to offer an accurate means of identification (Thompson *et al.*, 2004b). Procedures based on amplified fragment length polymorphism (AFLP) or multilocus sequence typing (MLST) (Thompson *et al.*, 2005) have been proposed while sequencing of the 16S rRNA gene or DNA:DNA hybridisation are alternative strategies. Chemical signatures have been explored as a non-genetic method for identification of the *Vibrionaceae* using pyrolysis-metastable atom bombardment-mass spectrometry (Wilkes *et al.*, 2005). These procedures have the potential to achieve high levels of

identification accuracy, but the major drawback to their use is cost of materials and labour (Millar & Moore, 2004), and for mass-spectrometry and AFLP, the high capital cost of equipment (Janssen, 2001).

The alternative to molecular methods is phenotyping, which has been, and remains for the foreseeable future, the principal means by which many laboratories routinely identify bacterial cultures, particularly those providing medical and veterinary diagnostic services. The utility of such an approach is determined by two factors: availability of compact panels of reproducible tests and phenotypic data that is differential of species in the target genera.

Molecular taxonomic approaches have achieved much in establishing a stable framework for the *Vibrionaceae* (Thompson *et al.*, 2001; Thompson *et al.*, 2004b) resulting in the recognition of many new species (Thompson *et al.*, 2002a; 2003a; 2003b; 2003c; 2003d; 2003e). What is increasingly apparent however is that development of a unified and differential phenotypic approach for identification of *Vibrionaceae* has not kept pace with the rapid advances made with phylogenetic based classification. Indeed it has been argued that phenotyping as a means of identification is not possible for such a large group of taxa (Sugita & Ito, 2006) because of the phenotypic diversity that exists across the *Vibrionaceae* (Thompson *et al.*, 2004b). The validity of this view is questionable. Using a numerical taxonomic approach as a means of phenotypic discovery, sufficient differential tests have been identified for the *Vibrionaceae* (Bryant *et al.*, 1986a) and a probability matrix constructed for use with computer assisted identification (Bryant *et al.*, 1986b).

Numerical methods for identification with computer assistance was first considered by Beers and Lockhart (1962) with a probabilistic approach developed later by Willcox and colleagues (1973). A characteristic of numerical identification is the ability to utilise simultaneous data without weighting particular tests and is well suited for large groups of taxa with heterogenous phenotypes. Probabilistic identification methods for *Vibrionaceae* have proved useful, but at present they remain unwieldy due to the large number of tests required to achieve a reliable identification (Bryant *et al*. 1986b; Farto *et al*. 1999) and lack currency as they do not cover many of the more recently described species. Alternative approaches using commercially available diagnostic kits developed for bacteria of medical importance appear unreliable and of limited use (Austin & Austin, 1999; O'Hara *et al.*, 2003; Vandenberghe *et al.*, 2003; Martinez-Urtaza *et al.*, 2006) because the range of tests are not sufficiently differential and, most

species of *Vibrionaceae* are not included in commercial databases. A simplified approach based on dichotomous keys has been developed for the identification of *Vibrionaceae* (Alsina & Blanch, 1994a; 1994b) and, though widely used (Ottaviani *et al.*, 2003; Zorrilla *et al.*, 2003a; Maugeri *et al.*, 2004), has not proved sufficiently accurate (Montes *et al.*, 2003; Thompson *et al.*, 2004b) or reliable.

A further complication of phenotyping, whether for describing new species or for identification, is the absence of agreed standard methods (Nair & Holmes, 2005) for the *Vibrionaceae*, a deficiency that underlies the inadequacy and inefficiency that characterises existing identification procedures (Benediktsdóttir *et al*. 1998). At best, phenotyping of the *Vibrionaceae* is piecemeal and tends to consider subsets of near related species. What is clearly evident is the lack of a unified approach with standardised tests that considers all species of the *Vibrionaceae*.

While phenotyping, using appropriate methodologies and databases, represents a practical approach to routine identification, molecular methods based on DNA amplification by PCR is an alternative but specialised means of identification. The power of PCR is only truly realised as a tool of detection or confirmation of species nominated *a priori*. Identification *a posteriori* is achievable only by sequencing the 16S rRNA gene. Where the specificity of primers are well established, PCR is particularly useful as a means of screening samples for the presence of nominated species or as a means of confirming the identity of strains with aberrant or poorly defined phenotypes (Oakey *et al.*, 2003).

The purpose of this study is to consider the *Vibrionaceae* as an entire group of species and, using numerical taxonomy as a means of phenotypic discovery, identify a compact panel of differential tests. The integrity of the species defined by numerical taxonomy will be assessed independently using partial 16S rDNA sequence identities. Data from the numerical study will be used to obtain estimates of species diversity so that a database can be established for probabilistic identification. Implementation of computer-assisted identification will be achieved by development of a software package for this purpose. PCR primers as a means of secondary confirmation of species identity will be evaluated for *Ph. damselae* ssp. *piscicida, Ph. damselae* ssp. *damselae, V. anguillarum, V. harveyi* and *V. vulnificus*, species of interest in the Australian context. The planned outcome is the creation of a practical and robust means of identifying species of the *Vibrionaceae*.

4 NEED

The need for better diagnostics in aquatic animal health is recognised internationally with the growing awareness of the impact of disease in the natural environment. Acquisition of microbiological and molecular tools for detection and identification of pathogens has been nominated as a key research priority by the Marine Disease Working group of the National Centre for Ecological Analysis and Synthesis, University of California (Harvell *et al.*, 2004).

Nationally, the importance of improved diagnostic capabilities is recognised by the Standing Committee for Fisheries and Aquaculture (SCFA) and was specifically identified as a key requirement for national research priorities (Standing Committee on Fisheries and Aquaculture, 1998). Under *Aquaplan* (National Office of Animal and Plant Health, 1999) improved diagnostic capacity in aquatic animal diseases was identified as a major national goal under Projects 3.1 Surveillance & Monitoring, 4.2.9 Diagnostic Resources and 6.2.3 Development of New Diagnostic Tests. The Fish Health Management Committee report on the *Workshop on Aquatic Animal Health Technical Issues* (Standing Committee on Fisheries and Aquaculture, 1999a) identified improved diagnostic capacity for *Vibrio* species as a matter of priority. Following consultation with aquatic animal industry stakeholders, the identification of *Vibrionaceae* was nominated as a priority need for funding under the Federal Government Budget Initiative, *Building A National Approach To Animal And Plant Health* (Fish Health Management Committee, Steering Group of the FRDC Aquatic Animal Health Sub-Program).

Of major concern is the drive to establish health surveillance programs for aquatic animals, identified as a key objective in *Aquaplan* for 1998-2003 and 2005-2010 (National Office of Animal and Plant Health, 1999; 2005). The ability to provide an appropriate level of diagnostic capacity in the absence of suitable diagnostic tests represents a significant deficiency in achieving the key goals identified in *Aquaplan* (Standing Committee on Fisheries and Aquaculture, 1999b; National Office of Animal and Plant Health, 2002).

Competence in identification of *Vibrionaceae* provides the basis to assess the significance of diagnostic findings, is a means of monitoring populations for the emergence of specific pathogens and underpins successful disease management strategies through the selection of appropriate antibiotics, probiotics or vaccines.

5 OBJECTIVES

- Undertake a definitive numerical taxonomic analysis to describe and characterise the phenotypes of aquatic animal Vibrios in Australia
- Develop practical, robust phenotypic identification systems for *Vibrio* species using computer assisted identification software
- Develop and implement PCR gene probes for the rapid identification of key bacterial pathogens
- Determine the range of *Vibrio* species associated with farmed fish, shellfish and crustacea in Australia
- Technology transfer workshop for state veterinary diagnostic microbiologists
- Develop an ANZSDP for the Identification of *Vibrio* species in aquatic animals

6 METHODS

6.1 UNDERTAKE A DEFINITIVE NUMERICAL TAXONOMIC ANALYSIS TO DESCRIBE AND CHARACTERISE THE PHENOTYPES OF AQUATIC ANIMAL VIBRIOS IN AUSTRALIA

6.1.1 Development of *Vibrio* culture library

Strain definition

The term *isolate* is used where a culture is obtained from the natural environment while *strain* is used in the taxonomic sense to indicate a pure culture derived from a single colony (Dijkshoorn *et al.*, 2000).

Framework

A library of 871 strains was developed using three main resources: type and reference cultures obtained from established culture collections; wild type isolates from aquatic animals in Australia; and wild type isolates from aquatic animals from countries outside Australia. Some contributors also provided type and reference cultures held in their collections so that duplicate cultures were obtained for some species.

Type and reference cultures

Species were obtained or purchased from the following collections:

ATCC: American Type Culture Collection, Manassas, VA, USA

CECT: Colección Española de Cultivos Tipo, Valencia, Spain

CIP: Collection de l'Institut Pasteur, Paris, France

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany

NCIMB: National Collection of Industrial & Marine Bacteria, Aberdeen, UK

NCTC: National Collection of Type Cultures, London, UK

Strain details of the cultures obtained directly from these collections are given in Tables 6.1-6.6.

Table 6.1 Type and reference strains obtained from ATCC

Species	Strain no.	Status	Origin
Photobacterium iliopiscarium	ATCC 51760	Type	Herring
Photobacterium iliopiscarium	ATCC 51761	Reference	Atlantic salmon
Vibrio alginolyticus	ATCC 17749	Type	Horse mackerel
Vibrio campbellii	ATCC 25920	Type	Seawater
Vibrio campbellii	ATCC 25921	Reference	Seawater
Vibrio fischeri	ATCC 7744	Type	Not known
Vibrio gazogenes	ATCC 43066	Reference	Hypersaline lagoon
Vibrio harveyi	ATCC 14126	Type	Amphipod
Vibrio ichthyoenteri	ATCC 700024	Reference	Japanese flounder
Vibrio mimicus	ATCC 33653	Type	Man

continued...

Species	Strain no.	Status	Origin
Vibrio mimicus	ATCC 33654	Reference	Lake water
Vibrio mimicus	ATCC 33655	Reference	Man
Vibrio natriegens	ATCC 14048	Type	Salt marsh mud
Vibrio natriegens	ATCC 33788	Reference	Seawater
Vibrio nereis	ATCC 25917	Type	Seawater
Vibrio nereis	ATCC 33893	Reference	Seawater
Vibrio nigripulchritudo	ATCC 33900	Reference	Seawater
Vibrio orientalis	ATCC 33933	Reference	Shrimp
Vibrio orientalis	ATCC 33935	Reference	Seawater
Vibrio parahaemolyticus	ATCC 17802	Type	Man
Vibrio pelagius biovar I	ATCC 25916	Type	Seawater
Vibrio pelagius biovar I	ATCC 33781	Reference	Seawater
Vibrio pelagius biovar II	ATCC 33504	Reference	Seawater
Vibrio pelagius biovar II	ATCC 33784	Reference	Seawater
Vibrio splendidus biovar II	ATCC 25914	Reference	Seawater
Vibrio splendidus biovar II	ATCC 33789	Reference	Seawater
Vibrio vulnificus biovar I	ATCC 27562	Type	Man
Vibrio vulnificus biovar II	ATCC 33148	Reference	Eel

Table 6.2 Type and reference strains obtained from CECT

Species	Strain no.	Status	Origin
Vibrio agarivorans	CECT 5084	Reference	Seawater
Vibrio agarivorans	CECT 5085	Type	Seawater
Vibrio agarivorans	CECT 5085	Type	Sea water
Vibrio lentus	CECT 5110	Type	Oyster
Vibrio lentus	CECT 5293	Reference	Oyster
Vibrio mediterranei	CECT 621	Type	Seawater
Vibrio mediterranei	CECT 622	Reference	Seawater
Vibrio mediterranei	CECT 623	Reference	Seawater
Vibrio mytili	CECT 633	Reference	Mussels
Vibrio mytili	CECT 634	Reference	Mussels
Vibrio scophthalmi	CECT 4638	Type	Turbot
Vibrio scophthalmi	CECT 5965	Reference	Turbot
Vibrio scophthalmi	CECT 5966	Reference	Turbot
Vibrio scophthalmi	CECT 5967	Reference	Turbot
Vibrio scophthalmi	CECT 5968	Reference	Turbot
Vibrio scophthalmi	CECT 5969	Reference	Turbot

Table 6.3 Type and reference strains obtained from CIP

Species	Strain no.	Status	Origin
Grimontia hollisae	CIP 104354	Reference	Man
Grimontia hollisae	CIP 101886	Type	Man
Moritella marina	CIP 102861	Type	Seawater
Vibrio campbellii	CIP 70.67	Reference	Seawater
Vibrio cyclitrophicus	CIP 106644	Type	Marine sediment
Vibrio halioticoli	CIP 106285	Reference	Abalone
Vibrio halioticoli	CIP 106284	Reference	Abalone
Vibrio logei	CIP 103204	Type	Arctic mussel
Vibrio mediterranei	CIP 103203	Type	Marine sediment
Vibrio nigripulchritudo	CIP 103195	Type	Seawater
Vibrio penaeicida	CIP 104417	Type	Kuruma prawn
Vibrio proteolyticus	CIP 102892	Type	Marine isopod
Vibrio salmonicida	CIP 103166	Type	Atlantic salmon
Vibrio tapetis	CIP 104856	Type	Clam

Table 6.4 Type and reference strains obtained from DSMZ

Species	Strain no.	Status	Origin
Vibrio ichthyoenteri	DSMZ 14397	Type	Japanese flounder
Vibrio penaeicida	DSMZ 14398	Type	Kuruma prawn
Vibrio calviensis	DSMZ 14347	Type	Sea water

Table 6.5 Type and reference strains obtained form NCIMB

Species	Strain no.	Status	Origin
Moritella viscosa	NCIMB 13484	Reference	Atlantic salmon
Moritella viscosa	NCIMB 13485	Reference	Atlantic salmon
Moritella viscosa	NCIMB 13584	Type	Atlantic salmon
Photobacterium damselae ssp. *damselae*	NCIMB 2181	Reference	Seawater
Photobacterium damselae ssp. *damselae*	NCIMB 2183	Reference	Man
Photobacterium damselae ssp. *damselae*	NCIMB 2184	Type	Damsel fish
Photobacterium damselae ssp. *piscicida*	NCIMB 2058	Reference	Yellowtail
Vibrio aestuarianus	NCIMB 2236	Type	Oyster
Vibrio ordalii	NCIMB 2168	Reference	Coho salmon
Vibrio ordalii	NCIMB 2167	Type	Coho salmon
Vibrio orientalis	NCIMB 2195	Type	Seawater
Vibrio penaeicida	NCIMB 13386	Type	Kuruma prawn
Vibrio tubiashii	NCIMB 1340	Type	Clam
Vibrio vulnificus biovar II	NCIMB 2136	Reference	Eel
Vibrio vulnificus biovar II	NCIMB 2137	Reference	Eel
Vibrio vulnificus biovar II	NCIMB 2139	Reference	Eel
Vibrio wodanis	NCIMB 13582	Type	Atlantic salmon

Table 6.6 Type and reference cultures obtained from NCTC

Species	Strain no.	Status	Origin
Vibrio fluvialis	NCTC 11327	Type	Man
Vibrio metschnikovii	NCTC 8443	Type	Fowls
Vibrio parahaemolyticus	NCTC 10884	Reference	Not specified
Vibrio parahaemolyticus	NCTC 10885	Reference	Oyster

Additional type and reference strains were obtained either directly from authors or research laboratories. The species are listed in Table 6.7.

Table 6.7 Type and reference strains obtained from other sources

Species	Strain no.	Status	Origin
Grimontia hollisae	CIP 101886	Type	Man
Photobacterium angustum[1]	ATCC 25915	Type	Seawater
Photobacterium damselae ssp. *damselae*	ATCC 33539	Type	Damsel fish
Photobacterium damselae ssp. *damselae*[1,2]	ATCC 51805	Reference	Labracoglossid
Photobacterium damselae ssp. *piscicida*	ATCC 51736	Type	Yellowtail
Photobacterium iliopiscarium	ATCC 51760	Type	Herring
Photobacterium leiognathi[1]	ATCC 25521	Type	Teleost fish
Photobacterium phosphoreum	NCIMB 1282	Type	Not known
Vibrio aestuarianus	ATCC 35048	Type	Oyster
Vibrio alginolyticus (2 cultures)	ATCC 17749	Type	Horse mackerel
Vibrio anguillarum	ATCC 14181	Reference	Brown trout
Vibrio anguillarum (2 cultures)	ATCC 19264	Type	Cod
Vibrio campbellii	ATCC 25920	Type	Seawater

continued...

Species	Strain no.	Status	Origin
Vibrio cholerae[1]	ATCC 14547	Reference	Fish (not specified)
Vibrio cincinnatiensis	LMG 7891	Type	Man
Vibrio diazotrophicus (2 cultures)	ATCC 33466	Type	Sea urchin
Vibrio fischeri[1]	ATCC 14546	Reference	Not specified
Vibrio fischeri[1]	ATCC 25918	Reference	Seawater
Vibrio fischeri[1]	ATCC 33715	Reference	Seawater
Vibrio fischeri[1]	ATCC 33984	Reference	Rockfish
Vibrio fischeri	ATCC 7744	Type	Not known
Vibrio fluvialis	NCTC 11327	Type	Man
Vibrio furnissii (2 cultures)	ATCC 35016	Type	Man
Vibrio furnissii	NCTC 11328	Reference	River water
Vibrio gazogenes (2 cultures)	ATCC 29988	Type	Marine mud
Vibrio halioticoli[3]	IAM 14596	Type	Abalone
Vibrio harveyi	ATCC 14126	Type	Amphipod
Vibrio harveyi[4] (2 cultures)	ATCC 35084	Reference	Brown shark
Vibrio ichthyoenteri	ATCC 700023	Type	Japanese flounder
Vibrio lentus	CECT 5110	Type	Oyster
Vibrio logei (2 cultures)	ATCC 29985	Type	Arctic mussel
Vibrio mediterranei[5,6]	ATCC BAA-91	Reference	Bleached coral
Vibrio mediterranei	CIP 103203	Type	Marine sediment
Vibrio metschnikovii	NCTC 11170	Reference	Cockles
Vibrio mytili	CIP 103929	Type	Mussels
Vibrio natriegens	ATCC 14048	Type	Salt marsh mud
Vibrio navarrensis	CIP 103381	Type	Sewage
Vibrio nereis	ATCC 25917	Type	Seawater
Vibrio ordalii	ATCC 33509	Type	Coho salmon
Vibrio orientalis	ATCC 33934	Type	Seawater
Vibrio parahaemolyticus	CECT 511	Type	Man
Vibrio parahaemolyticus	CECT 588	Reference	Mussels
Vibrio parahaemolyticus	CECT 611	Reference	Seawater
Vibrio parahaemolyticus	CECT 612	Reference	Seawater
Vibrio pectenicida[7]	CIP 105190	Type	Scallop larvae
Vibrio pelagius biovar I (2 cultures)	ATCC 25916	Type	Seawater
Vibrio rumoiensis[8]	NIBHT	FERM P-14531	Drain pool water
Vibrio splendidus biovar I[1]	ATCC 33125	Type	Fish (not specified)
Vibrio splendidus biovar II	ATCC 25914	Reference	Seawater
Vibrio tasmaniensis	LMG 20012	Type	Atlantic salmon
Vibrio tasmaniensis	LMG 21575	Reference	Atlantic salmon
Vibrio tubiashii (2 cultures)	ATCC 19109	Type	Hard clam

[1]Professor Paul Dunlap, University of Michigan, Ann Arbor, Michigan, USA
[2]Formerly the type strain of *Ph. 'histaminum'* a junior synonym of *Ph. damselae* ssp. *damselae*
[3]Dr Tomoo Sawabe, Hokkaido University, Hokkaido, Japan
[4]Formerly the type strain of *Vibrio 'carchariae'* a junior synonym of *V. harveyi*
[5]Dr Eugene Rosenberg, Tel Aviv University, Ramat Aviv, Israel
[6]Formerly the type strain of *Vibrio 'shilonii'* a junior synonym of *V. mediterranei*
[7]Dr Jean-Louis Nicolas, IFREMER, Plouzane, France
[8]Dr Isao Yumoto, National Institute of Advanced Industrial Science and Technology, Sapporo, Japan

NIBHT: National Institute of Bioscience and Human Technology, Tsukuba, Japan
LMG: Laboratorium voor Microbiologie, University of Ghent, Belgium

Wild type cultures

Wild type cultures, isolated mostly from aquatic animals with signs of disease, were obtained from the laboratories listed in Table 6.8. Cultures were submitted as either named species, unspeciated isolates of the genus *Vibrio* and *Photobacterium* or isolates identified presumptively as members of the *Vibrionaceae*.

Table 6.8 Laboratories providing wild type isolates of *Vibrionaceae*

Donor	Institution	Country
TCFB	DPIW, Tasmania	Australia
Morris Pizzuto	DPIFM, Northern Territory	Australia
Dr Nicky Buller	Department of Agriculture, Perth, Western Australia	Australia
Dr Annette Thomas	Department of Primary Industry, Townsville, Queensland	Australia
Dr Frederique Le Roux	IFREMER, La Tremblade	France
Dr Amedo Manfrin	Istituto Zooprofilattico delle Venezie, Padua	Italy
Dr Ben Diggles	NIWA, Wellington	New Zealand
Dr Luc Grisez	Intervet Norbio	Singapore
Professor Maria Pujalte	Universitat de Valencia	Spain
Professor Maria Figueras	Universitat Rovira i Virgili	Spain
Professor Brian Austin	Heriot-Watt University, Edinburgh	UK
Dr David Bruno	Marine Research Laboratory, SOAFD, Aberdeen	UK
Professor Paul Dunlap	University of Michigan, Ann Arbor	USA

DPIFM: Department of Primary Industry, Fisheries and Mines
DPIW: Department of Primary Industries & Water
IFREMER: Institut Français de Recherche pour l'Exploitation de la Mer
NIWA: National Institute for Water & Atmospheric Research
SOAFD: Scottish Office Agriculture Fisheries Department
TCFB: Tasmanian Collection of Fish Bacteria

Strain coding and data management

Cultures were allocated a consecutive number from 1 onwards with the prefix V. Primary details of the cultures including storage location were held in a controlled access, activity traceable database for the culture collection of the Fish Health Unit, DPIW. A subsidiary working database of cultures specific to phenotyping was in the form of a spreadsheet.

Culture recovery and maintenance

All cultures were maintained on Johnson's marine agar (Johnson, 1968) (JMA) although ZoBell's marine agar 2216E (Oppenheimer & ZoBell, 1952) (Amyl Media, Melbourne) (ZMA) was found to be equally suitable; fastidious species were grown on blood agar enriched with 7% defibrinated sheep's blood and 2% NaCl (Blood Agar Base no. 2, CM271, Oxoid, Adelaide) (SBA2). Cultures received freeze-dried were revived following the guidelines of the supplying culture collection; incubation temperature for cultures was either 25°C or 15°C as appropriate for the species; a summary of culture media requirements is given in Table 6.9. In most cases the

Table 6.9 Culture medium requirements and incubation temperatures for Vibrionaceae

Species	Recovery		Maintenance	
	Medium	Temperature	Medium	Temperature
Moritella marina	SBA2	15°C	JMA/ZMA/ SBA2	15°C
Moritella viscosa	NCIMB 252	15°C	SBA2	15°C
Ph. damselae ssp. *damselae*	SBA	25°C	JMA/ZMA	25°C
Ph. damselae ssp. *piscicida*	ZMA	25°C	JMA/ZMA	25°C
Photobacterium leiognathi	ZMA	25°C	JMA/ZMA	25°C
Photobacterium phosphoreum	ZMA	15°C	JMA/ZMA	15°C

continued...

Species	Recovery		Maintenance	
	Medium	Temperature	Medium	Temperature
Photobacterium angustum	ZMA	25°C	JMA/ZMA	25°C
Photobacterium iliopiscarium	ZMA	15°C	JMA/ZMA	15°C
Vibrio aestuarianus	ZMA	25°C	JMA/ZMA	25°C
Vibrio alginolyticus	ZMA	25°C	JMA/ZMA	25°C
Vibrio anguillarum	SBA	25°C	JMA/ZMA	25°C
Vibrio agarivorans	ZMA	25°C	JMA/ZMA	25°C
Vibrio calviensis	ZMA	25°C	JMA/ZMA	25°C
Vibrio campbellii	ZMA	25°C	JMA/ZMA	25°C
Vibrio cholerae	SBA	30°C	JMA/ZMA/SBA	25°C
Vibrio cincinnatiensis	ZMA	25°C	JMA/ZMA	25°C
Vibrio cyclitrophicus	ZMA	25°C	JMA/ZMA	25°C
Vibrio diabolicus	ZMA	25°C	JMA/ZMA	25°C
Vibrio diazotrophicus	ZMA	25°C	JMA/ZMA	25°C
Vibrio fischeri	ZMA	25°C	JMA/ZMA	25°C
Vibrio fluvialis	ZMA	25°C	JMA/ZMA	25°C
Vibrio furnissii	ZMA	25°C	JMA/ZMA	25°C
Vibrio gazogenes	ZMA	25°C	JMA/ZMA	25°C
Vibrio halioticoli	ZMA	25°C	JMA/ZMA	25°C
Vibrio harveyi	ZMA	25°C	JMA/ZMA	25°C
Vibrio hollisae	ZMA	25°C	JMA/ZMA	25°C
Vibrio ichthyoenteri	ZMA	25°C	JMA/ZMA	25°C
Vibrio lentus	ZMA	25°C	JMA/ZMA	25°C
Vibrio logei	ZMA	15°C	JMA/ZMA	15°C
Vibrio mediterranei	ZMA	25°C	JMA/ZMA	25°C
Vibrio metschnikovii	ZMA	25°C	JMA/ZMA	25°C
Vibrio mimicus	ZMA	25°C	JMA/ZMA	25°C
Vibrio mytili	ZMA	25°C	JMA/ZMA	25°C
Vibrio natriegens	ZMA	25°C	JMA/ZMA	25°C
Vibrio navarrensis	ZMA	25°C	JMA/ZMA	25°C
Vibrio nereis	ZMA	25°C	JMA/ZMA	25°C
Vibrio nigripulchritudo	ZMA	25°C	JMA/ZMA	25°C
Vibrio ordalii	SBA2	25°C	JMA/ZMA	25°C
Vibrio orientalis	ZMA	25°C	JMA/ZMA	25°C
Vibrio parahaemolyticus	ZMA	25°C	JMA/ZMA	25°C
Vibrio pectenicida	ZMA	25°C	JMA/ZMA	25°C
Vibrio pelagius biovar I	ZMA	25°C	JMA/ZMA	25°C
Vibrio pelagius biovar II	ZMA	25°C	JMA/ZMA	25°C
Vibrio penaeicida	ZMA	25°C	JMA/ZMA	25°C
Vibrio proteolyticus	ZMA	25°C	JMA/ZMA	25°C
Vibrio rumoiensis	ZMA	25°C	JMA/ZMA	25°C
Vibrio salmonicida	NCIMB 252	15°C	SBA2	15°C
Vibrio scophthalmi	ZMA	25°C	JMA/ZMA	25°C
Vibrio splendidus biovar I	ZMA	25°C	JMA/ZMA	25°C
Vibrio splendidus biovar II	ZMA	25°C	JMA/ZMA	25°C
Vibrio tapetis	ZMA	25°C	JMA/ZMA	25°C
Vibrio tubiashii	ZMA	25°C	JMA/ZMA	25°C
Vibrio vulnificus biovar I	ZMA	25°C	JMA/ZMA	25°C
Vibrio vulnificus biovar II	ZMA	25°C	JMA/ZMA	25°C
Vibrio wodanis	SBA2	15°C	SBA2	15°C

JMA: Johnson's marine agar; **SBA**: sheep blood agar; **SBA2**: sheep blood agar+2% NaCl;
NCIMB 252: Seawater agar+10% foetal calf serum; **ZMA**: ZoBell's marine agar 2216E

recovery medium was the same as that used for maintenance, but for fastidious species enrichment with 10% foetal calf serum of the recovery medium was of value (A. Baxter, NCIMB, pers. comm.).

All culture stocks were held frozen at -80°C either as a single use volume of a 50µl suspension in peptone-glycerol cryopreservative (Ward & Watt, 1971) or on MicroBank beads (Pro-Lab Diagnostics, Richmond Hill, Ontario). Frozen cultures were revived on JMA or SBA2 as appropriate and in *Vibrio* Recovery Medium (VRM), a formulation developed for this study. This medium was prepared with pyruvate and bacteriological charcoal, components known to assist in the recovery of damaged cells, including *Vibrio* species (Brewer *et al.*, 1977; Hoffman *et al.*, 1983; Mizunoe *et al.*, 2000; Wai *et al.*, 2000) and appeared useful in recovering cultures damaged by freeze-thawing.

6.1.2 Miniaturised tests for phenotyping

Test selection strategy

Macro-format tests known to have discriminating power for species of the *Vibrionaceae* were identified based on several numerical taxonomic studies (West *et al.*, 1983; Dawson & Sneath, 1985; Bryant *et al.*, 1986a; 1986b; West *et al.*, 1986; Kämpfer *et al.*, 1987; Grimes *et al.*, 1993; Farto *et al.*, 1999). Tests were selected to ensure a diversity of traits, thereby reducing the occurrence of test interdependence which can lead to a narrowing of the phenotypic description (Colwell, 1970; O'Brien & Colwell, 1987). Based on published data (Baumann *et al.*, 1971; Bryant *et al.*, 1986a), tests were selected in anticipation that a range of reaction from nearly all positive to nearly all negative would be achieved as a means of increasing the likelihood of finding tests that could define taxa. While tests at either end of the range may have limited discriminatory power, if the presence or absence of a trait is unique to a particular taxon, then it assumes greater importance for species definition.

Test miniaturisation

Test technology was based on the MicroSys® format (DPIW, Launceston) which was developed to provide test equivalence to conventional macro-format tests. Use of miniaturised tests allows high-throughput testing to be undertaken in a convenient and cost-effective manner. Tests were formulated based on procedures for the identification of *Aeromonas* (Carson *et al.*, 2001) and *Vibrio* species (Baumann *et al.*, 1971; Furniss *et al.*, 1978; Kämpfer *et al.*, 1987; Baumann *et al.*, 1984; West & Colwell, 1984; MacFaddin, 2000). The final working volume of each test was 100µl in a 96 micro-well plate.

Refinement of miniaturised tests

Tests for lysine and ornithine decarboxylase, fermentation and carbon source utilisation (CSU) tests in the MicroSys V48 panel of tests for *Vibrionaceae* were

improved. Changes were made to the test chemistry for decarboxylase and fermentation tests to prevent oxidative deamination which can cause false positive reactions with some species. The decarboxylase medium, based on Møller's method (Møller, 1955), was further modified by replacement of the double indicators, bromocresol purple and cresol red, with a single indicator, phenol red (Hansen & Sørheim, 1991) which improved test sensitivity and readability. Formulation of the CSU basal medium was modified to increase nutrient availability and stability, which resulted in improved growth and made test interpretation less ambiguous.

Novel miniaturised tests

Additional tests thought likely to be useful for phenotyping are listed in Table 6.10.

Table 6.10 New tests miniaturised for phenotyping the Vibrionaceae library

Test	Activity
NaCl tolerance	Growth at 4, 7 & 10% NaCl
Bile tolerance	Growth in the presence of 8% ox bile
Cobalt chloride tolerance	Growth in the presence of 0.0008% cobalt chloride
Crystal violet tolerance	Growth in the presence of 0.0003% crystal violet
Gelatinase	Gelatin hydrolysis
Alkaline phosphatase	Hydrolysis of 3-indoxyl phosphate to blue indoxyl
Acid phosphatase	Hydrolysis of 4-nitrophenyl phosphate to yellow nitrophenol
α-galactosidase	Hydrolysis of 4-nitrophenyl α-D-galactopyranoside to yellow nitrophenol
β-galactosidase	Hydrolysis of 2-nitrophenyl β-D-galactopyranoside to yellow nitrophenol
Prolyl aminopeptidase	Hydrolysis of L-proline 4-nitroanilide to yellow nitroaniline
γ-glutamyl transpeptidase	Hydrolysis of L-glutamic acid 5-(4-nitroanilide) to yellow nitroaniline
Sulphatase	Hydrolysis of 4-nitrophenyl sulfate to yellow nitrophenol
Amylase	Hydrolysis of starch
TTC reduction	Reduction of 2,3,5 triphenyltetrazolium chloride to red tetrazolium
Amino acid accumulation	Hydrolysis of peptones to yield free amino acids in culture media
Amino acid decomposition	Alkaline end products from leucine, serine, threonine and citrulline

Tests for specific enzymes were based on the formulation used for assessing hydrolysis of ONPG (2-nitrophenyl β-D-galactopyranoside) (Lowe, 1962); amino acid decomposition to produce alkaline end products was assessed in the basal medium for arginine dihydrolase activity (Thornley, 1960).

Performance of tests for amylase, gelatinase and tolerance to NaCl was assessed using species of known positive and negative reactions as listed in Table 6.11. The same panel of organisms was used to evaluate the remaining novel tests. A positive reaction in tolerance tests was determined as growth; production of specific enzymes was determined by the release of a chromogen from the colourless substrate; and alkaline end products from the decomposition of specific amino acids seen as a change in phenol red, the pH indicator, from tan to deep pink. Gelatin hydrolysis was determined by the addition of saturated ammonium sulphate (Pitt & Dey, 1970) to the

Table 6.11 Species used for test development and assessment of performance

Species	Strain no.
V. alginolyticus	ATCC 17749
V. anguillarum	ATCC 19264
V. furnissii	ATCC 35016
V. harveyi	ATCC 14126
V. metschnikovii	NCTC 8443
V. mimicus	ATCC 33653
V. parahaemolyticus	ATCC17802
V. halioticoli	IAM 14596
Ph. damselae ssp. *damselae*	V797
G. hollisae	CIP 101886
V. splendidus biovar II	ATCC 25914
V. fischeri	ATCC 7744

centrifuged supernatant of the test; if no precipitation occurred the test was positive while precipitation of un-hydrolysed gelatin was a negative result. Accumulated amino acids, not deaminated following proteolysis, were detected by the addition of ninhydrin; formation of a deep purple colour in the organic solvent layer was positive while colourless or a very pale purple was negative.

Test investigations

Nutrient supplementation. Some strains of *Vibrionaceae* are known to be nutritionally fastidious and require the addition of nutrient supplements. Typically this is in the form of Casamino acids (an acid hydrolysate of casein) (Baumann *et al.*, 1971), yeast extract (Smith *et al.*, 1991), tryptone or casein (Baumann *et al.*, 1984). Selection of a nutrient supplement for use with the CSU medium was investigated for a panel of carbon sources (Table 6.12) supplemented with a range of yeast extract and Casamino acid concentrations. The effect of supplementation was assessed with strains of *Ph. damselae* ssp. *damselae, Ph. damselae* ssp. *piscicida, Ph. iliopiscarium, V. ordalii* and *V. lentus*, which are known to be nutritionally fastidious. Growth at the

Table 6.12 Carbon source utilisation nutrient supplement test combinations

Nutrient	Concentration	Carbon sources	Species and strain number
Casamino acids	0.15-1.0 g L^{-1}	Cellobiose	*Ph. damselae* ssp. *damselae* NCIMB 2183
Yeast extract	0.05-0.5g L^{-1}	Citrulline	*Ph. damselae* ssp. *damselae* NCIMB 2184^T
		Galactose	*Ph. damselae* ssp. *damselae* V795
		Glucosamine	*Ph. damselae* ssp. *piscicida* V372
		Glucose	*Ph. iliopiscarium* ATCC 51760^T
		Glycerol	*V. ordalii* V439
		Lactate	*V. ordalii* NCIMB 2168
		Mannose	*V. lentus* CECT 5110^T
		Succinate	
		Sucrose	
		Control	

different concentrations of supplement for the panel of carbon sources and species was determined. Growth or no growth for each supplement and concentration was determined for the range of species and strains listed. The optimum concentration and nutrient was identified and used for phenotyping fastidious strains.

Arginine dihydrolase. During phenotyping the library of *Vibrionaceae* some strains were found to have unexpected positive reactions. These strains were retested in the miniaturised test and by conventional tube format using the medium of Thornley (1960), modified by adjusting the pH to 6.8 (West and Colwell, 1984) and raising the NaCl concentration to 1.5% (Furniss *et al.*, 1978).

Oxaloacetate CSU. Initial phenotyping found a high level of positive reactions for oxaloacetate utilisation. As more of the library was phenotyped the number of positive reactions declined and in retesting a proportion of the library the error rate for oxaloacetate of 53% was the highest of the tests. New stock of reagents were prepared and tested with the species listed in Table 6.13 to determine if the substrate was labile.

Table 6.13 Species tested for oxaloacetate utilisation

Species and strain number
Vibrio sp. Phenon 57 V16
Vibrio cyclitrophicus V127
Vibrio chagasii V160
Vibrio halioticoli CIP 106284
Vibrio splendidus biovar II ATCC 25914
Photobacterium damselae ssp. *damselae* V797
Photobacterium damselae ssp. *damselae* V817
Vibrio alginolyticus V858

Phenotyping panel

The panel of miniaturised tests for phenotyping is given in Table 6.14 and were accommodated in a single 96 micro-well tray. Tests for alginase, amino acid accumulation, gelatinase, gluconate oxidation, indole, nitrate reduction, phenylalanine deaminase and acetoin required the addition of detection reagents, listed in Table 6.15.

Table 6.14 Panel of miniaturised tests for phenotyping Vibrionaceae

Type	Test	Method Source
Tolerance tests	Growth 0% NaCl	
	Growth 4% NaCl	
	Growth 1% NaCl	
	Growth 7% NaCl	
	Growth 10% NaCl	
	Growth bile 0.8%	West *et al.*, 1983
	Growth crystal violet 0.0003%	West *et al.*, 1983
	Growth cobalt chloride 0.0008%	
	Growth tellurite 0.0005%	West *et al.*, 1983

continued...

Type	Test	Method Source
Miscellaneous	Gluconate oxidation	Cowan, 1974
	Voges Proskauer (Acetoin)	Furniss *et al.*, 1978
	Indole	Cowan, 1974
	TTC reduction	
	Nitrate reduction	Cowan, 1974
Amino acid metabolism	Arginine dihydrolase	Thornley, 1960
	Lysine decarboxylase	Møller, 1955
	Ornithine decarboxylase	Møller, 1955
	Leucine deamination	
	Serine deamination	
	Threonine deamination	
	Citrulline deamination	
	Amino acid accumulation	
	Phenylalanine deamination	Cowan, 1974
Hydrolysis tests	Aesculin hydrolysis	Cowan, 1974
	Alginase	Kitamikado *et al.*, 1990
	Gelatinase	Cowan, 1974
	Amylase	Cowan, 1974
	Urease	Maslen, 1952
Specific enzymes	Alkaline phosphatase	
	Acid phosphatase	
	α-D-galactosidase	
	β-D-galactosidase	Lowe, 1962
	Prolyl aminopeptidase	
	γ-glutamyl transpeptidase	
	Sulphatase	
Fermentation Tests	Amygdalin	Carson *et al.*, 2001
	Arbutin	
	Cellobiose	
	D-galactose	
	D-glucose	
	D-mannitol	
	D-mannose	
	D-sorbitol	
	Inositol	
	L-arabinose	
	L-rhamnose	
	Raffinose	
	Salicin	
	Sucrose	
	Gentiobiose	
Carbon source utilisation tests	1-propanol	Baumann *et al.*, 1984
	Acetamide	
	Acetate	
	Adenine	
	Allantoin	
	Amygdalin	
	Arbutin	
	Butyramide	
	Cellobiose	
	Citrate	
	D-alanine	
	D-arabitol	
	D-galacturonate	
	D-galactose	
	D-glucosamine	
	D-glucose	

continued...

Type	Test	Method Source
	D-glucuronate	
	DL-3-hydroxybutyrate	
	DL-lactate	
	DL-malate	
	D-mannose	
	D-sorbitol	
	D-xylose	
	Ethanol	
	Gluconate	
	Glycerol	
	Inulin	
	Lactose	
	L-arabinose	
	L-aspartate	
	L-citrulline	
	L-histidine	
	L-hydroxyproline	
	L-leucine	
	L-serine	
	Melezitose	
	Melibiose	
	Oxaloacetate	
	Propionate	
	Putrescine	
	Succinate	
	Sucrose	
	Urocanate	
	Valerate	
	α-ketoglutarate	
	Water (negative control)	

Table 6.15 Detection reagents

Test	Reagent	Reference
Alginase	Acidified bovine serum albumin	Kitamikado *et al.*, 1990
Amino acid accumulation	Ninhydrin	MacFaddin, 2000
Gelatinase	Saturated ammonium sulphate	Pitt and Dey, 1970
Gluconate oxidation	Benedict's reagent	Barrow & Feltham, 1993
Indole	Kovács' reagent	Cowan, 1974
Nitrate reduction	Combined reagent	Kamphake *et al.*, 1967
Phenylalanine deaminase	Acid ferric chloride	Barrow & Feltham, 1993
Voges-Proskauer (acetoin)	Coblentz reagents	MacFaddin, 2000

The culture media were prepared double strength and stored at 2-8°C. Aliquots of media were transferred to 96 well 2.2ml storage plates (ABgene, UK, cat. no. AB-0661) and sealed with a sterile silicone rubber mat (ABgene, cat. no. AB-0674). Phenotyping panels were prepared by transferring 50µl of medium to sterile flat bottomed 96 micro-well plates (Sarstedt, Adelaide, cat. no. 82.9923.150) by multi-channel pipette; plates were used the same day as aliquoted. The NaCl concentration of the test media after inoculation was 1.5% w/v.

6.1.3 Phenotyping of *Vibrionaceae* library

Test protocol

Cultures were revived from storage at -80°C on JMA and VRM and subcultured twice on JMA before use to check purity and to establish culture vigour. Cell suspensions were prepared in distilled water and 2% NaCl to a density equivalent to McFarland 0.5 and then diluted 1:5 in their respective diluents. The test well for growth in 0% NaCl was inoculated with 50µl of diluted suspension in distilled water and the remaining wells inoculated with 50µl of the saline suspension. Tests for fermentation, urease, decarboxylase, arginine dihydrolase and amino acid decomposition were overlayed with ~75µl of sterile liquid paraffin and the plates sealed with adhesive plate film (MP Biomedicals, cat. no. 7742000). Cultures at 25°C were incubated for 48 h and those at 15°C for 5 d. After incubation, detection reagents were added and reactions read as colour changes. CSU tests were read with an inverted magnifying mirror with indirect light; any signs by eye of turbidity, the result of growth, was considered positive. CSU results were considered void if there were signs of turbidity in the negative control well without carbon source.

Supplementary test procedures

Additional tests are listed in Table 6.16. Gram reaction was determined by the KOH lysis method (Gregersen, 1978) and oxidase by Kovács' method (Cowan, 1974); swarming was tested on freshly prepared ZMA and agarolysis was determined by

Table 6.16 Supplementary identification tests in macro-format

Test	
Gram reaction	
Oxidase	
Swarming	
Agarolysis	
Antibiotic resistance:	0/129 10µg
	0/129 150µg
	Ampicillin 10µg
	Polymyxin B 50iu
	Novobiocin 5µg
	Carbenicillin 100µg

evidence of colony pitting after incubation for 48 hours. Antibiotic sensitivity testing was performed on Mueller-Hinton agar supplemented by the addition of 2% w/v NaCl. Plates were inoculated using a cotton swab with a McFarland 0.5 suspension in 2% w/v NaCl prepared from a 24 hr culture on JMA. Ampicillin, carbenicillin and novobiocin discs at the potencies stated (Table 6.16) were obtained from Oxoid

(Adelaide). Discs of the vibriostat 0/129 (Collier *et al.*, 1950) at 10 and 150 µg (Furniss *et al.*, 1978) were prepared using 6mm blank sterile discs placed in a 96 micro-well plate and loaded with 20µl of 2,4-diamino-6,7-di-*iso*propylpteridine phosphate (Sigma-Aldrich, Sydney) at either 0.5mg ml^{-1} (10 µg discs) or 7.5mg ml^{-1} (150 µg discs). Polymyxin B discs were prepared using a stock solution of 2,500 iu ml^{-1}. Plates of discs were freeze-dried for 4 hours, sealed with plate tape (MP Biomedicals, Sydney, cat. no. 7742000) and stored at 2-8°C. Zones of inhibition were measured and interpreted as sensitive or resistant using the criteria listed in Table 6.17 (Furniss *et al.*, 1978; Kelly *et al.*, 1991; Hendrickson & Krenz, 1991); interpretation of zone size for novobiocin was deduced from species known to be resistant or sensitive (West *et al.*, 1983; 1986).

Table 6.17 Zone size interpretation for diagnostic antibiotics

Antibiotic	Resistant	Sensitive	Reference
Ampicillin 10µg	≤13mm	≥ 14mm	Kelly *et al.*, 1991
Carbenicillin 100µg	≤22mm	≥ 23mm	Kelly *et al.*, 1991
Novobiocin 5µg	≤16mm	≥ 17mm	West *et al.*, 1983; 1986
0/129 10µg	≤15mm	≥ 16mm	Furniss *et al.*, 1978
0/129 150µg	No zone	Zone of any size	Furniss *et al.*, 1978
Polymyxin B 50iu	<7mm	≥ 7mm	Furniss *et al.*, 1978

Test supplementation

Sodium requirement. Where growth was poor on Mueller-Hinton agar with 2% NaCl and in the 1% NaCl growth tolerance well, strains were considered to be obligate halophiles and retested using an inoculum prepared in 3% NaCl. The final concentration of NaCl in tests was increased from 1.5% w/v to 2% w/v by this means.

Nutrient supplementation. For nutritionally fastidious strains the inoculum was supplemented with 0.3g L^{-1} Casamino acids (Difco Laboratories, Becton-Dickinson, Sydney) to give a final test concentration after inoculation of 0.15g L^{-1}. Strains requiring the addition of Casamino acids were identified by the absence of growth in the glucose CSU control well or if very few substrates were utilised.

6.1.4 Genotyping of *Vibrionaceae* library

Transfer and storage of isolates

A duplicate set of strains of the library was transferred to the CSIRO Australian Animal Health Laboratory, Geelong in batches of sterile plastic screw capped tubes containing a small quantity of bacterial suspension (50 to 200µl) and frozen on dry-ice for transport. On receipt the samples were stored at –80°C prior to culture.

The purity and viability of the bacterial suspensions was assessed prior to preparing working stock and archive samples. Approximately 50µl of the frozen suspension was cultured on ZMA or SBA2 as appropriate for the species as listed in Table in 6.9. Cultures were stored at -80°C in MicroBank vials. Supernatant from the vials was not discarded but retained and frozen at -80°C as a backup.

DNA extraction for sequencing

Bacterial DNA for sequencing was prepared using PrepMan Ultra Sample Preparation Reagent™ (Applied Biosystems P/N 4318925), with some minor modification to the manufacturers' instructions to accommodate the use of microtitre format plates.

A 1µl inoculating loop was used to pick a small loopful of cells from a culture plate and cells resuspended by twirling the loop in a well of a twin.tec™ 96-well PCR plate (Eppendorf cat. no. 0030 128.605) containing 70-100µl of PrepMan™ reagent. The plates were then covered with PCR Film (Eppendorf cat. no. 0030 127.480) and incubated at 100°C for 10 minutes in a GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus) with the heated lid open. The plates were then removed, allowed to cool to ambient temperature and centrifuged at 3,000 x *g* for 15 minutes in an IEC Centra-7R bench-top centrifuge fitted with PCR plate adaptors (Applied Biosystems P/N N801-0531). A 50µl volume of supernatant containing DNA was transferred to a new PCR plate and diluted 1:10 (v/v) with autoclaved Milli-Q® (Millipore Australia, Sydney) high purity water (Milli-Q water). All the plates were then covered with PCR foil (Eppendorf cat. no. 0030 127.471) and stored at -20°C.

Amplification of the 16S rRNA gene

PCR amplification of the 16S rRNA gene was undertaken using the specific universal primers 27f and 1492r (Lane, 1991). Reactions were performed in 200µl thin-walled Twin.tec™ PCR plates in a GeneAmp® 9600 thermal cycler (Perkin Elmer-Cetus). Each 50µl reaction contained 2µl of diluted PrepMan™ DNA sample, 1.0 U of DNA Polymerase (Platinum® *Taq*, Invitrogen cat. no. 10966-034), 50µl of 10x PCR buffer, 1.5 mM $MgCl_2$, 18 pmoles each of the amplification primers 27f and 1492r (see Table 6.20 for sequences) and all four deoxyribonucleotide triphosphates at 200 µM each. Reaction mixes were held for 5 min. at 95°C then amplified for 30 cycles with denaturation for 90 sec. at 95°C, annealing for 60 sec. at 55°C and elongation for 120 sec. at 72°C, with a final extension step at 72°C for 10 min. The expected PCR product

size was 1465 base pairs. See Table 6.18 for a summary of the master mix and Table 6.19 for the thermal cycling profile used.

Table 6.18 Master mix for 16S rDNA PCR (27f/1492r) – 1 plate (96 wells), 50µl reaction mix per well

Reagent	Stock Solution	Volume (µl) for x1 50µl reaction
H_2O	Sterile Milli-Q	38
$MgCl_2$	50mM	1.5
RB 10X	Reaction Buffer (as supplied with polymerase)	5
dNTPs	dNTP stock - 4mM each dNTP or 16mM total	2.5
Primer - 27f	250ng/µl working stock	0.4
Primer - 1492r	250ng/µl working stock	0.4
Taq	Platinum® *Taq* (5U/µl)	0.2
DNA template	PrepMan™ sample diluted 1:10	2
	Volume (including DNA template)	50

Table 6.19 Thermal Profile for 16S rDNA PCR (27f/1492r) using GeneAmp 9600®

Temperature	Time	No. of Cycles
95ºC	5 min.	1cycle
95ºC	90 sec.	
55ºC	60 sec.	29 cycles
72ºC	120 sec.	
95ºC	90 sec.	
55ºC	60 sec.	1cycle
72ºC	8 min.	
4ºC	hold	

Purification of PCR products

Amplified 16S rDNA PCR products were subjected to batch purification using either Nucleon or Qiagen UF membranes. In order to maintain consistency between sample batches the Qiagen MinElute 96 UF PCR Purification Kit (cat. no. 28051) with a Qiagen QIAVac Multiwell Unit (cat. no. 19504) and Vacuum Regulator (cat. no. 19530) used according to the manufacturers' instructions was adopted as the standard method for purification. Where possible the PCR products were purified immediately following completion of the PCR, however on a few occasions PCR plates were maintained overnight at 4°C without any apparent ill effect.

After 50µl of PCR sample was applied to the membrane surface, a constant vacuum of between -700 and -800 mbar was maintained for 10 minutes. Usually most of the liquid associated with the samples had passed through the membranes, if not, the vacuum was maintained for a further 5 minutes or until the wells were free of liquid. At this stage an additional rinse of 50µl of sterile Milli-Q water was used to wash the DNA

samples. The purified PCR product was eluted in a 20µl volume of sterile Milli-Q water after 2 minutes agitation on a Titertek Microplate Shaker (Flow Laboratories).

Electrophoresis of purified PCR products

The purified 16S rDNA PCR amplification products were checked by gel electrophoresis to verify successful amplification and assess the consistency of yield between samples prior to proceeding with the batch preparation of the cycle sequencing reaction. Electrophoresis of each purified sample was carried out using the E-Gel® 96 High-Throughput Agarose Electrophoresis System (Invitrogen cat. no. G7008-02) and power supply (cat. no. G7100-01) using 2% agarose gels.

Typically, 5µl of the purified aqueous PCR product was added to 20µl of water which had been previously added to each well of the gel. The last well in each row of the gel was loaded with molecular size markers (Promega™ 100bp ladder, cat. no. G210A). As the gel system employed was easily overloaded with DNA the standards had to be diluted. In order to achieve the correct dilution using existing reagents, 30µl of working strength standard solution (130 ng/µl) was added to 16µl of water; 5µl of this diluted standard was then put into 20µl of water, which had been loaded to each well of the gel, as with the PCR samples. Gels were run for approximately 12 minutes or until the dye front had migrated sufficiently into the gel. Gel images were captured using a Kodak™ EDAS290 digital imaging system mounted over a UV transilluminator at 254nm. For samples where the procedure failed to produce a clear band when assessed by agarose gel electrophoresis, the strain was re-cultured and the procedure repeated until DNA suitable for cycle sequencing was obtained.

DNA quantification

Twenty randomly selected 20µl samples from one plate of purified DNA from the 27f/1492r PCR was quantified using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech) to determine the volume required in the cycle sequencing reaction. Thereafter, a clear band of similar intensity in the agarose gel was used to confirm that a suitable amount of template was added to the sequencing reactions.

Sequencing of the 16S rDNA gene

Partial sequencing of the first 685 bp of the 16S rRNA gene of all strains in the study was carried out using the antisense primer 685r2 (Table 6.20) using the purified 27f/1492r amplicon as a source of DNA template.

Where a more complete 16S rDNA sequence was required, for example in the case of HMOs of unidentified phenons or other exemplars of specific phenons, a combination of 6 universal primers were used (Lane 1991, Dorsch & Stackebrandt 1992). In each case the primers were used as a 2 µM stock solution in reaction mixes based on that summarised in Table 6.21. These primers provided overlapping sequence data from both sense and antisense strands of the bacterial genomic DNA, providing a high level of confidence in the reliability of the sequence data obtained and are summarised in Table 6.20.

Table 6.20 Summary of primers used for 16S sequencing

Primer	Sequence (5′ to 3′)	Length (bp)	Orientation	Reference
27f	AgAgTTTgATCMTggCTCAg	20	sense	Lane (1991)
503f	gTgCCAgCMgCCgCgg	16	sense	Dorsch & Stackebrandt (1992)
1114f	gCAACgAgCgCAACCC	16	sense	Lane (1991)
685r2	TCTACgCATTTCACYgCTAC	20	antisense	Lane (1991)
907r	CCgTCAATTCMTTTRAgTTT	20	antisense	Lane (1991)
1492r	TACggYTACCTTgTTACgACTT	22	antisense	Lane (1991)

Degenerate versions of primers. M=C:A, Y=C:T; used 1:1

Over the life of the project cycle sequencing of the 16S PCR products was performed using a variety of reaction mixtures, thermal profiles and ABI Prism™ BigDye™ Terminator Chemistries. Minor modifications were made to the sequencing protocol between batches of samples in order to minimise costs while at the same time endeavouring to maintain the reliability and accuracy of the sequence obtained.

Early reactions were performed using ¼ strength 20µl reactions using an ABI Prism Big Dye v1.1 Cycle Sequencing Kit (Applied Biosystems P/N 4336774); the reactions were analysed with an Applied Biosystems Model 377 gel format sequencer. At the end of the project good quality results were obtained using ⅛ strength 20µl reactions using Big Dye v3.1 Cycle Sequencing Chemistry (Applied Biosystems cat. no. 4337456). These samples were analysed using an Applied Biosystems 3730S capillary sequencer. Where less than a full plate of samples were processed, correspondingly less master mix was prepared according to the master mix summary outlined in Table 6.21and Table 6.22 for the thermal cycling profile used.

Table 6.21 Summary of the Master mix used for sequencing reactions using Applied Biosystems Big Dye Cycle Sequencing Chemistries

Reagent	X1 Reaction (µl)	X110 Reactions (µl)
Big Dye Reaction Ready Premix	1	110
5X Sequencing Buffer	3.5	385
Primer (2 µM)	0.8	88
Water	13.7	1507
DNA	1	1µl per well (20µl)

Table 6.22 GeneAmp 9600® thermal cycling conditions used for 16S rDNA cycle sequencing reactions.

Temperature	Time	No. of Cycles
96ºC	1 min.	1cycle
96ºC	10 sec.	
50ºC	5 sec.	25 cycles
60ºC	4 min.	
4ºC	hold	

Purification of sequencing reaction products

Following cycle sequencing reactions, unincorporated dye terminators were removed from the 20µl samples using a Qiagen DyeEx 96 Kit (cat. no. 63181) according to the manufacturer's instructions. Samples were eluted into a 96-well format plate compatible with the Applied Biosystems 3730S capillary sequencer (Axygen® 96 well half skirt PCR Microplate cat. no. PCR-96M2-HS-C). Plates containing the purified sequence reaction products were dried in the open heating block of a PCR thermal cycler at 70°C for 50 minutes, or until visibly dry. The plates were then stored at -20°C for 1 to 5 days until sufficient plates were ready for a batch to be submitted for analysis.

Data analysis

The short partial 16S rDNA sequence data obtained using the 685r2 primer were processed by first visually assessing the quality of the sequence trace files using either Chromas Version 2.23 (Technelycium) or FinchTV Version 1.1.0 (Geospinza Inc.) software. Data from failed reactions were discarded and the sequencing reaction repeated. Data from files where the trace files appeared clear and the peaks well resolved were retained for further processing and analysis. Where the sequencer base calling function failed to resolve peaks or inserted no calls (N) into the sequence output, the trace files were examined, a judgement made regarding the most likely result and the sequence edited manually to reflect the changes required. The trailing ends of unreadable sequence were then manually trimmed and eliminated from the sequence.

To reduce errors resulting from repetitive manual processing of the large numbers of sequence files, several small programs were written in MS-DOS. These correctly annotated the sequences using sample information embedded in the sequence trace files, took the reverse complement of the sequence derived from the antisense primer and saved it in a single labelled concatenated FASTA format file. Where more comprehensive sequence coverage was required as was the case with the HMOs or

exemplars of the unidentified phenons, the sequence contigs derived from the use of the 6 sequencing primers were assembled using MegAlign Version 6.00 (DNAStar Inc.).

The data was then either submitted directly to the National Center for Biotechnology Institution (NCBI) or the Australian National Genomic Information Service (ANGIS) using the BioManager Interface for BLASTn (nucleotide-nucleotide) analysis (Altschul *et al.*, 1997).

Criteria for identification

For the HMOs, species identity was accepted when the 16S rDNA sequence similarity was ≥99% with the nominated taxon in GenBank; genus identity was accepted where the sequence similarity was ≥97% (Drancourt *et al.*, 2000). Identifications are based on the GenBank database as at November 2004.

6.1.5 Numerical taxonomic analysis

Data integrity

Phenotyping. Quality control and guidelines for standardising characterisation tests were developed as a strategy for maintaining data quality (Snell, 1991). Prior to commencement of phenotyping standard procedures for testing were developed, including assessment of culture purity, preparation of cultures, use of supplements and incubation times and temperatures. Guidelines were also established for test interpretation as a means of limiting drift in reading tests which has the potential to introduce over time a systematic bias in the data. Culture media quality control was undertaken using the species listed in Table 6.23 to confirm test performance in the phenotyping panel.

Table 6.23 *Vibrio* species used for culture media quality control

Species	Strain no.	Species	Strain no.
G. hollisae	ATCC 33595	*V. halioticoli*	IAM 14596
Ph. damselae ssp. *damselae*	TCFB 86/0665	*V. harveyi*	ATCC 14126
V. alginolyticus	ATCC 17749	*V. metschnikovii*	NCTC 8443
V. anguillarum	ATCC 19264	*V. mimicus*	ATCC 336531
V. fischeri	ATCC 7744	*V. parahaemolyticus*	ATCC 17802
V. furnissii	ATCC 35016	*V. splendidus* bv II	ATCC 25914

Data acquisition. All tests were read twice independently and discrepancies of interpretation resolved at the time of reading. Results were transcribed to a paper record and the transcription verified independently. Hardcopy results were managed electronically in an Access database (Microsoft Corporation, Redmond, WA) developed for the purpose. Data was double entered and any discrepancies or missing

values identified by comparison of the two databases. Inconsistencies were rectified by reference to the hardcopy of results.

Estimate of test error. A random selection of 10% of the library was made for retesting. Random numbers were generated using the Research Randomizer (Urbaniak & Plous, 1997) to identify cultures in the collection for retesting. A total of 92 cultures were tested using the complete panel of phenotyping tests. Tests were read and the data managed as described previously. Test variance, as the frequency of mismatches, was determined by comparing test results obtained from phenotyping the library of *Vibrionaceae* and the randomly selected subset of cultures. The probability of error for an individual test, p_i and probability of error in the pooled tests, p was calculated by the method of Sneath & Johnson (1972).

Data handling

The primary data repository was in the form of an Access database. Data was exported from Access to Excel in preparation either for use by other software applications or for data analysis or manipulation using Excel.

Data analysis

All the phenotypic tests were binary in form, either positive or negative and were scored as either 1 or 0 respectively; missing or indeterminate results were scored 9. Tests that were either all positive or negative were excluded from the data set as were those with a test error greater than 15% (Sneath & Johnson, 1972); the final data matrix for analysis comprised 797 OTUs (operational taxonomic units) and 98 tests. Cluster analysis was undertaken using ClustanGraphics 7.04 (Clustan, Edinburgh). Proximities were calculated using the Jaccard similarity coefficient, S_J, (Sneath & Sokal, 1973) and transformed to dissimilarities as: $d = 1 - S_J$. The dissimilarity matrix was clustered by Ward's method (Increase in Sum of Squares) (Ward, 1963). The resulting phenogram was serialised by re-ordering proximities to achieve the best possible rank order of the OTUs so that clusters were arranged around strongest proximities (Wishart, 2004). The phenogram was scaled using E values, the sum of square index, calculated from the cluster fusion points (Romesburg, 2004). The scale, which is non-linear, is a relative index of dissimilarity which ranges from zero (least dissimilar) for individual OTUs to maximum dissimilarity at the point at which all clusters join. Fusion point values were expressed as a percentage of the maximum value of E. Clusters were defined on the basis of inclusion of type and reference strains and then validated by assessing

homogeneity of cluster membership with reference to partial 16S rDNA sequence of each OTU. The HMO of each cluster was identified using ClustanGraphics.

6.2 DEVELOP PRACTICAL, ROBUST PHENOTYPIC IDENTIFICATION SYSTEMS FOR *VIBRIO* SPECIES USING COMPUTER ASSISTED IDENTIFICATION SOFTWARE

6.2.1 Computer assisted identification software

The bacterial identification software PIB-DOS (Bryant, 1991a, 1995) was rewritten as PIBWin for Microsoft Windows, versions 98 to XP (Bryant, 2004). The software was developed using Borland Delphi ver. 5.0 (Scott's Valley, CA) with additional components produced by TMS Software (Wevelgem, Belgium), Axolot (Gothenburg, Sweden) and The Helpware Group (Melbourne). Identification is based on an implementation of Bayes Theorem (Willcox *et al.*, 1973; Willcox *et al.*, 1980) with additional routines to calculate an identification score as the Willcox probability value *P* and a modal likelihood score as the modal likelihood fraction (Dybowski & Franklin, 1968), a qualification statistic for the identification reached. Tools for the evaluation of probability matrices based on GBEST and IDSC programs (Bryant, 1991b) were integrated with PIBWin.

6.2.2 Creation of the probability matrix

By inspection, a small number of clusters were found to be heterogenous on the basis either of type strains or partial 16S rDNA gene sequence data. These clusters were homogenised by re-allocation of OTUs to sub-clusters on the criterion of sequence data. Clusters containing three or less OTUs which did not include a type strain were not included in development of the matrix. For each cluster, the percentage of strains positive for each test was determined using a query constructed in Access and the results exported to Excel in the form of a matrix of tests and phenons. For tests that were either all positive or all negative, probability values of 0.99 and 0.01 respectively were assigned (Willcox *et al.*, 1973).

6.2.3 Test selection

The most useful tests to differentiate phenons in the matrix were found using an implementation of GBEST, a software tool in PIBWin. Based on the ideas of Gyllenberg (1963) and further developed by Rypka and colleagues (1967), a phenon pair is separated by at least two tests where test difference was ≥70%. Tests were ranked in order of Gyllenberg's Separation value S_i and the sum of Absolute Difference value for tied S_i values (Bryant, 1991b).

6.2.4 Evaluation of the probability matrix

Defining the identification panel and matrix

Using the implementation of IDSC in PIBWin, identification scores as Willcox probability values were calculated for the HMO of each phenon using all 98 tests. To keep a putative identification panel to manageable size the number of miniaturised tests was restricted to 36 with an additional maximum of 5 antibiotic sensitivity tests. Tests were selected based on rank order of S_i value and the revised matrix evaluated by calculating matrix identification scores for the HMO of each phenon. Where the score for an individual phenon was less than 0.99, tests best able to differentiate the phenon were identified using the best test selection tool in PIBWin. Tests with low S_i values in the matrix were substituted for those best able to differentiate low scoring phenons and the matrix re-evaluated. Iterations of the matrix were continued until all phenons had Willcox scores ≥0.99. Some tests, historically used for the identification of the *Vibrionaceae*, had low discrimination values and were not selected for use in the matrix. For reference purposes however, the panel was expanded to include tests for lysine and ornithine decarboxylase, urease and resistance to 0/129 150µg. The probability matrix constructed was labelled VibEx7 and the corresponding phenotyping panel as MicroSys V36.

Distribution of tests by resolving value

The Gyllenberg Separation value S_t for individual tests was calculated as $S_t=n_1n_0$ where n_1 is the number of tests positive and n_0 the number of tests negative. Positive tests were defined as values ≥85% and negative as ≤15%; intermediate values were not included (Rypka *et al.*, 1967). The data was normalised as a percentage of S_{MAX} defined as the theoretical maximum value for a test (Lapage *et al.*, 1973), given as $S_{MAX}=(N/2)^2$ where N is the number of taxa in the data set. A dot-plot of S_{MAX}% was used to assess the distribution of the resolving power of tests in the VibEx7 identification panel.

Cluster overlap

The amount of overlap between phenons defined in VibEx7 was investigated using an implementation of the program OVERMAT (Sneath, 1980). The critical overlap value V_O was set at 5% and significance assessed at P=0.95; statistics determined by the analysis include V_G the index of overlap, F the effective degrees of freedom for a pair-wise comparison, and variance and standard deviations of OTU distributions for each phenon. For phenons with only one strain, the number of strains was set to n=2 for computation purposes (Sneath, 1980).

Cluster separation

An assessment of the separation of taxa defined by the probability matrix was made by cluster analysis using ClustanGraphics and construction of a phenogram. As the probability data in the matrix are continuous variables, proximities were calculated by the Euclidean sum of squares procedure and clustered by the average linkage or unweighted pair-group method with arithmetic average (UPGMA).

Identification scores for cluster members based on phenotyping data

A data set for each OTU matching the MicroSys V36 identification panel defined by VibEx7 was extracted from the phenotyping data set. Each OTU was examined using PIBWin and the VibEx7 probability matrix. Data acquired for each OTU was as the most likely identification, Willcox probability score and modal likelihood value.

Practical identification scores based on re-test phenotyping data

A subset of the bacterial library (*n*=92) had previously been re-tested to obtain an estimate of test error. As the OTUs had been selected at random, data from this assessment was considered suitable for a practical assessment of the matrix. Data matching the MicroSys V36 identification panel was extracted from the retest data set and examined using PIBWin and the VibEx7 probability matrix. The results of identification were evaluated in terms of Willcox probability scores and modal likelihood scores exceeding the threshold values of 0.99 and 0.01 and 0.001 respectively.

Prospective testing of the matrix based on wild type isolates

Vibrionaceae obtained from diagnostic cases (*n*=222) over a period of six months were identified using MicroSys V36 and the VibEx7 probability matrix. The results of identification were evaluated in terms of Willcox probability and modal likelihood scores exceeding threshold values of 0.99 and 0.01 and 0.001 respectively.

6.2.5 Test panel performance investigations

Quality control of V36 identification panel

At six monthly intervals, five batches of identification media were prepared and tested with a panel of QC control organisms as listed in Table 6.24. For each batch of medium the QC strains were recovered from stocks held at -80°C, subcultured twice on JMA, the test panels inoculated and incubated at 25°C for 48 hr. Test error, using as reference profiles from the phenetic taxonomy, was calculated by the method of Sneath & Johnson (1972) for individual species and for pooled tests.

Table 6.24 Quality control strains used for testing the performance of the MicroSys V36 panel for the identification of *Vibrionaceae*

Species	Strain no.
Vibrio anguillarum	ATCC 19264^T
Vibrio fluvialis	NCTC 11327^T
Vibrio parahaemolyticus	ATCC 17802^T
Vibrio tubiashii	NCIMB 1340^T
Vibrio mediterranei	CIP 103203^T

Freeze-drying phenotyping panels.

Double strength media for a complete panel of 95 tests were aliquoted as 50µl volumes into sterile 96 micro-well trays and frozen at -80°C for one hour and freeze-dried (Dynavac FD5, Adelaide) at 0.2 Torr for 18-24 hr. The trays were sealed with tape

Table 6.25 Species used for assessing stability of freeze-dried phenotyping panels

Species and strain designation
V. mimicus ATCC 33653^T
V. furnissii ATCC 35016^T
V. wodanis NCIMB 13582^T
V. metschnikovii NCTC 8443^T

and stored refrigerated at 2-8°C in ziplock plastic bags. Wells were reconstituted with 50µl of distilled water and inoculated as described with suspensions in 2% NaCl. Stability of dried panels were assessed over a five month period with testing undertaken at monthly intervals. Panels were tested with the four species listed in Table 6.25. Test error (Sneath & Johnson, 1972) was calculated for each species by determining the frequency of test difference compared to the reference profile generated for the test strain on JMA. The effect of freeze-drying and stability over time on identification outcome was assessed from a subset of the phenotyping data using PIBWin and VibEx7 identification database to determine a most probable identification, Willcox probability scores and modal likelihood scores.

Phenotype investigations

Effect of pre-inoculum medium on phenotype. Cultures of the species listed in Table 6.26 were subcultured twice on plates of JMA, ZMA and SBA2 at 25°C or 15°C as appropriate. Phenotyping panels of 96-well tests were inoculated as described with suspensions of test organisms previously grown on the three media; testing was repeated on three consecutive days. Only data where there was a difference in

Table 6.26 Species used for assessing inoculum effect on phenotype

Species and strain designation
M. viscosa NCIMB 13584
V. anguillarum NCIMB 6^T
V. lentus CECT 5110^T
V. harveyi ATCC 14126^T

reaction for the different inoculum media were considered. Differences in reaction were considered pair-wise for each medium and reactions compared against the original phenotype previously determined for each species. Test error (Sneath & Johnson, 1972) was calculated for each medium by determining the frequency of test difference for all replicates and test strains compared to the reference profiles generated for the test strains on JMA. The effect of medium on identification outcome was assessed from a subset of the data using PIBWin and VibEx7 identification database to determine a most probable identification, Willcox probability scores and modal likelihood scores.

Effect of inoculum size on phenotype. Cultures of the four *Vibrio* species listed in Table 6.25 were grown on plates of JMA, ZMA and SBA2. Cell suspensions for each species were made in 2% NaCl to a density equivalent to McFarland 0.5 from which decimal dilutions of 10^{-1} to 10^{-4} were prepared. Phenotyping panels of 96 wells were inoculated with the dilutions and incubated for 48 hr at 25°C. Test error (Sneath & Johnson, 1972) was calculated for each species by determining the frequency of test difference compared to the reference profile generated for the test strain on JMA. The impact of inoculum and medium on identification outcome was assessed from a subset of the data using PIBWin and VibEx7 identification database to determine a most probable identification, Willcox probability scores and modal likelihood values.

Effect of culture condition on phenotype. The effect of freezing and recovery on the immediate phenotype of cultures was assessed for the species listed in Table 6.27. Cultures were revived from storage at -80°C on JMA. Bacterial growth after 24 hr incubation (Day 1) was used as inoculum for the MicroSys V36 identification panel; subcultures were made on consecutive days and identification panels inoculated for Day 2 and Day 3 subcultures. The phenotypes of the cultures on the three successive days were assessed using PIBWin and the VibEx7 database.

Table 6.27 Species used to assess the effect of culture condition on expression of phenotype

Species	Strain	Supplements
M. viscosa	NCIMB 13584^T	
V. anguillarum	NCIMB 6^T	Casamino acids
V. halioticoli	ATCC 700680^T	3% NaCl
V. ichthyoenteri	ATCC 700023^T	
V. lentus	CECT 5110^T	Casamino acids

6.3 DEVELOP AND IMPLEMENT PCR GENE PROBES FOR THE RAPID IDENTIFICATION OF KEY BACTERIAL PATHOGENS

Reference sources for the primer sets for the nominated pathogens are listed in Table 6.28. The primer sequences, reaction mixes, cycle conditions and expected amplicon size of the systems as published are summarised in Appendix 4, Table A4.6.

Table 6.28 Source references for PCR primer sets evaluated for functionality

No.	Source
1	Arias *et al.* (1995) – 23S rRNA nested, *V. vulnificus*
2	Brasher *et al.* (1998) – Shellfish multiplex, including *cth* gene of *V. vulnificus*
3	Coleman *et al.* (1996) Cytolysin/haemolysin, *cth* gene of *V. vulnificus* biotypes 1 & 2
4	Coleman & Oliver (1996) PCR optimisation of *cth* cytolysin/haemolysin gene of *V. vulnificus*
5	Conejero & Hedreyda (2003) *V. harveyi tox*R gene
6	Dalla Valle *et al.* (2002) *Photobacterium damselae* ssp. *piscicida*, unique RAPD
7	González *et al.* (2003) *Vibrio anguillarum rpoN* gene
8	Hirono *et al.* (1996) *Vibrio anguillarum* haemolysin *vah*1 gene
9	Iwamoto *et al.* (1995) *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment
10	Kim & Jeong (2001) *Vibrio vulnificus* 16S rRNA gene, tri-primer
11	Kvitt *et al.* (2002) *Photobacterium damselae ssp. piscicida* 16S rRNA gene
12	Lee *et al.* (1998) *V. vulnificus vvh* cytolysin/haemolysin gene
13	Oakey *et al.* (2003) *Vibrio harveyi* 16S rRNA gene
14	Osorio *et al.* (1999) *Photobacterium damselae* 16S rRNA, hemi-nested
15	Osorio *et al.* (2000) *Ph. damselae* subspecies, multiplex PCR for *ureC* and 16S rRNA

6.3.1 BLASTn Analysis of PCR Primer Sequences

The specificity of the primer sets, particularly those designed prior to the mid-1990s when GenBank was a 1000 times smaller than it is now (Benson *et al.*, 2004), was assessed by checking that sequences remained unique. Sequences were submitted in FASTA format for a sequence database nucleotide similarity search of GenBank using BLASTn (Altschul *et al.* 1997) and BioManager as provided by the Australian National Genomic Information Service. The BioManager interface for BLASTn defaults to a word size of 11, this means that no segment pair can be scored unless it contains a perfect match of this length. As these searches could not be conducted at the amino acid level the word size of primer BLASTn searches was reduced to 5 in order to increase the sensitivity of analysis, otherwise the default values for cost-to-open gap penalty (5) and cost-to-extend a gap (2) were used for all searches.

6.3.2 NetPrimer® Analysis

NetPrimer (Premier Biosoft International) is a Web based Java® script application (www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) which analyses primer sequences for amplification related properties, useful for revealing fundamental underlying flaws associated with the design of primer pairs. All primers were analysed for melting temperature using nearest neighbour thermodynamics for T_m prediction.

Primers were analysed for secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs as these influence the availability of the primer for the reaction as well as the formation of primer dimers. Each primer was given a rating based on the stability of its secondary structures.

6.3.3 PCR Evaluation

Standardisation of PCR mixes

To make evaluation of the PCR systems more practical, published PCR reaction mixtures were converted to a standard volume and set of stock reagents. Unless an error in the original description of the reaction mixture was suspected the final concentrations of all basic components were equivalent to the original description. Stock reagents used were as follows: 50mM $MgCl_2$, 16mM dNTPs (4mM with respect to each dNTP, Promega 100mM stock), 20µM primers (Geneworks) and Platinum® Taq DNA Polymerase 5U/µl (Invitrogen). Autoclaved Milli-Q 18MΩ water was used as diluent for all reagents and aqueous solutions used for PCR. In the course of testing the standard PCR reaction volume was reduced from 50µl to 25µl to reduce the cost of reagents. As the source of DNA template was highly purified and derived directly from cells cultured *in-vitro* in the absence of host tissues, additional reaction components such as gelatin, Tween 20 or Triton-X were omitted and substituted with the standard 10X reaction buffer containing 200mM Tris/HCl (pH 8.4) and 500mM KCl supplied with the DNA polymerase.

Due to the large number of PCR systems and the constant need to prepare different volumes of master mix, a calculator was developed in Excel spreadsheet format. The volume of standard reagent required to reproduce each of the PCR reaction mixtures was entered and used to automatically calculate the volume of each component of the master mix when the number of reactions required for an experiment was entered. The formulations used for the 15 PCR systems are summarised in Table 6.29.

Reaction Conditions

Preliminary assessment of each system was carried out using the thermal profile and cycle conditions as published. A Perkin Elmer GeneAmp 9600 thermal cycler and 200µl PCR tubes (Astral Scientific Cat: I3240-09) were used throughout except where some conditions were checked by gradient PCR in a Molecular Biosystems 96-well gradient cycler.

Table 6.29 Summary of the composition of PCR reaction mixtures (volumes in µl). The quantities shown are for a 25µl reaction volume using the standard reagents described above. The PCR number refers to the numbered key given in Table 6.28.

PCR	Water	Mg^{2+} 50mM	10x RB[1]	dNTP 16mM	P1 20µM	P2 20µM	P3 20µM	P4 20µM	Taq	DNA	Total (25µL)
1a	6.4	1.25	2.5	1.25	6.25	6.25	-	-	0.1	1	25
1b	6.4	1.25	2.5	1.25	-	-	6.25	6.25	0.1	1	25
2	16.375	1.25	2.5	1.25	1.25	1.25	-	-	0.125	1	25
3a	16.654	2	2.5	1.565	0.5625	0.5625	-	-	0.156	1	25
3b	17.904	0.75	2.5	1.565	0.5625	0.5625	-	-	0.156	1	25
4	16.9125	3.25	2.5	0.3125	0.45	0.45	-	-	0.125	1	25
5	17.375	1.5	2.5	1.25	0.625	0.625	-	-	0.125	1	25
6a	19.575	0.6	2.5	0.625	0.3125	0.3125	-	-	0.075	1	25
6b	19.575	0.6	2.5	0.625	-	-	0.3125	0.3125	0.075	1	25
7	17.8	0.75	2.5	1.25	0.75	0.75	-	-	0.2	1	25
8	14.4	0.75	2.5	1.25	2.5	2.5	-	-	0.1	1	25
9	18.125	0.75	2.5	1.25	0.625	0.625	-	-	0.125	1	25
10	15.625	0.75	2.5	1.25	1.25	1.25	1.25	-	0.125	1	25
11a	18.775	0.75	2.5	1.25	0.3125	0.3125	-	-	0.1	1	25
11b	18.775	0.75	2.5	1.25	-	-	0.3125	0.3125	0.1	1	25
12a	16.985	1.5	2.5	1.565	0.625	0.625	-	-	0.2	1	25
12b	14.985	2	2.5	1.565	-	-	0.625	0.625	0.2	2.5	25
13	18.15	1.5	2.5	1.25	0.25	0.25	-	-	0.1	1	25
14a	16.89	1	2.5	1.25	1.115	1.145	-	-	0.1	1	25
14b	16.82	1	2.5	1.25	-	-	1.115	1.215	0.1	1	25
15	11.15	1	2.5	1.25	2	2	2	2	0.1	1	25

[1]reaction buffer

PCR assessment

The PCRs were assessed visually after agarose gel electrophoresis in 2% (w/v) agarose gels (Promega Cat: V3125) in 0.5X TBE buffer prepared from a 10X stock (Promega Cat: V4251) with 0.5 µg/ml w/v ethidium bromide (Promega Cat: H5041) added to both gel and electrophoresis buffer. After PCR 1 µl of 6X Gel Loading (Promega Cat: G190A) was added for every 5 µl of PCR reaction, mixed briefly by repetitive pipetting and 10µl of the resulting mixture then loaded onto the gel. Electrophoresis was carried out at 7 volts per cm constant voltage using gels of varying sizes. The gels were run until sufficient separation was achieved for photography. Gel images were captured using a Kodak EDAS 290 system.

DNA template for PCR

The DNA used for PCR was prepared from actively growing Type cultures (Table 6.30) in 2ml volumes of ZoBell's marine broth (ZMB) incubated in an Eppendorf Comfort Thermomixer® at 18°C for 48 hours, with continuous agitation at 400 rpm. Genomic DNA was isolated with the QIAGEN DNeasy system (Cat: 69504). RNA was removed by digesting all samples with 4µl of a 100mg/ml solution of RNase A (Qiagen) for 2 min at room temperature, during DNA extraction. DNA concentration was measured using a GeneQuant II DNA calculator (Pharmacia), where the mean value of

six measurements of each sample was used. Using this stock solution, six 10-fold dilutions covering the range from 500pg/µl to 5fg/µl of DNA were prepared for use in PCR (500pg, 50pg, 5pg, 500fg, 50fg, 5fg per µl). In each case 1µl of diluted DNA containing the required amount of DNA was added to the PCR reaction mixture.

Table 6.30 Type cultures used for PCR test evaluation

Species	Isolate Number
V. anguillarum	ATCC 19264
Ph. damselae ssp. *damselae*	ATCC 33539
Ph. damselae ssp. *piscicida*	ATCC 51736
V. harveyi	ATCC 14126
V. ordalii	ATCC 33509
V. vulnificus	ATCC 27562

Negative control DNA for PCR

Performance of the 15 PCR systems was challenged with genetically similar species using a series of seven pools, each containing DNA from five non-target *Vibrio* species, Table 6.31. Purified DNA from Type or reference cultures was prepared and quantified as previously described and adjusted to make a mixture such that 1µl of the sample contained 1 ng of DNA from each of the five species represented in the sample. In this way specificity with respect to 35 non-target *Vibrio* species could be tested in an eight reaction panel (seven test and one reagent control blank).

Table 6.31 Summary of non-target negative control DNA from Type and reference cultures, used for PCR validation

Pool no.	Species	Pool no.	Species
1	*V. salmonicida*	5	*V. mytili*
	Ph. iliopiscarium		*V. fischeri*
	V. logei		*V. tubiashii*
	V. nereis		*V. mediterranei*
	V. nigripulchritudo		*V. penaeicida*
2	*V. tapetis*	6	*V. cholerae*
	V. pelagius bv I		*V. aestuarianus*
	V. orientalis		*V. parahaemolyticus*
	V. gazogenes		*V. mediterranei*
	V. metschnikovii		
3	*V. mimicus*	7	*V. lentus*
	V. alginolyticus		*V. pectenicida*
	V. pelagius bv II		*V. fluvialis*
	V. splendidus bv I		*V. furnissii*
	V. ichthyoenteri		*G. hollisae*
4	*V. diazotrophicus*		
	V. campbellii		
	G. hollisae		
	V. natriegens		
	V. navarrensis		

Additional PCR optimisation

Where generic PCR conditions were used and amplicon yield was low or extraneous bands were evident, limited optimisation was undertaken of Mg^{2+} concentration and annealing temperature by gradient PCR.

The Mg^{2+} concentration of the PCR reaction mix was titrated in 1mM increments from 1 to 6mM. The annealing temperature was examined by temperature gradient PCR using the original denaturation and extension times and extending the range of annealing temperatures to see if the PCR was improved by reference to the homologous template and a pooled negative control sample (either DNA negative control Pool 1 or Pool 2).

6.4 Determine the range of *Vibrio* species associated with farmed fish, shellfish and crustacea in Australia

The range of *Vibrionaceae* associated with aquatic animals in Australia was determined from cultures submitted by veterinary diagnostic laboratories in Australia. No programme of isolation was undertaken specific for this study. All strains submitted were collected in the main from diagnostic cases either of farmed or wild aquatic animals. The isolates submitted formed a major component of the library used for this taxonomic study.

6.5 Technology transfer workshop for state veterinary diagnostic microbiologists

A laboratory based training programme for the identification of *Vibrionaceae* was developed for probabilistic methods using phenotypic tests and molecular procedures in the form of polymerase chain reaction (PCR) for the identification of bacterial cultures. Invitations to attend the workshop were sent to veterinary diagnostic laboratories in each State and Territory in Australia and to New Zealand.

6.6 Develop an ANZSDP for the Identification of *Vibrio* species in aquatic animals

A protocol for the identification of *Vibrionaceae* using probabilistic phenotypic methods and molecular PCR methods was derived directly from this taxonomic study. The protocols developed are intended to be in a format that can be readily adopted by diagnostic laboratories and to be sufficiently generic to ensure the least number of barriers to implementation.

7 RESULTS

7.1 UNDERTAKE A DEFINITIVE NUMERICAL TAXONOMIC ANALYSIS TO DESCRIBE AND CHARACTERISE THE PHENOTYPES OF AQUATIC ANIMAL VIBRIOS IN AUSTRALIA

7.1.1 Library composition

The library of cultures comprised 51 type strains (Table 7.1). There were duplicate holdings for some type strains either from the same culture collection or from a different culture collection. In addition, reference strains were held for some species. As a proportion of the total library, type and reference strains comprised 16% of the holdings.

Table 7.1 Type strains of *Vibrionaceae* in the library of isolates used for taxonomic analysis

Species	Species	Species
Grimontia hollisae	*Vibrio diazotrophicus*	*Vibrio nigripulchritudo*
Moritella marina	*Vibrio fischeri*	*Vibrio ordalii*
Moritella viscosa	*Vibrio fluvialis*	*Vibrio orientalis*
Photobacterium angustum	*Vibrio furnissii*	*Vibrio parahaemolyticus*
Photobacterium leiognathi	*Vibrio gazogenes*	*Vibrio pectenicida*
Photobacterium phosphoreum	*Vibrio halioticoli*	*Vibrio pelagius* I
Ph. damselae ssp. *damselae*	*Vibrio harveyi*	*Vibrio penaeicida*
Ph. damselae ssp. *piscicida*	*Vibrio ichthyoenteri*	*Vibrio proteolyticus*
Photobacterium iliopiscarium	*Vibrio lentus*	*Vibrio rumoiensis*
Vibrio aestuarianus	*Vibrio logei*	*Vibrio salmonicida*
Vibrio agarivorans	*Vibrio mediterranei*	*Vibrio scophthalmi*
Vibrio alginolyticus	*Vibrio metschnikovii*	*Vibrio splendidus* I
Vibrio anguillarum	*Vibrio mimicus*	*Vibrio tasmaniensis*
Vibrio calviensis	*Vibrio mytili*	*Vibrio tapetis*
Vibrio campbellii	*Vibrio natriegens*	*Vibrio tubiashii*
Vibrio cincinnatiensis	*Vibrio navarrensis*	*Vibrio vulnificus* I
Vibrio cyclitrophicus	*Vibrio nereis*	*Vibrio wodanis*

For species where biovars are recognised, type strains for the junior biovar does not exist even though they may be taxonomically important; in such instances reference cultures for the biovars were obtained. For other species, type strains could not be obtained but well defined reference strains were included and taken to be representative of the species; these are listed in Table 7.2. The balance of isolates were submitted by research and diagnostic laboratories and considered to be wild type strains isolated from aquatic animals.

Table 7.2 Species represented with reference strains but not type strains

Species	Species
Vibrio chagasii	*Vibrio splendidus* biovar II
Vibrio cholerae	*Vibrio vulnificus* biovar II
	Vibrio pelagius biovar II

7.1.2 Culture recovery and size of library phenotyped

The failure rate of cultures to recover from freeze-dried or frozen stocks was 3.8% representing a loss of 33 cultures. A further 15 cultures or 1.7% of the library were abandoned as they had characteristics not consistent with the *Vibrionaceae*. The most frequent basis for rejection was either failure to ferment glucose or strains were Gram positive. The final size of the library phenotyped was 823 cultures or 94.5% of the original library assembled.

7.1.3 Test investigation

Test supplementation

Nutrient supplementation. Strains which failed to grow in the control CSU glucose test or those which utilised two or less substrates were considered nutritionally fastidious. Nutrient supplementation was assessed with yeast extract or Casamino acids at final test concentrations between 0.05 to 0.5g L^{-1} yeast extract and 0.1 to 1.0g L^{-1} respectively. Results of testing are given in Tables 7.3 and 7.4. The minimum concentration of yeast extract for growth varied between species and even between strains of a species. Significant variation was noted in the requirement for supplements.

Table 7.3 Minimum concentration of yeast extract necessary to support growth in sole carbon substrate utilisation tests.

Supplement: Yeast extract	Reaction	Test substrates									
		Concentration g L^{-1}	Citrulline	Galactose	Glucosamine	Glucose	Glycerol	Lactate	Succinate	Sucrose	Control
Species	Expected		-	+	V	+	V	V	V	-	-
Ph. damselae ssp. *damselae*											
Strain: NCIMB 2183	Observed	0.3	-	+	+	+	+	+	+	+	-
Strain: NCIMB 2184^T	Observed	0.05	+	+	+	+	+	+	+	+	+
Strain: V795	Observed	0.05	+	+	+	+	+	+	+	+	+
Species	Expected		-	+	-	+	+	-	+	-	-
Ph. damselae ssp. *piscicida* V372	Observed	0.1	-	+	-	+	+	-	+	-	-
Species	Expected		-	+	+	+	+	-	+	-	-
Ph. iliopiscarium ATCC 51760^T	Observed	0.5	-	-	-	-	-	-	-	-	-
Species	Expected		-	-	V	+	V	-	-	V	-
V. ordalii V439	Observed	0.1	-	-	-	+	-	-	-	+	-
V. ordalii NCIMB 2168	Observed	0.1	-	-	-	+	-	-	-	+	-
Species	Expected		-	-	-	+	+	+	+	-	-
V. lentus CECT 5110^T	Observed	0.1	-	+	-	+	+	+	+	-	-

V: Variable reactions for strains of the species

For two strains of *Ph. damselae* ssp. *damselae* the minimum amount of yeast extract was sufficient to act as a nutrient source on its own while for *Ph. iliopiscarium* ATCC 51760, the type strain, none of the concentrations of yeast extract were sufficient to

Table 7.4 Minimum concentration of Casamino acids necessary to support growth in sole carbon substrate utilisation tests.

Supplement: Casamino acids	Reaction	Test substrates									
		Concentration g L^{-1}	Citrulline	Galactose	Glucosamine	Glucose	Glycerol	Lactate	Succinate	Sucrose	Control
Species	Expected		-	+	V	+	V	V	V	-	-
Ph. damselae ssp. *damselae*											
Strain: NCIMB 2183	Observed	0.1	-	+	+	+	+	+	+	-	-
		0.15	-	+	+	+	+	+	+	-	-
Strain: NCIMB 2184^T	Observed	0.1	-	+	+	+	+	+	+	-	-
		0.15	-	+	+	+	+	+	+	-	-
Strain: V795	Observed	0.1	-	+	+	+	+	+	+	-	-
		0.15	-	+	+	+	+	+	+	-	-
Species	Expected		-	+	-	+	+	-	+	-	-
Ph. damselae ssp. *piscicida* V372	Observed	0.1	-	+	-	+	+	-	+	-	-
		0.15	-	+	-	+	+	-	+	-	-
Species	Expected		-	+	+	+	+	-	+	-	-
Ph. iliopiscarium ATCC 51760^T	Observed	0.1	-	+	+	+	+	-	+	-	-
		0.15	-	+	+	+	+	-	+	-	-
Species	Expected		-	-	V	+	V	-	-	V	-
V. ordalii V439	Observed	0.1	-	-	+	+	+	-	-	+	-
		0.15	-	-	+	+	+	-	-	+	-
V. ordalii NCIMB 2168	Observed	0.1	-	-	-	-	-	-	-	+	-
		0.15	-	-	+	+	-	-	-	+	-
Species	Expected		-	-	-	+	+	+	+	-	-
V. lentus CECT 5110^T	Observed	0.1	-	-	-	-	+	+	+	-	-
		0.15	+	-	-	+	+	+	+	-	-

V: Variable reactions for strains of the species

meet the nutritional requirements of the strain. In contrast, Casamino acids appeared to meet the requirements of a nutritional supplement for all strains and species tested and at a single concentration. Most strains tested responded well at 0.1g L^{-1} but notably *V. ordalii* NCIMB 2168 was largely inactive at this concentration but at 0.15g L^{-1} gave results expected for the species; similarly the type strain for *V. lentus* CECT 5110 was less reactive at the lower concentration of Casamino acid but gave expected results with the higher concentration. A total of 60 strains, or 6.9% of the library, required supplementation with Casamino acids. Species with nutritionally fastidious strains are listed in Table 7.5.

Table 7.5 Species with strains that are nutritionally fastidious in sole carbon source utilisation tests. Casamino acids provided essential growth factors.

Species	Species	Species
M. viscosa	Phenon 52	*V. lentus*
Ph. damselae ssp. *damselae*	*V. agarivorans*	*V. mimicus*
Ph. damselae ssp. *piscicida*	*V. anguillarum*	*V. natriegens*
Ph. phosphoreum	*V. fischeri*	*V. navarrensis*
Phenon 27	*V. halioticoli*	*V. ordalii*

Sodium requirement. Standard testing suitable for most strains used an inoculum of 2% NaCl to achieve a final test concentration of 1.5% NaCl in the miniaturised media. Strains of some species grew poorly in the medium for the indole test (final NaCl concentration of 1%) and on Mueller-Hinton agar containing 2% NaCl. These were considered to be moderate halophiles and were retested using an inoculum containing 3% NaCl. At this level of NaCl in the inoculum the final concentration in tests was 2% NaCl. A total of 33 (3.8%) of strains were classed as moderate halophiles; species with strains requiring additional NaCl are listed in Table 7.6.

Table 7.6 Species with strains that required testing at an elevated NaCl concentration of 2% w/v.

Species	Species	Species
M. marina	Phenon 52	*V. ordalii*
Ph. damselae ssp. *damselae*	Phenon 53	*V. splendidus* biovar I
Phenon 27	*V. halioticoli*	*V. tapetis*
Phenon 36	*V. harveyi*	
Phenon 46	*V. lentus*	

Combined supplements. Some strains of *V. lentus* not only required additional NaCl but also required supplementation with Casamino acids. This mixed requirement by strains was not observed with any other species.

Arginine dihydrolase

Unexpected positive results in the miniaturised format for arginine dihydrolase were obtained for *V. calviensis, V. mediterranei, V. 'shilonii', V. salmonicida* and *M. viscosa*. The strains were retested in the miniaturised format and by tube test using the West and Colwell modification to Thornley's medium (West & Colwell, 1984). Results of testing are given in Table 7.7.

Table 7.7 Comparison of arginine dihydrolase results by miniaturised and tube format for species with unexpected results in miniaturised format.

Species	Strain	Expected	Tube	Miniaturised
M. viscosa	NCIMB 13485	-	-	+
	NCIMB 13584^T	-	-	+
V. calviensis	DSMZ 14347^T	-	-	+
V. mediterranei	CECT 615	-	+	+
	CECT 621^T	-	I	+
	CECT 622	-	+	+
	CECT 623	-	+	+
	CRL 15^T	-	+	+
V. salmonicida	CIP 103166^T	-	-	I
V. 'shilonii'	ATCC BAA-91	-	+	+

I: indeterminate reaction; may be read as weak positive

Utilisation of oxaloacetate as a sole carbon source

Validation of initial results of testing the library for utilisation of oxaloacetate was undertaken because of the surprisingly high number of positive tests based on previously published data for species of *Vibrionaceae* (Kämpfer *et al.*, 1987). Testing of the initial batch of medium together with a new batch was undertaken with a range of apparently positive strains in the library. On re-test with both batches of medium all the strains were negative; see Table 7.8. No further investigation was undertaken.

Table 7.8 Investigation of sole carbon source test for the utilisation of oxaloacetate

Species and strain number	Oxaloacetate csu			Controls	
	1st test batch 1	2nd test batch 1	Batch 2	Glucose csu	Negative csu
Vibrio sp. Phenon 57 V16	+	-	-	+	-
V. cyclitrophicus V127	+	-	-	+	-
V. chagasii V160	+	-	-	+	-
V. halioticoli CIP 106284	+	-	-	+	-
V. splendidus biovar II ATCC 25914	+	-	-	+	-
Ph. damselae ssp. *damselae* V797	+	-	-	+	-
Ph. damselae ssp. *damselae* V817	+	-	-	+	-
V. alginolyticus V858	+	-	-	+	-

Zone size interpretation for novobiocin 5µg disc potency

Zone sizes for determining resistance or sensitivity for novobiocin at 5µg have not been published although the test has been found useful for speciation of *Vibrionaceae* (Bain & Shewan, 1968; West *et al.*, 1983, 1986). Zone sizes were measured for all strains tested and interpretation guidelines established *a posteriori*. Ranges for the species are given in Table 7.9; using the test data and expected results from published data (West *et al.*, 1983, 1986) resistance was set at ≤16mm zone of inhibition and sensitive as ≥17mm, measured as the diameter of the zone of inhibition based on a 6mm disc.

Table 7.9 Zone size data in millimetres for species reported to be sensitive or resistant to novobiocin at 5µg using a 6mm disc

Species	Reported[1] resistance	No. strains tested	No. resistant	Observed range	No. sensitive	Observed range
E. coli ATCC 10418	Resistant	1	1	7mm[2]	0	-
V. anguillarum	21%	13	3	14-15 mm	10	19-27mm
V. cholerae	1%	10	1	8 mm	9	18-28 mm
V. fluvialis	96%	11	11	7-13 mm	0	-
V. furnissii	100%	10	10	6-12 mm	0	-
V. metschnikovii	5%	4	1	13mm	3	18-26 mm
V. natriegens	91%	8	8	6-10 mm	0	-
V. nereis	25%	3	1	14 mm	2	17-19 mm
V. ordalii	0%	8	0	-	8	22-32 mm
V. pelagius biovar I	75%	6	3	13-16 mm	3	17-26 mm
V. tubiashii	50%	7	4	11-14 mm	3	19-21 mm

[1]Data from West *et al.*, 1983, 1986; [2]testing at 37°C, for all other species data obtained at 25°C

7.1.4 Test performance

Test reaction

The number of tests in the panel for phenotyping the library of cultures was 107 of which 97 were in miniaturised format and the remainder of tests in macro-format. The proportion of tests positive and negative is shown in Figure 7.1. The almost linear distribution of tests ranging from nearly all positive to all negative indicates a

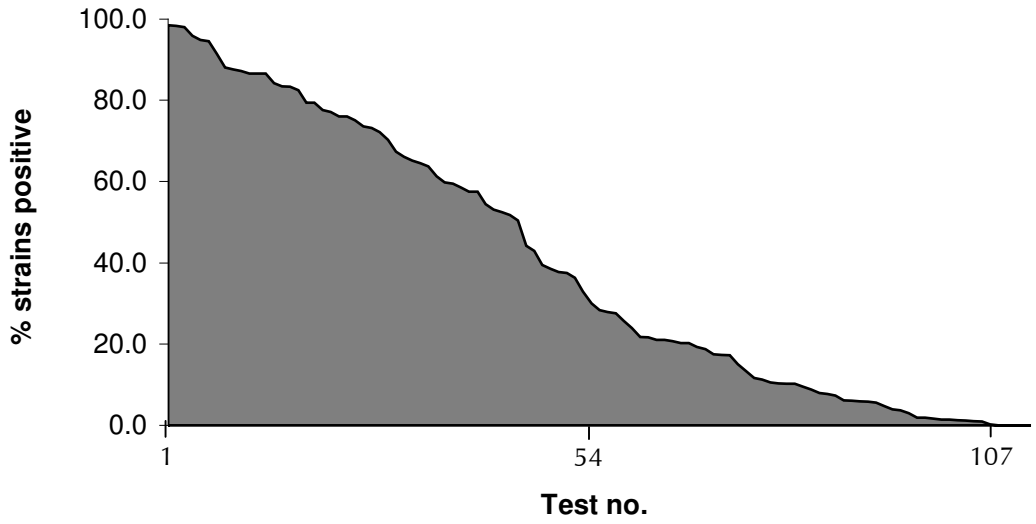

Figure 7.1 Ranked frequency distribution of positive reactions for the panel of 107 tests used for phenotyping the library of *Vibrionaceae*.

reasonably balanced distribution of reaction across the range of tests selected. No tests that were all positive; the highest scoring tests were oxidase and glucose fermentation at 98.5% and 98.4% respectively. Tests that were all negative were of two types: tests expected to be negative and those of unknown reaction. Expected negative results were obtained for Gram reaction and controls for decarboxylase reactions and carbon source utilisation basal medium; deamination of serine and threonine by *Vibrionaceae* was unknown but was found to be consistently negative for all strains tested. All other tests yielded positive reactions at varying frequencies. For the data set the proportion of tests that were positive was 41.5% with 58.5% of tests negative.

Test error

Estimates of test error were calculated for individual tests and for the entire data set. For the library of strains the average test error p using data for all tests was 4.68%. Individual test error p_i and percent positive reaction is summarised in Table 7.10.

Table 7.10 Individual test error p_i and percent strains positive for phenotyping tests. Data in rank order of individual test error p_i

Test	Percent strains positive	Individual test error p_i
Gram reaction	0%	0.0%
Growth 1% NaCl	95.9%	0.0%
0/129 150µg resistance	1.2%	0.0%
Raffinose fermentation	1.1%	0.0%
Serine deamination	0%	0.0%
Threonine deamination	0%	0.0%
Arabitol csu	6.0%	0.5%
Butyramide csu	0.2%	0.5%
Glucose fermentation	98.4%	0.5%
Inulin csu	0.9%	0.5%
Melezitose csu	1.4%	0.5%
Growth 4% NaCl	98.0%	0.5%
Oxidase	98.5%	0.5%
Hydroxybutyrate csu	11.3%	1.1%
Leucine deamination	1.0%	1.1%
Ornithine decarboxylase	19.4%	1.1%
Xylose csu	5.8%	1.1%
Agarolysis	1.6%	1.7%
Amygdalin fermentation	7.9%	1.7%
Citrulline deamination	27.5%	1.7%
Glucose csu	94.5%	1.7%
Glycerol csu	86.6%	1.7%
Malate csu	1.4%	1.7%
Nitrate reduction	94.9%	1.7%
Polymyxin 50 iu resistance	9.6%	1.7%
Rhamnose fermentation	5.6%	1.7%
Urease	17.2%	1.7%
Amygdalin csu	1.9%	2.2%
Galacturonate csu	7.7%	2.2%
Hydroxyproline csu	17.5%	2.2%
Inositol fermentation	4.0%	2.2%
Melibiose csu	7.3%	2.2%
Growth 10% NaCl	20.7%	2.2%
Sucrose fermentation	57.4%	2.2%
Valerate csu	4.7%	2.2%
Acetoin (VP)	27.9%	2.2%
Adenine csu	3.0%	2.8%
Alanine csu	72.2%	2.8%
Arabinose csu	18.7%	2.8%
Lactate csu	83.5%	2.8%
Lactose csu	10.2%	2.8%
Mannitol fermentation	77.7%	2.8%
Mannose fermentation	86.6%	2.8%
Allantoin csu	1.9%	3.4%
Arbutin csu	11.7%	3.4%
Arbutin fermentation	11.7%	3.4%
Bile tolerance	88.1%	3.4%
Cellobiose fermentation	73.6%	3.4%
Citrate csu	79.4%	3.4%
Indole	70.3%	3.4%
Phenylalanine deamination	66.1%	3.4%
Salicin fermentation	25.7%	3.4%
Swarming	6.1%	3.4%

continued...

Test	Percent strains positive	Individual test error p_i
Urocanate csu	3.7%	3.4%
α-ketoglutarate csu	53.1%	4.0%
Cellobiose csu	59.4%	4.0%
Ethanol csu	8.8%	4.0%
Gluconate oxidation	10.2%	4.0%
Putrescine csu	21.7%	4.0%
Ampicillin 10μg resistance	38.5%	4.6%
Arginine dihydrolase	59.8%	4.6%
Galactose fermentation	79.4%	4.6%
Gentiobiose fermentation	21.1%	4.6%
Histidine csu	37.5%	4.6%
Sorbitol csu	17.4%	4.6%
Succinate csu	87.2%	4.6%
TTC reduction	23.9%	4.6%
Carbenicillin 100μg resistance	44.2%	5.2%
Citrulline csu	20.2%	5.2%
Lysine decarboxylase	32.9%	5.2%
Arabinose fermentation	20.2%	5.8%
Galactose csu	67.4%	5.8%
Leucine csu	13.4%	5.8%
Acid phosphatase (pNPP)	91.4%	5.8%
Serine csu	77.2%	5.8%
Glucosamine csu	86.6%	6.4%
Glucuronate csu	21.1%	6.4%
Mannose csu	83.4%	6.4%
Acetamide csu	10.5%	7.0%
Acetate csu	76.1%	7.0%
Cobalt chloride resistance	84.2%	7.0%
γ-glutamyl transpeptidase (LGN)	61.3%	7.0%
Novobiocin 5μg resistance	39.5%	7.0%
Starch hydrolysis	75.1%	7.0%
Sucrose csu	57.4%	7.0%
Gluconate csu	73.2%	7.7%
Aspartate csu	51.7%	8.3%
Gelatin hydrolysis	63.8%	8.3%
Growth 0% NaCl	10.3%	8.3%
Growth 7% NaCl	76.1%	8.3%
Sulphatase (NPS)	42.9%	8.3%
0/129 10μg resistance	30.0%	8.3%
Propanol csu	15.0%	8.3%
Sorbitol fermentation	21.6%	8.3%
Aesculin hydrolysis	64.5%	9.0%
α-galactosidase (PNPG)	58.6%	9.6%
β-galactosidase (ONPG)	65.1%	10.3%
Prolyl aminopeptidase (LPN)	82.5%	11.0%
Amino acid accumulation	28.4%	11.7%
Propionate csu	54.5%	13.1%
Alkaline phosphatase (IXP)	87.6%	13.9%
Crystal violet resistance	52.5%	15.4%
Alginase	37.7%	20.5%
Potassium tellurite resistance	36.4%	42.6%
Oxaloacetate csu	50.4%	50.0%

csu: carbon source utilisation

Tests for resistance to crystal violet, alginase, resistance to potassium tellurite and utilisation of oxaloacetate had individual test errors greater than 15% and were excluded from the taxonomic analysis (Sneath & Johnson, 1972). After removal of these tests, the overall test error *p* was 4.02%.

7.1.5 Cluster analysis

Data review

Data for tests that were all negative and all positive were deleted together with tests that had a test error p_i greater than 15%. The final data set for cluster analysis comprised 98 tests.

Cluster analysis

The taxonomic relationship of clusters, which equate to phenons or taxospecies, is shown in the phenograms (Figures 7.2-7.7) based on Jaccard's similarity coefficient (Sneath & Sokal, 1973) and Ward's method for clustering (Ward, 1963).

At a relative index of dissimilarity of 5%, 97 clusters were delineated and were generally well defined taxonomically with clusters centred around type strains. Inspection of the data found 28 wild type strains either could not be allocated to clusters, or formed clusters that contained between one and three members. For clarity, these strains were deleted and not considered further; the total number of strains considered for analysis was 785. Further inspection of the phenogram revealed some minor heterogeneity of membership in a few clusters that contained type and reference strains. These clusters were further investigated by assessing partial 16S rDNA sequences of the member OTUs. Clusters were split into sub-clusters according to genotyping data and the presence or absence of type strains. Clusters that required additional assessment by this means are listed in Table 7.11. Only clusters 62 and 67 could not be resolved this way. Cluster 62 contained wild type strains of

Table 7.11 Cluster composition assessed using 16S rDNA sequence data

Cluster no.	Action	Components
16-17	Split	*V. calviensis* and *V. tubiashii*
18-19	Split	*V. orientalis* and Phenon 19 *Vibrio* sp.
22-23	Split	*V. rumoiensis* and *V. aestuarianus*
26-27	Split	Phenon 26a - *V. cyclitrophicus*, Phenon 26b - *Vibrio* sp., Phenon 27 - *Vibrio* sp.
35	Split	*V. vulnificus* biovar I, *V. vulnificus* biovar II
37-38	Split	*V. nigripulchritudo* and *V. penaeicida*
48	Split	*V. campbellii* and *V. splendidus* II
60-61	Split	*V. logei* and *Ph. phosphoreum*
62	Split	*V. fischeri* biovar II and *Ph. angustum*
80-81	Split	*V. wodanis* and *V. tapetis*

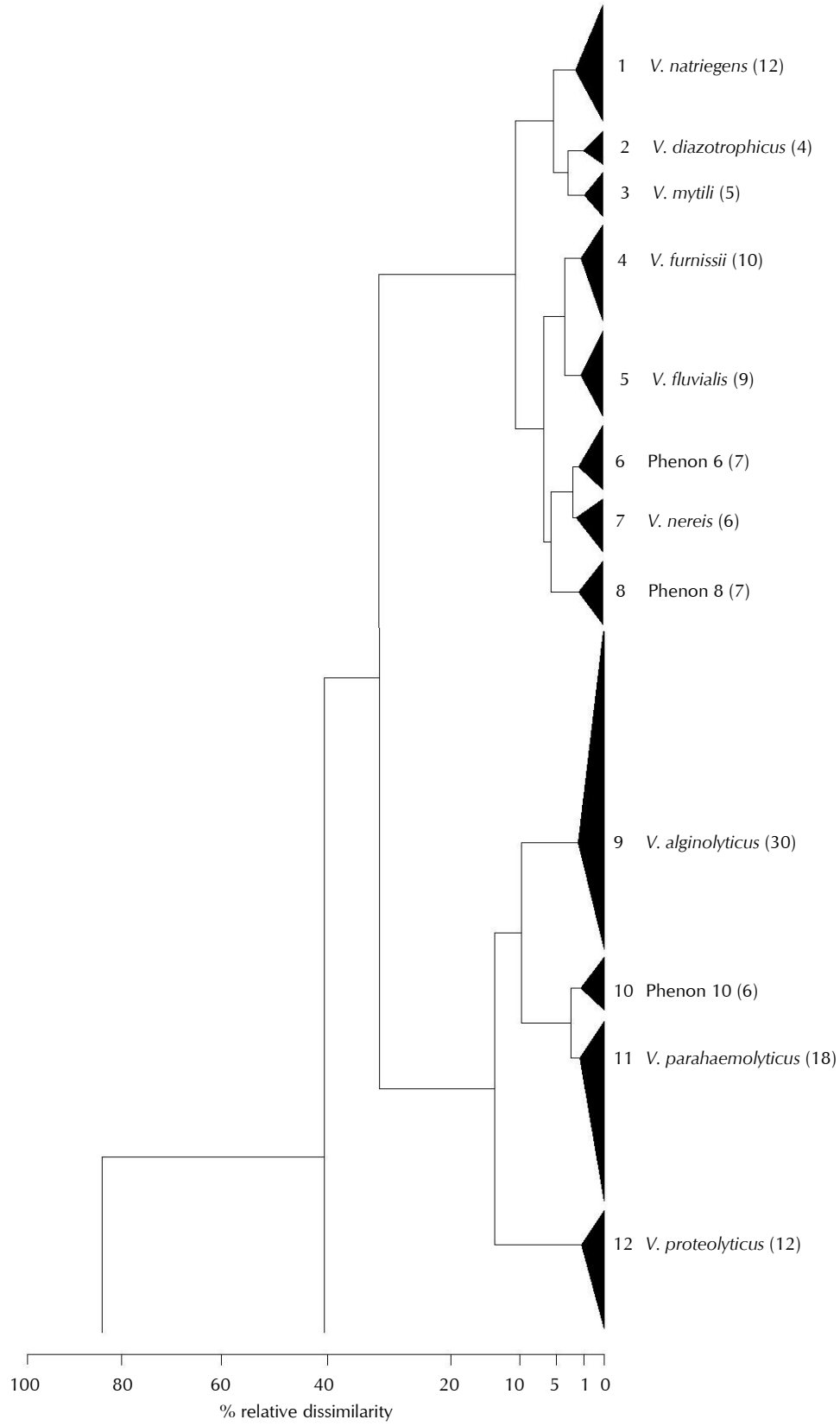

Figure 7.2 Condensed phenogram for clusters 1-12; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

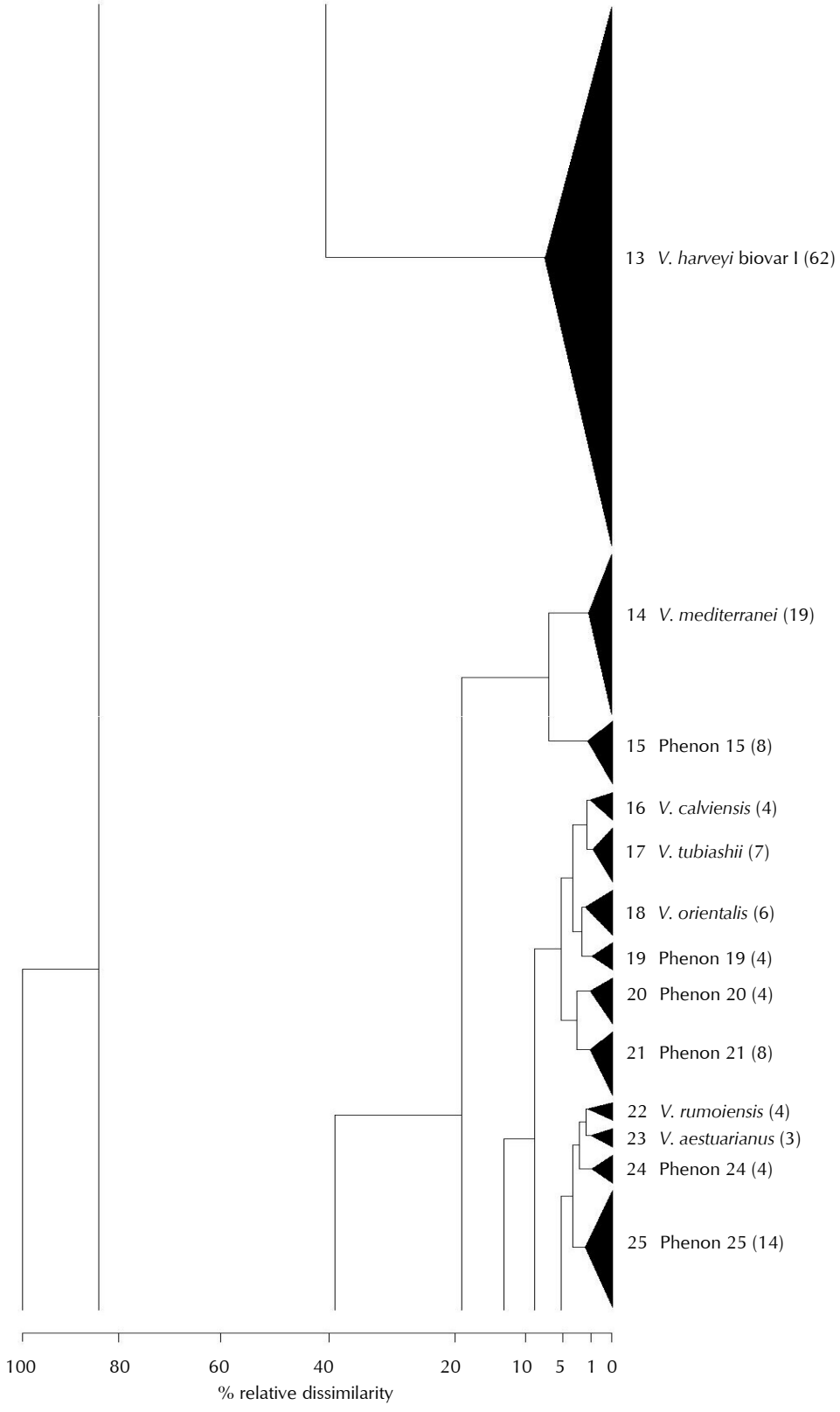

Figure 7.3 Condensed phenogram for clusters 13-25; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

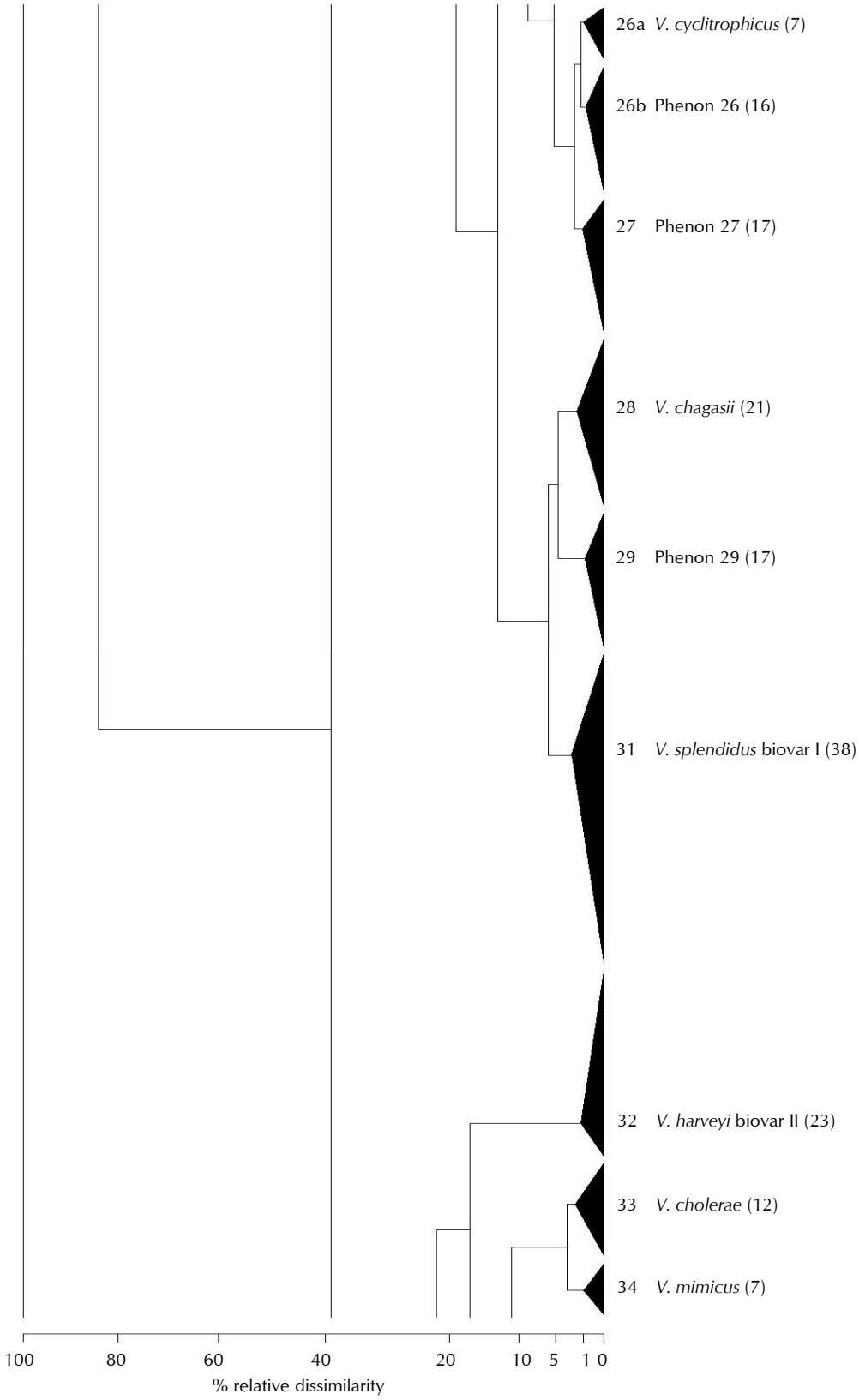

Figure 7.4 Condensed phenogram for clusters 26a-34; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

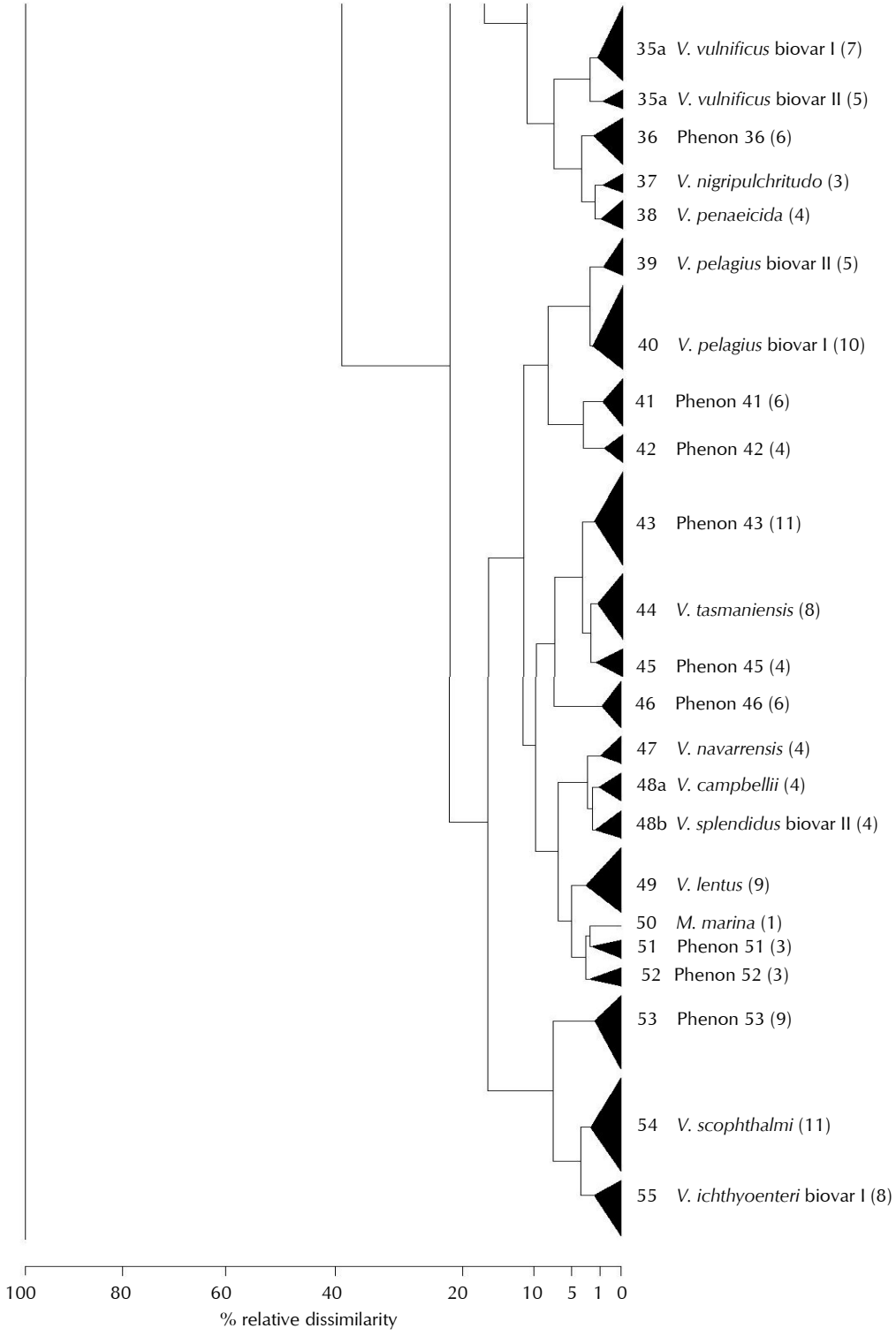

Figure 7.5 Condensed phenogram for clusters 35-55; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

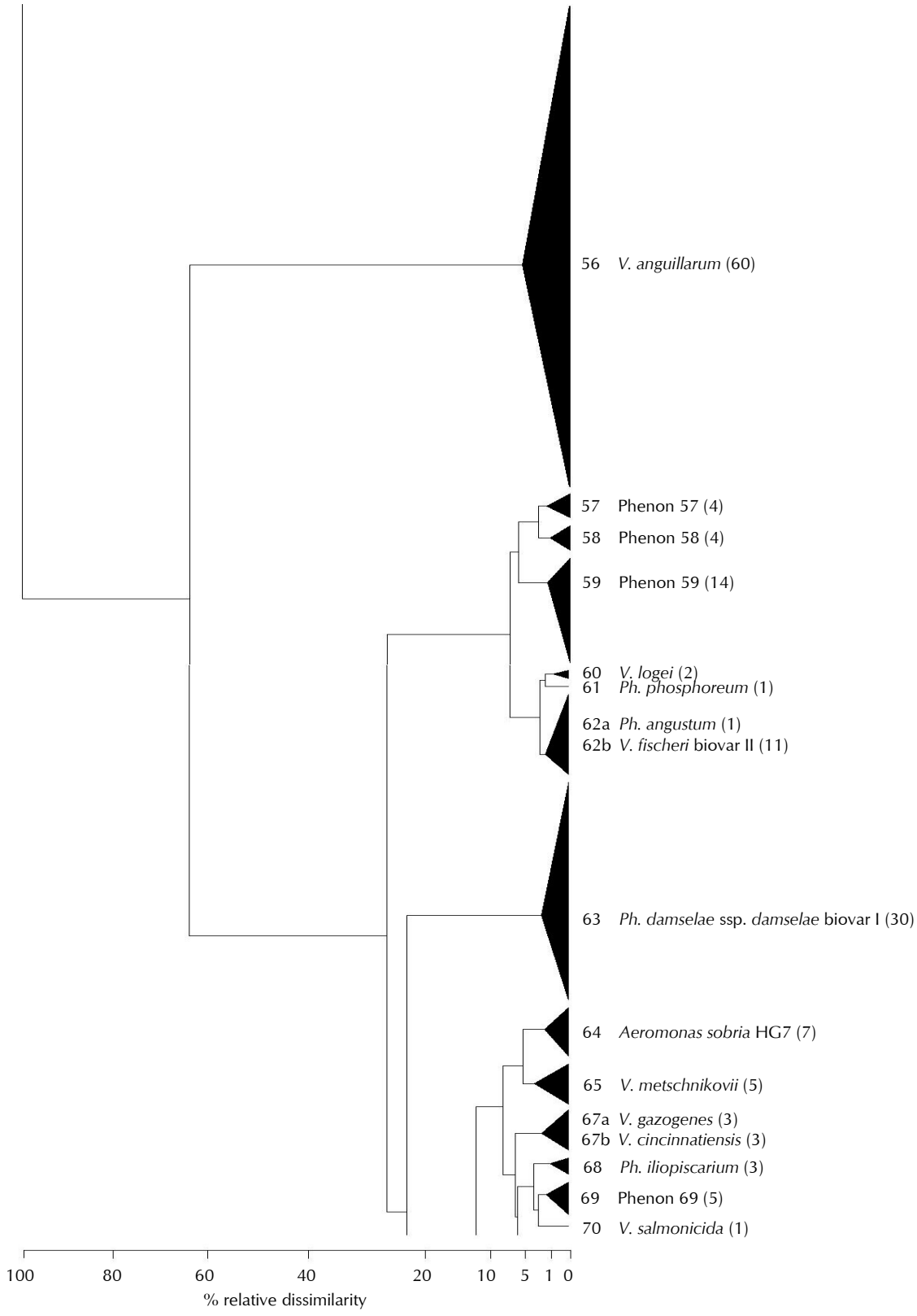

Figure 7.6 Condensed phenogram for clusters 56-70; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

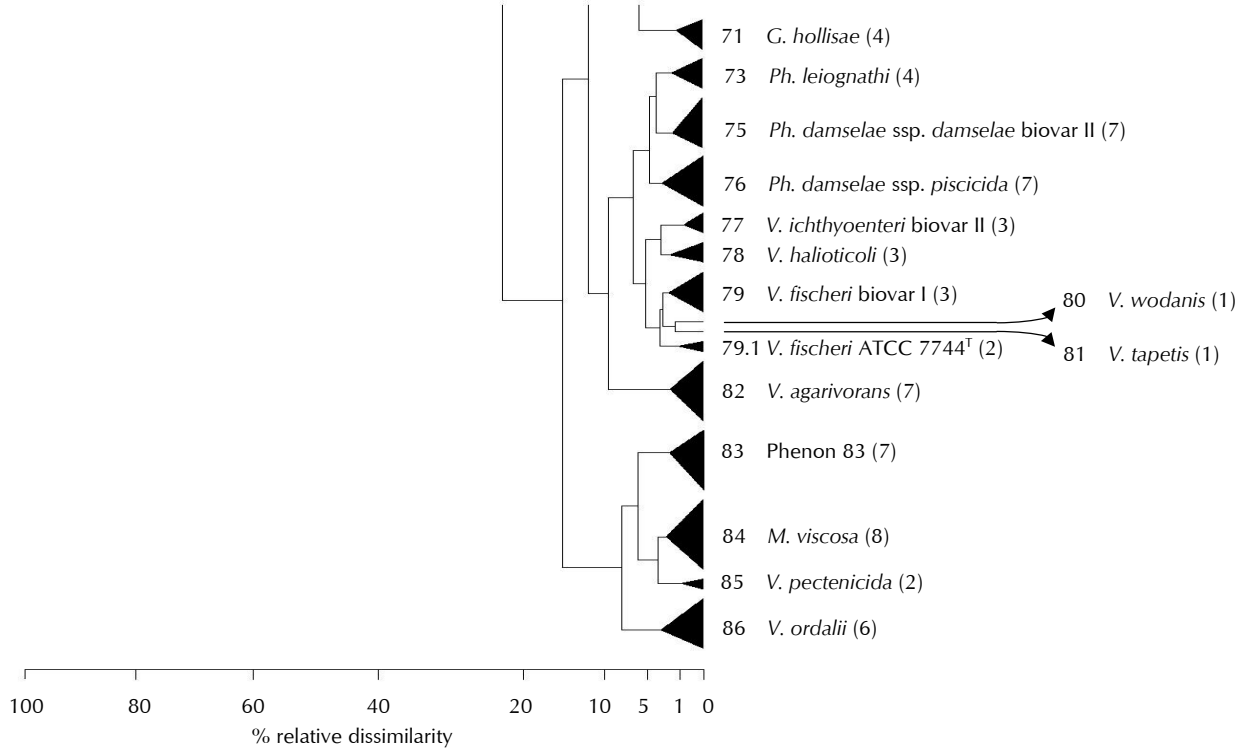

Figure 7.7 Condensed phenogram for clusters 71-86; proximities calculated by Jaccard's matching coefficient and clustered by Ward's method; clusters identified by phenon number, phenon name and number of strains in the cluster

V. fischeri and the type strain *Ph. angustum*; while 16S rDNA sequences confirmed their identities, partitioning into logical subclusters could not be achieved. Similarly, cluster 67 contained type and reference strains of both *V. gazogenes* and *V. cincinnatiensis* that had partial 16S rDNA sequences consistent with their respective species. Logical partitioning into subclusters was not possible because of the arbitrary distribution of OTUs within the cluster. The final phenogram after cluster assessment comprised 86 phenons. Of these, 59 contained type and reference strains corresponding to formally proposed species of *Vibrionaceae*, 25 phenons represent putative new species or biovars of existing species and one phenon, which formed a tight group of strains with a partial 16S rDNA sequence consistent with *Aeromonas sobria*, hybridisation group 7. The phenons defined and the number of OTUs in each cluster is given in Table 7.12.

Table 7.12 Phenons defined by cluster analysis of the library of *Vibrionaceae*

Species	Cluster	No. OTUs in cluster	Species	Cluster	No. OTUs in cluster
V. natriegens	1	12	Phenon 43	43	11
V. diazotrophicus	2	4	*V. tasmaniensis*	44	8
V. mytili	3	5	Phenon 45	45	4
V. furnissii	4	10	Phenon 46	46	6
V. fluvialis	5	9	*V. navarrensis*	47	4
Phenon 6	6	7	*V. campbellii*	48a	4
V. nereis	7	6	*V. splendidus* biovar II	48b	4
Phenon 8	8	7	*V. lentus*	49	9
V. alginolyticus	9	30	*M. marina*[1]	50	1
Phenon 10	10	6	Phenon 52	52	3
V. parahaemolyticus	11	18	Phenon 53	53	9
V. proteolyticus	12	12	*V. scophthalmi*	54	11
V. harveyi biovar I[4]	13	62	*V. ichthyoenteri* biovar I[4]	55	8
V. mediterranei	14	19	*V. anguillarum*	56	60
Phenon 15	15	8	Phenon 57	57	4
V. calviensis	16	4	Phenon 58	58	4
V. tubiashii	17	7	Phenon 59	59	14
V. orientalis	18	6	*V. logei*[1]	60	2
Phenon 19	19	4	*Ph. phosphoreum*[1]	61	1
Phenon 20	20	6	*Ph. angustum*[1]	62a	1
Phenon 21	21	8	*V. fischeri* biovar II	62b	11
V. rumoiensis	22	3	*Ph. damselae* ssp. *damselae* biovar I[4]	63	30
V. aestuarianus	23	3	*A. sobria* HG7[3]	64	7
Phenon 24	24	4	*V. metschnikovii*	65	5
Phenon 25	25	14	*V. gazogenes*	67a	3
V. cyclitrophicus	26a	7	*V. cincinnatiensis*	67b	3
Phenon 26b	26b	16	*Ph. iliopiscarium*	68	3
Phenon 27	27	17	Phenon 69	69	5
V. chagasii[2]	28	21	*V. salmonicida*[1]	70	1
Phenon 29	29	17	*G. hollisae*	71	4
V. splendidus biovar I[4]	31	38	*Ph. leiognathi*	73	4
V. harveyi biovar II	32	23	*Ph. damselae* ssp. *damselae* biovar II	75	8
V. cholerae	33	12	*Ph. damselae* ssp. *piscicida*	76	7
V. mimicus	34	7	*V. ichthyoenteri* biovar II	77	3
V. vulnificus biovar I[4]	35a	7	*V. halioticoli*	78	3
V. vulnificus biovar II	35b	5	*V. fischeri* biovar I[4]	79	6
Phenon 36	36	6	*V. wodanis*[1]	80	1
V. nigripulchritudo	37	3	*V. tapetis*[1]	81	1
V. penaeicida	38	4	*V. agarivorans*	82	7
V. pelagius biovar II	39	5	Phenon 83	83	7
V. pelagius biovar I[4]	40	10	*M. viscosa*	84	8
Phenon 41	41	6	*V. pectenicida*[1]	85	2
Phenon 42	42	4	*V. ordalii*	86	6

[1]Phenon contains only the type strain
[2]Phenon contains reference strains but not type strain
[3]Phenon does not contain type strain
[4]Biovar includes type strain

For the un-named phenons, HMOs were identified by ClustanGraphics and are listed in Table 7.13.

Table 7.13 Hypothetical median organisms for un-named phenons

No.	Phenon no.	HMO strain no.	No.	Phenon no.	HMO strain no.
1	Phenon 6	V357	16	Phenon 42	V35
2	Phenon 8	V201	17	Phenon 43	V781
3	Phenon 10	V261	18	Phenon 45	V810
4	Phenon 15	V759	19	Phenon 46	V632
5	Phenon 19	V51	20	Phenon 52	V205
6	Phenon 20	V322	21	Phenon 53	V859
7	Phenon 21	V20	22	Phenon 57	V68
8	Phenon 24	V140	23	Phenon 58	V776
9	Phenon 25	V69	24	Phenon 59	V48
10	Phenon 26b	V102	25	Phenon 64	V606
11	Phenon 27	V624	26	Phenon 69	V615
12	Phenon 29	V4	27	Phenon 75	V798
13	Phenon 32	V639	28	Phenon 77	V170
14	Phenon 36	V31	29	Phenon 83	V125
15	Phenon 41	V515			

Orphan type and reference strains

Some type and reference strains appeared in unexpected clusters. Reference strains as listed in Table 7.14 appeared in clusters that did not contain the type strain of the species. For three of the strains it would appear that they are misplaced.

Table 7.14 Orphan reference strains that did not appear in a cluster containing the type strain of the species

Species as submitted	Strain no.	Cluster no. and definition	16S rRNA ID	GenBank
V. fischeri	ATCC 33984	73 *Ph. leiognathi*	*Ph. leiognathi*	AY292944
V. ichthyoenteri	ATCC 700024	76 *Ph. damselae* ssp. *piscicida*	*V. ichthyoenteri*	AJ437192
Ph. damselae ssp. *damselae*	ATCC 51805	79 *V. fischeri* biovar I	ND	-
V. logei	CIP 103204	79 *V. fischeri* biovar I	*V. logei*	AY292934

ND: not determined

Vibrio ichthyoenteri ATCC 700024 and *V. logei* both had partial 16S rDNA sequences consistent with their respective species designation. A partial sequence was not generated for *Ph. damselae* ssp. *damselae* ATCC 51805 (formerly *Ph. 'histaminum'*; Kimura *et al.*, 2000) but was tested by multiplex PCR and was positive for both 16S rRNA and urease genes, confirming the species identity. *Vibrio fischeri* ATCC 33984 clustered with *Ph. leiognathi* and of note is that the partial 16S rDNA sequence for ATCC 33984 matched that for *Ph. leiognathi*, a measure of agreement of phenotype and genotype indicating that the strain is perhaps not misplaced. Duplicate type strains of *V. fischeri* ATCC 7744 appeared in a unique subcluster 79a and adjacent to cluster 79 that contains several reference strains of *V. fischeri*. Clusters 79a and 79 are however separated by clusters 80 and 81 that contained single OTUs of the type strains *V. wodanis* NCIMB 13582 and *V. tapetis* CIP 104856, respectively .

7.1.6 Phenon characteristics

The phenotype of each phenon for all 98 characterisation tests is given in Appendix 4, Table A4.1. A detailed description of each phenon has not been given as this was presented in the phenogram and the phenotype tables. Of note is the proposal to define new biovars for *Photobacterium damselae* ssp. *damselae, Vibrio fischeri, V. harveyi* and *V. ichthyoenteri* on the basis of phenotype and genotype. Phenon 63 contains 30 strains of *Ph. damselae* ssp. *damselae* including the type strain NCIMB 2184 and the reference strains NCIMB 2181 and NCIMB 2183. Phenon 75 contained strains submitted as *Ph. damselae* ssp. *damselae* that were all urease positive. All members of the cluster were positive for both the 16S rRNA primers as well as the urease primers when tested by multiplex PCR specific for the species (Osorio *et al.,* 2000); in addition, all members of the phenon had sequences that matched *Ph. damselae* ssp. *damselae*, [AY147861] for 16S rDNA. On the basis of this evidence two phenotypic biovars could be recognised for *Ph. damselae* ssp. *damselae*.

Strains of *V. fischeri* occurred in clusters, Phenon 62b, 79 and 79a. Duplicate type strains of ATCC 7744 and reference strains of ATCC 14546 ATCC 25918 and ATCC 33715 occurred in phenons 79a and 79 respectively and had partial sequence alignments that matched *V. fischeri* [X74702]. In Phenon 62b, nine of the ten members had sequences matching *V. fischeri* [AY292920, AY292922, AY292926, AY292946 and X74702]. One strain, V579, had a sequence aligned with *V. logei*, strain SR181 [AY292934]. Phenon 79a and 79 are phenotypically similar and distinctly different from those in Phenon 62b. As the Phenon 79 sub-clusters contain the type strain it was designated *V. fischeri* biovar I and those strains in Phenon 62b as *V. fischeri* biovar II.

Phenon 13, which consisted of 6 sub-clusters, included duplicate type strains of *V. harveyi* ATCC 14126 and CRL 30 together with duplicate strains of ATCC 35084 as *V. 'carchariae'*, a junior synonym for *V. harveyi*. Sequences were obtained for 33 of the 62 members of the phenon and matched the 16S rDNA sequence for *V. harveyi* [X74693 and X74706]. Phenon 32 comprised 23 strains submitted as atypical *V. harveyi* associated with blister disease in farmed abalone (*Haliotis* species). All 23 strains were sequenced and found to match the 16S rDNA sequence for *V. harveyi* [X74693] except for V832 which did not. Testing by PCR of three strains, V639, V783 and V784 with 16S rDNA primers (Oakey *et al.*, 2003) was positive and provided further evidence that strains of Phenon 32 can be classified as *V. harveyi* but represent a distinct biovar.

Strains of *V. ichthyoenteri* were located in Phenon 55 and 77. Phenon 55 contained duplicate type strains of the species ATCC 700023 and DSMZ 14397. There was agreement with the 16S rDNA sequence of *V. ichthyoenteri* for three of the seven cluster members [AJ421445] but not for the remaining strains. There were only three members of Phenon 77 but for each strain there was high sequence homology with *V. ichthyoenteri* [AJ421445]. Phenon 55 was designated *V. ichthyoenteri* biovar I and Phenon 77 as *V. ichthyoenteri* biovar II.

A type strain for *V. chagasii* was not available for this study but two strains submitted as TNEMF6 (V452) and TN1117 (V470) have subsequently been shown, by DNA:DNA hybridisation, to be *V. chagasii* (Le Roux *et al.*, 2004). Both strains appeared in Phenon 28. Members of the cluster had sequence similarity primarily to a complex of *V. splendidus-pomeroyi-lentus-chagasii*. Separation of the *V. splendidus* complex by phylogenetic analysis of 16S rDNA sequences is unreliable (Le Roux *et al.*, 2004) and on the basis that 16S rDNA sequence of cluster members is consistent with the taxonomic complex. Accordingly, Phenon 28 was designated as *V. chagasii*.

Phenon 64 contains seven strains all of which are sensitive to 0/129 at 150µg, ferment glucose and are oxidase positive, characteristics consistent with the *Vibrionaceae*. Strains in the group had 16S rDNA sequence similarity to *Aeromonas sobria* hybridisation group 7. Sensitivity to 0/129 by *A. sobria* has been reported previously (Carnahan & Joseph, 1993). The strains were all isolated from rainbow trout and Atlantic salmon at freshwater hatcheries and in sodium chloride tolerance/requirement tests 71% of the strains did not require NaCl for growth. This cluster was considered to represent *Aeromonas sobria, sensu stricto.*

A small cluster of three OTUs, designated Phenon 51 was excluded from further analysis as they were found to be resistant to 0/129 at 150µg and did not ferment glucose. Sequence analysis of the three OTUs found matches for *Pseudoalteromonas* sp. EH-2-1 (GenBank accession no. AF295034), *Pseudoalteromonas* sp. SM9913 (GenBank accession no. AY305857) and *Shewanella baltica* (GenBank accession no. AF173966).

7.1.7 Analysis of putative species

Strains were received with the designation of *V. 'piscailia', V. 'piscium', V. 'morestus'* and *V. 'baumanii'*. The single strain of *V. 'piscailia'* (V371) clustered in Phenon 63, *Ph. damselae* ssp. *damselae*, but appeared to be a distinct outlier phenotypically and

genotypically. Sequence analysis for V371 matched *Ph. phosphoreum* (GenBank accession no. X74687) and by multiplex PCR for *Ph. damselae* ssp. *damselae* was negative for both the 16S rDNA and urease primers, consistent with a negative urease reaction by phenotyping. *V. 'piscium'* (V374) clustered with *V. anguillarum* in Phenon 56 but by sequence analysis the strain matched *V. ordalii* (GenBank accession no. X74718) although by phenotyping, using a critical panel of tests (see section *7.2.2 Creation of the probability matrix*), it did not have characteristics consistent with the species. The four strains of *V. 'baumanii'* (V452, V461, V470, V471) all clustered in Phenon 28 defined as *V. chagasii*. None of the strains had a sequence similarity matching *V. chagasii* and as a group appeared heterogenous; V452 resembling an uncultured *Photobacterium* sp. (GenBank accession no. AY494613), V461, *Vibrio* sp. TK327 (GenBank accession no. AB038025) and for V470 and V471, *Vibrio* sp. 3d clone 3d2 (GenBank accession no. AF388391). The three strains of *V. 'morestus'* occurred in two clusters: Phenon 27 (V455 and V456) and *V. splendidus* biovar I, Phenon 31 (V460). By partial 16S rDNA sequencing all three strains appeared different to each other and there were no matches to the clusters in which they appeared. V455 resembled *V. mytili* (GenBank accession no. X99761), V456 as *V. natriegens* (GenBank accession no. X74714) and V460 as *V. splendidus* biovar II (GenBank accession no. AB038030). The status of Phenon 10 is unclear. Phenotypically members of the phenon have properties of *V. alginolyticus* and five of the six members also have partial sequences that match *V. alginolyticus*. Phenon 10 however is more closely linked to *V. parahaemolyticus* (Phenon 11) than to the *V. alginolyticus* cluster of Phenon 9, and there was no basis to combine Phenons 9 and 10 as a super cluster of *V. alginolyticus* (see Figure 7.2).

7.1.8 Genotyping of *Vibrionaceae* library

DNA extraction for sequencing

In general, the use of PrepMan™ Ultra reagent provided a reliable and rapid means of obtaining bacterial genomic DNA for PCR amplification and genotyping. Initial attempts at PCR using PrepMan™ Ultra derived template were unsuccessful. This reagent was originally developed for rapid preparation of DNA templates from Gram-negative pathogens present in food. These samples often have high lipid content or other PCR inhibitors which may be co-purified with DNA and inhibit amplification. The initial failed attempts at PCR were thought to be due to either a PCR inhibitor, which was co-purified in the samples or PCR amplification was failing due to the high

concentration of nucleic acid present in samples despite only a small amount of culture being used for extraction.

In an attempt to either dilute co-purified PCR inhibitors or the large amount of DNA present, dilutions ranging from 1:2 to 1:10,000, of a few representative PrepMan™ DNA preparations that had previously failed in PCR (primers 27f/1492r) were prepared in sterile 18MΩ water. These samples were then subjected to PCR; Figure 7.8 shows the PCR results of four PrepMan samples diluted from 1:1 to 1:16. A dilution of 1:2 to

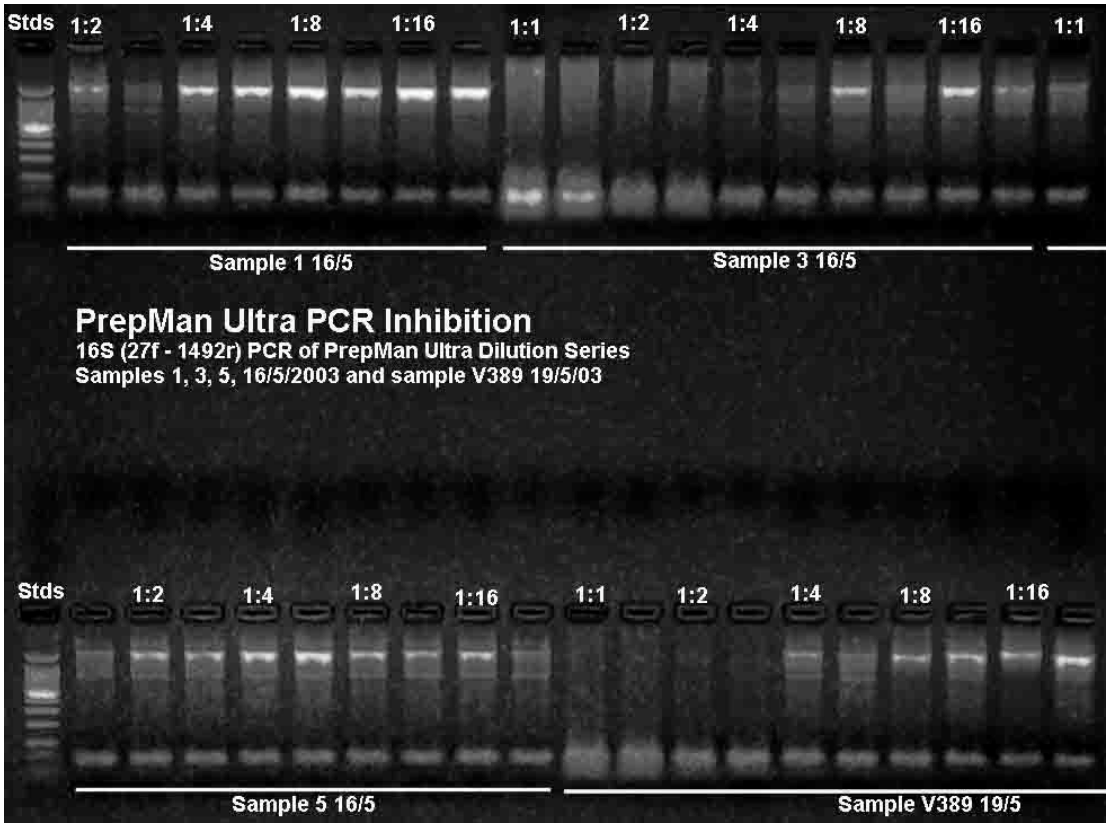

Figure 7.8 16S rDNA (27f/1492r) PCR of serially diluted PrepMan Ultra samples from four previously failed reactions. Dilutions shown range from 1:1 to 1:16.

1:4 was sufficient to obtain a PCR product with some samples, however these results were inconsistent but samples diluted 1:8 resulted in reliable amplification. While the precise reason for PCR inhibition with undiluted samples was not determined, it was most likely due to the high concentration of DNA in the preparations. As a result, extracts were standardised on an aqueous dilution of 1:10 of the original PrepMan™ Ultra sample and found this to provide reliable and consistent results for 16S rDNA PCR.

Reactions prepared using template diluted 1:10 yielded similar band intensities when products were assessed by agarose gel electrophoresis, Figure 7.8. During sample

preparation it was found that a significant saving in both time and cost of reagents could be achieved by halving the sample volume from the recommended 200µl of reagent to 70-100µl and processing the samples in 96-well microtitre plate format in a PCR thermal cycler.

Amplification of the 16S gene and purification of PCR products

Following amplification with the 27f/1492r primer pair and concentration and purification of the product by ultrafiltration, an initial failure rate of 3.6% was observed. As familiarity with the procedure and equipment increased this was reduced to 2 or 3 reactions per 96-well plate. After the procedure was established, all preparations from failed samples were discarded, the strains re-cultured and the procedure repeated until purified 16S rDNA for cycle sequencing was obtained from all the strains.

Electrophoresis of purified PCR products

Figure 7.9 shows a typical result obtained by this method. Gel results for first round amplification of all the strains are shown in Appendix 4, Table A4.2.

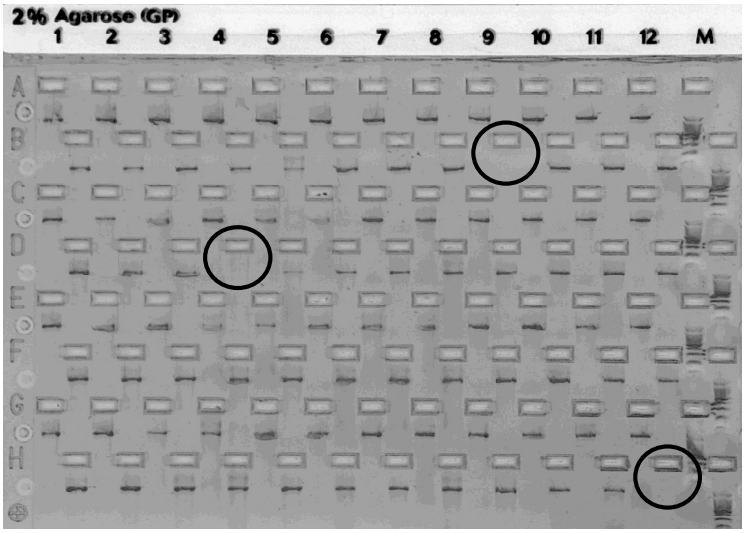

Figure 7.9 Gel electrophoresis of purified 16S rDNA products. 5µl samples of 1456 bp purified 16S rDNA PCR products were run for 12 minutes in an Invitrogen E-Gel® 96 well system using a 2% agarose gel. The final staggered row on the right (M) contains molecular size markers (Promega ™ 100bp ladder). Note the uniformity of yield in most lanes and the failed reactions at B9, D4 and H12 (circled).

DNA quantification

The quality and amount of DNA template used in the sequencing reaction has a direct effect on the quality of the resulting sequence data. When sequencing PCR products, the size of the product has direct bearing on the amount of DNA required in

the sequencing reaction. In this case, when using 1000-2000 bp targets, between 10 and 40 ng was found optimal.

The relatively consistent yield and concentration of the purified 16S rDNA observed in gels meant that the DNA concentration of the samples could be estimated by measuring a few select trial samples from each batch. Twenty samples were assayed and revealed a mean DNA yield of 49.4 ng/µl for every 50µl of purified sample, with concentrations ranging from 35.1 to 67.9 ng/µl for individual samples. The appearance of the amplification product in the gel was used as the main guide to assess the suitability of a sample for the standard processing procedure. Provided a relatively uniform yield was observed in the gel, a set volume of 1µl was shown to provide approximately 50 ng of 1,465 bp purified 27f/1492r amplicon for template in the 685r2 sequencing reactions.

Sequencing the 16S rDNA gene

Over the life of the project 1,439 sequencing reactions were performed. Before being accepted for analysis all sequence trace files were visually assessed using either Chromas Version 2.23 (Technelycium) or FinchTV Version 1.1.0 (Geospinza Inc.). Failed reactions either appeared blank or as a series of N's in the base calling display. In some cases the sequencer output assigned bases to individual peaks, despite the fact that trace files appeared irregular and the chromatograms failed to differentiate individual traces from each other or from the background. In either case these data were discarded and where possible the sequencing reaction repeated.

Early sequences derived from the AB Model 377 gel based sequencer did not contain information on sequence quality. While it might have been possible to analyse this data with a base-calling program such as Phred (Ewing. *et al*. 1998; Ewing & Green 1998), which reads DNA sequencer trace data, calls bases, assigns quality values to the bases, and writes the base calls and quality values to an output file, it would not have been practical to analyse such a large number of sequences this way. It was therefore accepted that beyond the level of visually assessing the trace files there was an undetermined level of error of individual bases and sequences.

Later sequence derived from the AB Model 3730S capillary sequencer contained qualitative information on the reliability of base-calls which could be displayed as a small histogram above each base in the FinchTV program window. While this did not provide a quantitative measure of the level of error, it was very useful for rapidly

identifying potential trouble spots when screening and modifying sequences. Figure 7.10 shows the same sequence viewed with Chromas (above) and FinchTV (below); the most likely result and the sequence was edited manually to reflect the changes required. The trailing ends of unreadable sequence were then manually trimmed and eliminated from the sequence.

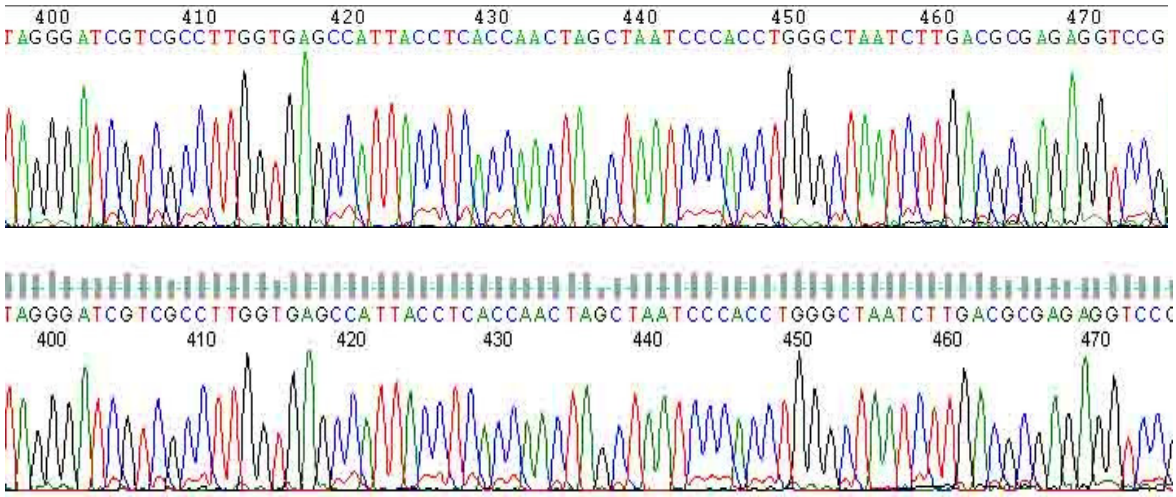

Figure 7.10 Sequence trace file for V166 as viewed with Chromas (above) and FinchTV (below) showing data quality as small bars above the sequence.

FinchTV was capable of displaying most of the sequence in one screen by "wrapping" the display. This facilitated much faster inspection of the data and more rapid location of the start and end of useful sequence, Figure 7.11. Data from files where the trace files appeared clear and the peaks were well resolved were retained for further processing and analysis. Where the sequencer base calling function failed to resolve peaks or inserted no calls (N) into the sequence output, the trace files were examined, a judgement made regarding the most likely result and the sequence either edited manually to reflect the changes required or trimmed from the final sequence, as was the unreadable sequence at either end of most reads.

The strain number and primer used for each reaction were used to name the sequence output files. This information was also embedded inside the sequence data file. This made it possible to write a series of DOS batch files in Excel which were used to convert the individual edited sequence files to a single concatenated file in FASTA format, where the annotated sequence identifiers were free of transcription error. These files were then transferred to ANGIS using BioManager and submitted for

BLASTn analysis by batch process. This largely automated process ensured that the sequencing samples were consistently labelled with the same sample numbers at all stages after the sequence result files were generated and the corresponding numbers were used in the output files from the BLAST analysis.

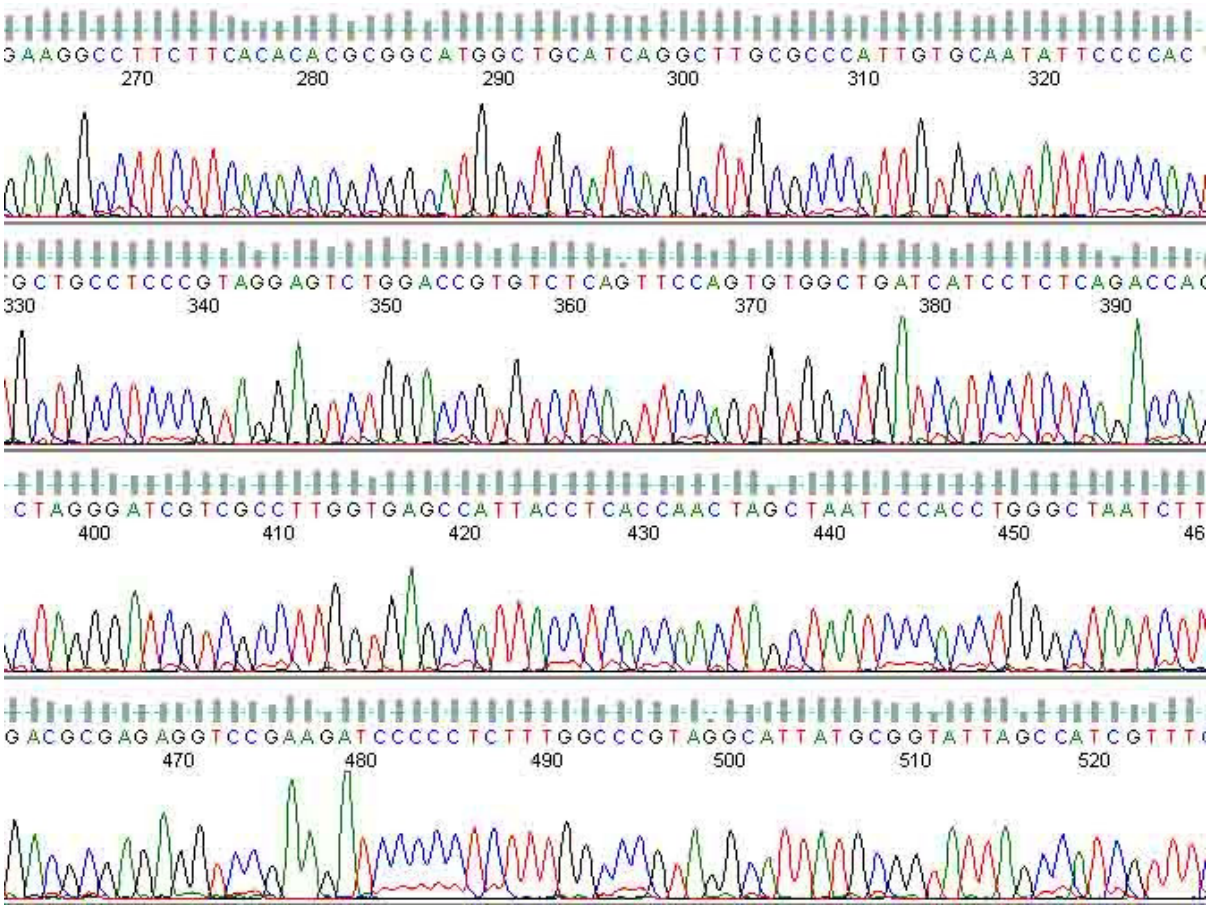

Figure 7.11 Sequence trace file for V166, *V. ichthyoenteri*, viewed with FinchTV showing the expanded display function.

Partial sequencing of the library with primer 685r2

To obtain basic sequence information on all the strains in the library, partial sequencing of the first 670 bp of the 16S rRNA gene was undertaken. The antisense primer 685r2 was used in conjunction with the purified 1,465 bp fragment as a source of DNA template. The antisense primer 685r2 is similar to other primers designed for the bacterial 16S rDNA gene but includes some degeneracy (CT) at position 670 in order to ensure more universal activity.

Good quality sequence for BLASTn analysis was obtained for 790 strains. After visual inspection and editing, these sequences ranged in length from 562 to 673 bp (data not shown). There were very large amounts of data generated by the BLASTn search of the

partial 16S rDNA sequence. A simple one line summary was generated for the purpose of this report, but does not fully reflect the detail and volume of data obtained, Appendix 4, Table A4.3.

Sequence performance

The utility of a partial sequence of 16S rDNA to identify species was assessed by reference to type and reference strains. Most type strains were correctly identified on the basis that the best alignment gave a match for the expected species, Table 7.15.

Table 7.15 Type strains of *Vibrionaceae* which have partial 16s rDNA sequences based on the 685r2 primer that are in agreement with the species designation

Species	Type Strain	Species	Type Strain
G. hollisae	CIP 101886	*V. metschnikovii*	NCTC 11170
M. marina	CIP 102861	*V. mimicus*	ATCC 33653
M. viscosa	NCIMB 13584	*V. mytili*	CIP 103929
Ph. damselae ssp. *damselae*	NCIMB 2184	*V. natriegens*	ATCC 14048
Ph. damselae ssp. *piscicida*	NCIMB 2058	*V. navarrensis*	CIP 103381
Ph. iliopiscarium	ATCC 51760	*V. nereis*	ATCC 25917
Ph. leiognathi	ATCC 25521	*V. nigripulchritudo*	CIP 103195
V. aestuarianus	NCIMB 2236	*V. ordalii*	NCIMB 2167
V. calviensis	DSMZ 14347	*V. orientalis*	NCIMB 2195
V. campbellii	ATCC 25920	*V. parahaemolyticus*	ATCC 17802
V. cincinnatiensis	LMG 7891	*V. pelagius* biovar I	ATCC 25916
V. diazotrophicus	ATCC 33466	*V. penaeicida*	DSMZ 14398
V. fischeri	ATCC 7744	*V. proteolyticus*	CIP 102892
V. furnissii	ATCC 35016	*V. rumoiensis*	NIBHT FERM P-14531
V. gazogenes	ATCC 29988	*V. tapetis*	CIP 104856
V. halioticoli	ATCC 700680	*V. tubiashii*	NCIMB 1340
V. harveyi	ATCC 14126	*V. vulnificus* bv I	ATCC 27562
V. mediterranei	CIP 103203	*V. wodanis*	NCIMB 13582

Not all type strains had alignments that were the first choice for the expected species designation but did occur at a lower rank order of matching, Table 7.16

Table 7.16 Unexpected alignments for type strains based on 562-673 nt sequence of 16S rDNA.

Species	Strain	Closest alignment	Rank match [a]
V. tasmaniensis	CRC D39^T	AY262019 *Vibrio* sp. YWA	3: *V. tasmaniensis*
V. scophthalmi	CECT 4638^T	AJ421445 *V. ichthyoenteri* DSMZ 14397^T	2: *V. scophthalmi*
V. salmonicida	CIP 103166^T	AJ278426 *Vibrio* sp. Torgnes 1	8: *V. salmonicida*
V. pectenicida	ATCC 700783^T	AY374411 *Vibrio* sp. clone 6-29	2: *V. pectenicida*
V. logei	ATCC 29985^T	AJ278426 *Vibrio* sp. Torgnes 1	2: *V. logei*
V. lentus	CECT 5110^T	AJ316200 *Vibrio* sp. R-3884	4: *V. lentus*
V. fluvialis	NCTC 11327^T	AY345409 Bacterium K2-61	2: *V. fluvialis*
V. anguillarum	ATCC 19264^T	74718 *V. ordalii* ATCC 33509^T	3: *V. anguillarum*
V. alginolyticus	ATCC 17749^T	AF388389 *V. parahaemolyticus* clone Vp 27	6: *V. alginolyticus*
V. agarivorans	CECT 5085^T	AY654760 Mucus bacterium 11	2: *V. agarivorans*
V. splendidus bv I	ATCC 33125^T	AF388392 *Vibrio* sp. 3d clone	2: *V. splendidus* bv I

[a]rank order position of match

Reference strains, those obtained from culture collections as named species, had a similar pattern of identification as the type strains. Alignments that matched the designated species occurred for 86% of the reference strains (*n*=56) but for 14% of strains there was no corresponding match. Strains that had no match are listed in Table 7.17.

Table 7.17 Culture collection strains which did not have a partial sequence of 16S rDNA that matched the expected species designation

Species	Strain
V. agarivorans	CECT 5084
V. campbellii	ATCC 25921
V. lentus	CECT 5293
V. mediterranei	CECT 623
V. mediterranei	CECT 615
V. mytili	CECT 663
V. scophthalmi	CECT 5965
V. scophthalmi	CECT 5967

Sequence repeatability

Alignment fidelity was assessed for a subset of strains selected because of apparent anomalies of the expected matches; a summary of comparisons and agreements is given in Table 7.18. Differences were assessed solely on the basis of search outcome; no comparison was made of sequence similarity between the first and second BLASTn results to determine differences in nucleotides. The difference in search outcome between the first BLASTn search and a repeated search starting with the raw sequence data was calculated as 29%, measured as the proportion of searches where a different outcome was observed between the first search and a subsequent search. The level of agreement between the first BLASTn assessment and that for a sequence reaction based on a new culture was low with only a third of repeat reactions agreeing with the original BLASTn result. For most of the sequence reactions repeated *de novo* there was a 78% between the best match and the identity of the strain tested; in most cases these were type or reference strains where the identity of the strain was known *a priori*.

Table 7.18 Comparison of alignment outcomes for repeat sequences of selected isolates

Comparison	No. of comparisons	Agreement	Disagreement
Repeat BLASTn analysis from raw sequence data	41	71%	29%
BLASTn analysis based on repeat sequence reaction	12	32%	68%
Repeat BLASTn sequence reaction and expected alignment	21	78%	22%

Sequencing of Hypothetical Median Organisms and cluster exemplars

Hypothetical median organisms of 27 unidentified phenons were selected for more extensive sequence analysis as they represented either potential new or previously unnamed species or were problematic groups that did not fit easily into the phenotypic groupings. A minimum set of 6 primers were used to cover the whole 16S rRNA gene from both DNA strands. These primers provided overlapping sequence data for both sense and anti-sense strands, providing an increased level of confidence in the reliability of the sequence data. Sequencing of the 5'and 3' termini was carried out using an estimated 2 to 3 µg of genomic DNA per 20µl reaction. The termini were the only areas where there was no overlapping sequence data to verify the result and confirm the sequence was accurate. When the partial sequences were aligned in order to generate a continuous consensus sequence, only those areas where the sequence trace files appeared clear and unambiguous were included. The sequences were lodged with GenBank using the Sequin software supplied by GenBank. A summary of HMOs and their GenBank accession numbers is given in Table 7.19. For Phenon 42,

Table 7.19 GenBank accession numbers for 16S rDNA sequence data of Hypothetical Median Organisms (HMOs).

GenBank accession no.	Phenon no.	Isolate no.	Host origin of Isolate
DQ146984	6	V357	*Penaeus* species
DQ146980	8	V201	*Portunus pelagicus*
DQ146982	10	V261	Calanoid copepod
DQ146990	15	V759	*Haliotis* sp.
DQ146974	19	V051	*Latris lineata*
DQ146983	20	V322	*Scylla serrata*
DQ146971	21	V020	*Latris lineata*
DQ146978	24	V140	*Haliotis* sp.
DQ146976	25	V69	*Latris lineata*
DQ146970	29	V4	*Latris lineata*
DQ146989	32	V639	*Haliotis* sp.
DQ146972	36	V31	Seawater
DQ146985	41	V515	Unknown
DQ146993	42	V794[1]	Unknown
DQ146992	43	V781	*Salmo salar*
DQ146995	45	V810	*Catostylus mosaicus*
DQ146988	46	V632	*Haliotis* sp.
DQ146981	52	V205	*Phycodurus eques*
DQ146996	53	V859	*Lates calcarifer*
DQ146975	57	V68	*Latris lineata*
DQ146991	58	V776	*Oncorhynchus mykiss*
DQ146973	59	V48	*Latris lineata*
DQ146986	64	V606	*Oncorhynchus mykiss*
DQ146987	69	V615	*Crassostrea gigas*
DQ146994	75	V798	*Delphinus* sp.
DQ146979	77	V170	*Salmo salar*
DQ146977	83	V125	*Phycodurus eques*

[1]V35, the HMO failed in culture; V794 was used as the exemplar of Phenon 42

the HMO was identified as strain V35. This strain failed to recover from storage and V794 was used instead as an exemplar of the phenon.

BLASTn Analysis of hypothetical media organisms

A list of the first 10 one line summaries from each BLAST search for the 16S rDNA sequence data of HMOs is given in Appendix 4, Table A4.4. The matches are listed from best to worst, with high *S* scores and low E-values being better. A summary of the phenons designations and HMO sequences is given in Table 7.20.

Phenon 6 (HMO: V357) The sequence for HMO V357 aligned most closely with the marine gliding bacterium UWA-1; the sequence homology however was only 97%, a level insufficient to suggest species identity (Clarridge, 2004; Drancourt & Raoult, 2005). This alignment, based on an extended sequence of 1,489 nucleotides, is at significant variance with the partial sequence based on 685 nucleotides.

The shorter sequence, V357, was most closely aligned with *Vibrio xuii* (LMG 21346^T, strain R-15052) (Thompson *et al.*, 2003b) and phenotypically has characteristics consistent with the *Vibrionaceae* and not the *Flavobacteriaceae*. Of note is that for five of the seven members of the phenon (71%), all had partial sequences consistent with GenBank accession AJ316181 *Vibrio xuii* (LMG 21346^T, strain R-15052). The anomalous findings for the 685 and 1,489 nucleotide sequences for the HMO remains unresolved but based on consistent phenotypic and genotypic data based on partial sequences, Phenon 6 may represent the species *V. xuii*.

Phenon 8 (HMO: V201) V201 had a 99% sequence homology with *V. lentus* strain Sat 201 (Nishiguchi & Nair, 2003) over a 1,499 nucleotide length of the 16S rRNA gene; strain Sat 201 is not the type strain for *V. lentus*. Six of the seven strains comprising phenon 8 also listed *V. lentus* as possible matches based on partial sequences, although in only two cases did *V. lentus* list as the best match; the closest match was not to the type strain of *V. lentus*. The phenon may represent a new species.

Phenon 10 (HMO: V261) Phenon 10 is an enigmatic group with strong phenotypic characteristics of *V. alginolyticus* although the genotypic data was less definite. For 83% of the strains in the phenon, an alignment with *V. alginolyticus* occurred but only at a lower level of likelihood except for V262 where it was the best match. By contrast,

Phenon	Tentative Designation	Concordance[1]	HMO	HMO partial sequence 16S rDNA	HMO full sequence 16S rDNA	Homology
Phenon 6	*Vibrio xuii*	71%	V357	AJ316181 *Vibrio xuii* (LMG 21346^T [strain R-15052])	AB039966 Marine gliding bacterium UWA-1	97%
Phenon 8	*Vibrio* sp. Sat101	85%	V201	AY292936 *Vibrio lentus* strain Sat201	AY292936 *Vibrio lentus* strain Sat201	99%
Phenon 10	*Vibrio alginolyticus*	83%	V261	AF064637 *Vibrio* sp. NAP-4	AB026194 Alpha proteobacterium MBIC1876	97%
Phenon 15	Bacterium K2-61	83%	V759	AY345409 Bacterium K2-61	AB038026 *Vibrio* sp. OC25	96%
Phenon 19	*Vibrio* sp. C33	100%	V051	AY254040 *Vibrio hispanicus* strain LMG 13240^T	AF500207 *Vibrio* sp. CJ11052	97%
Phenon 20	Heterogenous	0%	V322	AF108137 Uncultured *Vibrio* sp. 'Artemia'	AJ440009 *Vibrio gallicus* (LMG 21330^T)	99%
Phenon 21	*Vibrio* sp. QY101	88%	V020	AY174869 *Vibrio* sp. QY101	AY174869 *Vibrio* sp. QY101	99%
Phenon 24	*Vibrio pomeroyi*	75%	V140	AJ491290 *Vibrio pomeroyi* LMG 20537^T	AJ491290 *Vibrio pomeroyi* (LMG 20537^T)	99%
Phenon 25	*Vibrio* sp. YWA	64%	V69	AY262019 *Vibrio* sp. YWA	AY174869 *Vibrio* sp. QY101	99%
Phenon 26	*Vibrio* sp. QY101	71%	V102	AY620969 *Vibrio tasmaniensis* strain 236.10	Sequence not determined	
Phenon 27	*Vibrio* sp. 3d clone 3d4	69%	V624	AJ316200 *Vibrio* sp. R-3884	Sequence not determined	
Phenon 29	*Vibrio* sp. R-3884	93%	V4	No sequence data	AJ316200 *Vibrio* sp. R-3884	99%
Phenon 32	*Vibrio harveyi* biovar II	96%	V639	X74693 *V. harveyi* (ATCC 35084^T)	AY750578 *Vibrio harveyi* strain S35	99%
Phenon 36	*Vibrio* sp. EN276	80%	V31	AY136119 *Vibrio* sp. MED103	AJ514911 *Grimontia hollisae* (LMG 21538)	95%
Phenon 41	*Vibrio coralliilyticus*	100%	V515	AJ316168 *Vibrio* sp. R-14968	AJ316167 *Vibrio coralliilyticus* (LMG 10953)	99%
Phenon 42	*Vibrio* sp. C33	100%	V794[2]	AY034144 *Vibrio* sp. C33	AJ316169 *Vibrio* sp. (LMG 19270)	98%
Phenon 43	Heterogenous	0%	V781	AY620969 *Vibrio tasmaniensis* strain 236.10	AY147861 *Ph. damselae* ssp. *damselae,* wild type	99%
Phenon 45	*Vibrio* sp. 3d clone 3d4	75%	V810	AF293974 Unidentified bacterium 4c	AF388394 *Vibrio* sp. 3d clone 3d8	99%
Phenon 46	*Vibrio* sp. QY101	100%	V632	AY174869 *Vibrio* sp. QY101	AY174869 *Vibrio* sp. QY101	99%
Phenon 52	*Pseudoalteromonas* sp.	50%	V205	AY305857 *Pseudoalteromonas* sp. SM9913	AY305857 *Pseudoalteromonas* sp. SM9913	99%
Phenon 53	*Vibrio* sp. LMG 20363	80%	V859	No sequence data	X76336 *V. furnissii* (ATCC 35016^T)	97%
Phenon 57	Heterogenous	0%	V68	X74723 *V. proteolyticus* (ATCC 15338^T)	AF513463 *Vibrio proteolyticus* K52 WT	99%
Phenon 58	*Vibrio fischeri* Clade A	100%	V776	AY494613 Uncultured *Photobacterium* sp. clone GAMMA5B	AY292924 *Vibrio fischeri* strain SA1G	99%
Phenon 59	*Photobacterium* sp. Gamma5B	100%	V48	AY494613 Uncultured *Photobacterium* sp. clone GAMMA5B	AY292932 *Vibrio logei* strain 15382	97%
Phenon 64	*Aeromonas sobria* HG7	71%	V606	X74683 *A. sobria* (ATCC 43979^T)	X60412 *Aeromonas sobria* 16S rRNA (NCIMB 12065^T)	99%
Phenon 69	Heterogenous	0%	V615	M22365 *Oceanospirillum linum* ATCC 11336	AF500207 *Vibrio* sp. CJ11052	99%
Phenon 75	*Ph. damselae* ssp. *damselae* bv II	67%	V798	AY147861 *Photobacterium damselae* ssp. *damselae*	AY174869 *Vibrio* sp. QY101	99%
Phenon 77	*Vibrio ichthyoenteri* bv II	100%	V170	AJ421445 *Vibrio ichthyoenteri* (DSMZ 14397^T)	AJ437192 *Vibrio ichthyoenteri* (LMG 19664^T)	98%
Phenon 83	*Photobacterium* sp. HAR72	71%	V125	AJ551094 *Hyphomicrobium* sp. wp13	AB038032 *Photobacterium* sp. HAR72	97%

Table 7.20 Summary of alignments for partial and full sequences of 16S rDNA for hypothetical median organisms for unnamed phenons.

[1]Concordance: Proportion of strains with partial sequence alignments that agree with phenon designation. For phenons with heterogenous sequences the concordance value was given as 0%
[2]The HMO for phenon 42 is V35 which failed on subsequent culture. V794 was used instead as the exemplar for the phenon

the sequence for the HMO V261 showed a 97% homology with Alpha proteobacterium MBIC1876 and a range of "Mucus bacterium" strains, which is distinctly at variance to the genotypes based on partial sequences for Phenon 10. The alignment anomaly for the 685 and 1,421 nucleotide sequences for the HMO remains unresolved but based on consistent phenotypic and genotypic data, Phenon 10 may represent a phenotypic variant of *V. alginolyticus*.

Phenon 15 (HMO: V759) Based on partial sequences, Phenon 15 consists of strains aligned with Bacterium K2-16 (AY345409) and *Vibrio* sp. LMG 20363 (AJ345064). A sequence of the HMO, V759 consisting of 1,517 nucleotides was most closely aligned with *Vibrio* sp. strain OC25 with a sequence similarity of 96%. On the basis of the phenotype and genotype data, Phenon 15 may represent a new species.

Phenon 19 (HMO: V51) The phenon is a small group comprising four strains, which on the basis of partial 16S rDNA sequencing, may be related to *V. hispanicus* or *V. tubiashii*. For the longer sequence however, V51 was most closely aligned to *Vibrio* sp. CJ11052 with a sequence similarity of 97%; there was no match with *V. hispanicus*, the alignment for the shorter sequence. Further investigation is required to resolve the status of the phenon, which based on the short sequence alignments may represent *V. hispanicus*.

Phenon 20 (HMO: V322) Partial 16S rDNA sequence data for Phenon 20 suggests the group is heterogenous in composition. The extended sequence of V322, the HMO shows a 99% level of similarity to both *Vibrio xuii* (LMG 21346^T, strain R-15052) and *V. gallicus* (LMG 21330^T). The short sequence for the V322 matched an uncultured *Vibrio* species designated 'Artemia' (AF108137). The taxonomic status of Phenon 20 is unresolved but may represent a new species.

Phenon 21 (HMO: V20) The phenon appears to be homogeneous phenotypically and genotypically. From the short sequence alignments, 88% of the strains matched *Vibrio* sp. QY101 an alginate lyase producing strain (AY174869). A longer sequence of the HMO strain V20 also aligned with *Vibrio* sp. QY101 (AY174869) with a sequence homology of 99%. The phenon appears to represent a new species designated *Vibrio* sp. QY101, phylogenetically similar to *V. splendidus* biovar I (Thompson *et al.*, 2004c).

Phenon 24 (HMO**: V140)** 16S rDNA partial sequencing suggests Phenon 24 is a heterogeneous group of four strains but with a common genotype matching *V. pomeroyi*. Both the short and extended sequences for HMO V140 were closely

aligned to *Vibrio pomeroyi* LMG 20537^T (AJ491290) with a sequence homology of 99% over a 1,486 nucleotide region. The phenotype of the group is tightly circumscribed, and based on phenotype and genotype, Phenon 24 probably represents *V. pomeroyi*.

Phenon 25 (HMO: V69) This is a group of 14 strains of which a number show similarity to *Vibrio* sp. YWA based on partial 16S rDNA analysis. With a longer sequence of 1,522 nucleotides, V69 most closely matched *Vibrio* sp. QY101 and *Vibrio* sp. YWA another alginate lyase species; the match for *Vibrio* sp. YWA was at a lower similarity score to that of *Vibrio* sp. QY101. The sequence homology for both strains was 99% The phenon appears to represent a new species best described either as *Vibrio* sp. QY101 or YWA. Further work is required to resolve the status of the phenon.

Phenon 26 (HMO: V4) An extended sequence was not generated for the HMO. A partial sequence had an alignment with an unpublished description of *V. tasmaniensis* strain 236.10 with a second match for *Vibrio* sp. QY101. Other members of the phenon also had partial sequences that matched *Vibrio* sp. QY101 and based on this information it would appear that the phenon may represent a new species.

Phenon 27 (HMO: V624) An extended sequence was not generated for the HMO. The best match for the partial sequence of the HMO was for *Vibrio* sp. R3884 although the most commonly occurring match, though not the highest ranked, was for clone 3d4 from *Vibrio* sp. 3d (Moreno *et al.*, 2002). Phenon 27 probably represents a new species.

Phenon 29 (HMO: V4) Phenon 29 appears to be a well defined group genotypically with 93% of the members having partial 16S rDNA sequences aligning with *Vibrio* sp. R-3884. The extended sequence of 1,519 nucleotides for HMO V4 has a 99% homology with *Vibrio* sp. R-3884 an unnamed member of Cluster 55 defined by fluorescent amplified fragment length polymorphism (FAFLP) analysis (Thompson *et al.*, 2001). Phenon 29 appears to be genotypically similar to the as yet unnamed *Vibrio* sp. R-3884.

Phenon 32 (HMO: V639) Members of the phenon have a uniform phenotype as well as genotype based on partial 16S rDNA sequence. Nearly all members (96%) of the phenon had sequence alignments that best matched with *V. harveyi* ATCC 35084, the type strain for *V. 'carchariae'*, now a junior synonym for *V. harveyi*. For V639 the HMO, there was a 99% homology with *V. harveyi*. Based on these findings, Phenon 32

can be defined as *V. harveyi*. According to phenotype it represents a biovar distinct from Phenon 13, designated as *V. harveyi* biovar I and comprises a wide range of strains from a variety of hosts including the type strain ATCC 14126^T. Phenon 32 has a distinct phenotype and was designated *V. harveyi* biovar II.

Phenon 36 (HMO: V31) Phenon 36 is typified as *Vibrio* sp. EN276 (AB038023), as determined by partial sequence analysis and comprised 80% of the strains. For the HMO V31 the short sequence aligned with *Vibrio* sp. MED103 followed by *Grimontia hollisae* ATCC 33564^T (X74707) as the next most likely match. For a sequence of 1,523 nucleotides, V31 aligned with *G. hollisae* LMG 10953 (Thompson *et al.*, 2003a) although the level of homology at 95% was low indicating that V31 is unlikely to be a strain of *G. hollisae*. There is a level of phenotypic and genotypic homogeneity in Phenon 36 to indicate that it may represent a new species.

Phenon 41 (HMO: V515) The genotype for Phenon 41, based on a sequence length of 685 nucleotides was determined for all members of the phenon as *Vibrio* sp. R-14968. This strain was grouped in Cluster A2, an unclassified taxon based on FAFLP analysis (Thompson *et al.*, 2001). For a longer sequence of 1,517 nucleotides, HMO V515 had a 99% homology to *Vibrio coralliilyticus* LMG 10953 (Ben-Haim *et al.*, 2003a); there was no alignment with *Vibrio* sp. R-14968 for the longer sequence. The status of the phenon is uncertain but may represent either *V. coralliilyticus* or *Vibrio* sp. R-14968.

Phenon 42 (Exemplar: V794) The phenon consists of four strains and based on partial sequences has homology with *Vibrio* sp. strain C33 (AY034144), a putative probiont with activity against unspecified mollusc pathogens. For a sequence of 1,505 nucleotides, V794 the HMO, aligned with *Vibrio* sp. LMG 19270 in FAFLP cluster A3 (Thompson *et al.*, 2001) with a sequence homology of 98%. There was no alignment with *Vibrio* sp. strain C3 with the longer sequence. Phenon 42 appears to represent a new species.

Phenon 43 (HMO: V781) Genotypically, the phenon appears heterogenous based on partial sequence data. For 685 nucleotides, the HMO V781 has a sequence alignment with *V. tasmaniensis* strain 236.10, a wild type (AY620969). For the longer sequence there was an unambiguous alignment with a wild type strain of *Ph. damselae* ssp. *damselae* with a 99% sequence homology. This finding however does not agree with the phenotype of V781 or the phenon as a whole, which is urease negative, while

Ph. damselae ssp. *damselae* is uniformly urease positive. The inconsistency of sequence results point to experimental error and the findings based on the extended sequence must be considered suspect.

Phenon 45 (HMO: V810) Although not the highest ranking match, 75% of strains had alignments, based on partial sequences, with *Vibrio* sp. 3d clone 3d4, an unnamed species isolated from seawater off the Chilean coast (Moreno *et al.*, 2002). For the HMO V810 with an extended sequence of 1,520 nucleotides, an alignment occurred with *Vibrio* sp. 3d clone 3d8 with a sequence homology of 99%. There is good evidence that phenon 45 should be considered to represent *Vibrio* sp. 3d, an unclassified new species.

Phenon 46 (HMO: V632) The best match for V632 was with *Vibrio* sp. QY101 with sequence homology of 99%. Based on partial sequences, the majority of strains in phenon 46 also matched with *Vibrio* QY101. Of note is that the HMO for phenon 21 and phenon 25 had matching sequence alignments for *Vibrio* sp. QY101 although each phenon had clearly different phenotypes. It is apparent that further investigation of genotype is required for these phenons to establish the relationships between the groups. Phenotypic data indicates that phenon 46 is a distinct entity but requires validation by genotypic analysis.

Phenon 52 (HMO: V205) Phenon 52 is a minor cluster of three strains. Partial sequence analysis of the group was ambiguous; V205 aligned with *Pseudoalteromonas* sp. SM9913 (Chen *et al.*, 2003) and V888 with *V. proteolyticus,* ATCC 15338^{T}; the third strain in the group was not sequenced. A longer sequence of 1,501 nucleotides for V205 aligned with *Pseudoalteromonas* sp. SM9913 with a sequence homology of 99%. Phenotypically however the three strains were confounding: all were sensitive to the vibriostat at 150µg but V343 and V888 failed to ferment glucose whereas V205 fermented glucose but no other substrate. *Pseudoalteromonas* species characteristically do not ferment glucose and are resistant to 0/129 (Romanenko *et al.*, 2003; Ivanova *et al.*, 2004) although some species, such as *Ps. tunicata* (Holmström *et al.*, 1998) and *Ps. ulvae* (Egan *et al.*, 2001), are sensitive. Phenon 52 appears heterogenous in composition and does not contain members of the *Vibrionaceae*.

Phenon 53 (HMO: V859) Using the criterion of best sequence match, phenon 53, based on partial sequences, appears heterogenous. The most frequently occurring

match, ranging in rank order from one to nine was *Vibrio* sp. LMG 20363. For the HMO V859, the best match for a sequence of 1,516 nucleotides was for *V. furnissii* ATCC 35016^T however the sequence homology was 97%, a level below the acceptance threshold for a species (Clarridge, 2004; Drancourt & Raoult, 2005). A partial sequence for V859 had not been generated and a comparison of alignments based on short and long sequences could not be made. As the partial sequence of the 16S rDNA had been determined for only half the members of the cluster it was not possible to obtain a true measure of genotypic homogeneity. Based on partial sequences, V290 and V315 had low level of match to *V. furnissii* though this was not apparent in the other strains that had been sequenced. Phenotypically the phenon did not have characteristics of *V. furnissii* which is well defined as phenon 4, both genotypically and phenotypically including duplicate type strains; there is little evidence to consider that phenon 53 is a phenotypic variant of *V. furnissii*. Without further sequence analysis of strains, the taxonomic status of the phenon remains unclear but may represent a new species.

Phenon 57 (HMO: V68) Phenon 57 is a small group of four strains that appears to be heterogeneous genotypically with no clear associations based on the partial 16S rDNA sequencing. The best match for V68 the HMO was *V. proteolyticus* K52 (Donachie *et al.*, 2004) with a sequence homology of 99%. Strain K52 in turn was reported to have a sequence similarity of 98.93% to *V. proteolyticus*. The second order match was *V. proteolyticus* ATCC 15338^T which also matches the alignment for the partial sequence. The phenon does not however have characteristics of *V. proteolyticus* which is well defined both genotypically and phenotypically in phenon 12 that includes the type strain of *V. proteolyticus* ATCC 15338^T. The status of Phenon 57 is unclear given the heterogeneity of genotypes despite a uniformity of phenotype.

Phenon 58 (HMO: V776) The phenon appears homogeneous genotypically with alignments for all strains matching an uncultured *Photobacterium* sp. clone Gamma5B (AY494613). An extended sequence of 1,508 nucleotides for V776 matched *V. fischeri* strain SA1G, clade A (Nishiguchi & Nair, 2003); the sequence homology was 99%. For the corresponding partial sequence for V776, although the closest match was for *Photobacterium* sp. clone Gamma5B, *V. fischeri* strain SA1G was ranked fourth. On the basis of the extended sequence, Phenon 58 appears to represent a distinct phenotype of *V. fischeri*.

Phenon 59 (HMO: V48) Members of the phenon form a homogenous group and on partial sequence analysis best match an uncultured *Photobacterium* sp. clone Gamma5B (AY494613). For the HMO V48, the best alignment for an extended sequence of 1,509 nucleotides was *Vibrio logei* ATCC 15382 a wild type strain with the remaining 14 matches as a range of *V. fischeri* strains (Nishiguchi & Nair, 2003). The sequence homology was 97%, suggesting that the HMO is an unknown taxon and unlikely to be *V. logei* which is represented in Phenon 60. Of note however is that the *V. logei* phenon lies adjacent to Phenon 59 in the phenogram but on separate branches of the tree. Phenon 59 probably represents a new species.

Phenon 64 (HMO: V606) This phenon was unusual in that it comprises strains with characteristics both genotypically and phenotypically consistent with the genus *Aeromonas*. Partial sequences for the group showed matches for 71% of strains with *Aeromonas sobria* hybridisation group 7, ATCC 43979^T and only two strains with matches for *Vibrio* sp. EN276 (AB038023) and *A. salmonicida* ssp. *salmonicida* (ATCC 33658^T). All strains in the group were isolated from salmonids held in freshwater, grew in the absence of NaCl and were sensitive to the vibriostat 0/129. Sensitivity has been reported for strains of *A. sobria* HG 7 (Carnahan & Joseph, 1993). An alignment matching *A. sobria* HG 7 was found for the HMO V606 with a longer sequence of 1,509 nucleotides having a 99% sequence similarity. The consistent phenotypic and genotypic results indicate that Phenon 64 can be designated as *A. sobria* hybridisation group 7.

Phenon 69 (HMO: V615) A small phenon of five strains, partial sequences were generated for three of the group. Genotypically the strains were heterogenous with only one strain aligning with an unnamed species, *Vibrio* R-3884, a member of FALFP cluster A55 (Thompson *et al.*, 2001). There was a major difference in sequence matches for the 685 and 1,517 nucleotide length sequences of the HMO V615. The partial sequence aligned with *Oceanospirillum linum* ATCC 11336 while for the longer sequence of 1,517 nucleotides, the highest match occurred with *Vibrio* sp. strain CJ11052 with a 99% sequence similarity. Given the genotypic heterogeneity and inconsistency, the status of Phenon 69 is unclear but may represent a new species.

Phenon 75 (HMO: V798) The partial sequence results for the phenon showed that 67% of the strains matched *Photobacterium damselae* ssp. *damselae* whereas the two remaining strains V122 and V371 matched an uncultured *Photobacterium* species clone Gamma 5A (AY494612) and *Ph. phosphoreum* ATCC 11040^T, respectively. All

members of the group, other than V371, were positive for urease and the multiplex PCR specific for *Ph. damselae* ssp. *damselae* (Osorio *et al.*, 2000). For the longer sequence of HMO V798, the best match occurred with *Vibrio* sp. QY101 (AY174869) with a 99% sequence similarity. The search outcome, however, is at variance with the partial sequence result as well as the phenotype and *Ph. damselae* ssp. *damselae* multiplex PCR reaction. Further investigation is required to verify the 16S rDNA sequence of V798 but on the basis of other data collected for the HMO and the phenon as a group, it appears to represent a distinct phenotype of *Ph. damselae* ssp. *damselae* designated biovar II.

Phenon 77 (HMO: V170) The phenon is a homogeneous taxon both genotypically and phenotypically. Partial sequences for all strains in the phenon gave the highest match with *V. ichthyoenteri* DSMZ 14397^T and for the corresponding sequence of 1,513 nucleotides for the HMO V170, with *V. ichthyoenteri* LMG 19664^T at a 98% similarity level. Phenon 77 represents a distinct phenotypic biovar designated *V. ichthyoenteri* biovar II.

Phenon 83 (HMO: V125) The most frequently occurring match with the partial sequences for strains of the phenon was with *Photobacterium* sp. HAR72 (71%) although this was not always the best match. For the HMO V125 however, the best match, with a similarity of 96-97% over 1,507 nucleotides, was *Photobacterium* sp. HAR72 while the best match for a partial sequence was *Hyphomicrobium* sp. wp13 isolated from a deep sea vent (AJ551094). The ecological niche for the genus is in freshwater environments and at least one species, *H. indicum,* has been transferred to the genus *Photobacterium* (Xie & Yokota, 2004) based on phenotypic, phylogenetic and chemotaxonomic characteristics. The taxonomic status of *Hyphomicrobium* sp. wp13 is unclear but may possess characteristics of the genus *Photobacterium* as suggested by the sequence data obtained for members of Phenon 83. *Photobacterium* sp. HAR72 is reported to be a luminous species, isolated off the coast of Japan, with an RFLP pattern that grouped the strain with *Ph. leiognathi* and *Ph phosphoreum* (Urakawa *et al.*, 1999). Phenon 83 has genotypic characteristics consistent with *Photobacterium* sp. HAR72 and represents a potential new species.

7.1.9 Concordance of genotype and phenotype

The phenons were established based on clusters of strains defined by a common phenotype and where present, inclusion of a type strain and strains of the species whose identity had been established independently. An assessment was made of the

level of agreement between the genotype, based on partial sequence of the 16S rRNA gene for members of each cluster, and the designation of the phenon defined by phenotype. Since only partial sequences were generated, a match in the first 10 alignments that agreed with the phenon definition was accepted as evidence of agreement. Justification for this approach is made on the basis of confirmatory species specific 16S rDNA PCR for *V. harveyi*, Phenon 13 (Oakey *et al.*, 2003) and *Ph. damselae* ssp. *piscicida*, Phenon 76 (Osorio *et al.*, 2000). For both species, strains were selected on the basis that there was either no match for the anticipated species by BLASTn search of 16S rDNA sequences or that the match was not the highest rank. In all cases, a positive PCR reaction was obtained for the expected species. Although this approach was used for only two species it provides sufficient justification for accepting a sequence match if it occurred in the first 10 likely matches.

Table 7.21 Level of agreement between phenotype and genotype for strains in phenons defined by cluster analysis

Phenon no. and designation	Agreement	Phenon no. and designation	Agreement
Phenon 4 *V. furnissii*	100%	Phenon 56 *V. anguillarum*	89%
Phenon 5 *V. fluvialis*	100%	Phenon 35a *V. vulnificus* bv I	86%
Phenon 7 *V. nereis*	100%	Phenon 64 *A. sobria* HG7	86%
Phenon 16 *V. calviensis*	100%	Phenon 86 *V. ordalii*	83%
Phenon 17 *V. tubiashii*	100%	Phenon 3 *V. mytili*	80%
Phenon 18 *V. orientalis*	100%	Phenon 39 *V. pelagius bv* II	80%
Phenon 23 *V. aestuarianus*	100%	Phenon 2 *V. diazotrophicus*	75%
Phenon 34 *V. mimicus*	100%	Phenon 38 *V. penaeicida*	75%
Phenon 35b *V. vulnificus* bv II	100%	Phenon 48a *V. campbellii*	75%
Phenon 40 *V. pelagius* bv I	100%	Phenon 48b *V. splendidus.* bv II	75%
Phenon 44 *V. tasmaniensis*	100%	Phenon 73 *Ph. leiognathi*	75%
Phenon 47 *V. navarrensis*	100%	Phenon 12 *V. proteolyticus*	73%
Phenon 50 *M. marina*	100%	Phenon 14 *V. mediterranei*	71%
Phenon 60 *V. logei*	100%	Phenon 37 *V. nigripulchritudo*	67%
Phenon 61 *Ph. phosphoreum*	100%	Phenon 75 *Ph. damselae* ssp *damselae* bv II	67%
Phenon 62b *V. fischeri* bv II	100%	Phenon 76 *Ph. damselae* ssp *piscicida*	67%
Phenon 63 *Ph. damselae* ssp *damselae* bv I	100%	Phenon 33 *V. cholerae*	64%
Phenon 65 *V. metschnikovii*	100%	Phenon 49 *V. lentus*	63%
Phenon 67a *V. gazogenes*	100%	Phenon 13 *V. harveyi* bv I	60%
Phenon 68 *Ph. iliopiscarium*	100%	Phenon 54 *V. scophthalmi*	55%
Phenon 70 *V. salmonicida*	100%	Phenon 71 *G. hollisae*	50%
Phenon 77 *V. ichthyoenteri* bv II	100%	Phenon 1 *V. natriegens*	45%
Phenon 78 *V. halioticoli*	100%	Phenon 55 *V. ichthyoenteri* bv I	43%
Phenon 79 *V. fischeri* bv II	100%	Phenon 82 *V. agarivorans*	43%
Phenon 80 *V. wodanis*	100%	Phenon 31 *V. splendidus* bv I	39%
Phenon 81 *V. tapetis*	100%	Phenon 22 *V. rumoiensis*	33%
Phenon 84 *M. viscosa*	100%	Phenon 67b *V. cincinnatiensis*	33%
Phenon 85 *V. pectenicida*	100%	Phenon 28 *V. chagasii*	10%
Phenon 32 *V. harveyi* bv II	96%	Phenon 26a *V. cyclitrophicus*	0%
Phenon 9 *V. alginolyticus*	92%	Phenon 62a *Ph. angustum*	0%
Phenon 11 *V. parahaemolyticus*	92%		

Using data only for phenons containing named species, the overall level of agreement between phenotype and genotype was 76%. For the unnamed phenons the level of agreement, using homogeneity of sequence outcome as the criterion of interest, was 67%. The level of agreement for the entire library of strains was 73%. The distribution of phylo-phenetic agreements by phenon is given in Table 7.21 and is an index of confidence in phenon definitions.

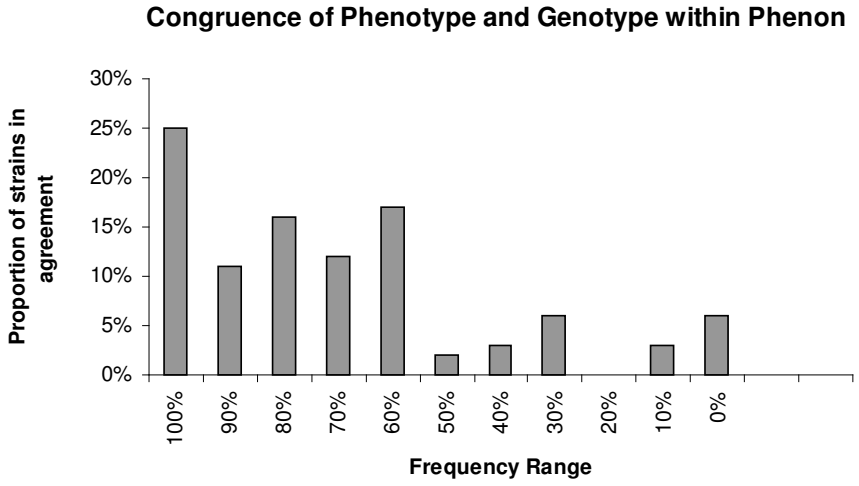

Figure 7.12 Percentile rank distribution of strains where phenotype and genotype are in agreement

The proportion of strains where phenotype and genotype agreed was arranged as percentile rank order to determine the distribution of agreement across the library of strains, Figure 7.12. At the 80th percentile there was an agreement of phenotype and genotype for 52% of the strains and at the 60th percentile, there was agreement between phenotype and genotype for 81% of the strains; less than 10% of strains were below the 30th percentile of agreement.

7.2 DEVELOP PRACTICAL, ROBUST PHENOTYPIC IDENTIFICATION SYSTEMS FOR *VIBRIO* SPECIES USING COMPUTER ASSISTED IDENTIFICATION SOFTWARE

7.2.1 Computer assisted identification software

Software features

The program PIBWin has three major functions: the identification of an unknown strain, the selection of additional tests to distinguish between possible strains if identification is not achieved, and the storage and retrieval of results. It also has some

utilities for assessing the usefulness of identification matrices and for converting matrices into different file formats.

PIBWin uses the Microsoft Excel file format to store identification matrices as well as test results in a separate archive file; other file formats are supported such as comma separated values and text format. Text format files provides for backward compatibility with PIB an MS-DOS predecessor of this program.

The flexibility of the Microsoft Excel format for probability matrices enables data to be extracted from published papers or exported from other electronic data files. Data can also be readily amended by the user and new data added as required.

Program options

Program options are set in the program registry and, if installed on a network that accommodates user profiles, PIBWin options will be specific for a logged-in user. Settings are in two categories: file access and identification parameters. File settings determine if the last identification matrix and archive file are loaded automatically on opening PIBWin and if identification results are sent to an output window for printing or copying into other documents. The Identification options tab sets parameters relevant to a particular identification matrix, see Figure 7.13. Thresholds can be set to

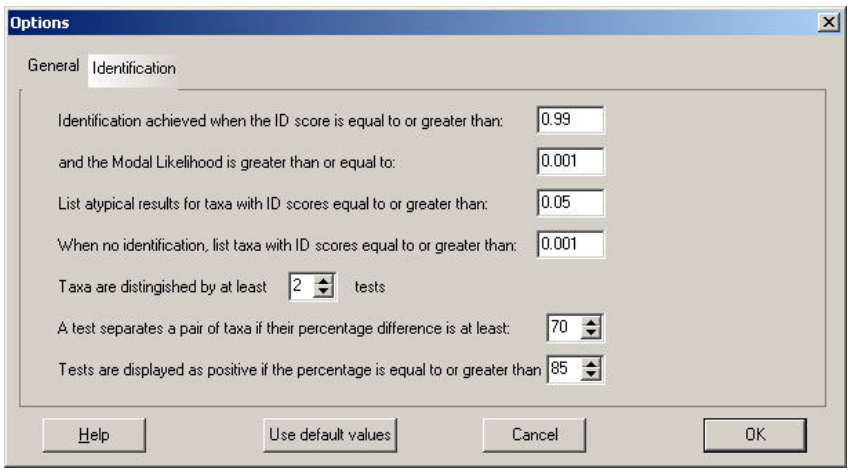

Figure 7.13 Identification options available in PIBWin

accept an identification for both the Willcox probability value *P* and the modal likelihood score. Other parameters include the number of test differences needed to accept an identification and cut-offs for defining positive and negative reactions. The number of likely taxa shown and the number of differences between likely taxa and the unknown isolate displayed can also be set.

Entering results

The programme opens by, either requesting that an identification matrix be selected or by opening the last identification matrix used. A Results tab is displayed (Figure 7.14) and the user can enter Key, Source and Detail information relating to the isolate. A Key must be entered if the results are to be saved to an archive file for recall at a later time; the Key is a unique identifier of up to fifteen characters in length. The source

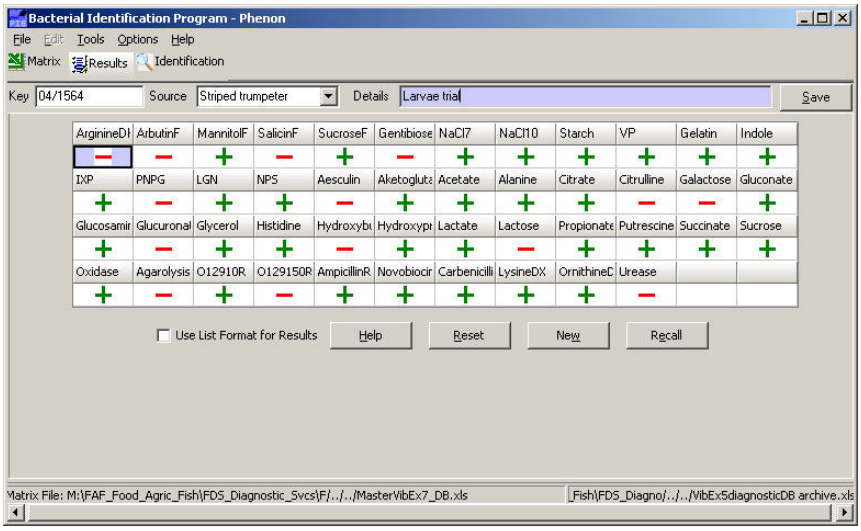

Figure 7.14 Data entry screen, grid format for PIBWin identification software

information is entered via drop down list box ,which allows text up to a maximum of 50 characters to describe the source from which the isolate was obtained. To achieve consistent entry of the source information, previous entries are used to construct the items shown in the drop down list, thus items offered in the list increase in number over time. Details provides for a description of the isolate of up to 255 characters.

Isolate results can be entered in a grid or list format as selected by the user. The grid format enables a 96 well micro-well plate format to be accommodated. The full name of each test is shown in a pop up box when the cursor is placed over the test name. Positive and negative results can be entered using the keyboard or left and right mouse clicks. Additionally a question mark character '?' can be entered to denote that a test has been carried out but the result is indeterminate; tests with no data are left blank.

Identification

When all test results have been entered, the Identification tab can be selected. The programme then suggests an identification for the unknown or lists the most likely taxa depending on the identification threshold selected by the user (Figure 7.15). Differences between the unknown isolate and the identified taxon, or likely taxa are displayed in a second grid. The program states if the identification reached exceeds the

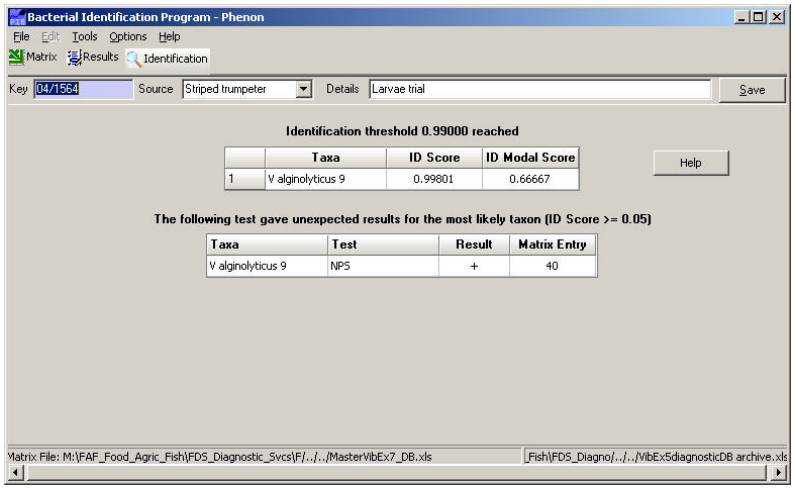

Figure 7.15 Identification results screen

Willcox probability threshold and also if it exceeds the modal likelihood threshold; the thresholds set are a function of the matrix quality. An unknown may have a high probability score indicating a good match across the species in the matrix but the modal likelihood score may be less than the threshold set for the matrix. In such instances the suggested identification is rejected unless there is additional evidence obtained independently that confirms the suggested identification. In such an event the organism can be considered an outlier. Where the Willcox probability score does not reach the matrix threshold then the most likely identifications are listed (Figure 7.16) together with tests at variance to that expected for the closest matching species.

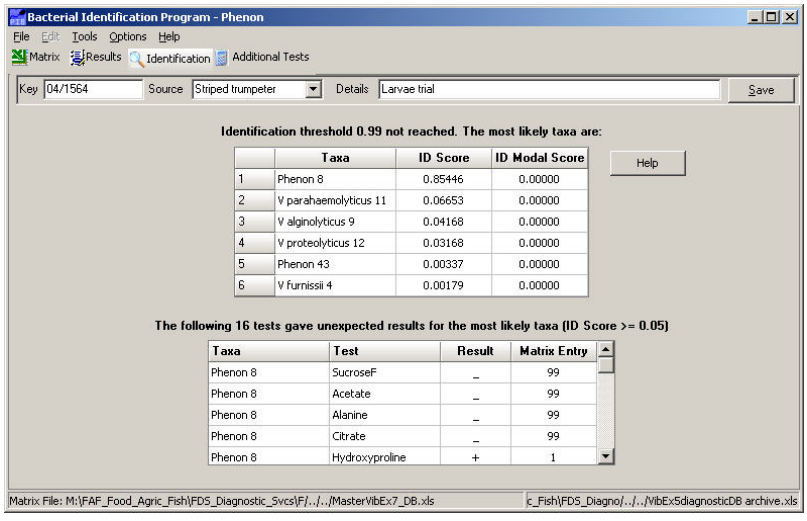

Figure 7.16 Results screen in the event that an identification is not reached

Additional tests

If an identification is not achieved and more than one taxon is a possible candidate, the Additional Tests tab is shown (Figure 7.17). Additional tests may be chosen in two ways; they may be selected so that the most likely taxon can be distinguished from

other likely taxa, or they can be selected to distinguish likely taxa from each other. An Exclude Tests button permits tests to be excluded before the selection process is performed. This may be necessary if one test takes longer to perform than other tests or is known to be difficult to interpret.

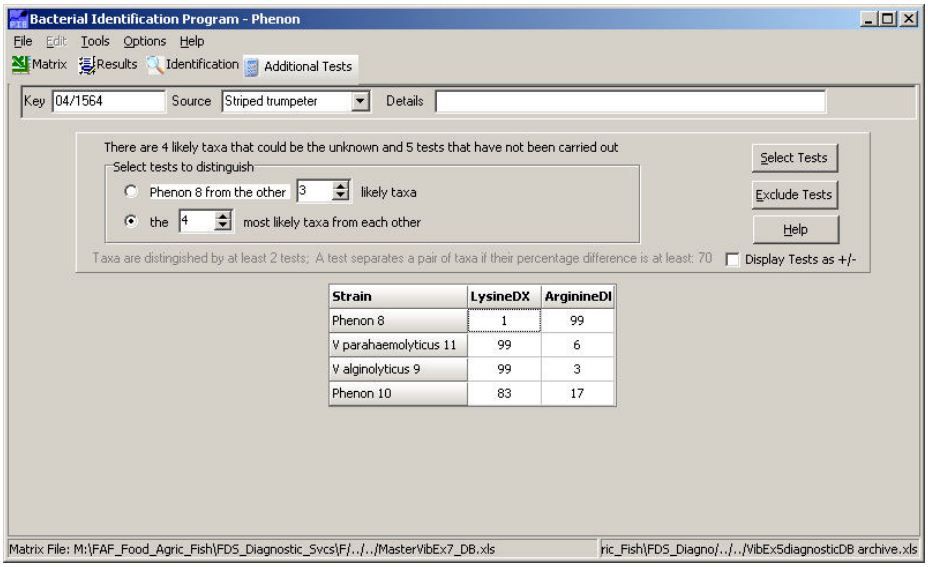

Figure 7.17 Other tests nominated when an identification is not reached

Archiving results

Tests results are saved to an Excel file by clicking the Save button. Different files can be set by the user for storing results for different purposes. The results for an isolate can be recalled, by clicking on the Recall button and a table of archived results displayed. The data can also be searched by selecting the Find button. Once the required isolate has been located its results can be recalled by double clicking on the row (Figure 7.18).

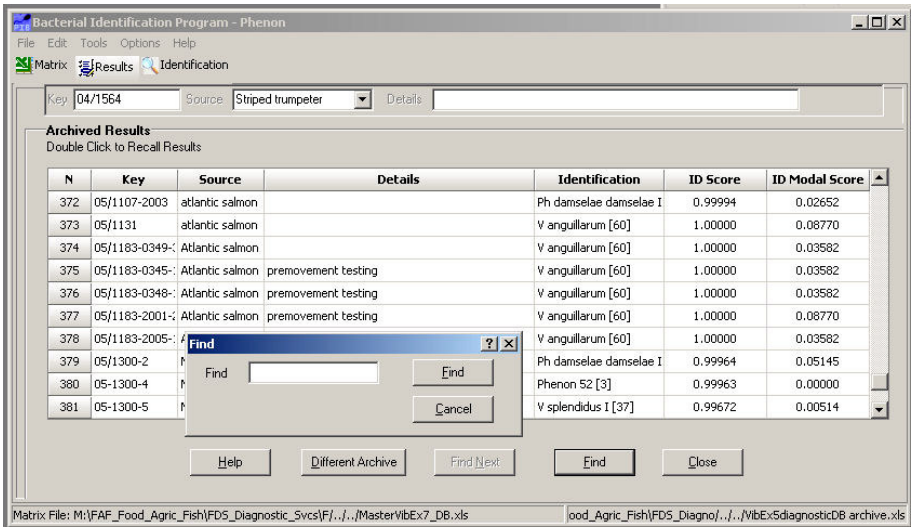

Figure 7.18 Archive screen to locate saved identifications

Printed output

A log of the test results for strains, identifications and additional tests suggested by the programme may be recorded in an Output window. The output can be printed or saved as a Rich Text File (RTF) that can be modified using word processing software.

Other features

In order to provide an upgrade path for users, PIBWin can read and convert to the Excel file format identification matrices and archive files used with earlier software versions (Bryant, 1991a; Bryant, 1995). PIBWin can also read and convert identification matrices created in a comma separated value (CSV) format. Users sometimes update an identification matrix supplied with probabilistic identification software by adding newly identified strains or modifying the probabilities for strains already in that matrix following the publication of new data. To accommodate this, the GBEST tool in PIBWin enables the user to determine which are the most important tests in a matrix and, using the IDSC tool, whether the identification matrix is capable of identifying each taxon (strain) that is contained within it. This latter procedure considers each taxon in turn; it uses each percentage probability for that taxon as a positive or negative result, creating a HMO. It then uses this HMO to calculate an Identification Score using Willcox probability values.

7.2.2 Creation of the probability matrix

The probability matrix VibEx7 was developed by selecting tests with high Gyllenberg S_i values for all phenons in the data set of all 98 tests. Where phenons were not well separated high scoring tests specific for those phenons were selected and substituted for less well scoring tests. The final matrix contains 46 tests based on 36 main miniaturised tests, three supplementary tests, three housekeeping tests for glucose fermentation, glucose carbon source utilisation test and negative control, five antibiotic sensitivity tests and two macro format tests: oxidase and agarolysis. The tests for the probability matrix are listed in Table 7.22. Data for probability matrix VibEx7 is given in Appendix 4, Table A4.5. Gyllenberg S_i values are given in Table 7.23. Tests which did not have value globally were the reference tests for lysine and ornithine decarboxylase, resistance to 0/129 150µg, oxidase and urease. Similarly, carbon source utilisation tests for hydroxybutyrate and glycerol had no overall separation value but did have importance in separating some species. The value of these tests in differentiating

Table 7.22 Panel of tests for the probabilistic identification of *Vibrionaceae*

Miniaturised Tests		
Test	**Test**	**Test**
Arginine dihydrolase	IXP alkaline phosphatase	Utilisation: Glucosamine
Acid: Arbutin	PNPG α-D-galactosidase	Glucuronate
Mannitol	LGN γ-glutamyl transpeptidase	Glycerol
Salicin	NPS sulphatase	Histidine
Sucrose	Aesculin hydrolysis	DL-3-hydroxybutyrate
Gentiobiose	Utilisation: α-ketoglutarate	Hydroxyproline
Growth: 7% NaCl	Acetate	DL-lactate
10% NaCl	Alanine	Lactose
Amylase	Citrate	Propionate
Voges Proskauer (Acetoin)	Citrulline	Putrescine
Gelatinase	Galactose	Succinate
Indole	Gluconate	Sucrose
Macro-format tests	**Sensitivity tests**	**Supplementary tests**
Oxidase	Resistance: 0/129 10µg	Lysine decarboxylase
Agarolysis	0/129 150µg	Ornithine decarboxylase
	Ampicillin 10µg	Urease
	Novobiocin 5µg	
	Carbenicillin 100µg	

Table 7.23 Ranked tests in order of decreasing resolving power for species of the probability matrix VibEx7.

Test	S_i	Test	S_i
Arginine dihydrolase	1673	Glucuronate csu	6
Sucrose fermentation	1490	Resistance: Ampicillin 10µg	5
PNPG α-D-galactosidase	1145	DL-lactate csu	5
α-ketoglutarate csu	843	Gelatinase	4
Indole	536	Sucrose csu	4
Histidine csu	380	Propionate csu	3
Resistance: Carbenicillin 100µg	270	Gentiobiose fermentation	3
NPS sulphatase	213	Acetate csu	2
Voges Proskauer (Acetoin)	172	Lactose csu	2
Gluconate	121	Succinate csu	2
Salicin fermentation	94	Amylase	1
Putrescine csu	70	Alanine csu	1
Citrate csu	58	Resistance: 0/129 10µg	0
Aesculin hydrolysis	45	Resistance: Novobiocin 5µg	0
LGN γ-glutamyl transpeptidase	32	Growth 10% NaCl	0
Galactose csu	25	Agarolysis	N
Arbutin fermentation	24	DL-3-hydroxybutyrate csu	N
Mannitol fermentation	17	Glycerol csu	N
Hydroxyproline csu	16	Lysine decarboxylase	N
Glucosamine csu	12	Ornithine decarboxylase	N
IXP alkaline phosphatase	10	Urease	N
Growth 7% NaCl	9	Oxidase	N
Citrulline csu	8	Resistance: 0/129 150µg	N

N: no global separating value

specific taxa are given in Table 7.24 together with S_i values for oxidase reaction and agarolysis for *V. metschnikovii* and *V. agarivorans,* respectively. The singularity of these characteristics in the *Vibrionaceae* makes them significant for these species but of little

differentiating value for other species. Resistance to 0/129 150µg has no value in separating species but is retained as a useful marker of the *Vibrionaceae*.

Table 7.24 Tests of low overall resolving power but useful in separating individual species

Taxon	Phenon	Test	S*i* value[1]
Ph. damselae ssp. *damselae*	63	Urease	74
V. nereis	7	Hydroxybutyrate utilisation	69
V. harveyi biovar II	32	Lysine decarboxylase	63
V. cholerae	33	Ornithine decarboxylase	77
V. scophthalmi	54	Glycerol utilisation	73
V. agarivorans	82	Agarolysis	84
V. metschnikovii	65	Oxidase	84

[1]S*i* value determined for tests that distinguish individual species

7.2.3 Evaluation of the probability matrix

Defining the identification matrix

The resolving power of the matrix was assessed initially using the IDSC tool in PIBWin to calculate HMOs for each taxon and to compute an identification score for the corresponding phenon. Several iterations were made of the matrix with different combinations of test to achieve Willcox probability values of ≥0.99 for each taxon. Of the 86 taxa, 75% had identification scores of 1.0000; those with a lower score are listed in Table 7.25.

Table 7.25 Willcox probability scores for cluster HMOs with scores less than 1.0000

Taxon	ID Score	Taxon	ID Score
Phenon 20	0.99999	Phenon 45	0.99989
Phenon 6	0.99998	*V. calviensis*	0.99989
Phenon 83	0.99998	*V. parahaemolyticus*	0.99982
Phenon 58	0.99997	*V. chagasii*	0.99941
V. aestuarianus	0.99997	Phenon 10	0.99870
V. pelagius biovar *II*	0.99997	*V. alginolyticus*	0.99867
V. scophthalmi	0.99997	*V. ichthyoenteri* biovar I	0.99852
V. rumoiensis	0.99996	Phenon 27	0.99819
Phenon 29	0.99995	*M. viscosa*	0.99810
V. nereis	0.99993	*V. cyclitrophicus*	0.99351
Phenon 43	0.99991	Phenon 26	0.99159

The data from this analysis indicates that each phenon is discrete for the identification panel. Since the *P* scores for all HMOs is greater than 0.99, this was used as the threshold identification score to accept an identification of an unknown.

The distribution of tests in respect of individual Separation value S_t is given in Table 7.26 and by dot-plot in Figure 7.19. The S_{MAX} for an individual test was calculated as 1849. Most tests fell within the range of 20-60% of S_{MAX} with only a few tests lying between 0 and 20% or in excess of 60%.

Table 7.26 Separation values S_t of individual tests as a percentage of S_{MAX} for the identification matrix VibEx7

Test	S_t	$\%S_{MAX}$		S_t	$\%S_{MAX}$
Arginine dihydrolase	1462	79%	Resistance: Ampicillin 10µg	624	34%
Sucrose fermentation	1258	68%	Gelatinase	624	34%
Indole	1216	66%	Gentiobiose fermentation	610	33%
Alanine csu	1081	58%	Resistance: Novobiocin 5µg	540	29%
Sucrose csu	1073	58%	Resistance: 0/129 10µg	520	28%
α-ketoglutarate csu	1023	55%	Ornithine decarboxylase	511	28%
PNPG α-D-galactosidase	1015	55%	Urease	504	27%
Histidine csu	980	53%	Growth 10% NaCl	496	27%
Mannitol fermentation	980	53%	Arbutin fermentation	490	27%
Propionate csu	980	53%	Growth 7% NaCl	480	26%
Aesculin hydrolysis	936	51%	Lactose csu	476	26%
Voges Proskauer (Acetoin)	896	48%	Hydroxybutyrate csu	448	24%
Gluconate csu	880	48%	Hydroxyproline csu	426	23%
LGN γ-glutamyl transpeptidase	841	45%	Glucuronate csu	420	23%
Galactose csu	840	45%	Glycerol csu	414	22%
Putrescine csu	832	45%	Citrulline csu	406	22%
Citrate csu	826	45%	IXP alkaline phosphatase	392	21%
Lysine decarboxylase	798	43%	Glucosamine csu	378	20%
Resistance: Carbenicillin 100µg	774	42%	Acetate csu	357	19%
NPS sulphatase	731	40%	Succinate csu	264	14%
Amylase	731	40%	Oxidase	166	9%
Lactate csu	696	38%	Agarolysis	84	5%
Salicin fermentation	672	36%	Resistance: 0/129 150µg	0	0%

S_t: Separation value of an individual test

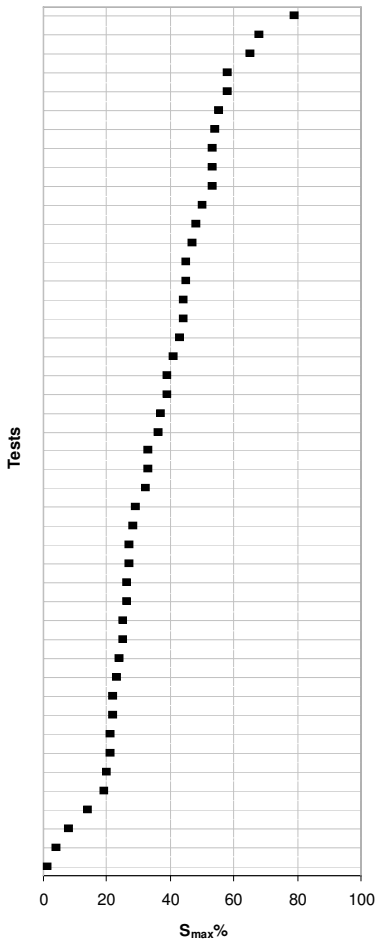

Figure 7.19

Dot-plot of separation values for individual tests S_t plotted against S_{MAX}.% for tests in the VibEx7 identification panel.

Cluster overlap

An assessment of cluster overlap was made with the critical overlap value V_O set at 5% and a confidence level of p=0.95 (Sneath 1980). Overlaps exceeding this level were found for four species in five taxon pairs listed in Table 7.27. In each case one of the taxa in the pair comprised only three strains and F, the effective degrees of freedom for a pair-wise comparison, was less than 4. Where F values are less than 4, estimates of

Table 7.27 Clusters with an overlap exceeding a critical value V_O of 5%

Taxon	No. strains	Taxon	No. strains	V_G index of overlap	F degrees c freedom
Taxon pair					
V. rumoiensis	3	*V. splendidus* biovar I	38	9.4%	2.2
V. rumoiensis	3	*V. anguillarum*	60	16.3%	2.1
V. aestuarianus	3	*V. anguillarum*	60	9.7%	2.2
V. cincinnatiensis	3	*V. anguillarum*	60	8.1%	2.1

overlap are considered inaccurate (On *et al.*, 1996) and usually occurs where a taxon contains less than four strains. Excluding these results, there was no overlap between clusters at the critical value of 5%. Standard deviations for individual taxa, ranged from 0.1407 for the single strain phenons to 0.3726 for Phenon 52. Phenons with standard deviations <0.3 are considered homogeneous and represent dense compact clusters while phenons with standard deviations >0.3 are heterogeneous and have a diffuse composition of strains with greater amounts of phenotypic variation (Priest & Alexander, 1988). Phenons of greater phenotypic variance are listed in Table 7.28.

Table 7.28 Phenons with cluster overlap standard deviations >0.3

Phenon	SD	Phenon	SD
V. natriegens	0.3006	*V. metschnikovii*	0.3381
Phenon 43	0.3113	*Ph. leiognathi*	0.3390
V. halioticoli	0.3122	*V. fischeri* bv I	0.3421
Phenon 36	0.3152	*A. sobria* HG7	0.3486
Phenon 45	0.3178	*V. lentus*	0.3499
Phenon 69	0.3246	*V. cincinnatiensis*	0.3565
Phenon 20	0.3254	*V. rumoiensis*	0.3632
V. calviensis	0.3355	Phenon 52	0.3726

Cluster separation

An assessment of phenon separation was made by cluster analysis of the probability matrix. A phenogram of the clusters (Figure 7.20) shows the similarity of phenons based on proximity to adjacent phenons as well as the effective level of separation determined by the depth of the branches before fusion. In nearly all cases phenons are well differentiated, shown by the extent of branch depth. Biovars of *Ph. damselae* ssp. *damselae*, *V. fischeri, V. vulnificus* and *V. pelagius* occur at adjacent locations indicating

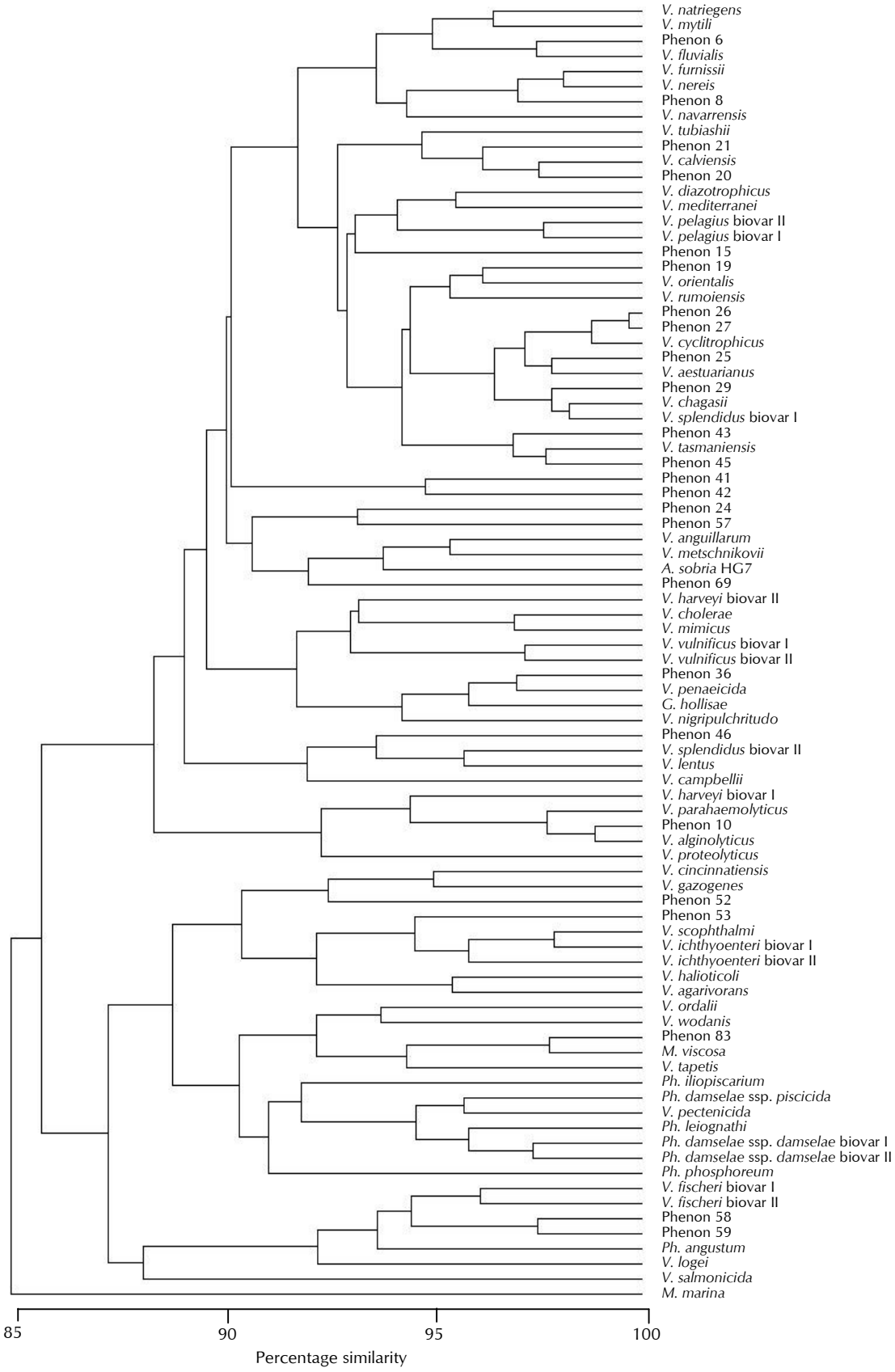

Figure 7.20 Phenogram of phenons based on the probability matrix VibEx7

overall phenotypic similarity but with sufficient difference that biovars can be distinguished. The relatedness of the biovars for *V. splendidus*, *V. harveyi* and

V. ichthyoenteri is less evident since their respective biovars do not appear adjacent to each other but occur in other well separated clusters. *Vibrio gazogenes* and *V. cincinnatiensis*, which had formed a single undifferentiated cluster as Phenon 67, appear well separated with deep branches indicating that there is sufficient phenotypic difference in the probability matrix to distinguish the two species. In contrast the branching of Phenon 10 and *V. alginolyticus* is shallow indicating that there is little phenotypic difference between these two taxa. Differences between Phenon 26 and 27 are even less pronounced and identification may be difficult to achieve where even minor variation in strain phenotype occurs.

Identification scores for cluster members based on phenotyping data

Phenotypes matching the tests in the VibEx7 probability matrix were extracted from the taxonomic profiles for 792 strains in the library. Identities, identification scores and modal likelihood scores were obtained with PIBWin and VibEx7 to assess the predictive quality of the matrix. Analysis of only the identification scores (Table 7.29) found that

Table 7.29 Willcox probability scores *P* for strains (n=792) of the taxonomic library identified using the VibEx7 probability matrix

ID Score Range	Frequency	%	Cumulative %
1.000-0.999	677	85%	85%
0.990-0.998	63	8%	93%
0.98-0.989	14	2%	95%
0.950-0.979	13	2%	97%
0.900-0.949	8	1%	98%
0.800-0.899	7	1%	99%
<0.79	10	1%	100%
Total	**792**	**100%**	

85% of strains had Willcox probability scores ≥0.999 while 93% of strains had scores ≥0.99. Using a threshold value of 0.01 for modal likelihood scores to indicate that strain variation is within acceptable limits, 70% of strains met or exceeded this value and at the less stringent threshold of 0.001, 87% of strains met this criterion, Table 7.30.

Table 7.30 Modal Likelihood scores for strains (n=792) of the taxonomic library identified using the VibEx7 probability matrix

Modal Likelihood Score Range	Frequency	%	Cumulative
1.00-0.1	333	42%	42%
0.09-0.01	219	28%	70%
0.009-0.001	136	17%	87%
<0.0009	104	13%	100%
Total	**792**	**100%**	

An assessment was made of the identifications reached by matching these to the parent phenon defined by cluster analysis. On the basis solely of an identification reached, 98.6% of strains were correctly identified and only 1.4% incorrectly identified (Table 7.31). Using a value of *P*=0.99 as the identification threshold then 93.2% of the strains were correctly identified and 0.3% incorrectly identified. The two strains incorrectly identified at *P*≥0.99 were V15, *Vibrio* sp. that clustered in Phenon 15 and identified as Phenon 27 (identification score, 0.999; modal likelihood score, 0.030) and V157 that clustered in *V. anguillarum*, Phenon 56 and identified as Phenon 8 (identification score, 0.991; modal likelihood score 0.000).

Table 7.31 Concordance of identification of strains by the probability matrix VibEx7 and their allocation to phenons by cluster analysis

	Correct phenon		Incorrect phenon	
ID Score Range	**Frequency**	**%**	**Frequency**	**%**
1.000-0.999	677	85.5%	0	0.0%
0.990-0.998	61	7.7%	2	0.3%
0.980-0.989	13	1.6%	1	0.1%
0.950-0.979	13	1.6%	0	0.0%
0.900-0.949	7	0.9%	1	0.1%
0.800-0.899	7	0.9%	0	0.0%
<0.79	3	0.4%	7	0.9%
Total	**781**	**98.6%**	**11**	**1.4%**

A more stringent analysis was undertaken of the identification results and statistics by considering identification scores and their corresponding modal likelihood scores. This provides a more realistic assessment of an identification reached as it takes into account both the identification score as well as modal likelihood score, the statistic used to assess the amount of deviation of the observed from expected phenotype. For identification scores ≥0.99 and a stringent level for the modal likelihood score of 0.01, 65% of the strains were correctly identified; using a less stringent level of ≥0.001, 77% of the library were correctly identified.

Phenotype definitions of clusters appeared well circumscribed. Typically, all members of a cluster had identification scores ≥0.99. For some clusters there appeared to be greater phenotypic variation with a corresponding reduction in the proportion of strains with identification scores ≥0.99. These taxa are listed in Table 7.32.

Practical identification scores based on re-test phenotyping data

The phenotype data generated for approximately 10% of the library as part of test error assessment was used with VibEx7 to determine the ability of the matrix to correctly identify strains characterised *de novo*. For the re-phenotyped subset there was

Table 7.32 Phenons containing strains with identification scores less than *P*=0.99

Taxon	Identification scores		
	***P*≥0.99**	***P*=0.980-0.989**	***P*<0.98**
V. harveyi biovar I	95%	3%	2%
V. anguillarum	95%	0%	5%
Ph. damselae ssp. *damselae* biovar *I*	93%	4%	3%
Phenon 59	93%	7%	0%
V. chagasii	90%	5%	5%
V. furnissii	90%	0%	10%
Phenon 29	88%	12%	0%
Phenon 26	88%	0%	12%
V. alginolyticus	87%	7%	6%
Phenon 83	86%	14%	0%
Phenon 25	86%	0%	14%
V. cyclitrophicus	86%	0%	14%
Ph. damselae ssp. *piscicida*	86%	0%	14%
V. nereis	83%	17%	0%
Phenon 20	83%	0%	17%
Phenon 36	83%	0%	17%
Phenon 43	82%	0%	18%
V. splendidus biovar I	79%	11%	11%
V. tasmaniensis	75%	0%	25%
V. ichthyoenteri biovar I	75%	0%	25%
Phenon 27	71%	12%	18%
Phenon 10	67%	0%	33%
Ph. damselae ssp. *damselae* biovar II	57%	14%	29%

a reduced level of performance compared to results obtained for the same strains tested in the phenotyping study. No corresponding strains from the phenotyping study were misidentified but for the re-phenotyped subset, 7.7% were allocated incorrectly. Identification error appeared to be random and not related to a specific phenon. Identification statistics were noticeably poorer for the re-phenotyped subset than that for corresponding strains from the phenotyping study (Table 7.33). A major difference

Table 7.33 Identification scores and modal likelihood scores using VibEx7 for a re-phenotyped subset of isolates.

Identification scores	Data from re-test study[1]	Corresponding data from phenotyping study[2]
ID scores P ≥ 0.99	77%	91%
Modal likelihood scores ≥ 0.01	33%	60%
Modal likelihood scores ≥ 0.001	56%	71%
ID score P ≥ 0.99 + modal score ≥ 0.01	33%	59%
ID score P ≥ 0.99 + modal score ≥ 0.001	49%	74%

[1]*n*=91; [2]data for strains corresponding to those from re-test study

in the data sets relates to modal likelihood scores which for the subset are lower than that achieved in the phenotyping study. Lower modal likelihood scores will be the result of minor variations in test reactions, a reflection of individual test error.

Prospective testing of the matrix based on wild type isolates

Identification statistics for a library of 222 wild type strains of *Vibrionaceae* from aquatic animals compared favourably with those obtained for both the subset and phenotyping libraries (Table 7.34). Although the frequency of identification scores was

Table 7.34 Identification statistics for a library of wild type isolates of *Vibrio* from aquatic animals.

Identification scores	Data from wild type isolates[1]	Data from phenotyping study[2]
ID scores P ≥ 0.99	79%	92%
Modal likelihood scores ≥ 0.01	57%	68%
Modal likelihood scores ≥ 0.001	70%	81%
ID score P ≥ 0.99 + modal score ≥ 0.01	52%	65%
ID score P ≥ 0.99 + modal score ≥ 0.001	63%	77%

[1]n=222; [2]n=792

uniformly lower for the wild type data set compared to that of the phenotyping study, differences were not large. Performance of the phenotyping study inevitably will be high as the identification data is to some extent self-referential as it is based on the phenotyping data used to create the identification matrix.

7.2.4 Panel of tests for the identification of *Vibrionaceae*

A panel of tests, MicroSys V36, based on the VibEx7 probability matrix for the identification of *Vibrionaceae* was developed using the miniaturised tests as described, Table 7.22. The layout of tests in micro-well format is shown in Figures 7.21a-c. The main panel of tests is across three rows and has been arranged for ease of set up. Arginine dihydrolase and the fermentation tests are grouped together as they require an oil overlay after inoculation. The last four tests in the row, starch hydrolysis, acetoin, gelatin hydrolysis and indole, require the addition of reagents following incubation. An empty well in the fourth row is used for the gelatin hydrolysis reaction using centrifuged supernatant and acidified bovine serum albumin. The fourth row has the supplementary tests for lysine and ornithine decarboxylase and urease as well as three housekeeping tests: glucose fermentation, glucose utilisation and a negative control for the carbon source utilisation medium. Typical appearance of tests following incubation at 48 hours is shown in Figures 7.22a and 7.22b.

7.2.5 Quality control of identification panel V36

Batch-to-batch variation was small and that which was seen appeared to be more a function of species instability rather than medium variation. Test error *p*, the pooled test variation for the five batches of media, was 1.9%. For individual species test error

Identification Tests

Arginine dihydrolase	Arbutin acid	Mannitol acid	Salicin acid	Sucrose acid	Gentiobiose acid	7% NaCl tolerance	10% NaCl tolerance	Starch hydrolysis	Acetoin	Gelatin hydrolysis	Indole
IXP[1]	PNPG[2]	LGN[3]	NPS[4]	Aesculin hydrolysis	α-ketoglutarate csu[5]	Acetate csu	Alanine csu	Citrate csu	Citrulline csu	Galactose csu	Gluconate csu
Glucosamine csu	Glucuronate csu	Glycerol csu	Histidine csu	Hydroxybutyrate csu	Hydroxyproline csu	Lactate csu	Lactose csu	Propionate csu	Putrescine csu	Succinate csu	Sucrose csu

Supplementary Tests **Housekeeping Tests**

Lysine decarboxylase	Ornithine decarboxylase	Urease	Glucose acid	Glucose csu	Control csu

[1]IXP: indoxyl phosphate; [2]PNPG: 2-nitrophenyl β-D-galactopyranoside; [3]LGN: L-glutamic acid 5-(4-nitroanilide); [4]NPS: 4-nitrophenyl sulfate; [5]csu: carbon source utilisation

Figure 7.21a MicroSys V36 panel of miniaturised tests for the identification of *Vibrionaceae* using the VibEx7 probability matrix

Figure 7.21b Detail of uninoculated miniaturised tests for arginine dihydrolase, sugar fermentation and hydrolysis of chromogenic substrates

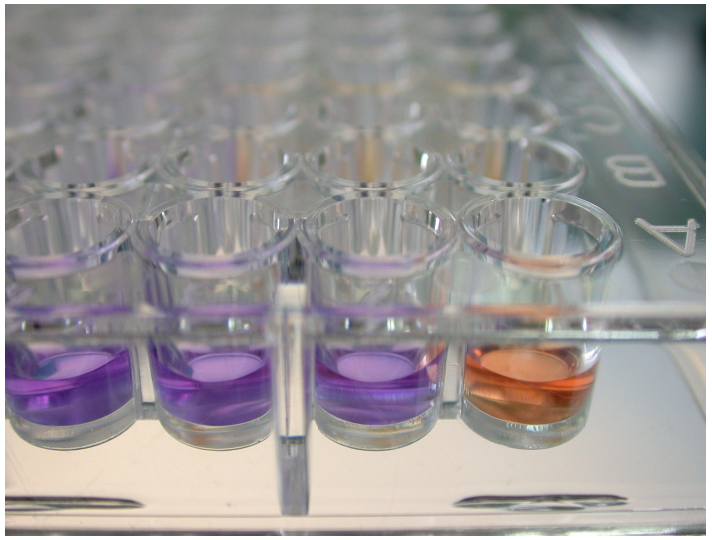

Figure 7.21c Detail of uninoculated miniaturised tests for arginine dihydrolase and sugar fermentation

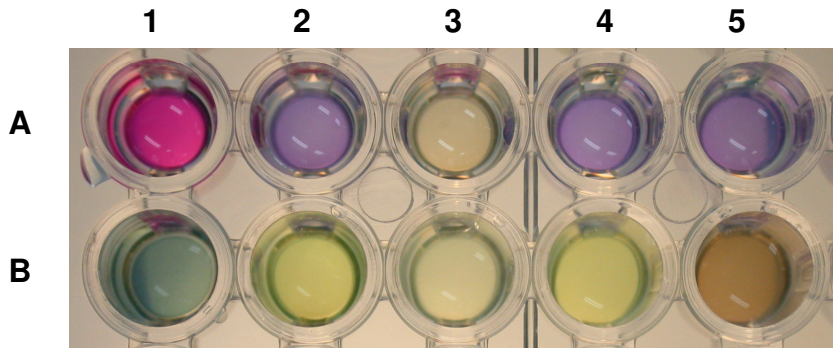

Figure 7.22a Miniaturised tests for the identification of *Vibrionaceae*

A1: Arginine dihydrolase - positive
A2: Fermentation of arbutin - negative
A3: Fermentation of mannitol - positive
A4: Fermentation of salicin - negative
A5: Fermentation of sucrose - negative
B1: Alkaline phosphatase - positive
B2: α galactosidase - positive
B3: Prolyl aminopeptidase - negative
B4: Sulphatase - positive
B5: Aesculin hydrolysis - weak positive

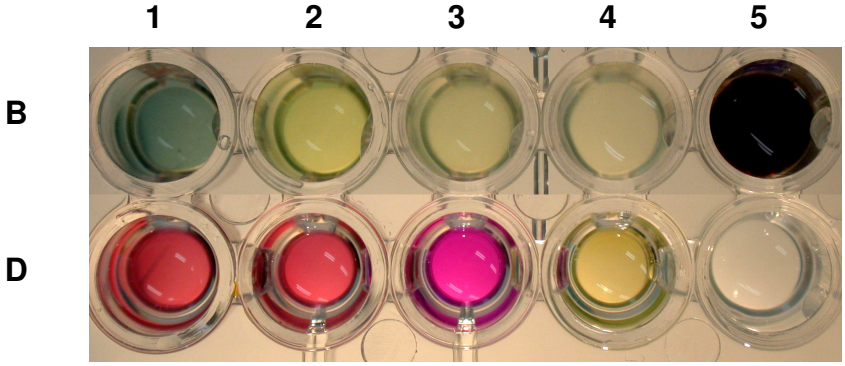

Figure 7.22b Miniaturised tests for the identification of *Vibrionaceae*. Composite picture with carbon source utilisation tests in row C omitted for clarity. Well D5 is a test for glucose utilisation; the test reaction as turbidity is not discernible in this picture.

B1: Alkaline phosphatase - positive
B2: α galactosidase - positive
B3: Prolyl aminopeptidase - weak positive
B4: Sulphatase - negative
B5: Aesculin hydrolysis - positive
D1: Lysine decarboxylase - positive
D2: Ornithine decarboxylase - positive
D3: Urease - positive
D4: Glucose fermentation - positive
D5: Glucose csu

ranged from 1.0% for *V. mediterranei* to 2.9% for *V. fluvialis*, see Table 7.35. All strains at each time interval were correctly identified with Willcox probability scores of $P \geq 0.99$ and modal scores all ≥0.001.

Table 7.35 Test error for five batches of media for the MicroSys V36 identification panel

Species	Test error *p*
Vibrio mediterranei CIP 103203^T	1.0%
Vibrio parahaemolyticus ATCC 17802^T	1.2%
Vibrio tubiashii NCIMB 1340^T	1.7%
Vibrio anguillarum ATCC 19264^T	2.7%
Vibrio fluvialis NCTC 11327^T	2.9%

The expected reactions for the five quality control strains appeared stable and are listed in Table 7.36. Most tests gave consistent results but some variation occurred within species; tests with variable results are listed in Table 7.37; there was no variation in the remaining tests.

Table 7.36 Expected test reactions for quality control of MicroSys V36 identification media

Well no.	Test	*V. anguillarum* ATCC 19264^T	*V. fluvialis* NCTC 11327^T	*V. mediterranei* CIP 103203^T	*V. parahaemolyticus* ATCC 17802^T	*V. tubiashii* NCIMB 1340^T
A1	Arginine dihydrolase	+	+	+	-	+
A2	Acid: Arbutin	-	+	-	-	-
A3	Mannitol	+	+	+	+	+
A4	Salicin	-	+	+	-	+
A5	Sucrose	+	+	+	-	+
A6	Gentiobiose	-	-	-	-	+
A7	Growth: 7% NaCl	+	+	+	+	+
A8	10% NaCl	-	+	-	+	-
A9	Amylase	+	+	+	+	+
A10	Voges Proskauer (Acetoin)	+	-	-	-	-
A11	Gelatinase	+	+	-	+	+
A12	Indole	+	+	+	+	+
B1	IXP alkaline phosphatase	+	+	+	+	+
B2	PNPG α-D-galactosidase	-	-	+	-	+
B3	LGN γ-glutamyl transpeptidase	+	+	+	+	+
B4	NPS sulphatase	-	-	+	-	-
B5	Aesculin hydrolysis	-	+	+	-	+
B6	Utilisation: α-ketoglutarate	-	+	+	+	-
B7	Acetate	+	+	+	+	+
B8	Alanine	+	+	+	+	+
B9	Citrate	-	+	+	+	+
B10	Citrulline	-	+	-	-	+
B11	Galactose	-	+	+	+	+
B12	Gluconate	+	+	-	+	+
C1	Glucosamine	+	+	+	+	+
C2	Glucuronate	-	+	+	-	+
C3	Glycerol	+	+	+	+	+
C4	Histidine	+	+	+	+	+
C5	DL-3-hydroxybutyrate	-	-	-	-	+
C6	Hydroxyproline	-	-	-	+	+
C7	DL-lactate	+	+	+	+	+
C8	Lactose	-	-	+	-	+
C9	Propionate	-	+	+	+	+
C10	Putrescine	-	-	+	+	-
C11	Succinate	+	+	+	+	+
C12	Sucrose	+	+	+	-	+
D1	Lysine decarboxylase	-	-	-	+	-
D2	Ornithine decarboxylase	-	-	-	+	-
D3	Urease	-	-	-	+	-
D4	Acid: Glucose	+	+	+	+	+
D5	Utilisation: Glucose	+	+	+	+	+
D6	Control	-	-	-	-	-

Table 7.37 Tests with variable reactions for individual species of quality control strains

Species	Test	No. tests variant *n*=5
V. anguillarum ATCC 19264^T	LGN: γ-glutamyl transpeptidase	1
	DL-lactate csu	1
	L-citrulline csu	1
	Propionate csu	1
	Aesculin hydrolysis	2
	D-galactose csu	2
	Citrate csu	3
V. fluvialis NCTC 11327^T	LGN: γ-glutamyl transpeptidase	1
	NPS: sulphatase	1
	L-hydroxyproline csu	1
	DL-lactate csu	1
	Indole	2
	L-citrulline csu	3
	Hydroxybutyrate csu	3
V. mediterranei CIP 103203^T	Gentiobiose acid	1
	Lysine decarboxylase	3
V. parahaemolyticus ATCC 17802^T	10% NaCl tolerance	1
	NPS: sulphatase	1
	D-galactose csu	1
	L-hydroxyproline csu	2
V. tubiashii NCIMB 1340^T	L-citrulline csu	1
	Hydroxybutyrate csu	1
	DL-lactate csu	1
	7% NaCl tolerance	2
	IXP: indoxyl phosphate	2

7.2.6 Stability of freeze-dried phenotyping panels

Performance of freeze-dried identification panels was assessed by changes in test reaction over time and the ability of the panels to correctly identify the four test strains: *V. furnissii* ATCC 35016^T, *V. metschnikovii* NCTC 8443^T, *V. mimicus* ATCC 33653^T and *V. wodanis* NCIMB 13582^T. There was no evidence of systematic test failure over the five months of testing but test failure associated with specific species was apparent. For the tests listed in Table 7.38, positive reactions occurred one month after freeze-drying but were consistently negative over the remaining four months; there was no evidence

Table 7.38 Tests that consistently failed two months after freeze-drying

V. furnissii	*V. metschnikovii*	*V. mimicus*	*V. wodanis*
1-propanol csu[1]	7% NaCl tolerance	Bile tolerance	β-galactosidase
DL-malate csu	$CoCl_2$ tolerance	D-glucuronate csu	
Oxaloacetate csu	DL-malate csu		
Propionate csu	D-mannose csu		
	Acid phosphatase		

[1]csu: carbon source utilisation test

of false positive reactions in any of the tests over the evaluation period. Test error *p* (Sneath & Johnson, 1972) for pooled data for all four species over the five-month test period was 3.5%. Variable error rates were found for each species over the test period and ranged from 2.9% for *V. wodanis* to 4.1% for *V. furnissii*. For a subset of 39 tests for

MicroSys V36, the pooled error was 3.5% and ranged from 2.6% for *V. metschnikovii* to 4.01% for both *V. wodanis* and *V. mimicus*.

The practical effect of test variation over the evaluation period was assessed by probabilistic identification using the VibEx7 matrix. At each time interval, the species were correctly identified with Willcox probability scores of ≥0.99 except for *V. wodanis* where at the three and five month intervals the identification scores were less than 0.99; Table 7.39. The effect over time on modal likelihood scores, the statistic for

Table 7.39 Performance of freeze-dried culture media over time determined by identification outcome.

Species	Month	VibEx6 ID	ID score	ID modal	ID threshold	ID threshold %
V. mimicus	1	*V. mimicus*	1.00000	0.02471	Y	
ATCC 33653^T	2	*V. mimicus*	1.00000	0.00153	Y	
	3	*V. mimicus*	0.99999	0.00000	N	60%
	4	*V. mimicus*	1.00000	0.01864	Y	
	5	*V. mimicus*	0.99999	0.00000	N	
V. furnissii	1	*V. furnissii*	1.00000	0.42857	Y	
ATCC 35016^T	2	*V. furnissii*	0.99883	0.00146	Y	
	3	*V. furnissii*	0.99990	0.00000	N	80%
	4	*V. furnissii*	0.99877	0.00340	Y	
	5	*V. furnissii*	0.99764	0.00433	Y	
V. wodanis	1	*V. wodanis*	1.00000	1.00000	Y	
NCIMB 13582^T	2	*V. wodanis*	1.00000	0.00010	N	
	3	*V. wodanis*	0.94947	0.00000	N	20%
	4	*V. wodanis*	1.00000	0.00010	N	
	5	*V. lentus*	0.77278	0.00000	N	
V. metschnikovii	1	*V. metschnikovii*	1.00000	0.03292	Y	
NCTC 8443^T	2	*V. metschnikovii*	1.00000	0.29630	Y	
	3	*V. metschnikovii*	1.00000	0.19753	Y	100%
	4	*V. metschnikovii*	1.00000	0.00299	Y	
	5	*V. metschnikovii*	1.00000	0.29630	Y	

estimating the deviation in phenotype, was variable for the species tested. For *V. metschnikovii*, modal likelihood scores at each time interval were ≥0.001 but for *V. wodanis* an acceptable modal likelihood score was achieved only after the first month but not subsequently indicating that changes over time had occurred in the media, sufficient to have eroded the ability to identify *V. wodanis* with certainty.

7.2.7 Inoculum effects on identification outcome

Effect of pre-inoculum medium on phenotype

Despite some apparent variation in test reactivity associated with types of pre-inoculum media, there was little difference in phenotype when using either ZMA or JMA to grow the inoculum but for SBA2 differences were more pronounced. Test variation, measured against a reference profile of 96 tests generated with an inoculum prepared on JMA, is summarised in Table 7.40.

Table 7.40 Test variation using three different media for generating inocula

Species	SBA2	JMA	ZMA	No. tests[1]
	No. tests at variance to reference profile on JMA			
M. viscosa NCIMB 13584^T	18	12	12	864
V. anguillarum NCIMB 6^T	10	10	9	864
V. harveyi ATCC 14126^T	26	23	16	864
V. lentus CECT 5110^T	18	13	25	864
Total no. tests at variance	72	58	62	
No. tests positive	65	75	71	
Test error *p* 96 panel of tests	4.02%	2.18%	2.81%	
Test error *p* VibEx7 panel of tests	4.36%	3.15%	3.26%	

[1]Testing in triplicate

Test variation was smallest with JMA with the biggest difference noted when inocula were prepared on SBA2. An effect of using SBA2 to generate the inoculum was to reduce subsequent test reactivity compared with either JMA or ZMA, though differences between the latter two media were marginal. When test error was calculated (Sneath & Johnson, 1972) for the panel of 96 tests, *p* was less than 5%, the threshold above which test variation is considered unacceptably high. Test error *p* was greater when only tests matching the VibEx7 identification matrix were considered. For JMA and ZMA test error were similar, but for SBA2 the error was appreciably higher indicating that test outcome may be less reliable when this medium is used as the basis for generating the inoculum. The practical effect on identification of test differences

Table 7.41 Willcox probability scores *P* for identifications reached with VibEx7 probability matrix for different pre-inoculum media

Medium	Replicate	*M. viscosa* NCIMB^T	*V. anguillarum* NCIMB 6^T	*V. harveyi* ATCC 14126^T	*V. lentus* CECT 5110^T
JMA	1	0.99999	0.99992	0.99995	0.99049
	2	0.99814	0.99972	0.99426	0.99970
	3	0.99814	0.99974	0.99982	**0.71081**
ZMA	1	1.00000	0.99992	0.99974	**0.87943**
	2	0.99814	0.99991	0.99685	**0.66709**
	3	0.99986	0.99990	0.99999	0.99999
BA+2% NaCl	1	1.00000	0.99969	0.99974	0.99940
	2	1.00000	0.99989	0.99865	**0.85615**
	3	0.99841	0.99983	0.99999	**0.92259**

P≤0.99 in red

associated with pre-inoculum medium was variable, Table 7.41. For the four species, the correct identification was reached in all cases but the identification scores were in some instances less than the threshold of *P*≥0.99. The type of medium did not appear to affect identification of *M. viscosa* and *V. anguillarum* but for both *V. lentus* and *V. harveyi*, identification was less reliable when the inoculum had been generated on either ZMA or SBA2. Where identification scores were <0.99 however, the most likely

identification was consistent with the known identity of the species indicating that test variation was not sufficient to cause misallocation to an incorrect taxon.

Effect of inoculum size on phenotype

Test performance appeared to be a function of inoculum size with fewer tests positive as test inoculum was diluted. Comparing test performance of a standard inoculum with diluted inocula, found that test difference increased as inocula became more dilute, irrespective of medium used to generate the inocula; Table 7.42. The effect of increasing inoculum dilution was to reduce test reactivity. The medium least affected by dilute inocula was JMA while differences were greatest when SBA2 was used to generate the inoculum.

Table 7.42 Effect of inoculum density and origin of inoculum on test performance compared to a standard inoculum dilution of 1:5

Inoculum dilution	**1:100**	**1:1,000**	**1:10,000**	**Total no. tests different by medium**
	No. tests different by dilution			
Medium		SBA2		
V. lentus CECT 5110^T	3	15	16	
V. anguillarum NCIMB 6^T	1	9	11	**91**
V. harveyi ATCC 14126^T	12	12	12	
Medium		ZMA		
V. lentus CECT 5110^T	9	12	18	
V. anguillarum NCIMB 6^T	5	8	11	**76**
V. harveyi ATCC 14126^T	3	4	6	
Medium		JMA		
V. lentus CECT 5110^T	9	9	11	
V. anguillarum NCIMB 6^T	5	5	7	**60**
V. harveyi ATCC 14126^T	5	4	5	
Total no. tests different by dilution	**52**	**78**	**97**	

Tests found to be susceptible to inoculum dilution effects are listed in Table 7.43 and tended to be species related rather than inoculum dilution or medium used for generating the inoculum. Most of the tests that failed with dilute inocula were those for sole carbon sources of which only succinate was common to all three species and appears the most susceptible to inoculum dilution. Failure of the hydrolysis tests using chromogenic substrates occurred almost exclusively with *V. anguillarum* but not for the other two species except for *V. harveyi* with the test for alkaline phosphatase.

Table 7.43 Tests found to give negative reactions when using dilute inocula in the range of 1:100 to 1:10,000

Species	Tests
V. lentus CECT 5110^T	α-ketoglutarate csu Alanine csu Citrate csu Glycerol csu Lactate csu Succinate csu
V. anguillarum NCIMB 6^T	Alkaline phosphatase (IXP) γ-glutamyl transpeptidase (LGN) α-D-galactosidase (PNPG) Succinate csu
V. harveyi ATCC 14126^T	Tolerance to 7% NaCl Alkaline phosphatase (IXP) Galactose csu Glucosamine csu Histidine csu Propionate csu Succinate csu

The practical effect of reduced inoculum concentration on identification outcomes was assessed using the VibEx7 probability matrix, Table 7.44. A correct identification was reached at all inoculum dilutions and on all media but at higher dilutions identification scores were less than *P*=0.99, an indication that test variation was unacceptably high. *Vibrio lentus* was more susceptible to low inoculum concentration effects than either *V. anguillarum* or *V. harveyi*. Medium effect on inocula was evident where SBA2 was used and was most noticeable with dilute inocula.

Table 7.44 Practical effect of inoculum concentration and source of inoculum on identification outcome using the probability identification matrix VibEx7.

Species		Identification	ID Score	Identification	ID Score	Identification	ID Score
Origin of inoculum		JMA		ZMA		SBA2	
V. lentus CECT 5110^T							
Inoculum	1:10	*V. lentus*	0.99669	*V. lentus*	0.99669	*V. lentus*	0.99902
	1:100	*V. lentus*	**0.98531**	*V. lentus*	0.99985	*V. lentus*	0.99427
	1:1,000	*V. lentus*	**0.82086**	*V. lentus*	**0.82086**	*V. lentus*	**0.82761**
	1,10,000	*V. lentus*	0.99260	*V. lentus*	**0.96293**	*V. lentus*	**0.82761**
V. anguillarum NCIMB 6^T							
Inoculum	1:10	*V. anguillarum*	0.99944	*V. anguillarum*	0.99998	*V. anguillarum*	0.99980
	1:100	*V. anguillarum*	0.99999	*V. anguillarum*	0.99982	*V. anguillarum*	0.99957
	1:1,000	*V. anguillarum*	0.99839	*V. anguillarum*	0.99839	*V. anguillarum*	**0.96512**
	1,10,000	*V. anguillarum*	0.99839	*V. anguillarum*	0.99839	*V. anguillarum*	0.99894
V. harveyi ATCC 14126^T							
Inoculum	1:10	*V. harveyi*	1.00000	*V. harveyi*	1.00000	*V. harveyi*	0.99998
	1:100	*V. harveyi*	1.00000	*V. harveyi*	1.00000	*V. harveyi*	0.99998
	1:1,000	*V. harveyi*	1.00000	*V. harveyi*	1.00000	*V. harveyi*	**0.97960**
	1,10,000	*V. harveyi*	1.00000	*V. harveyi*	0.99998	*V. harveyi*	0.99969

P≤0.99 in red

Effect of culture condition on phenotype

Culture vigour, measured as changes in phenotype of consecutive subcultures following recovery from a frozen stock, had little practical effect on identification outcome other than for *V. lentus*. For this species, identification and modal likelihood scores improved with consecutive subcultures; Table 7.45. Minor improvements in identification score were evident for *M. viscosa* and *V. ichthyoenteri* although in each case the identification scores *P* were in excess of 0.99 after the first subculture. The most noticeable effect of repeated subculture was the improvement in modal likelihood scores for most of the species, including *V. lentus,* indicating that the observed phenotype contained fewer outlier reactions and conformed more closely to the definition phenotype for the species.

Table 7.45 Effect of subculture on Willcox *P* and modal likelihood scores following revival from stock frozen at -80°C

Species	Subculture no.	Identification	ID score	Modal score
M. viscosa NCIMB 13584^T	1	*M. viscosa*	0.99994	0.00000
	2	*M. viscosa*	1.00000	0.00057
	3	*M. viscosa*	1.00000	0.05613
V. anguillarum NCIMB 6^T	1	*V. anguillarum*	1.00000	0.00019
	2	*V. anguillarum*	1.00000	0.00019
	3	*V. anguillarum*	1.00000	0.00871
V. halioticoli ATCC 700680^T	1	*V. halioticoli*	1.00000	0.00498
	2	*V. halioticoli*	1.00000	0.01010
	3	*V. halioticoli*	1.00000	0.00010
V. ichthyoenteri ATCC 700023^T	1	*V. ichthyoenteri*	0.99993	0.00035
	2	*V. ichthyoenteri*	0.99999	0.00068
	3	*V. ichthyoenteri*	0.99999	0.00037
V. lentus CECT 5110^T	1	*V. lentus*	**0.92869**	0.00000
	2	*V. lentus*	0.99615	0.00000
	3	*V. lentus*	0.99778	0.00034

P≤0.99 in red

Overall, repeated subculture resulted in a greater number of positive tests. The effect was variable across the species with the most changes occurring with *M. viscosa* while *V. ichthyoenteri* and *V. anguillarum* were the most stable and least affected of the species tested. There was no apparent trend for changes in the expression of phenotype with subculture other than sensitivity to 0/129 at 10 µg with both *M. viscosa* and *V. lentus* appearing sensitive on first subculture but resistant for subsequent subcultures; Table 7.46.

Table 7.46 Tests with negative reactions after first subculture but positive after subsequent subcultures.

Species	Tests
M. viscosa NCIMB 13584^T	Arginine dihydrolase Acetate csu Citrulline csu Lactate csu 0/129 10µg resistance
V. anguillarum NCIMB 6^T	Citrate csu Galactose csu
V. halioticoli ATCC 700680^T	7% NaCl tolerance Glycerol csu
V. ichthyoenteri ATCC 700023^T	None
V. lentus CECT 5110^T	α-ketoglutarate csu 0/129 10µg resistance

7.3 Develop and implement PCR gene probes for the rapid identification of key bacterial pathogens

7.3.1 BLASTn Analysis of PCR Primer Sequences

A summary of the first 10 matches of the BLASTn sequence similarity search is shown in Appendix 4, Table A4.7. At the end of each line the BLASTn score S followed by the Expect (E) values are shown. The alignments that made up the bulk of each report have been omitted for convenience.

Primers 118V and 1037R (Arias *et al.,* 1995) both had relative high E-values, indicating a relatively low significance threshold. This is not unexpected given the intended use of these universal primers as a general detection system for bacterial DNA. It was difficult to ascribe any significance to this result as there was a likelihood that the matches listed, although largely bacterial in origin, were chance events. Few *Vibrio* species where listed in the search results with the exception of *V. cholerae*. Although *V. cholerae* appeared to show some homology with primer Dvu9V, the only primers that displayed any potential common homology were Dvu9V and Dvu45R, the two primers for the second step of the nested reaction.

The primers L-CTH and R-CTH of Brasher *et al.* (1998) were directed towards *Vibrio* toxin genes with L-CTH showing homology with the *V. vulnificus* cytolysin *vvhA* and *vvhB* genes whereas R-CTH was closely related to the *V. parahaemolyticus* thermolabile haemolysin gene. No other bacterial species appeared in the top 10 matches for this primer pair.

The primers Vv-Primer1 and Vv-Primer2 of Coleman *et al.* (1996) revealed significant homology with the *V. vulnificus* cytolysin *vvhA* and *vvhB* gene (S=40, E=0.009). Some unrelated human and *Drosophila* sequences were listed but were clearly less relevant than *V. vulnificus*. Likewise, the only bacterial species listing with the primer pair of VvPCR1 and VvPCR2 of Coleman and Oliver (1996) was *V. vulnificus*.

The four primers of Dalla Valle *et al.* (2002), PdP1, PdP2, PdP5 and PdP6 gave the closest match with *Ph. damselae* ssp. *piscicida*. No other bacterial species matched, except PdP6 which showed some homology with *V. cholerae*, although both the BLAST score value S- and E-value indicated that the homology was low and there was better agreement with *Ph. damselae* ssp. *piscicida*.

The only bacterial species which listed with primer Vah1-P1 described by Hirono *et al.* (1996) was the *V. anguillarum vah*1 gene lodged by the authors. The other primer VaH1-P2 showed a high degree of homology with the *V. anguillarum vah*1 gene, but was also similar to a region on the haemolysin genes of *V. mimicus* and *V. cholerae*.

The three primers in the system of Kim and Jeong (2001) all revealed a high level of sequence homology with numerous other *Vibrio* species. This was not surprising given that these primers were designed to target the 16S rRNA genes. In each case it was not possible to differentiate the search scores or the E-values. Primer TriVib-1 showed similarity to *V. vulnificus, V. parahaemolyticus*, *Photobacterium* sp., an uncultured Proteobacterium and a *Vibrio* sp. Primer TriVib-2 displayed homology with the *V. vulnificus* target, but could not be separated from *V. mimicus*, Bacterium K23 or *V. cholerae* using these search parameters. Primer TriVib-3R, similarly could not be differentiated from *V. vulnificus, V. fortis, V. proteolyticus, V. nereis* and a range of other *Vibrio* sp. 16S rRNA genes.

The four primers of Kvitt *et al.* (2002) Pdp-P1f, Pdp-P2r, Pdp-P3f and Pdp-P4r were clearly similar to *Ph. damselae*. Both subspecies of *Ph. damselae* and *Ph. 'histaminum'*, a junior synonym of *Ph. damselae* ssp. *damselae*, (Kimura *et al.*, 2000) appeared most commonly. Primer Pdp-P4r was most similar to *'V. damselae'*, the basonym of *Ph. damselae* ssp. *damselae* (ATCC 33539^T), but a range of unidentified and uncultured bacteria were also listed.

BLASTn analysis of the four primers of Lee *et al.* (1998) listed one bacterial species only, corresponding with the homologous target of the *V. vulnificus* cytolysin gene;

some human, murine and *Drosophila* genes were listed but were clearly less homologous.

As was found with the four primers of Kvitt *et al.* (2002), the three hemi-nested primers of Osorio *et al.* (1999) were homologous with both subspecies of *Ph. damselae*. The only bacterial species listed when the primers targeting the urease gene (Osorio *et al.* 2000) were analysed, were *Yersinia kristensenii* and *Y. intermedia*.

Only one bacterial species listed with the *rpoN* primers of González *et al.* (2003), the *V. anguillarum* metabolic regulator σ^{54}, the target sequence for this PCR. Similarly the *toxR* primers of Conejero & Hedreyda (2003) were specific for the toxin gene of *V. harveyi* and were the only bacterial sequences that occurred.

The *Pst*1 generated primers of Iwamoto *et al.* (1995) were most related to the sequence lodged by the authors for *Vibrio 'trachuri'*, a junior synonym of *V. harveyi* (Thompson *et al.*, 2002b). A few other sequences were listed for species of *Corynebacterium*, *Pyrobaculum* and *V. vulnificus*, but each with lower search scores.

The *V. harveyi* primers VH-1 and VH-2 designed by Oakey *et al.* (2003) targeting the 16S rRNA gene revealed a range of *Vibrio* sequences all with identical scores and E-values. This was not unexpected since there is a high degree of similarity amongst species of the *Vibrionaceae* in the target region.

7.3.2 NetPrimer Analysis

NetPrimer is a Web based Java® script application which analyses primer sequences to optimise PCR, sequencing, and hybridization reactions. Primers are analysed for amplification related properties, such as melting temperature (T_m) and the theoretical stability of secondary structures. The primary purpose of the analysis was to conveniently determine if any of the amplification systems described were fundamentally flawed. An example of the typical NetPrimer output for the primer pair R-CTH and L-CTH of Brasher *et al.* (1998) for the *cth* gene in *V. vulnificus* is given in Figures 7.23a and b.

NetPrimer also provided information on the secondary structure of primers, based on temperature and solution concentrations. The secondary structures of primers can reduce the efficiency of amplification. For example, where secondary structure is formed within a primer or between primers and the energy to form or maintain the structure is high, the structure is likely be too unstable to interfere with the reaction. On the other hand, if the energy required to maintain the structure is low, the structure has

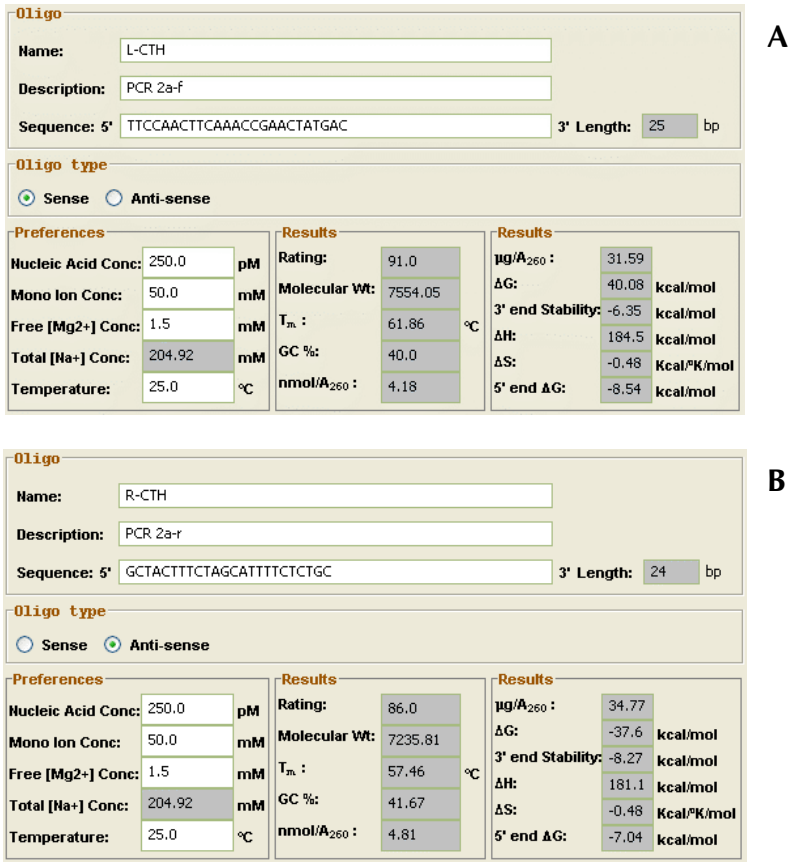

Figure 7.23 NetPrimer output for *V. vulnificus* (Brasher *et al.* [1998]) for primer L-CTH (A) and R-CTH (B).

a greater likelihood of being formed and may in turn affect the efficiency of amplification. Appendix 4, Table A4.8 contains a summary of the numbers of secondary structures determined for each primer pair. The graphical output of secondary structure is not shown.

Potential dimers (*n*= 61) and cross-dimers formed between primer pairs (*n*=82) and hair-pin structures (*n*=24) were the most common secondary structures identified. Only four primers with palindromic sequences were identified: Vv- Primer 2, Pdp-P1f, Pdp-P3f and Ure-5'. Although the overall rating of primers could not be used to rank or compare the primers from different systems, the additional information was useful when the PCRs were implemented. If a reaction failed it was possible to eliminate a range of potential primer associated defects as the cause.

7.3.3 PCR Evaluation

PCR 1. Arias *et al.* (1995) – *Vibrio vulnificus* 23S rRNA gene

Target: *Vibrio vulnificus*

Primers: 118V / 1037R – 23S rRNA gene

Primers: Dvu9V / 1037R – *V. vulnificus* specific primers

PCR products: *First round*: 1828bp amplicon

Second round: 978bp amplicon

PCR 1 was a nested primer system using a set of universal 23S rDNA targeted primers in combination with a set of primers specific for *V. vulnificus*. Figures 7.24a and 7.24b show the *V. vulnificus* species specific second round reaction. Using both specific homologous DNA as target (Figure 7.24a) and pooled non-target species DNA (Figure 7.24b). Despite numerous attempts, including manipulating the annealing temperature, varying the Mg^{2+} concentration and new primers it was not possible to get the first round universal 23S rDNA PCR reaction to work convincingly (results not shown). The

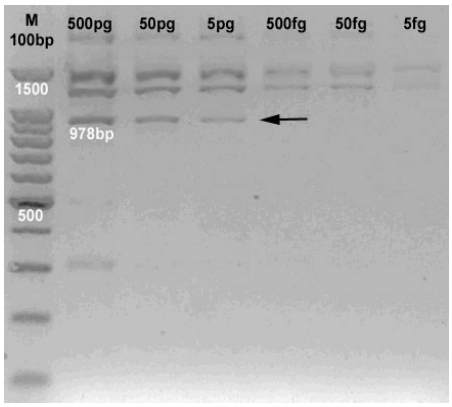

Figure 7.24a PCR 1: Second round PCR of *V. vulnificus* DNA dilution series, showing species specific 978bp amplicon (arrow)

species specific second round reaction was improved when purified DNA was used, however, several spurious bands were observed, including some prominent bands

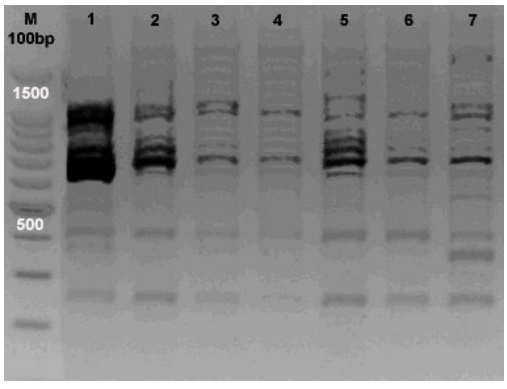

Figure 7.24b PCR 1 second round PCR of pooled non-target species DNA.

between 1,300 and 1,500bp. The intensity of these bands appeared related to the template dilution series as they became less intense with dilution of the DNA.

When pooled non-target DNA was tested using the same conditions numerous bands ranging in size from 250bp to over 1,500bp were observed. Given the number and intensity of bands it would not be possible to differentiate the species specific 978bp amplicon expected for this reaction.

PCR 2. Brasher *et al.* (1998) – *Vibrio vulnificus cth* (cytolysin/haemolysin) gene

Target: *Vibrio vulnificus cth* (cytolysin/haemolysin) gene

PCR product: 205bp amplicon

Initial attempts with establishing this PCR showed an apparent lack of efficiency as little PCR product was observed in the DNA dilution series. A gradient PCR was performed covering a range of annealing temperature from 53.5 to 58.7°C. Pool number 2 of non-target *Vibrio* species was used as a negative control for comparison, and did not display any bands over this temperature range. It appeared that the best yield of PCR product was obtained around 57°C, Figure 7.25a.

When the purified *V. vulnificus* DNA dilutions were tested a faint amplification product was observed at template dilutions down to 500fg, although 5pg was probably the practical limit of detection, Figure 7.25b.

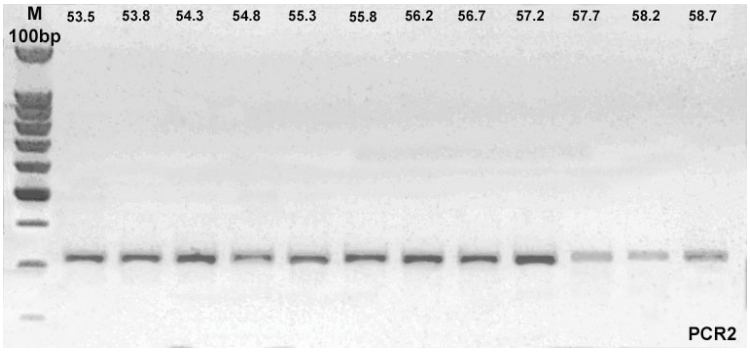

Figure 7.25a PCR 2 gradient PCR of annealing temperature, showing an apparent maximum yield at 57.2°C.

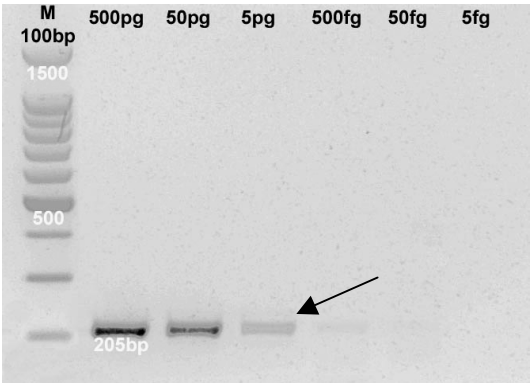

Figure 7.25b PCR 2 for the *cth* gene of *V. vulnificus*. Dilution series of purified DNA, showing species specific 205bp amplicon at 5pg of template DNA (arrow)

No amplification products were observed among the seven pooled non-target samples tested (not shown).

PCR 3. Coleman *et al.* (1996) - *Vibrio vulnificus cth* (cytolysin/haemolysin) gene

Target: *V. vulnificus* cytolysin/haemolysin gene

PCR product: 344bp amplicon

This PCR for *V. vulnificus* was able to detect purified DNA diluted to a concentration of 50fg, Figure 7.26a.

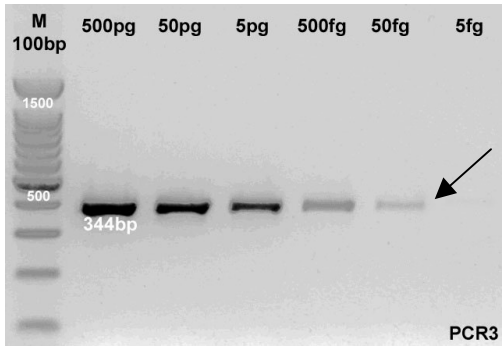

Figure 7.26a PCR 3: Dilution series of purified DNA, showing species specific 344bp amplicon at 50fg of template DNA (arrow)

When the seven negative controls (pooled non-target species DNA) were analysed two prominent amplification products were observed in samples 1 and 7 (Figure 7.26b). These bands are similar in size to the specific product obtained with *V. vulnificus* DNA. Initially they were thought to be the same, however on closer inspection they both appeared to be slightly smaller in size, around 320bp (Figure 7.26b). These bands are easily confused with the specific product.

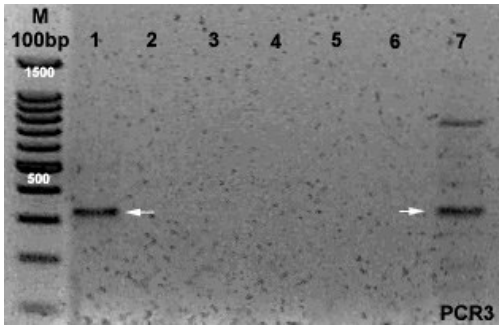

Figure 7.26b PCR 3: PCR of seven negative control pooled non-target DNA. Two bands approximately 320bp in size were observed in Pools 1 and 7 (arrowed).

PCR 4. Coleman & Oliver (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Target: *V. vulnificus* cytolysin/haemolysin gene

PCR product: 388bp amplicon

This PCR also targets the cytolysin/haemolysin gene of *V. vulnificus* and shows a lower limit of detection of around 50fg of purified DNA (Figure 7.27a). This result is very similar to that observed in PCR 3, which shares a common forward (sense) primer, but a slightly modified reverse primer. At the higher DNA concentrations an additional pair of faint bands was also observed at around 100 to 120bp. These bands were not previously observed in PCR 3.

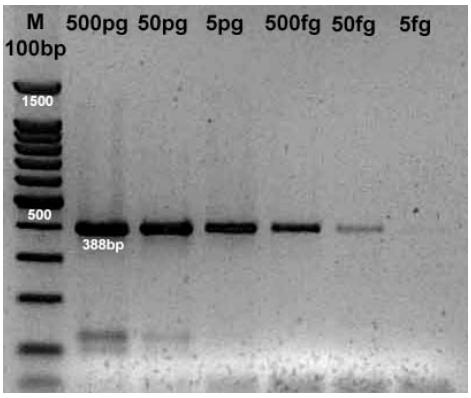

Figure 7.27a PCR 4: Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing a clear species specific 388bp band using down to 50fg of template DNA.

The results for the pooled non-target species DNA were surprising and displayed the most non-specific amplification seen throughout the testing. Pools 1, 2, 4 and 6 all displayed amplification products which could easily be confused with that expected for *V. vulnificus*, Figure 7.27b. This is in marked contrast to what was seen in the related system PCR 3.

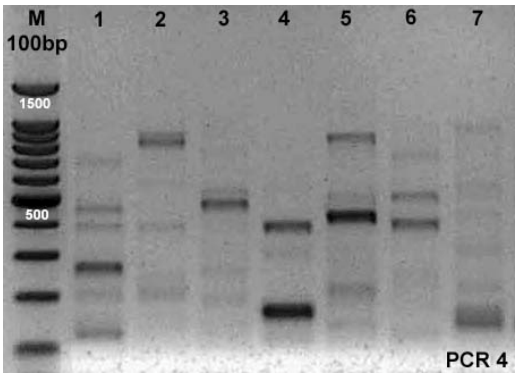

Figure 7.27b PCR 4: PCR of seven negative control pooled non-target DNA. A large number of non-specific bands of various sizes were observed in all the pooled samples tested.

PCR 5. Conejero & Hedreyda (2003) - *Vibrio harveyi tox*R gene

Target: *V. harveyi tox*R gene

PCR product: 390bp amplicon

PCR 5 for *V. harveyi* was based on the transmembrane transcription regulator *tox*R. Amplification of homologous serially diluted purified template yielded a 390bp amplification product with a lower limit of detection of approximately 5pg, a very faint band could be seen with 500fg of template, Figure 7.28.

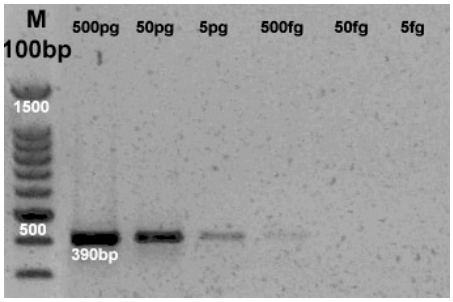

Figure 7.28 PCR 5. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 390bp amplicon using 500fg of template DNA.

When the seven pooled non-target species DNA were screened by PCR 5 a few faint bands were observed in negative control samples. These non-specific bands varied in size between different control samples and were not significant when compared with the obvious 390bp band observed when homologous template was used (not shown).

PCR 6. Dalla Valle *et al*. (2002) – *Photobacterium damsela*e ssp. *piscicida*, RAPD

Target: Not known - RAPD generated

PCR products: *First round*: 474bp amplicon

Second round: 323bp amplicon

This nested PCR system for *Photobacterium damselae* ssp. *piscicida* was able to detect as little as 5fg of DNA even in the first round reaction, Figure 7.29a. After the

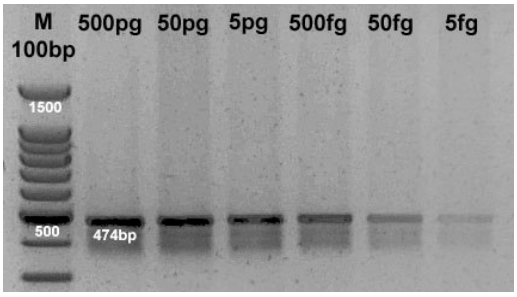

Figure 7.29a PCR 6. First step reaction of a dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 474bp amplicon with down to 5fg of template DNA.

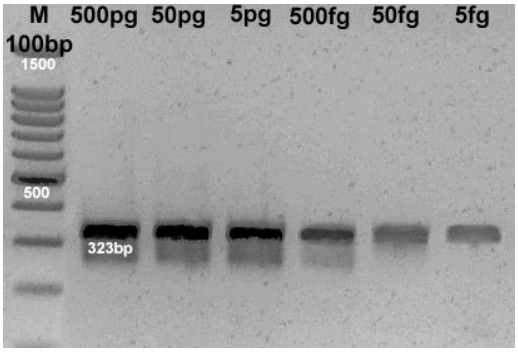

Figure 7.29b PCR 6 showing second round amplification from the samples corresponding to those shown in Figure 5a. A clear positive band of 323bp, the predicted size, can be seen with as little as 5fg of starting template.

second round of amplification, the previously weak band at 5fg was clearly positive, Figure 7.29b. Both the first and second round reactions displayed some laddering or smearing below the main product. While this may have been due to excess DNA in the nested reaction the reason for the additional bands in the first round reaction was not clear. In order to try and eliminate or reduce additional bands and smearing a gradient PCR covering annealing temperatures ranging from 54°C to 67.3°C was carried out (Figure 7.29c) using 5pg of starting template. A negative control sample consisting of pooled DNA sample 2 was included as a negative control. The annealing temperature appeared to have little effect on the intensity of the main band or the smearing below. Over 64°C the yield of specific product was reduced and the smearing resolved into a distinctly separate band approximately 390bp in size. Some non-specific banding was

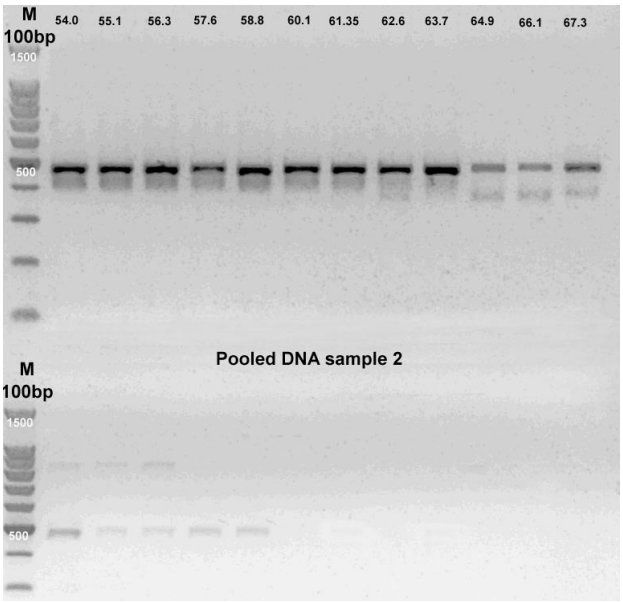

Figure 7.29c Gradient PCR of first round reaction, using 5pg of *Ph. damsela*e ssp. *piscicida* template (above) and DNA pool 2 as a non-target negative control.

evident in the pooled negative sample at temperatures below 58.8°C, but this disappeared completely at the annealing temperature of 60°C.

No amplification products were observed among the seven pooled non-target samples tested (not shown).

PCR 7. González ***et al.*** **(2003) -** ***Vibrio anguillarum rpoN*** **gene**

Target: *Vibrio anguillarum* metabolic regulator σ^{54}, *rpoN* gene

PCR product: 519bp amplicon

The design of PCR 7 was based on the metabolic regulator gene *rpoN* in *V. anguillarum*. Amplification of serially diluted purified template yielded a lower limit of detection of 500fg, Figure 7.30. No significant bands were seen with PCR 7 when the seven pooled non-target species DNA were tested (not shown).

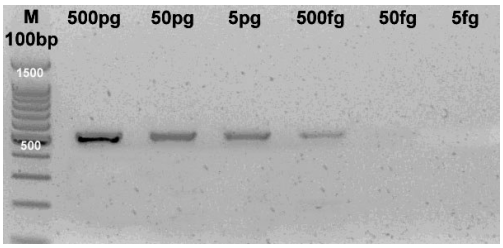

Figure 7.30 PCR 7 dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 519bp amplicon using 500fg of template DNA.

PCR 8. Hirono *et al.* (1996) - ***Vibrio anguillarum vah*****1 haemolysin gene**

Target: *V. anguillarum* haemolysin *vah*1 gene

PCR product: 490bp amplicon

This one-step PCR for the *V. anguillarum* haemolysin *vah*1 gene produced a very faint but clear product with as little as 5fg of starting template, although 50fg is

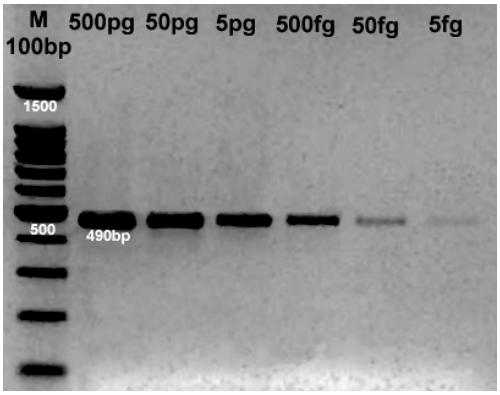

Figure 7.31a PCR 7. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 490bp amplicon down to 5fg of purified DNA template.

probably a more realistic lower limit, Figure 7.31a. The negative pooled species DNA showed no activity with this PCR, Figure 7.31b.

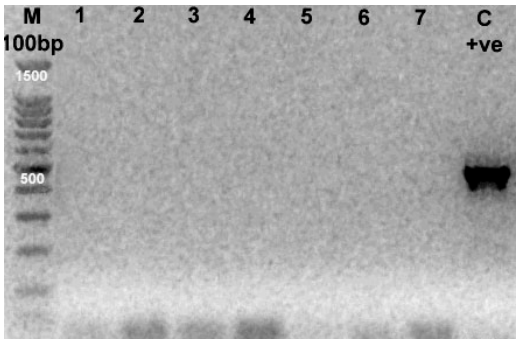

Figure 7.31b PCR 7. PCR of the seven negative control pooled non-target DNA samples. The last lane on the right side of the gel shows a strong positive reaction with 1ng of positive control template.

The PCR was developed so that the *V. anguillarum vah*1 gene could be cloned and sequenced; optimisation was not required. To determine if annealing temperature had much effect on the yield of product or on the specificity, a temperature gradient was assessed for this system, Figure 7.31c. The system appeared to work well over a range of annealing temperatures. Slightly more prominent bands were observed at 55°C to 55.5°C. No bands were observed in any of the negative control reactions.

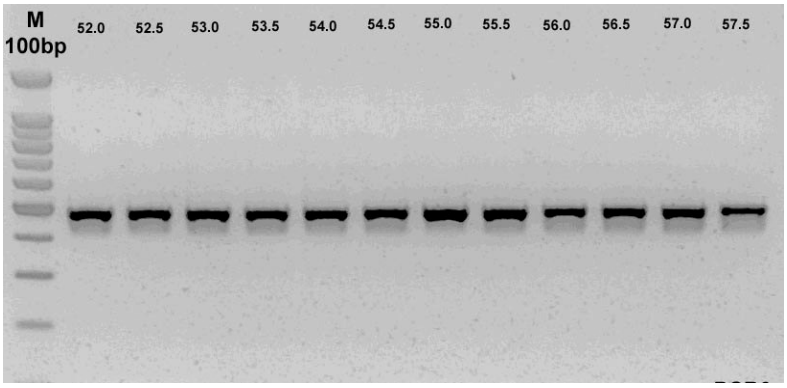

Figure 7.31c. Gradient PCR of annealing temperature from 52°C to 57.5°C for PCR 7, using 1ng of starting template; negative DNA control using pooled sample 2 not shown.

PCR 9. Iwamoto *et al.* (1995) - *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment

Target: *V. 'trachuri'* (*V. harveyi*) unique *Pst*I fragment

PCR product: 417bp amplicon

The PCR for *Vibrio 'trachuri'*, a junior synonym of *V. harveyi* (Thompson *et al.*, 2002b), was developed before the taxonomic status of *V. 'trachuri'* had been

determined. A *Pst*1 restriction fragment apparently specific for *V. 'trachuri'* was identified and PCR primers developed.

Amplification of serially diluted purified template from the type strain *V. harveyi* ATCC 14126, yielded a specific 417bp amplification product with a lower limit of detection of approximately 50pg, Figure 7.32a. When the seven pooled non-target species DNA were screened, no bands were observed, Figure 7.32b.

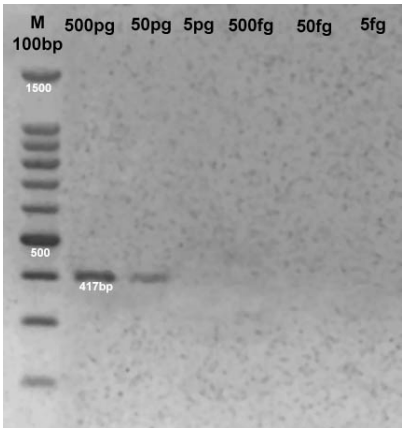

Figure 7.32a PCR 9. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing specific 417bp amplicon at a lower detection limit of 50pg of template DNA.

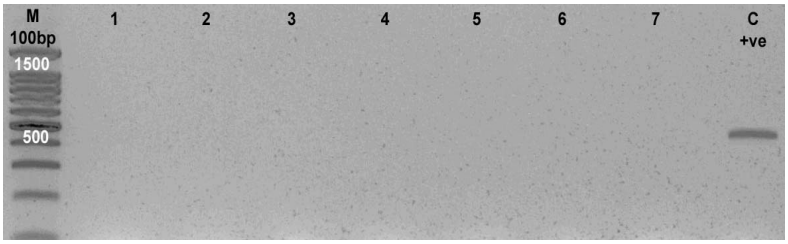

Figure 7.32b PCR 9. PCR of seven negative control pooled non-target species DNA.

PCR 10. Kim and Jeong (2001) – *Vibrio vulnificus* 16S rRNA gene, tri-primer

Target: *V. vulnificus* 16S rRNA gene

PCR products: 273bp and 825bp amplicons with *V. vulnificus* DNA
825bp amplicon from other bacteria (test negative)

PCR 10 is a multiplex system that relies on the use of three primers to produce two products in a one-step PCR reaction. Specific detection of *V. vulnificus* 16S DNA target is indicated by the presence of two amplification products of 825bp and 273bp.

Despite the fact that two reactions were taking place at the one time the system was able to produce bands of the correct size with as little as 50fg of purified DNA of starting template, Figure 7.33a. Concentrations however, of 5pg and 500fg were much

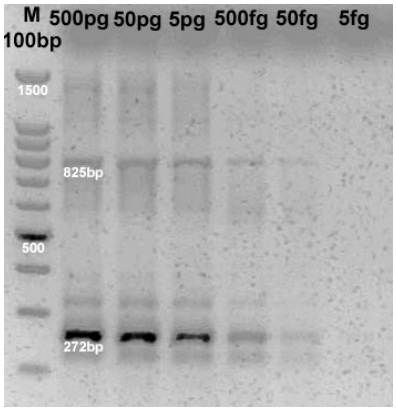

Figure 7.33a PCR 10. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 825 and 273bp amplicon using 500fg of purified template DNA.

clearer and easier to interpret with confidence. A positive test for *V. vulnificus* is accepted when a 273bp and 825bp is present. The smaller sized amplicon is indicative of *V. vulnificus* while the larger amplicon acts as a non-specific amplification control. The PCR is not clean and other, fainter bands were always present which has the potential for confusion and difficulty with interpretation even though the species specific amplicon of 273bp is prominent. The system appeared to be finely tuned with respect to the annealing temperature and attempts to reduce the number of non-specific bands by manipulating both the annealing temperature and the Mg^{2+} concentration failed to make the amplification profiles any clearer. A degree of smearing and faint extra bands were always present, especially at higher template concentrations.

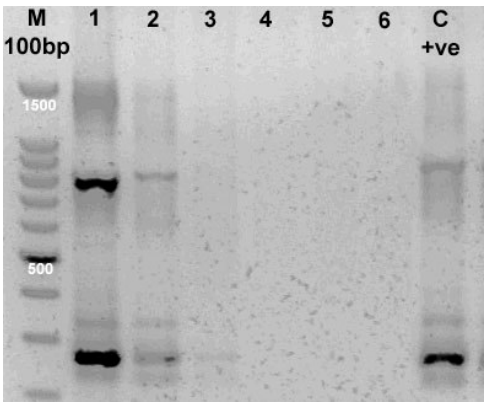

Figure 7.33b PCR 10. PCR of the seven negative control pooled non-target DNA samples.

When the pooled non-target DNAs were tested a number of non-specific amplification products were observed, although these appeared to vary in molecular weight. The 825bp band, which was intended to act as an internal control indicating the presence of bacterial DNA, was not always reproducible when the non-target DNA was tested. Figure 7.33b clearly shows the non-specific amplification products from samples one and two, but is not representative of the faint 825bp band that was sometimes, but not always, observed in samples three to six.

PCR 11. Kvitt et al. (2002) - *Photobacterium damsela*e ssp. *piscicida* 16S rRNA gene

Target: *Photobacterium damselae* ssp. *piscicida* 16S rRNA gene

PCR products: *First round*: 570bp amplicon

Second round: 270bp amplicon

This nested PCR protocol was based on 16S rRNA gene sequence with an annealing temperature at 64°C. After first round amplification it was possible to detect 500fg of purified DNA template, Figure 7.34a. After second round amplification the lower limit of detection fell to 5fg, Figure 7.34b.

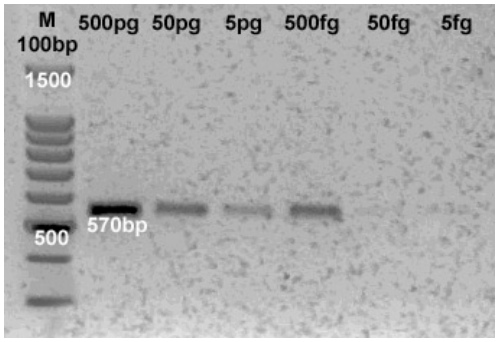

Figure 7.34a PCR 11. First step reaction of a dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 570bp amplicon with 500fg of template DNA.

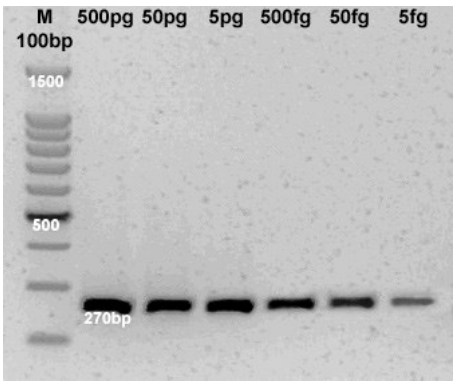

Figure 7.34b PCR 11. Second round amplification from the samples corresponding to those shown in Figure 7.23a. A clear positive band of 270bp can be seen with as little as 5fg of starting template.

No significant bands were seen in in the second round reaction when the seven pooled non-target species were tested (not shown).

PCR 12. Lee *et al.* (1998) - *Vibrio vulnificus vvh* cytolysin/haemolysin gene

Target: *V. vulnificus vvh* cytolysin/haemolysin gene

PCR products: *First round*: 704bp amplicon
Second round: 222bp amplicon

PCR 12 is a two-step nested PCR system designed to detect the cytolysin/haemolysin gene *vvh* of *V. vulnificus*. It was possible to detect as little as 500fg of purified DNA using the first round reaction and this limit of detection was

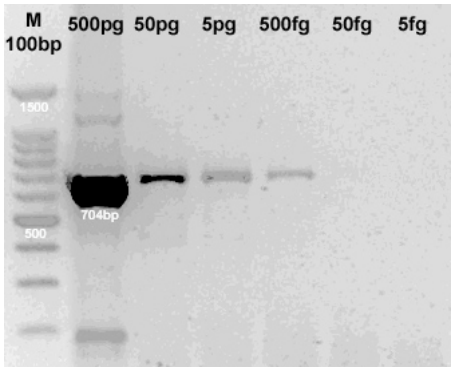

Figure 7.35a PCR 12. First step reaction of a 10-fold dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 704bp amplicon with 500fg of template DNA.

reduced to 5fg of DNA using the nested system, Figure 7.35a. Lee and colleagues (1998) reported a lower detection limit of 1fg for this system. The reactions appeared to be very efficient when the amount of amplicon was assessed by gel electrophoresis. In particular, the second round reactions produced so much DNA that some smearing and additional banding was observed at the trailing edge of the specific product, Figure 7.35b.

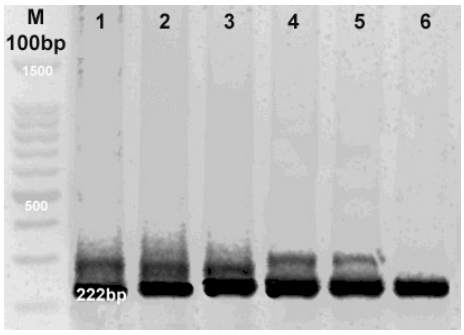

Figure 7.35b PCR 12. Second round amplification from the samples corresponding to those shown in Figure 7.33a. A clear positive band of 222bp can be seen with 5fg of template.

No significant amplification products were seen after the second round reaction for PCR 12 when the seven pooled non-target species DNA were tested (not shown).

PCR 13. Oakey *et al.* (2003) – *Vibrio harveyi* 16S rRNA gene

Target: *V. harveyi* 16S rRNA gene

PCR product: 413 bp amplicon

PCR 13 for *V. harveyi* was based on hypervariable regions in the 16S rRNA gene. Amplification of serially diluted purified *V. harveyi* DNA yielded a 413bp amplification product with a lower limit of detection of approximately 5fg, Figure 7.36a.

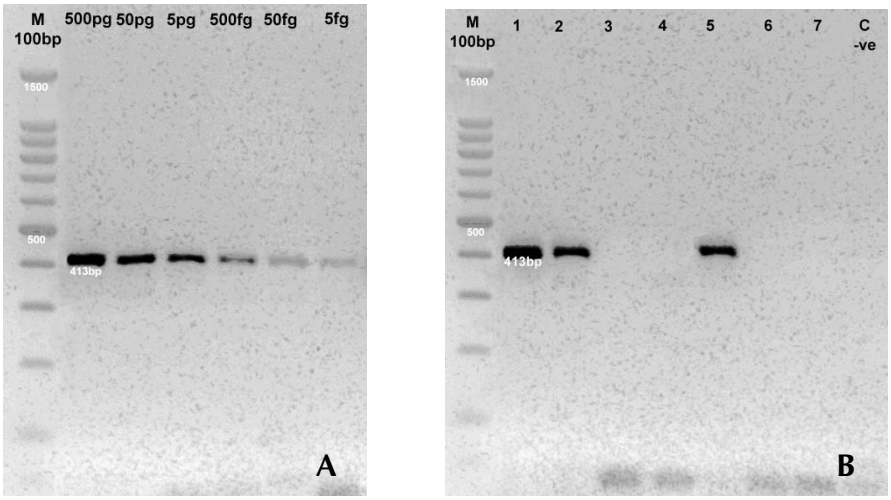

Figure 7.36 **(A) PCR 13. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 413bp amplicon down to 5fg of template DNA. (B) PCR of seven negative control pooled non-target DNA samples. Strong bands of amplification similar in size to the 413bp expected for *V. harveyi* were observed in three of the seven pools. Lanes 1, 2 and 5.**

Screening of the pooled non-target species DNA with primer VH-1 and VH-2 revealed strong amplification products similar in size to the 413bp expected for *V. harveyi* were observed in three of the seven pools, Figure 7.36b.

PCR 14. Osorio *et al.* (1999) - *Photobacterium damselae* 16S rRNA gene, hemi-nested

Target: *Photobacterium damselae* 16S rRNA gene

PCR products: *First round*: 267bp amplicon
Second round: 259bp amplicon

PCR 14 is a hemi-nested system for *Ph. damselae* based on the 16S rRNA gene sequence. The system relies on the use of three primers, where the forward primer (Car1) is the same for both the first and second rounds of amplification. Although the

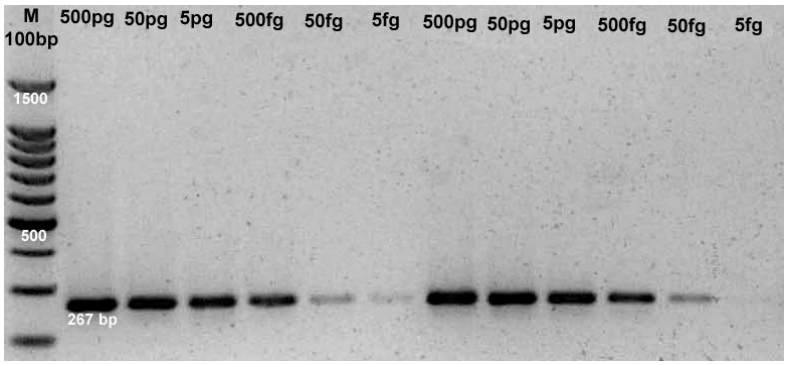

Figure 7.37a PCR 14. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing species specific 267bp amplicon for both *Ph. damselae* ssp. *piscicida* ATCC 51736^T (V906 left panel) and *Ph. damselae* ssp. *damselae* ATCC 33539^T (V912 right panel) using 50fg of DNA template

practical limit of detection of the first round reaction was 50fg of purified template DNA, a faint band could usually be seen at 5fg, Figure 7.37a. After the second round amplification a very clear band of 259bp provided clear evidence of specific amplification from subspecies of *Ph. damselae* at template concentration of 5fg, Figure 7.37b. Osorio *et al.*, (1999) also reported little or no increase in analytical sensitivity after the second round of amplification, although the weaker bands observed here after the first round reaction were much clearer after the second round of amplification and provided an unequivocal result.

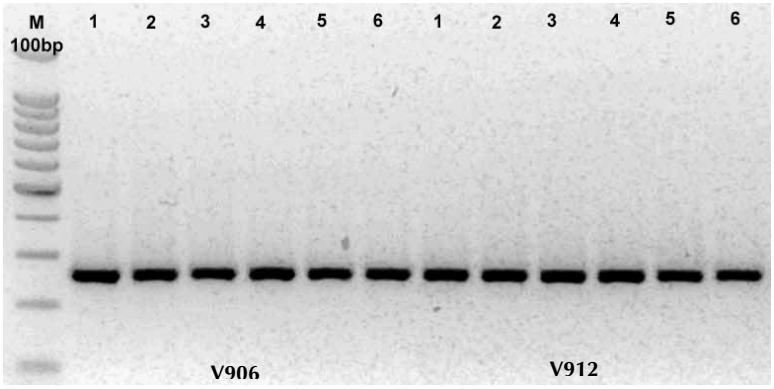

Figure 7.37b PCR14. Second round of amplification of hemi-nested PCR of corresponding samples from above (Figure 10a). Showing dilution series of purified DNA template ranging from 500pg to 5fg (samples 1-6), showing species specific 259bp amplicon for both *Ph. damselae* ssp. *piscicida* ATCC 51736^T (V906) and *Ph. damselae* ssp. *damselae* ATCC 33539^T (V912)

Screening of the pooled non-target species DNA with the Car1 and Car2 primer pair did produce a number of faint non-specific bands (Figure 7.38b for PCR 15). The non-specific multiple banding pattern was very similar in Pools 1 to 4, with Pools 5 to 7 displaying a much simpler pattern or no bands at all (Pool 6). However the non-specific

bands were larger than the 267bp specific amplicon and much fainter and less prominent, making them easy to differentiate from the specific amplification product.

PCR 15. Osorio *et al.* (2000) - *Photobacterium damselae* ssp. *damselae* and *piscicida*, *ureC* and 16S genes, multiplex format

Target: *Ph. damselae* ssp. *piscicida* and *damselae* 16S rRNA and *ureC* gene multiplex

PCR products: Ure: 448bp amplicon
Car: 267bp amplicon

The purpose of PCR 15 is to both identify and differentiate the two subspecies of *Ph. damselae*. Four primers were used in a multiplex format. The first two primers were the same as those described for PCR 14, while the second set targeted the large sub-unit of the *Ph. damselae* ssp. *damselae* urease gene. Interestingly these urease gene

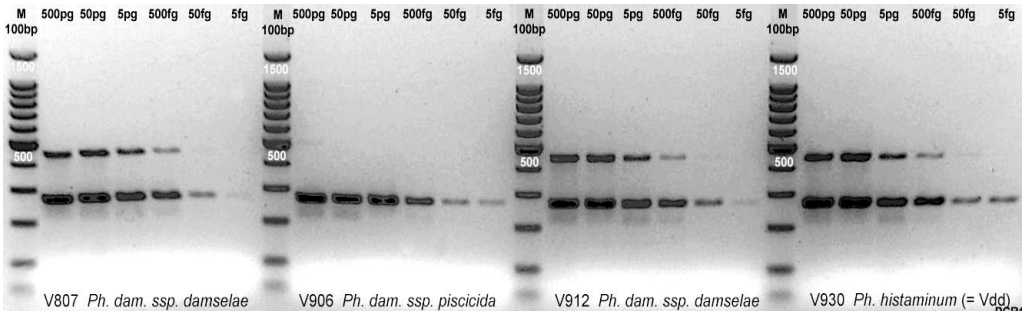

Figure 7.38a PCR 15. Dilution series of homologous purified DNA ranging from 500pg to 5fg, showing *Photobacterium damselae* specific 267bp amplicon down to 5 or 50fg of purified template DNA. The urease specific product (448bp) can be seen in three of the reaction panels, corresponding to *Ph. damselae* ssp. *damselae* NCIMB 2184^T (V807), *Ph. damselae* ssp. *damselae* ATCC 33539^T (V912) and *Ph. damselae* ssp. *damselae* ATCC 51805 (V930, *Ph. 'histaminum'*).

primers did not produce any other matches for bacterial urease genes in the BLASTn search apart from *Ph. damselae* ssp. *damselae*.

Despite the potential for competition for reaction components, the limits of detection for the *Ph. damselae* specific primers were similar when run individually (first round - PCR 14) or in multiplex format (PCR 15) using similar reaction mixture and cycling conditions. The urease PCR was, however, 10 to 100 times less sensitive in the multiplex format compared to the stand-alone PCR, Figure 7.38a.

Screening of the pooled non-target species DNA with the Car1 and Car2 primer pair produced a number of faint non-specific bands, Figure 7.38b. In each case, the non-specific multiple banding pattern was very similar in negative pools 1 to 4, with pools 5 to 7 displaying a much simpler pattern or no bands at all for pool 6. The non-specific

bands were larger than the 267bp specific amplicon but much fainter and less prominent, making them easy to differentiate from the *Ph. damselae* specific amplification product.

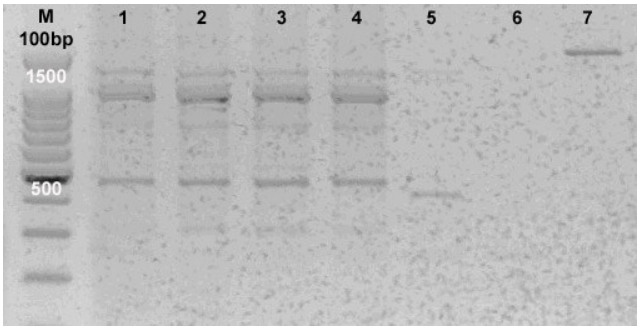

Figure 7.38b PCR 15 testing of 7 negative control pooled "non-target" DNA samples. All pooled samples, with the exception of pool 6, displayed some "non-specific" banding. These bands where easily differentiated from the specific amplicons expected.

To determine if the non-specific banding observed in pooled non-target samples could be reduced or eliminated, a range of Mg^{2+} concentrations were assessed in PCR 15, Figure 7.38c. At low Mg^{2+} concentrations of 1 to 2mM, the non- specific

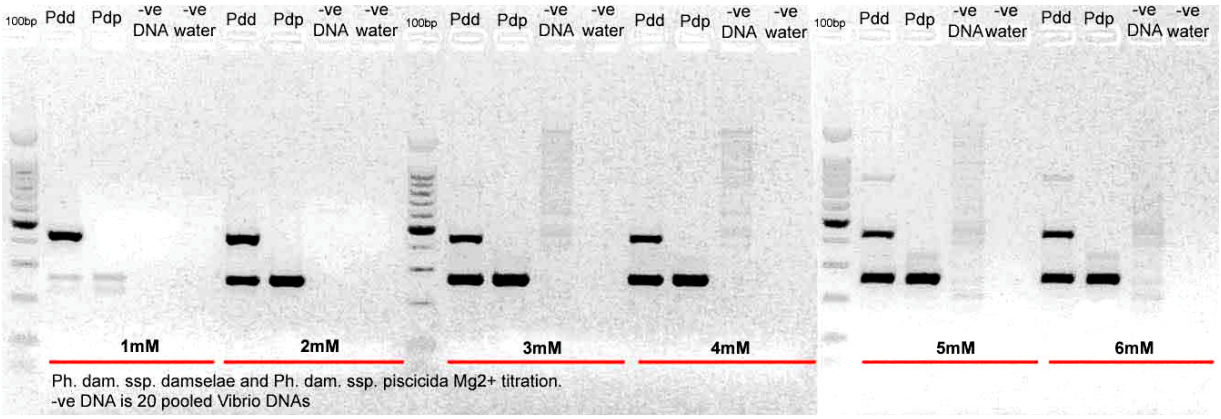

Figure 7.38c Optimisation of Mg^{2+} in the PCR 15 multiplex system for *Ph. damselae* subspecies differentiation. Six panels are shown, each consisting of increasing amounts of Mg^{2+} from 1 to 6mM. Each reaction mix was assessed with three DNA template samples: *Ph. damselae* ssp. *damselae* (Pdd), *Ph. damselae* ssp. *piscicida* (Pdp) and a pooled negative control (-ve DNA).

banding of a pooled non-target sample (a mixture of 20 non-target species DNA) was eliminated. As the Mg^{2+} concentration was increased to 3mM and beyond the non-specific banding returned. At 1mM Mg^{2+} the 267bp *Ph. damselae* specific band was severely inhibited. At a Mg^{2+} concentration of 2mM, both *Ph. damselae* amplicons were clear, and the non-specific banding of the non-target sample was greatly reduced, but

not entirely eliminated. Higher Mg^{2+} concentrations appeared to produce a better yield of *Ph. damselae* specific amplicons, but with a concomitant increase in non-specific banding. The best conditions for amplification with this system were achieved with 2mM Mg^{2+}.

7.4 DETERMINE THE RANGE OF *VIBRIO* SPECIES ASSOCIATED WITH FARMED FISH, SHELLFISH AND CRUSTACEA IN AUSTRALIA

Development of the library was based principally on cultures originating from aquatic animals that had been submitted for disease investigation or health surveillance. There was no systematic approach to collecting cultures and accordingly a level of bias exists in the collection that reflects disease episodes and more commonly encountered aquatic animals. Most of the animal species are either farmed or held in captivity but others are wildlife. There is a bias towards Northern Territory and the States of Western Australia, Tasmania and Queensland, which have committed resources to investigating fish diseases and thus provided the majority of cultures. The range of *Vibrionaceae* occurring in New South Wales, Victoria and South Australia remains largely unknown and unassessed.

The species of *Vibrionaceae* associated with aquatic animals was determined using the phenotypic data from the numerical taxonomic study using tests corresponding to the identification panel, MicroSys V36 and the VibEx7 database. The flora associated with hosts is given in Table 7.47 and the range of hosts associated with species of *Vibrionaceae* is listed in Table 7.48.

Table 7.47 Species of *Vibrionaceae* associated with aquatic animals in Australia, arranged by host taxonomic group

Group	Common name	Latin name	Host associated *Vibrionaceae*
Teleosts	Angel fish	*Pomacanthidae* Species not known	*V. fluvialis*
	Atlantic salmon	*Salmo salar*	*A. sobria* HG7 *Ph. damselae* ssp. *damselae* bv I *Ph. damselae* ssp. *damselae* bv II *Ph. leiognathi* Phenon 6 Phenon 8 Phenon 25 Phenon 26 Phenon 27 Phenon 36 Phenon 43 Phenon 45 Phenon 57

continued...

Group	Common name	Latin name	Host associated *Vibrionaceae*
			Phenon 59 Phenon 83 *V. anguillarum* *V. cyclitrophicus* *V. fischeri* bv II *V. furnissii* *V. harveyi* bv I *V. ichthyoenteri* bv II *V. scophthalmi* *V. splendidus* bv I *V. tasmaniensis*
	Barramundi	*Lates calcarifer*	*Ph. damselae* ssp. *damselae* bv I Phenon 53 *V. cholerae* non-O1 *V. harveyi* bv I *V. ichthyoenteri* bv I *V. vulnificus* bv I
	Barred grunter	*Amniataba percoides*	*Ph. damselae* ssp. *damselae* bv I
	Black bream	*Acanthopagrus butcheri*	*Ph. damselae* ssp. *damselae* bv I *Ph. damselae* ssp. *damselae* bv II *Ph. iliopiscarium* *V. harveyi* bv I
	Blue tang	*Acanthurus leucosternon*	*V. mediterranei*
	Coral trout	*Cephalopholis miniata*	*Ph. damselae* ssp. *damselae* bv I *V. harveyi* bv I
	Dhufish	*Glaucosoma hebraicum*	*Ph. damselae* ssp. *damselae* bv I *Ph. damselae* ssp. *damselae* bv II
	Discus	*Symphysodon discus*	*V. cholerae* non-O1 *V. fluvialis*
	Eastern black bream	*Acanthopagrus australis*	*V. anguillarum*
	Goldfish	*Carassius auratus*	*V. cholerae* non-O1
	Greenback flounder	*Rhombosolea tapirina*	*V. alginolyticus*
	John Dory	*Zeus faber*	Phenon 21
	Leafy sea dragon	*Phycodurus eques*	Phenon 10 Phenon 42 Phenon 52 Phenon 83 *V. alginolyticus* *V. splendidus* bv I
	Mahi Mahi	*Coryphaena equiselis*	*Ph. damselae* ssp. *damselae* bv I *V. chagasii* *V. fischeri* bv II *V. harveyi* bv I *V. ichthyoenteri* bv I
	Mangrove jack	*Lutjanus argentimaculatus*	*V. harveyi* bv I
	Mullet	*Mugil cephalus*	*V. anguillarum* *V. diazotrophicus* *V. natriegens* *V. splendidus* bv I
	Mulloway	*Argyrosomus japonicus*	*V. navarrensis*
	Murray catfish	*Tandanus tandanus*	*Ph. damselae* ssp. *damselae* bv I

continued...

Group	Common name	Latin name	Host associated *Vibrionaceae*
	Rainbow trout	*Oncorhynchus mykiss*	*A. sobria* HG7 *Ph. damselae* ssp. *damselae* bv I Phenon 58 Phenon 59 *V. anguillarum* *V. mediterranei* *V. parahaemolyticus*
	Ramirezi	*Mikrogeophagus ramirezi*	*V. fluvialis*
	Sea hare	*Aplysia* sp. Species not known	Phenon 57 *V. alginolyticus* *V. chagasii*
	Sea perch	*Helicolenus percoides*	*V. alginolyticus*
	Seahorse	*Hippocampus* sp. Species not known	*V. alginolyticus* *V. cyclitrophicus*
	Snapper	*Pagrus auratus*	Phenon 43 *V. chagasii* *V. harveyi* bv I *V. mediterranei* *V. pelagius* bv II
	Striped trumpeter	*Latris lineata*	Phenon 19 Phenon 21 Phenon 25 Phenon 26 Phenon 27 Phenon 29 Phenon 57 Phenon 59 *V. anguillarum* *V. chagasii* *V. rumoiensis* *V. splendidus* bv I
	Summer whiting	*Sillago ciliata*	*V. harveyi* bv I
	Southern bluefin tuna	*Thunnus maccoyii*	*Ph. damselae* ssp. *damselae* bv I *V. fischeri* bv II
	Weedy sea dragon	*Phyllopteryx taeniolatus*	*V. alginolyticus*
	Whiting	*Sillago* sp. Species not known	*V. chagasii*
	Yellowtail	*Seriola lalandi*	*V. alginolyticus* *V. chagasii* *V. nereis* *V. proteolyticus*
Mammals	Dolphin	*Delphinidae* Species not known	*Ph. damselae* ssp. *damselae* bv I *Ph. damselae* ssp. *damselae* bv II
	Dugong	*Dugong dugong*	Phenon 43 *V. harveyi* bv I *V. parahaemolyticus* *V. vulnificus* bv I
	Humpbacked dolphin	*Sousa chinensis*	Phenon 10 *V. harveyi* bv I
	Pilot whale	*Globicephala melas*	*Ph. damselae* ssp. *damselae* bv II
Cephalopods	Nautilus	*Nautilus* sp.	*V. harveyi* bv I
	Southern dumpling squid	*Euprymna tasmanica*	*V. fischeri* bv II
Cnidaria	Blue blubber jellyfish	*Catostylus mosaicus*	Phenon 45

continued...

Group	Common name	Latin name	Host associated *Vibrionaceae*
Crustacea	Artemia	*Artemia* sp. Species not known	*V. alginolyticus* *V. harveyi* bv I *V. natriegens* *V. proteolyticus*
	Black tiger prawn	*Penaeus monodon*	Phenon 6 *V. harveyi* bv I
	Blue manna crab	*Portunus pelagicus*	*Ph. damselae* ssp. *damselae* bv I Phenon 8 Phenon 20
	Brown tiger prawn	*Penaeus esculentus*	*V. harveyi* bv I
	Marron	*Cherax tenuimanus*	*V. mimicus*
	Mud crab	*Scylla serrata*	*Ph. damselae* ssp. *damselae* bv I Phenon 6 Phenon 20 Phenon 36 *V. alginolyticus* *V. pelagius* bv I
	Packhorse lobster	*Jasus verreauxi*	*V. harveyi* bv I
	Rotifer	*Brachionus* sp. Species not known	*V. anguillarum*
	Southern rock lobster	*Jasus edwardsii*	Phenon 52 Phenon 53 Phenon 83 *V. alginolyticus* *V. fischeri* bv II *V. harveyi* bv I *V. penaeicida* *V. tasmaniensis*
	Yabby	*Cherax destructor*	*V. mimicus*
Molluscs	Abalone	*Haliotis* sp. Species not known	Phenon 8 Phenon 10 Phenon 15 Phenon 24 Phenon 25 Phenon 26 Phenon 27 Phenon 43 Phenon 46 *V. chagasii* *V. cincinnatiensis* *V. halioticoli* *V. harveyi* bv I *V. harveyi* bv II *V. mediterranei* *V. mytili* *V. rumoiensis* *V. splendidus* bv I
	Mussels	*Mytilus edulis*	Phenon 24 Phenon 27 Phenon 43 Phenon 59 *V. fischeri* bv II *V. natriegens* *V. parahaemolyticus* *V. splendidus* bv I

continued...

Group	Common name	Latin name	Host associated *Vibrionaceae*
	Pacific oyster	*Crassostrea gigas*	Phenon 19 Phenon 26 Phenon 69 *V. cyclitrophicus* *V. lentus* *V. tubiashii*
	Pearl oyster	*Pinctada maxima*	*Ph. leiognathi* Phenon 20 *V. alginolyticus* *V. pelagius* bv I *V. pelagius* bv II
	Sydney rock oyster	*Saccostrea glomerata*	*V. natriegens*
Reptiles	Flatback turtle	*Natator depressus*	*Ph. damselae* ssp. *damselae* bv I Phenon 53 *V. alginolyticus* *V. harveyi* bv I *V. nereis*
	Green turtle	*Chelonia mydas*	*Ph. damselae* ssp. *damselae* bv I Phenon 53 *V. fluvialis* *V. harveyi* bv I *V. parahaemolyticus* *V. vulnificus* bv I
	Leatherback turtle	*Dermochelys coriacea*	*Ph. damselae* ssp. *damselae* bv I
	Loggerhead turtle	*Caretta caretta*	*V. harveyi* bv I
	Marine turtles	*Cheloniidae* Species not known	*Ph. damselae* ssp. *damselae* bv I *V. alginolyticus* *V. furnissii* *V. tubiashii*

Table 7.48 Species of *Vibrionaceae* associated with aquatic animals in Australia, arranged by bacterial species. Number of host species from which a bacterial species has been isolated is given as no.

Bacterial species	No.	Host species
A. sobria HG7	2	Atlantic salmon Rainbow trout
Ph. damselae ssp. *damselae* biovar I	17	Atlantic salmon Barramundi Barred grunter Black bream Blue manna crab Coral trout Dhu fish Dolphin Flatback turtle Green turtle Leatherback turtle Mahi Mahi Marine turtles Mud crab Murray catfish Rainbow trout Southern bluefin tuna

continued...

Bacterial species	No.	Host species
Ph. damselae ssp. *damselae* biovar II	5	Atlantic salmon Black bream Dhu fish Dolphin Pilot whale
Ph. iliopiscarium	1	Black bream
Ph. leiognathi	2	Atlantic salmon Pearl oyster
Phenon 6	3	Atlantic salmon Black tiger prawn Mud crab
Phenon 8	3	Abalone Atlantic salmon Blue manna crab
Phenon 10	3	Abalone Humpbacked dolphin Leafy sea dragon
Phenon 15	1	Abalone
Phenon 19	2	Pacific oyster Striped trumpeter
Phenon 21	2	John Dory Striped trumpeter
Phenon 24	2	Abalone Mussel
Phenon 25	3	Abalone Atlantic salmon Striped trumpeter
Phenon 26	4	Abalone Atlantic salmon Pacific oyster Striped trumpeter
Phenon 27	4	Atlantic salmon Abalone Mussel Striped trumpeter
Phenon 29	1	Striped trumpeter
Phenon 36	2	Atlantic salmon Mud crab
Phenon 42	1	Leafy sea dragon
Phenon 43	5	Abalone Atlantic salmon Dugong Mussel Snapper
Phenon 45	2	Atlantic salmon Blue blubber jellyfish
Phenon 46	1	Abalone
Phenon 52	2	Leafy sea dragon Southern rock lobster
Phenon 53	4	Barramundi Flatback turtle Green turtle Southern rock lobster

continued...

Bacterial species	No.	Host species
Phenon 57	3	Atlantic salmon Sea hare Striped trumpeter
Phenon 58	1	Rainbow trout
Phenon 59	4	Atlantic salmon Mussel Rainbow trout Striped trumpeter
Phenon 69	1	Pacific oyster
Phenon 83	3	Atlantic salmon Leafy sea dragon Southern rock lobster
V. alginolyticus	12	Artemia Flatback turtle Greenback flounder Marine turtles Mud crab Pearl oyster Sea hare Sea perch Seahorse Southern rock lobster Weedy sea dragon Yellowtail
V. anguillarum	6	Atlantic salmon Eastern black bream Mullet Rainbow trout Rotifer Striped trumpeter
V. chagasii	6	Abalone Mahi Mahi Sea hare Snapper Whiting Yellowtail
V. cholerae non-O1	3	Barramundi Discus Goldfish
V. cincinnatiensis	1	Abalone
V. cyclitrophicus	3	Atlantic salmon Pacific oyster Seahorse
V. diazotrophicus	1	Mullet
V. fischeri biovar II	6	Atlantic salmon Southern dumpling squid Mahi Mahi Mussel Southern bluefin tuna Southern rock lobster
V. fluvialis	4	Angel fish Discus Green turtle Ramirezi
V. furnissii	2	Atlantic salmon Marine turtles

continued...

Bacterial species	No.	Host species
V. halioticoli	1	Abalone
V. harveyi biovar I	19	Abalone Artemia Atlantic salmon Barramundi Black bream Black tiger prawn Brown tiger prawn Coral trout Dugong Flatback turtle Green turtle Humpbacked dolphin Mahi Mahi Mangrove jack Mud crab Nautilus Packhorse lobster Snapper Southern rock lobster Summer whiting
V. harveyi biovar II	1	Abalone
V. ichthyoenteri biovar I	2	Barramundi Mahi Mahi
V. ichthyoenteri biovar II	1	Atlantic salmon
V. lentus	1	Pacific oyster
V. mediterranei	4	Abalone Blue tang Rainbow trout Snapper
V. mimicus	2	Marron Yabby
V. mytili	1	Abalone
V. natriegens	4	Artemia Mullet Mussel Sydney rock oyster
V. navarrensis	1	Mulloway
V. nereis	2	Flatback turtle Yellowtail
V. parahaemolyticus	4	Dugong Green turtle Mussel Rainbow trout
V. pelagius biovar I	2	Pearl oyster
V. pelagius biovar II	2	Pearl oyster Snapper
V. penaeicida	1	Southern rock lobster
V. proteolyticus	2	Artemia Yellowtail
V. rumoiensis	2	Abalone Striped trumpeter
V. scophthalmi	1	Atlantic salmon

continued...

Bacterial species	No.	Host species
V. splendidus biovar I	5	Abalone Atlantic salmon Mullet Mussel Striped trumpeter
V. tasmaniensis	2	Atlantic salmon Southern rock lobster
V. tubiashii	2	Marine turtles Pacific oyster
V. vulnificus biovar I	3	Barramundi Dugong Green turtle

7.5 TECHNOLOGY TRANSFER WORKSHOP FOR STATE VETERINARY DIAGNOSTIC MICROBIOLOGISTS

Invitations to attend a workshop were sent to State Government veterinary diagnostic and private veterinary laboratories in Australia by direct mail and the fish health newsgroup "Belly Up" managed by the Aquatic Animal Health Unit of the Department of Agriculture, Fisheries & Forestry; workshop details were also sent to The National Centre for Disease Investigation, Ministry of Agriculture & Forestry, Wallaceville, New Zealand. The workshop was attended by four scientists from: Primary Industries Research, Victoria, Attwood; Gribbles Veterinary Pathology, Adelaide, South Australia; Aquatic Veterinary Services, Victoria and NCDI, MAF, New Zealand. There were no participants from Western Australia, Northern Territory, Queensland or New South Wales.

The workshop consisted of two lectures: one on the identification of *Vibrio* species and the probabilistic approach to identification and the second on PCR and its role as a tool for the identification of *Vibrio* species. The practical component of the workshop enabled participants to undertake the identification of *Vibrionaceae* using both miniaturised tests and PCR. Details of the course provided are given in Appendix A4.9, *Vibrio* Identification Workshop - Laboratory Schedule.

Participants were given a range of cultures for identification using the MicroSys V36 identification panel (Table 7.49) as well as some simulated cases as listed in Table 7.50.

Table 7.49 Species identified by workshop participants using MicroSys V36

Species	Species
Grimontia hollisae	*Vibrio metschnikovii*
Vibrio alginolyticus	*Vibrio mimicus*
Vibrio anguillarum	*Vibrio penaeicida*
Vibrio mediterranei	*Vibrio scophthalmi*

The cases required participants to assess significance of primary plates that had been synthesised to mimic typical cases of Vibriosis and follow through on their evaluation by identifying cultures using MicroSys V36. Participants correctly identified the cultures provided and in most cases the Willcox probability score exceeded 0.99 with modal likelihood scores greater than 0.001. Where identification scores were less than the

Table 7.50 Simulated diagnostic cases of infection with *Vibrio* species

Case	Organisms
Septicaemia in freshwater ornamental angel fish	*V. fluvialis*
Septicaemia in farmed Atlantic salmon	*V. ichthyoenteri* (with *V scophthalmi*)
Crash of rock lobster phyllosoma	*V. penaeicida* (with *V. proteolyticus*)
Crash of Pacific oyster larvae	*V. tubiashii* (with *V. alginolyticus*)

threshold values for acceptance, this appeared to be the result of test interpretation error, arising from insufficient experience with MicroSys V36. Participants were given the opportunity of using freeze-dried identification panels which were well received because of the simplicity of use.

Participants at the workshop were introduced to multiplex PCR using the primer sets for *Ph. damselae* that discriminates between the subspecies *damselae* and *piscicida*. A threshold of detection assay was undertaken to demonstrate the robustness of the PCR and the utility of the assay assessed by showing that no difference in test outcome was apparent when using cultures that were two and seven days old. In addition, participants were shown how DNA from bacterial cultures can be rapidly extracted and stored dry on FTA® cards (Whatman Asia Pacific, Singapore) for later analysis by PCR.

7.6 Develop an ANZSDP for the Identification of *Vibrio* species in aquatic animals

A draft Australian New Zealand Standard Diagnostic Procedure is given in Appendix 5. The procedure is intended to be a procedures manual for the identification of bacterial strains and is not intended to provide guidance on clinical disease in aquatic animals. PCR protocols should be used for confirmation purposes; primers have not been evaluated for specificity for direct detection of target species in animal or environmental samples. While use of primers in this manner will have obvious application, confirmation of specificity and establishing test parameters was beyond the scope of the project.

8. DISCUSSION

8.1 UNDERTAKE A DEFINITIVE NUMERICAL TAXONOMIC ANALYSIS TO DESCRIBE AND CHARACTERISE THE PHENOTYPES OF AQUATIC ANIMAL VIBRIOS IN AUSTRALIA

8.1.1 Numerical taxonomy as a descriptive tool

Numerical taxonomic analysis has been used as a means of establishing a classification of bacterial groups based on common phenotypes. This approach was used extensively until the late 1980s when the advent of gene based technologies led to the development of molecular systematics and elucidation of phylogenetic relationships (Goodfellow & O'Donnell, 1993). Classification of the *Vibrionaceae* was developed initially through numerical taxonomic studies (Bryant *et al.*, 1986a, West *et al.*, 1986) but is now largely based on 16s rDNA analysis (Dorsch *et al.*, 1992; Ruimy *et al.*, 1994; Wiik *et al.*, 1995) augmented by amplified fragment length polymorphism (AFLP) analysis (Urakawa *et al.*, 1998; Thompson *et al.*, 2001) to establish a stable classification of the family. The *Vibrionaceae* currently consists of five genera: *Enterovibrio, Grimontia, Moritella, Photobacterium* and *Vibrio* consisting in total of 89 validly published species (Euzéby 1997, Euzéby, 2006). Species are defined on the basis of 16S rDNA sequences, DNA:DNA hybridisation and for some, such as members of the *V. splendidus* complex where 16S rDNA sequence is not sufficiently discriminatory, *gyrB* sequences (gyrase B subunit) have been used (Le Roux *et al.*, 2004). With increasing reliance on molecular systematics as a means of classification there has been a corresponding decline in descriptive phenotyping of the *Vibrionaceae*.

While phenotypic numerical taxonomy has all but been abandoned as a means of classifying *Vibrionaceae* it remains nevertheless a powerful tool for describing and analysing bacterial communities. The precepts of numerical taxonomy impose rigour to the collection of data to yield objective and descriptive phenotyping of taxa. This approach has been used extensively to assess *Vibrionaceae* in aquatic environments particularly as a means of cataloguing species and determining distributions and habitat associations (Simidu & Tsukamoto, 1985; Grimes *et al.*, 1993; Barbieri *et al.*, 1999; Martin-Kearley & Gow, 1994; Ortigosa *et al.*, 1994; Blanch *et al.*, 2001; Castro *et al.*, 2002; Alcaide, 2003; Battaglene *et al.*, 2006). As a structured unbiased approach to phenotyping, numerical taxonomy is a valid means of defining phenospecies of the *Vibrionaceae* but remains an under-utilised tool for generating data for the development of identification schemes (Priest & Austin, 1993).

8.1.2 Library composition

Since data generated by numerical taxonomy of taxospecies is both objective and descriptive it can be the basis of predictive and stable identification schemes (Bryant *et al.*, 1986b; On *et al.*, 1996). The utility of the data is dependent on the number of strains and diversity of phenotypic tests used for numerical taxonomy. It has been advocated that the number of strains tested be at least 10-20 times the anticipated number of taxa (Stanier *et al.*, 1966). For this study of *Vibrionaceae* the likely number of taxa encountered could not be reasonably estimated since a comprehensive survey of species from aquatic animals in Australia had not been undertaken previously and the diverse range of habitats represented would likely yield more species than hitherto recognised. The target number of strains was instead based on the number of validly published species. At the time of assembling the library, 53 species were formally recognised which meant a target range of 530 - 1,060 strains should be obtained. The working library assembled contained 871 strains, within the required range to ensure the library was representative of the anticipated diversity of the flora.

A known deficiency in the library is the absence of 35 species (Table 8.1) all of which were formally described after the library was assembled. Some species were included fortuitously such as *Vibrio tasmaniensis*, which was classified using strains obtained from the TCFB, DPIW as normal flora of Atlantic salmon (Thompson *et al.*, 2003e) and strains of unnamed species which were later identified by 16S rDNA sequencing and DNA:DNA hybridisation as *V. chagasii* (Le Roux, 2004). Since the species listed in Table 8.1were not included in the library, the phenotypic

Table 8.1 Species of *Vibrionaceae* not included in the numerical taxonomic analysis

Species	Species	Species
Enterovibrio coralii	*Vibrio aerogenes*	*Vibrio pacinii*
Enterovibrio norvegicus	*Vibrio brasiliensis*	*Vibrio pomeroyi*
Moritella abyssi	*Vibrio coralliilyticus*	*Vibrio ponticus*
Moritella japonica	*Vibrio crassostreae*	*Vibrio rotiferianus*
Moritella profunda	*Vibrio diabolicus*	*Vibrio ruber*
Moritella yayanosii	*Vibrio ezurae*	*Vibrio superstes*
Photobacterium aplysiae	*Vibrio fortis*	*Vibrio xuii*
Photobacterium frigidiphilum	*Vibrio gallicus*	
Photobacterium ganghwense	*Vibrio gigantis*	
Photobacterium halotolerans	*Vibrio hepatarius*	
Photobacterium indicum	*Vibrio hispanicus*	
Photobacterium lipolyticum	*Vibrio kanaloae*	
Photobacterium profundum	*Vibrio neonatus*	
Photobacterium rosenbergii	*Vibrio neptunius*	

description of the *Vibrionaceae* is incomplete, a known limitation of this study. The likely effect of these missing species on those defined by the numerical taxonomic study reported here is unknown. If the panel of phenotypic tests is sufficiently broad, there should be adequate phenotypic depth to differentiate new species from those included in the current taxonomic analysis. Only by profiling new species can the robustness and predictive qualities of the phenotypic panel be assessed by determining its capacity to accommodate and differentiate new taxa.

8.1.3 Phenotyping

Test selection and format

The type and number of tests for a taxonomic study is to some extent dependent on the taxa to be typed and the logistics of handling a large number of strains and tests. For the study reported here 107 tests were used, which is within the guidelines of a minimum of 50 tests and an optimum of 100-200 tests considered suitable for a numerical taxonomic study (O'Brien & Colwell, 1987). For 50 tests or less sampling error can be quite large but for 100-200 tests is greatly reduced (Sneath, 1962). Using more tests will decrease the sampling error but much beyond 300 tests the gain achieved is disproportionate to magnitude of testing. Selection of tests for phenotyping the library of strains was based on earlier numerical taxonomic studies of the *Vibrionaceae* (West *et al.*, 1983; Dawson & Sneath, 1985; Bryant *et al.*, 1986a; West *et al.*, 1986; Kämpfer *et al.*, 1987; Grimes *et al.*, 1993; Farto *et al.*, 1999). Selecting tests based on previous studies is not generally recommended (Sneath, 1978a) as there is a risk that the tests may be poor at resolving the underlying taxonomic structure of the library. In practice it is not possible to avoid tests commonly used for phenotyping and where there is good evidence that a range of tests have been shown to differentiate species (Grimes *et al.*, 1993; Martin-Kearley & Gow, 1994), including them will increase the likelihood of partitioning taxa, and limit the use of tests of unknown or limited resolving power.

An estimate of the distribution of all positive and negative reactions for the panel of tests was determined by assessing the linearity of the frequency of positive reactions. A panel of tests is more likely to reveal taxonomic structure where the number of positive to negative tests are in roughly equal proportion compared to a panel with a preponderance of either positive or negative tests. For the panel of 107 tests a good gradation was achieved in the number of positive tests in the range of 100% to 50% though the number of positive tests below 50% was less than the expected trend

(Figure 8.1). The distribution of tests achieved compares well with the large data set of Bryant *et al.* (1986a) which showed an equally linear range of tests with only slight deviations from the expected in the high and low percentage ranges.

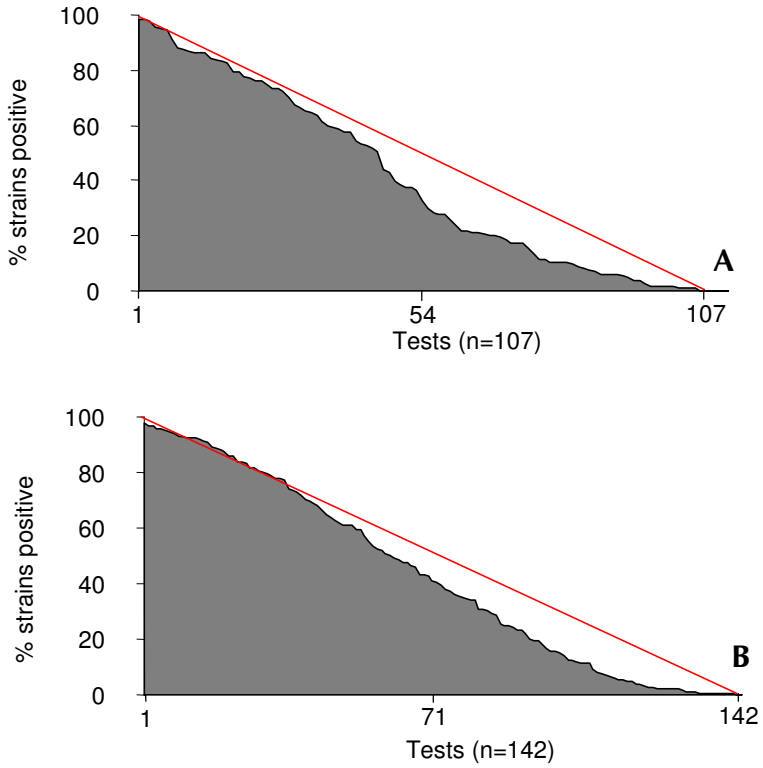

Figure 8.1 Distribution of percentage positive tests in phenotyping panels for the characterisation of *Vibrionaceae* by numerical taxonomy. A: Data this study; B: Data from Bryant *et al.*, 1986a

Phenotyping was undertaken using conventional tests in miniature form so that high volume testing could be undertaken conveniently. Miniaturisation has been used for phenotyping *Vibrionaceae* to a limited extent only (Kämpfer *et al.*, 1987; Austin *et al.*, 1997) with earlier classifications based entirely on macro-format tests (West *et al.*, 1983, West *et al.*, 1986) as indeed have most taxonomic studies of *Vibrionaceae* (Martin-Kearley & Gow, 1994; Ortigosa *et al.*, 1994; Castro *et al.*, 2002; Alcaide, 2003). More recent studies of *Vibrionaceae* have used the Biolog™ GN panels (Hayward, CA, USA) for phenotyping, principally for ease of use and availability. Characterisation by this means, however, has limited value in describing species (Austin *et al.*, 1997; Vandenberghe *et al.*, 2003) because the fixed range of tests in the panels have only limited resolving power for species of the *Vibrionaceae*. While conventional tests in whatever format are inconvenient to make and quality control effectively, they enable nevertheless great flexibility in the choice of tests and provides for the possibility of phenotypic discovery not possible with fixed format panels.

Test performance

A key objective of miniaturisation was to ensure test reaction mirrored as closely as possible that obtained in conventionally formatted tests. Congruence is important as it ensures that testing can be done independently by conventional means and is not tied necessarily to using miniaturised tests in MicroSys phenotyping panels, which are only available commercially. This provides a realistic basis for independent comparative testing when new species are described or for characterisation with the intention of bacterial identification. To have true applicability, testing needs to be independent of commercial systems.

Characterisation tests were undertaken at 25°C for mesophiles and at 15°C for obligate psychrophiles and incubated for 48 hr and seven days, respectively. These time frames were selected so that a bacterial identification scheme could be based on realistic incubation times, important for disease diagnosis. The phenotypes given in this report are those explicitly for the specified incubation times. A distinction can be made between *realised* phenotype and *latent* phenotype. Realised phenotypes are those observed at a predetermined time, the test *chronometric*. Strains may have a negative reaction, the realised phenotype, for the test chronometric, but on continued incubation may become positive to yield the latent phenotype. Failure to distinguish between realised and latent phenotypes can lead to significant misunderstanding of strain characteristics and test performance particularly when describing species characteristics. The effect of incubation time and temperature on numerical taxonomic practice has been explored by Sneath (1968) who determined that expression of phenotype comprises two components: vigour and pattern. Vigour was defined as the general metabolic activity or achievement and is influenced by temperature and rates of reaction. Metabolic achievement is equivalent in concept to *latent* phenotype. Sneath established a pattern coefficient D_P that removes the effect of vigour when calculating similarity of taxa. The effect of vigour is more noticeable when comparing strains that are taxonomically similar than for more distantly related taxa and exclusion of this component may reduce taxonomic differences between species (Sneath, 1968; Sodell & Seviour, 1998). Setting the test chronometric is a balance between expediency of a short incubation time and achieving expression of the latent phenotype and increased reliability of the test. In this study of *Vibrionaceae* vigour is an important component of phenotype given the high level of species similarity and as a consequence careful consideration was given to setting the test chronometric. Using

48 hr for test incubation time appears sufficient to express sufficient strain and species vigour and remains practical as the basis for identification, particularly in a diagnostic setting.

A range of incubation times and temperatures have been used for numerical taxonomic studies of *Vibrionaceae* from 24 hr (Dawson & Sneath, 1985) to 48 hr (McNicol *et al.*, 1983; Montes *et al.*, 1999) through to two weeks (West *et al.*, 1983; West *et al.*, 1984; Nair *et al.*, 1985) and incubation temperatures from 20°C (Martin-Kearley & Gow, 1994) to 30°C (Kämpfer *et al.*, 1987) though most studies have used 25°C. The range of incubation times and temperatures highlights the need for standardisation and the problem of comparing data from studies that have used different metrics.

Test error

Where test error is excessive, the underlying taxonomic structure may be distorted and mask the relationships of taxa in a numerical taxonomic study. Test error has the effect of reducing the similarity between phenetically similar strains, making clusters more diffuse and less coherent. The average test error (p) for all tests in the data set was 4.7%, and is less than the recommended maximum error of 5% for a numerical taxonomic data set (Sneath & Johnson, 1972). It is recommended that individual test error (p_i) should not exceed 0.1 although strategically it is better to include more tests in a taxonomic study even if some are unreliable (Sneath & Johnson, 1972). Accordingly, tests with variance greater than 0.15 were excluded from the data set, which reduced the average test error to 4.0%. In this study, five tests (see Table 7.10) had a p_i between 0.1 and 0.15. Of these, alkaline phosphatase (indoxyl phosphate) and utilisation of propionate were found to be useful in discriminating between species (*vide infra*), and demonstrated the value of including tests which are less reproducible. The cause of error in these tests is not immediately apparent but as four of the tests involved an assessment of colour, variance may have been due to interpretation of weak reactions. Bryant *et al.* (1986a) found that by including tests with a p_I of 0.15, clusters were less well defined than if tests were used with a p_I of 0.10 or less. Although the effect on cluster outcome of using tests with a p_I of 0.15 was not assessed in this study, the impact is likely to be small since these more variable tests represent only 4.8% of the tests used compared with 14.0% in the study of Bryant *et al.* (1986a).

Overall, performance of characterisation tests for the *Vibrionaceae* are reproducible. Average test errors are reported to be as low as 1.5% (West *et al.*, 1983) and as high as

5.4% (West *et al.*, 1986), though values of 4.1% (McNicol *et al.*, 1983), 4.5% (Dawson & Sneath, 1985) and 4.3% (Bryant *et al.*, 1986a) are more typical and compare well with the error level reported in this study.

8.1.4 Genotyping

Intended purpose of partial sequences

Comparison of 16S rRNA gene sequences has become a central element of bacterial molecular systematics and has resulted in improved classifications for many groups of bacteria (Ludwig & Klenk, 2001). While ribosomal genes are highly conserved and can provide much useful phylogenetic information (Woese, 1992) only the 16S rRNA gene has been found useful. The small size of 5S rRNA and extensive secondary and tertiary structure of the 23S rRNA genes make them less suitable for sequencing and taxonomic analysis (Raghava *et al.*, 2000). With well established protocols for obtaining 16S rDNA sequences from conserved regions within the gene (Lane, 1991; Dorsch & Stackebrandt, 1992; Cai *et al.*, 2003;) extensive sequence catalogues have been generated (Benson *et al.*, 2004), which can be used to infer phylogenetic relationships and create stable classifications.

Partial sequencing of the 16S rRNA gene was used primarily as a means of verifying the composition of clusters based on phenotype. By using an independent means of determining the likely identity of a strain, it was possible to achieve an additional level of assurance that the composition of clusters was well founded and accurate. Confidence in cluster composition is important as the quality of the phenotypic data for the identification scheme derived from the cluster analysis is predicated on the homogeneity of the taxospecies, represented as clusters.

Utility of partial sequences to reveal taxonomic affinities

The partial sequence generated using the 685r2 primer represents approximately 40% of the 16S rRNA gene. This is a short segment containing three hypervariable regions with sequence motifs unique for species of the *Vibrionaceae* (Dorsch *et al.*, 1992) yet has considerable sequence variation that is defining for species of the *Vibrionaceae* (Kita-Tukamoto *et al.*, 1993). While many taxonomic studies have used almost complete sequences of the 16S rRNA gene, there is good evidence that shorter sequences can be effective in species identification. Such sequences, typically around 500-800 bp in length, use information rich areas of the gene specific for the target species (Song *et al.*, 2005). Short sequences may also have more universal application

and be suitable for identifying a range of species across several genera (Domann *et al.*, 2003; Christensen *et al.*, 2005).

Data integrity

The library of 792 strains was sequenced in several large batches and so the quality and reliability of the sequence data was a significant concern throughout the project. A number of strategies were adopted to minimise coding and labelling errors considered to be potential sources of inaccuracy. Strain numbers were used to label data files and also embedded within the metadata associated with the sequences. All the sequence trace files were visually assessed and manually edited prior to sequence searching. While this was to some extent a subjective process, where the sequence was trimmed according to the appearance of the chromatogram, in most cases the choice was clear cut.

Given the number of strains sequenced a level of error is expected. Sources of error are of two types: firstly, that inherent with the process of sequencing and second, process error associated with handling large data sets. Of the former, sequence error has been reported as high as 1.25% with a further 1% due to random error (Clayton *et al.*, 1995) while alignment tools can have calculation errors as high as 2.8% for sequence lengths of 500 nt (Clarridge, 2004). Marginal improvements in sequence fidelity have been found when both forward and reverse sequences are used but assigning a species identification can be achieved using either sequence direction (Clarridge, 2004). For this study, only the reverse sequence was used but given the number of strains assessed, the intended use of the data and the marginal error rate of using only a unidirectional sequence, this less rigorous approach is justified. An assessment of the total error inherent in sequencing is beyond the scope of this study other than recognising that it may occur but is likely to have only a marginal effect on identification outcome.

A formal estimate of process error was not undertaken in this study but in a biased investigation of 9% of the genotype library that had noticeable anomalous results, there was an overall 33% disagreement in re-sequence outcomes. The cause of the observed difference could not be readily identified but was thought likely to be due to operational transcription or handling errors of culture materials. Projection of this error rate to the sequence data as a whole is not justified given that the estimate was from a biased sample and there was no evidence based on subsequent analysis that the data set was significantly compromised. A level of caution nevertheless needs to be applied

to the data but since the purpose of genotyping in this study was to provide information to confirm the phenetic taxonomic structure, a degree of error in sequence outcomes can be tolerated.

Identifications based on significant sequence alignments were made using data in the public repository GenBank. As has been observed (Bosshard *et al.*, 2004; Clarridge, 2004; Hoshino *et al.*, 2005; Song *et al.*, 2005), the accuracy of nomenclature for sequences lodged in public repositories is questionable and may not represent the nominated species. In the extreme this may have a profound effect on strain identification with matches ranging from 100% to 23% depending on the database used (Turenne *et al.*, 2001). An assessment of the quality of sequence data in public repositories has shown that 5% of 16S rDNA sequences contain chimeras or regions of degeneracy (Ashelford *et al.*, 2005). Most of the anomalous data is chimeric and has arisen from culture-independent studies but there was also evidence of sequence degeneracy in cultured samples including the 16S rDNA sequence for the type strain of *V. rumoiensis* [AB013297].

Species identification

An identification was accepted if the expected match occurred within the first 10 ranked results of the BLASTn search. For clusters containing a type strain, it was anticipated that the expected result would match the cluster definition based on the type strain; for un-named phenons, the expected match was defined as the strain designation that occurred most frequently amongst the cluster members. In many instances the match expected for a cluster was ranked first but not in all cases when the expected match occurred at a lower rank or did not appear in the first 10 possibilities.

A relaxed approach to accepting a species identification was taken for several reasons. Although partial 16S rDNA sequences have been found useful for bacterial identification, this approach may be less robust for the *Vibrionaceae* given the high level of species relatedness that ranges from 91 to ≥ 99% similarity (Kita-Tsukamoto *et al.*, 1993; Ruimy *et al.*, 1994), so that some taxa are indistinguishable by 16S rDNA sequence (Thompson *et al.*, 2004a). Although the region of the 16S rRNA gene sequenced is known to be information rich, it clearly, given the high level of similarity that exists between species, may not be sufficient for species differentiation in all instances.

Anomalies were clearly evident with some data, which tended to support the view that a sequence in the first 10 ranked matches was sufficient to accept an identification. Phenon 13, defined as *V. harveyi*, contained the type strain of the species ATCC 14126. Of the 62 members of the cluster, 32% of strains had a first rank order match and 45% of strains with a match within the first 10 rank order of possibilities, Table 8.2. Strains which either no match or a low match rank were tested by species specific PCR and were all found to be positive. Of note is the finding that strain V236 which had a

Table 8.2 Sequence matches with *Vibrio harveyi* in phenon 13 and confirmation of identity using the species specific PCR primers of Oakey *et al.* (2003)

Category of match with *Vibrio harveyi*	No. of strains	% strains	No. tested by PCR	% positive by PCR
Rank order match of 1	20	32%	2	100%
Rank order match of 2	5	8%	-	-
Rank order match of 5	2	3%	-	-
Rank order match of 9	1	2%	1	100%
Strains with no match with *V. harveyi*	24	39%	6	100%
No. strains with no sequence data	10	16%	2	100%
Total no. strains in cluster	62	100%		

sequence match ranked ninth for *V. harveyi*, was PCR positive as were all the strains tested that did not list *V. harveyi* as a possible match. One of these strains, V213, was submitted as *V. parahaemolyticus*; the best sequence match was with the type strain for *V. natriegens* but had matches with *V. parahaemolyticus* from ranks 6-10. Nevertheless, the strain clustered with *V. harveyi* on the basis of phenotype and its position confirmed by species specific PCR.

Sequence lengths used for this study were variable and ranged form 652 to 673nt depending on degeneracy at the ends. To improve species identification where a library of strains is assessed, it has been recommended that sequences of the same length are used with the ends cut at predetermined and consistent locations (Song *et al.*, 2005). In addition, sequences should be mapped in a phylogenetic tree using data from type strains as points of reference as a means of independently assessing similarity with the sequences of the unknowns. Anomalies associated with names of sequences lodged in GenBank are seen with Phenon 5, designated as *V. fluvialis*. The first ranked match for all nine strains, including duplicate type strains, was for bacterium K2-61 [AY345409] with the second ranked match for the type strain of *V. fluvialis*, ATCC 33809^T [X74703]. Bacterium K2-61 is a clone which is reported to have a sequence match of 99.4% with the type strain of *V. furnissii* ATCC 35016^T (Donachie *et al.*, 2004). A BLASTn search of the sequence for bacterium K2-61, however, shows a match of

99.9% with *V. vulnificus* strain MP-4 [AY911393], a plasmid bearing *Vibrio* from an unpublished study and not *V. furnissii* as reported. There is no apparent phenotypic overlap between *V. fluvialis* and *V. furnissii* in Phenon 4; both taxa form unique clusters. All 10 strains of *V. furnissii* have a first rank sequence match with the type strain ATCC 35016^T, further evidence of genotypic homogeneity of the strains that show no similarity to either *V. fluvialis* or *V. vulnificus*. Clearly there are some uncertainties surrounding the status and designation of bacterium K2-61 and use of the second ranked match of *V. fluvialis* is justified as it best describes the isolates both genotypically and phenotypically.

Using a relaxed approach to assessing strain identification partial sequences generally appeared to contain sufficient information to identify species, although for clades containing less well-differentiated taxa, a partial sequence appeared less reliable as a means of identification. Where data appeared confounding and the causes not clearly evident, sequence error may have arisen from the use of the 27f and 1492r primers to amplify the 16S rRNA gene. This primer pair has been shown to introduce artefacts consisting of concatenated primer sequences, particularly when PCR conditions are sub-optimal (Osborne *et al.*, 2005) and may be enough to introduce degeneracy sufficient to degrade sequence matches. Experimental error, particularly given the volume of sequencing undertaken, would explain some of the disparity, and nomenclatural ambiguity for strains in GenBank must account for the remainder of the observed differences. Overall, the strategy used for strain identification represents a reasonable compromise between accuracy and expediency given the size of the library to be surveyed and the intended purpose of sequencing.

Sequence outcomes

Nearly all the type strains had alignments that matched the species designations; the exceptions were *Ph. angustum* ATCC 25915 and *V. cyclitrophicus* CIP 106644 which did not appear as a match in the first ten alignments. The best match for *Ph. angustum* was *V. fischeri* strain ES915 [AY292920] belonging to a distinct clade of the species (Nishiguchi & Nair, 2003), while *V. cyclitrophicus* appeared most closely aligned with an unpublished description of *V. tasmaniensis* strain 236.10 [AY620969]. Failure to obtain the expected alignment for these two type strains is not clear though for *V. cyclitrophicus* this may be due to the close sequence similarity that exists between it and members of the *V. splendidus* complex (Thompson *et al.*, 2004b) and in particular *V. tasmaniensis* (Thompson *et al.*, 2003e).

For several type strains a match was obtained for the expected designation but the alignment was not the first choice. For *V. anguillarum*, the best alignment was with *V. ordalii* but this is not unexpected since only a partial sequence was used and there is a 99.4% sequence similarity between the two species (Wiik *et al.*, 1995). A similar case applies for *V. scophthalmi* and *V. ichthyoenteri* where there is close 16S rDNA sequence similarity (Thompson *et al.*, 2002a; Sugita & Ito, 2006). Using FASTA for sequence comparison (Pearson, 1990) and GenBank accessions U46579 and AJ421445 for the respective species there is, over a 1,357 nt region of 16S rDNA, a 99.3% similarity equivalent to a 5 nt difference. The high level of similarity between the two species and short sequence used for alignments would account for the misidentification of *V. scophthalmi* by sequence. Reasons why alignments for the remaining species did not match the expected species designation as the best match is not clear but may be the result of using only partial sequences; further investigation by re-sequencing new strains is required to resolve this uncertainty.

Vibrio harveyi, based on the partial sequence was best aligned with *V. harveyi* ATCC 35084, formerly the type strain for *V. 'carchariae'* but now a junior synonym of *V. harveyi*. The better match with *V. 'carchariae'* rather than the type strain as would be expected is probably the result of using only a partial sequence.

Most of the reference strains could also be correctly identified using partial sequences of 16S rDNA and mirrored the performance of type strains. A group of six species *V. agarivorans, V. campbellii, V. lentus, V. mediterranei V. mytili* and *V. scophthalmi* had partial sequences that did not match the expected species designation. All the strains, except *V. campbellii*, were obtained from the CECT culture collection. All the strains, including *V. campbellii*, were correctly identified by phenotyping. Of note is that these strains all had similar working strain numbers and it is likely that failure to identify these strains by sequencing is due to experimental error from a labelling inconsistency during sequencing.

While most of the type strains were successfully allocated to their designated species on the basis of a partial sequence, there is sufficient evidence to suggest that for some species this not sufficiently large for the purpose of identification. The *Vibrionaceae* consists of species that span a range of sequence similarities that reflect the phylogenetic development of the family (Kita-Tsukamoto *et al.*, 1993) with some species clearly being closely related and others more distant (Ruimy *et al.*, 1994, Thompson *et al.*, 2004a; Thompson *et al.*, 2004b). High levels of similarity for instance

are seen with the *V. splendidus* clade that contains species which have sequence similarities in excess of 99% and are difficult to define even by DNA:DNA hybridisation (Gay *et al.*, 2004).

8.1.5 Cluster analysis

Cluster approach

Many strategies are available for cluster analysis but the most common for bacterial numerical taxonomy is to use either the simple matching coefficient S_{SM} or Jaccard's coefficient S_J, with matrices clustered using UPGMA. In this study the approach that best partitioned the data to reveal taxonomic structure was the Jaccard coefficient and Ward's method for clustering.

Jaccard's similarity coefficient is one of several association coefficients that have been used for bacterial taxonomic studies. Unlike the simple matching coefficient that considers both positive and negative matches, Jaccard's coefficient ignores negative matches and calculates proximities solely on positive associations (Priest & Austin 1993). Sneath and Sokal (1973) observed that Jaccard's coefficient is appropriate for microbial taxonomic data as omitting negative matches can be justified on the basis they may be the result of unexpressed latent phenotypes rather than negative traits that have biological and taxonomic meaning. In a review of similarity coefficients and their relative value for bacterial numerical taxonomy, Jaccard's similarity coefficient was found to be good at revealing subtle associations that were not apparent using the more commonly used simple matching coefficient S_{SM} and related monotonic coefficients (Austin & Colwell, 1977). Jaccard's coefficient, therefore, is seen as an appropriate strategy for calculating proximities for bacterial taxonomic data.

A variety of hierarchical agglomerative procedures are available for clustering dis(similarity) matrices of which single linkage and average linkage or UPGMA is most commonly used for bacterial taxonomic studies. A characteristic of this procedure is the creation of well defined clusters that tend not to distort the underlying taxonomic structure (Priest & Austin, 1993; Romesburg, 2004). The conservative approach inherent with UPGMA may produce a more diffuse structure less able to distinguish clusters in dense areas of character space (van Ooyen, 2001). Ward's method is better at recovering cluster structure from dense data to yield a unique and exact hierarchy. Characteristically, clusters, defined by Ward's method, are compact and well defined with a clearly evident arrangement (Romesburg, 2004). The method can be sensitive to

outliers and some clusters may contain OTUs that have little in common other than they are less dissimilar to other clusters (van Ooyen, 2001).

Jaccard similarity data transformed to the Jaccard distance as a dissimilarity metric remains non-Euclidean (Anderson, 2006) yet can be accommodated by Ward's method to calculate cluster distances in Euclidean space and is sufficiently robust to yield high rates of cluster recovery (Finch, 2005). Although not widely applied, this approach has been used to assess the distribution of marine mollusca in Arctic fjords (Aitken & Gilbert, 1996), bacterial population dynamics in bioreactors (Saikaly *et al.*, 2005) and for a numerical taxonomic study of *Vibrionaceae* in seawater (Martin-Kearley & Gow, 1994) which used squared Euclidean distance for proximities and Ward's method for clustering.

Structure of the phenogram

The selection of tests and choice of cluster analysis yielded an unexpectedly well differentiated taxonomic structure of the *Vibrionaceae*. Phenotyping is reported to have limited value in differentiating species (Vandenberghe *et al.*, 2003; Gomez-Gil *et al.*, 2004; Le Roux *et al.*, 2004; Thompson *et al.*, 2004b), which is surprising given that numerical taxonomy has previously proved a useful means of classifying the *Vibrionaceae* (Bryant *et al.*, 19861a). Discovery of taxonomic structure was undertaken by first applying a cut-off at a 5% dissimilarity level to establish a framework which was then refined by sequential inspection of potential clusters and setting boundaries on a case-by-case basis (Sackin & Jones, 1993; On & Holmes, 1995). This approach yields clusters that better reflect phenotypic variation within taxospecies so that tight, well separated clusters are recognised alongside more diffuse and loosely defined clusters which exhibit greater phenotypic variation. These more phenotypically diverse clusters appear in the phenogram as deeply projecting clusters with fusion points at higher levels of dissimilarity compared to compact clusters which are phenotypically more homogeneous.

Boundaries to clusters were, on the whole, well defined on the basis of inclusion of type and reference strains within clusters and the phenogram appears to reflect a good natural taxonomy for species of *Vibrionaceae* included in this study. In 10 instances clusters appeared well-defined (Table 8.3) but contained type strains for two species. In these instances, clusters were partitioned on the basis of 16S rDNA sequence identity of the cluster members. Phenon 48 consisted of two subclusters containing type and wild type strains of *V. campbellii* and *V. splendidus* biovar II. Sequence data confirmed

Table 8.3 Clusters defined on the basis of 16S rRNA sequence data of cluster OTUs

Phenon no.	Components
16-17	*V. calviensis* and *V. tubiashii*
18-19	*V. orientalis* and Phenon 19 *Vibrio* sp.
22-23	*V. rumoiensis* and *V. aestuarianus*
26-27	Phenon 26a - *V. cyclitrophicus*, Phenon 26b - *Vibrio* sp., Phenon 27 - *Vibrio* sp.
35	*V. vulnificus* biovar I and *V. vulnificus* biovar II
37-38	*V. nigripulchritudo* and *V. penaeicida*
48	*V. campbellii* and *V. splendidus* II
60-61	*V. logei* and *Ph. phosphoreum*
62	*V. fischeri* biovar II and *Ph. angustum*
80-81	*V. wodanis* and *V. tapetis*

that the subclusters formed distinct taxa and justified their separation into two clusters. Bryant *et al.* (1986a) had reservations regarding the phenotype of *V. splendidus* biovar II, which differed from that given by Baumann *et al.*, (1984), and observed that the published description was more like *V. campbellii*, a characteristic also noted by Baumann *et al.* (1984). Like Bryant *et al.* (1986a), it was found that *V. splendidus* biovar II was arginine dihydrolase positive but reported by Baumann *et al.* (1984) to be negative. Of note is that in the original description of *'Beneckea' campbellii* (Baumann *et al.*, 1973), which included strains later classified as *V. splendidus* biovar II, 65% of the strains were not tested for arginine dihydrolase. Also, where strains were tested, an enzymic method was used (Stanier *et al.*, 1966) and not Thornley's method (Thornley, 1960), the procedure used by Bryant *et al.* (1986a) and in this study. The phenotype generated for both *V. splendidus* biovar II and *V. campbellii*, while clearly similar could be readily differentiated using the phenotyping panel developed in this study.

Only two clusters could not be resolved logically by phenotype and genotype. Cluster 62 contained the type strain for *Ph. angustum* ATCC 25915 and wild type strains of *V. fischeri*. Phenotypically the strains appear similar and members of the cluster, including *Ph. angustum*, had 16S rDNA sequences that aligned with *V. fischeri*. A sequence comparison however of *Ph. angustum* ATCC 25915 [D25307] and the sequence obtained for *Ph. angustum* ATCC 25915 used in this study showed only a 92.4% sequence similarity over a 1,476 nt overlap. Clearly there is some doubt as to the provenance of the type strain of *Ph. angustum* that was used in this study. Phenotypically and genotypically the cluster appears to be a discrete taxon consisting of strains of *V. fischeri*. Members of the cluster had sequence matches with strains of *V. fischeri* from a clade representing symbiotic forms of *V. fischeri* (Nishiguchi & Nair, 2003). Of note is that four strains in Cluster 62 were isolated from the light organ of

Euprymna tasmanica, E. moresie, E. scolopes and *Monocentris japonicus*, all species of squid and is further evidence of the taxonomic integrity of the phenon.

A second cluster, Phenon 67 contained six strains comprising type and reference strains of *V. gazogenes* and the type strain of *V. cincinnatiensis*. On the basis of cluster analysis the species appear to have highly similar phenotypes, a characteristic found by Montes *et al.* (1999) in a numerical taxonomic study of *Vibrionaceae* from the skin of turbot. The type and reference strains for both species had partial 16S rDNA sequences that matched the expected species identity but by cluster analysis no logical subclusters could be defined to represent the two species. It was possible to separate both species phenotypically by partitioning OTUs on the basis of 16S rDNA sequence data. Failure of cluster analysis to differentiate the species in this instance is not immediately obvious. It may reflect the use of Ward's method, which allocates OTUs to clusters that cause the minimum increase in variance. In doing so there is a risk that outliers in character space are allocated to clusters of least dissimilarity even though as a result taxonomic affinity between some members of the cluster may be low (van Ooyen, 2001).

While it is possible to create heterogenous clusters from disparate outliers using Ward's method, the strength of using Jaccard's similarity coefficient with Ward's method is the ability to create well defined and hierarchic taxonomic structures. The methodology appears particularly sensitive with the known biovars of *V. splendidus, V. pelagius* and *V. vulnificus* clearly delineated in separate clusters. In addition to these recognised biovars, distinct phenotypic biovars, supported by 16S rDNA sequence data were evident for *V. harveyi*, *V. ichthyoenteri, V. fischeri* and *Ph. damselae* ssp. *damselae*. Recognition of biovars is implicit with a numerical taxonomic approach to phenotyping (McNichol *et al.*, 1983) and consideration needs to be given as to their taxonomic meaning and whether sufficient characteristics exist to effectively delineate them to have diagnostic value. The biovars nominated in this study could be differentiated phenotypically and were generally associated with particular hosts or disease processes. In particular, *V. harveyi* biovar II was isolated exclusively from abalone with blister disease, an acute to chronic form of septicaemia while *V. ichthyoenteri* biovar II was recovered only from Tasmanian farmed Atlantic salmon infected with a *Rickettsia*-like organism (Corbeil *et al.*, 2005). *Vibrio fischeri* biovar II represents symbiotic strains while the second biovar of *Ph. damselae* ssp. *damselae* is characteristically less reactive phenotypically than strains of biovar I.

The library of strains for numerical taxonomy contained only non-O1 strains of *V. cholerae* and consequently did not include the type strain which is a classical O1 biovar. The non-O1 strains formed a discrete cluster that was readily distinguished from the closely related species *V. mimicus*. Although the type strain of *V. cholerae* was not included in this study, omission of this and other O1 strains is unlikely to modify the phenon description as the taxon is considered phenotypically homogeneous irrespective of serotype (Colwell, 1970; McNicol *et al.*, 1983; West *et al.*, 1983; Dawson & Sneath, 1985; Bryant *et al.*, 1986a).

Assessment of taxonomic structure, inferred from the phenogram, revealed four orphan reference strains, which clustered in phenons unrelated to their apparent species designation. Misallocation appeared to be of two types: phenotyping aberration or strain provenance. Phenotype aberration occurred with *Vibrio logei* CIP 103204, *V. ichthyoenteri* ATCC 700024 and *Ph. damselae* ssp. *damselae* ATCC 51805 (the type strain for *Ph. 'histaminum'*, a junior synonym of *Ph. damselae* ssp. *damselae*) all of which had 16S rDNA data that confirmed their species designation. Occurrence of these three strains in unexpected clusters appears to be due to atypical phenotypes. Interestingly, when a subset of phenotype data was used for identification purposes the three strains were correctly identified, indicating that the atypical phenotypes occur outside the core characteristics that defines the respective species. Conversely, *V. fischeri* ATCC 33984 clustered in Phenon 73, which is defined as *Ph. leiognathi* and contains the type strain of the species, ATCC 25521. The partial sequence of *V. fischeri* ATCC 33984 aligned with *Ph. leiognathi*, [AY292944] indicating that the phenotype and genotype of the strain is consistent with the designation of *Ph. leiognathi* for Phenon 73. Using the identification panel developed for the *Vibrionaceae* the strain was identified as *Ph. leiognathi*, further evidence that the strain, as acquired, has core phenotypic characteristics consistent with *Ph. leiognathi*. From the data obtained it would appear that *V. fischeri* ATCC 33984 may be misclassified, however further investigation using a newly acquired strain is necessary to confirm this conclusion.

The major purpose of the numerical taxonomy of the *Vibrionaceae* was to obtain phenotypic data that would be sufficiently discriminating for use in probabilistic identification. It has been proposed that sufficient phenetic depth to locate the centre and radius of a cluster in character space (Sneath, 1977) could be obtained from at least 25 strains or at minimum 10 strains; clusters containing 3-4 OTUs are considered barely acceptable (Sneath, 1976). Of the 86 phenons defined only five contain at least

25 strains and a further 17 phenons contain the minimum number of strains recommended. While no significant cluster overlap was detected it does highlight a weakness in the data set as a whole. Clearly, further strains are required to obtain more robust descriptions of those phenons containing small numbers of strains.

Characteristics of phenons defined as taxospecies

A detailed analysis of phenon characteristics was not undertaken because a comparative analysis with published data is of little value given the diversity of techniques used. Many of the newer species described have only limited phenotypic descriptions, and that which is provided is based on identification panels such as API 20E (bioMérieux, Mercy-l'Etoile, France) and Biolog GN, which have been found to be of little value for the identification of *Vibrionaceae* (Austin *et al.*, 1997; Vandenberghe *et al.*, 2003). Comparison is made more difficult because there is little consistency in the tests used and the phenotypes described across species (for comparison see Sawabe *et al.*, 1998; Macián *et al.*, 2001; Thompson *et al.*, 2003d; Hjelm *et al.*, 2004). The lack of uniformity of tests is recognised as weakness in the description of the *Vibrionaceae* and there have been recommendations over a period of years to establish a minimum standard for the description of species (Nair & Holmes, 1999; Nair & Holmes, 2002; Nair & Holmes, 2005) though as yet no standard has been developed or proposed.

The effect on phenotype of different methodologies is illustrated by procedures used in just three tests: the determination of sensitivity to the vibriostat 0/129, deamination of tryptophan to form indole and arginine dihydrolase activity. *Vibrio lentus* is reported to be uniformly resistant at 150µg (Macián *et al.*, 2001) but in the study reported here, all strains, including the type, were found to be sensitive including several used in the study of Macián *et al.*, (2001). The difference in observed reaction is likely due to the choice of medium for sensitivity testing. Macián *et al.* (2001) used the medium STB based on tryptone and yeast extract. In this study, Mueller-Hinton agar supplemented with 2% NaCl was used, as recommended for sensitivity testing of aquatic animal pathogens (Miller *et al.*, 2003), because it is a defined medium low in thymidine content and because there is no apparent interference due to increased levels of NaCl (Ottaviani *et al.*, 2001). Assessment of the folate inhibitor diaminopyrimidine and its analogues trimethoprim and the pteridine compound 0/129 (Matsushita *et al.*, 1984), needs to be made with media low in thymidine which otherwise is used by alternate pathways that avoid folate metabolism in the synthesis of purines and pyrimidines

(Koch & Burchall, 1971). The apparent resistance of *V. lentus* to 0/129 may be due to the presence of thymidine in the medium STB derived from the tryptone and in particular yeast extract which contains significant amounts of the pyrimidine nucleoside. Medium interference in sensitivity testing of trimethoprim-sulphamethoxazole has also been observed with strains of *Ph. damselae* ssp. *piscicida* which were resistant on tryptone soy agar but sensitive with Mueller-Hinton agar (Thyssen & Ollevier, 2001), further evidence of the need to use defined media with a low thymidine content for testing sensitivity to 0/129.

In the original descriptions for *V. fluvialis* (Lee *et al.*, 1981) and *V. furnissii* (Brenner *et al.*, 1983), both species were reported to be almost uniformly negative in the test for indole. The inability of the two species to form indole by deamination of tryptophan has been confirmed in other numerical taxonomic studies (Ortigosa *et al.*, 1994; Montes, *et al.*, 1999). In all instances the test was based on meat infusion broths but these can give inconsistent results (MacFaddin, 2000) because the amount of tryptophan present in batches of meat is variable. A more reliable medium is based on trypsinised casein which contains consistent and higher concentrations of tryptophan compared to meat based media. When tryptone water (Oxoid) is used a weak positive reaction has been observed for *V. furnissii* (Buller, 2004) although *V. fluvialis* remains negative (data not shown). The ability of both species to form indole is however readily apparent in the API 20E test format (Dawson & Sneath, 1985; Tall *et al.*, 2003) and Flow Laboratories TTE-AS miniaturised system (Kämpfer *et al.*, 1987). In this study of the *Vibrionaceae* all strains of *V. fluvialis* (*n*=9) and *V. furnissii* (*n*=10) were positive in the MicroSys V36 test for indole and in the conventional tube test using tryptone water (Oxoid) supplemented with tryptophan. The reaction for *V. fluvialis* is weakly positive but readily discernible (Figure 8.2B), appears stable and does not fade with time. With unsupplemented tryptone water *V. fluvialis* is negative for indole (Figure 8.2A) while *V. anguillarum* has a weak positive reaction that is just discernible. These findings are consistent with the observation of Benediktsdóttir and colleagues (1998) who also found that *V. fluvialis* was indole positive in media supplemented with tryptophan. To reduce test variability with species that form only small amounts of indole, supplementation with tryptophan is recommended (MacFaddin, 2000) and for the *Vibrionaceae* this would appear to be a prudent strategy to obtain consistent results that are independent of batch-to-batch variations in medium compositon.

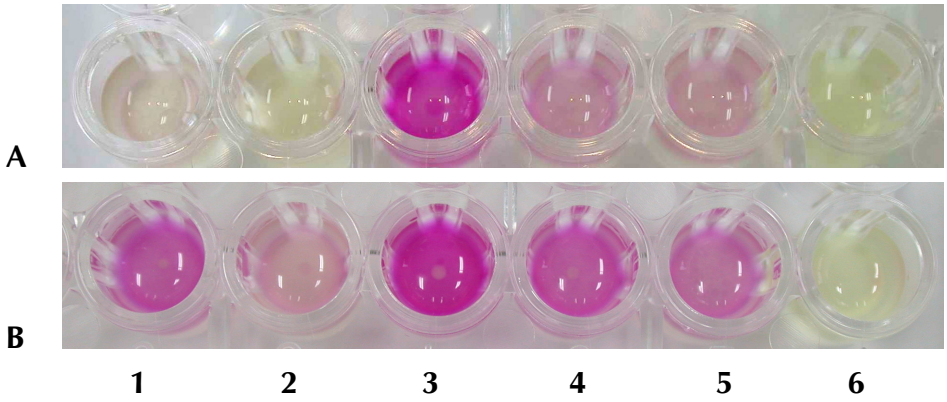

Figure 8.2 Range of intensity for indole test for species of *Vibrionaceae*. Sub-samples from conventional tube tests of tryptone water. A: un-supplemented; B: supplemented with tryptophan. 1: *V. anguillarum*; 2: *V. fluvialis*; 3: *V. mediterranei*; 4: *V. tubiashii*; 5: *V. parahaemolyticus*; 6: *Ph. damselae* ssp. *damselae*. Test after 48 hours incubation at 25°C.

In the original description of *V. mediterranei*, strains of the species were reported to be arginine dihydrolase negative (Pujalte & Garay, 1986). Subsequently, *V. 'shilonii'*, also arginine dihydrolase negative (Kushmaro *et al.*, 2001), was found to be a junior synonym of *V. mediterranei* and reported to have phenotypes consistent with the original descriptions of the type strain and its junior synonym (Thompson *et al.*, 2001). All three descriptions however are based on different methods for determining arginine dihydrolase. The description of *V. 'shilonii'* and its reclassification as *V. mediterranei* used API 20NE and API 20E phenotyping panels, respectively, while the description of the type strain was based on either the ornithine assay of Ratner or a modified formulation of Thornley's medium (Baumann & Baumann, 1981); which of the two procedures used was not specified by Pujalte & Garay (1986). In the study of *Vibrionaceae* reported here all the type and reference strains of *V. mediterranei* and the type strain of *V. 'shilonii'* were found to be arginine dihydrolase positive by the West & Colwell (1984) modification of Thornley's method (1960), consistent with the results of Macián *et al.* (1996) who used the methods of Ratner and the Baumann modification to Thornley's medium. Of note was the finding that arginine dihydrolase in *V. mediterranei*, like the streptococci and pseudomonads, is induced by arginine and suppressed by glucose (Marquis *et al.*, 1987) so that by Møller's method for arginine decarboxylation, strains are consistently negative because of glucose in the medium. A consistent form of testing is important, particularly for arginine dihydrolase, which has particular value as a differential test for species of the *Vibrionaceae*. Analysis of tests in this study found that arginine dihydrolase had the greatest resolving power of all the tests evaluated (S_{max}=79%; *Section 7.2.2 Creation of the probability matrix*) and serves to

illustrate the importance of using standardised tests, particularly when used with well defined identification schemes. Test failure through inappropriate substitution of methods can have severe consequences on identification outcomes, particularly with tests of high resolving power.

Clearly, test procedure is critical for assessing phenotypes within the *Vibrionaceae* and test equivalence cannot be assumed without prior knowledge and testing. The lack of standardisation in recent years, and the practice of uncontrolled test substitution has lead to markedly inconsistent descriptions of phenotypes. Of further concern is the practice of using published phenotypes of existing species to discover characteristics that can differentiate new species. If inappropriate methodologies have been used for the nominated tests then this can lead to unjustified and misleading comparisons.

16S rDNA sequences of Hypothetical Median Organisms of unnamed phenons

Cluster analysis revealed 29 phenons that did not contain a type strain of a known species. Since 35 valid species were not included in this study there was a likelihood that some of the phenons may represent one or more of the newly described species not included in this study. The identity of phenons was investigated by determining 16S rRNA gene sequences of the HMO for each cluster, with homogeneity of clusters determined from partial sequences for the cluster members. For some phenons there was good agreement between the partial and full sequences of the HMO and evident homogeneity within clusters. Phenons whose identity was more clearly defined were limited to Phenon 32, classified as *V. harveyi* biovar II, Phenon 64, identified as *Aeromonas sobria* (*sensu stricto*), Phenon 75, classified as *Ph. damselae* ssp. *damselae* biovar II and Phenon 77, classified as *V. ichthyoenteri* biovar II. The sequence similarity for *V. ichthyoenteri* was 98%, which is less than the 99% threshold proposed for species identification (Clarridge 2004) and so the status of the phenon remains tentative. Other phenons which could be provisionally identified were Phenon 6 as *V. xuii* and Phenon 24 as *V. pomeroyi*. For the remaining phenons the HMOs and partial sequences matched unspeciated strains of *Vibrio*. Attempting to determine the identity of these unnamed phenons using 16S rDNA sequences is problematic because of the lack of consistency in the sequence data. Incongruence was evident in two forms: either as lack of agreement between partial and full sequences or, poor homogeneity of cluster membership based on partial sequences.

Of the 29 unnamed phenons, there were only eight agreements between the partial and full sequence for the HMO. For the remaining sequences there was no agreement.

In some instances, such as Phenon 25, cluster members had alignments either as *Vibrio* sp. YWA or *Vibrio* sp. QY101. For these two strains sequence similarity is 99.5% over 1,520 nt and differences between partial and full sequences can be attributed to sequence length. Of more concern are gross differences between partial and full sequences such as V357 the HMO for Phenon 6. The partial sequence matches *V. xuii* but the full sequence aligns with a marine gliding bacterium UWA-1 [AB039966] and only at 97% similarity. Using a FASTA comparison of the two sequences for a 1,440 nt overlap, there is only a 73% sequence similarity, a difference sufficiently large to indicate experimental error. Similar anomalies are apparent for V781 the HMO for Phenon 43. The partial sequence matches *V. tasmaniensis* strain 236.10 while the best alignment for the full sequence is for a wild type strain of *Ph. damselae* ssp. *damselae* (AY147861). A comparison of sequences found only a 93% similarity over a 1,020 nt overlap, a clear indication of an anomalous finding . The species identity of [AY147861] was confirmed by sequence comparison with the type strain of *Ph. damselae* ssp. *damselae* ATCC 33539 [X74700], which had a similarity of 99.5%. Phenon 43 appears homogeneous phenotypically and all members of the cluster, including V781 the HMO, are urease negative a characteristic that is universally positive for *Ph. damselae* ssp. *damselae*. These data suggest experimental error and the status of Phenon 43 is unresolved.

The second form of incongruence is heterogeneity of cluster membership based on identities derived from partial sequences of 16S rDNA. For most phenons strain sequence similarity was high, typically more than 70% of strains had the same sequence but for phenons 20, 43, 57 and 69 there was no intra-phenon uniformity in sequence identity suggesting that cluster membership is heterogenous. These clusters however, when assessed by phenotype appear largely homogeneous. Using a core set of identification tests, all strains in phenons 57 and 69 were correctly identified while for clusters 20 and 43 over 80% of strains were correctly identified.

Tentative designations were given to the phenons using sequence identity of the HMO and partial sequences of the cluster members. Some caution is required with this approach. Several phenons have designations, *pro tempore*, that are the same. Phenons 21, 26 and 46 were designated as *Vibrio* sp. QY101 on the basis of intra-phenon homogeneity of sequence identity though each phenon has a distinct phenotype. Of note is that *Vibrio* sp. QY101 has been placed within the *V. splendidus* clade (Thompson *et al.*, 2004b), a group of species with high sequence similarity and strains

which have confounding levels of interspecies DNA:DNA hybridisation (Gay *et al.*, 2004; Le Roux *et al.*, 2004); the three phenons, therefore, may represent distinct taxa on the basis of phenotype. Strain designations are considered tentative as they are based on details given by the submitter to GenBank of the matching sequence. In many cases sequences are from unpublished data so it is not possible to determine the basis on which the stated identity was reached. Phenon 8 appears to be a homogeneous cluster phenotypically and genotypically. A sequence similarity with a 99% match was found with *V. lentus* strain Sat101 [AY292936] (Nishiguchi & Nair, 2003). *Vibrio lentus* is defined as Phenon 49, which contains both type and reference strains of the species; designation of a second phenon as *V. lentus* is problematic. The basis for identifying strain Sat101 as *V. lentus* is not given but using a FASTA comparison of sequences for *V. lentus* Sat101 and the type strain of *V. lentus* (Macián *et al.*, 2001) [AJ278881] there is only 97.9% agreement over a 1,549 nt overlap. This level of agreement is not sufficient to conclude that Sat101 is a strain of *V. lentus* and it is likely that the phenon represents a new species.

Phenon 10 has been tentatively designated as a biovar of *V. alginolyticus* based on sequence similarity of cluster members to this species. This is an interesting group since Phenon 9 was unambiguously designated as *V. alginolyticus* because of genotype and phenotype and inclusion of the type strain. The phenotypes of Phenon 9 and 10 are very similar and several strains from diagnostic samples from Tasmanian aquatic animals have been isolated that share properties of both phenons (data not included). While the phenotypes appear similar, Phenon 9 occurs on a separate branch to Phenon 10 suggesting that it has closer phenotypic affinities to *V. parahaemolyticus* (Phenon 11) than *V. alginolyticus* (Phenon 9). The similarity of *V. alginolyticus* and *V parahaemolyticus* phenotypically and genotypically is well recognised (Baumann *et al.*, 1973; Robert-Pillot *et al.*, 2002) and at one time *V. alginolyticus* was classified as *V. parahaemolyticus* biotype 2 (Sakazaki, 1968). The close proximity of the two species in the phenogram and the position of Phenon 10 in relation to them is not unexpected. Distinct biovars of *V. alginolyticus* have been described (Zorrilla *et al.*, 2003b), which suggests that Phenon 10 may represent a discrete phenotype of the species. Further investigation is required to establish the status of Phenon 10 using a species specific PCR for *V. alginolyticus* (di Pinto *et al.*, 2005) to resolve the taxonomy of the two phenons.

The level of intra-phenon sequence heterogeneity and difference between partial and almost complete sequences for individual HMOs may be due to experimental

error in some instances, but consideration should be given to the known polymorphism of the 16S rRNA gene in species of the *Vibrionaceae* (Moreno *et al.*, 2002). Sequence variation as a result of this polymorphism was found to be as high as 2%, which could have a significant effect on identification outcome particularly since the overall sequence variation in 16S rDNA across the entire *Vibrionaceae* family is less than 9% (Kita-Tsukamoto *et al.*, 1993). While species may have multiple copies of the 16S rRNA gene, for *V. parahaemolyticus* up to 10 copies (Tagomori *et al.*, 2002), any sequence heterogeneity that may exist across alleles does not appear to have a profound effect on the phylogeny of γ-proteobacteria that includes species of the *Vibrionaceae* (Coenye & Vandamme, 2003; Olivier *et al.*, 2005). A better assessment of the sequence data for strains allocated to the unnamed phenons might have been achieved using multiple sequences alignments to obtain a more objective measure of uniformity based on similarity rather than supposed sequence labels. Similarly, construction of phylogenetic trees might also provide useful information about the position of these unclassified strains relative to the known species of the *Vibrionaceae*.

Despite some misgivings as to the integrity of genotypes obtained, the data nevertheless clearly shows that many of the unnamed phenons form distinct taxa, not represented by any of the known species of *Vibrionaceae*. For some phenons, sequence data provided unambiguous evidence that they represent a distinct biovar of an existing species such as *V. harveyi* biovar II, the cause of blister disease in abalone. Bacterial infection in Japanese abalone, *Sulculus diversicolor supratexta*, is associated with *V. 'carchariae'* (Nishimori *et al.*, 1998). Of particular interest is that the reported phenotype for 9 strains is a good match with *V. harveyi* biovar II (Willcox *P*=0.9991; MLS=0.20482) and for an almost complete 16S rDNA sequence there was a 99.9% similarity with *V. 'carchariae'* and 99.4% to *V. harveyi*. These phenotypic and genotypic findings are entirely consistent with the description of *V. harveyi* biovar II determined for this study. For other unclassified phenons such as Phenon 6 and 24, there was good evidence that genotypically they were consistent with the recently described species *V. xuii* (Thompson *et al.*, 2003b) and *V. pomeroyi* (Thompson *et al.*, 2003d), respectively. Confirmation is required by phenotyping the type strains of these species and undertaking DNA: DNA hybridisation.

8.1.6 Congruence of phenome and genome

While phylogenetics as a means of establishing the framework of prokaryote taxonomic structure has become increasingly important, it should not be used as the

sole means of delineating taxa (Goodfellow & O'Donnell, 1993). In preference, genotypic and phenotypic data should be integrated and used as the basis for defining taxa, a polyphasic approach to bacterial systematics (Vandamme *et al.*, 1996; Owen, 2004). A convincing polyphasic taxonomy needs to achieve a high level of agreement between the two approaches such that there must be mutual consistency between phylogenetic and phenotypic based taxonomies (Wayne *et al.*, 1987). It has been noted that while phenotype is a key element of prokaryote taxonomy, acquisition of data is burdensome and often compromised by the use of inappropriate tests, commercial kit systems and lack of standardisation (Vandamme *et al.*, 1996). It has been suggested that description of the taxon must be exhaustive as it is the basis of a discriminative classification and a means of determining the diversity within a taxon (Rosselló-Mora & Amann, 2001). Integration of phenotype and genotype and assessing the degree of concordance or overlap of taxonomies is the basis for the concept of a phylo-phenetic species (Rosselló-Mora & Amann, 2001).

Phenotyping as a means of coherent description of *Vibrionaceae* is reported to be problematic (Gomez-Gil *et al.*, 2004; Thompson *et al.*, 2004b), yet on the basis of the polyphasic approach taken in this study there is good overall agreement between phenotype and genotype. It would appear that phenotype can be used to delineate and describe species of the *Vibrionaceae*. The evidence generally is that phenotypic data invariably supports and validates a phylogenetic taxonomy where adequate phenotypic data has been obtained (Goodfellow & O'Donnell, 1993). For 52% of the phenons defined phenotypically, there was a high level of congruence, better than 80% of strains, between the identity of OTUs determined by 16S rDNA sequencing and the species phenotype. For the remaining portion of the library the level of agreement was progressively less convincing. The true level of agreement may be better than it appears due to ambiguities and uncertainties in the sequence data generated in this study. From an assessment of the clusters for *V. harveyi* biovar II (Phenon 32) and *Ph. damselae* ssp. *piscicida* (Phenon 76), strains which did not have a match by sequence for the anticipated phenospecies were positive by species specific PCR targeting 16S rDNA. The apparent lack of agreement between sequence identity, PCR identification and species cluster clearly indicates a level of uncertainty in 16S rDNA sequence data generated for the library, and further work is required to validate the sequence findings obtained. It is likely that phylo-phenetic congruence may be higher than is evident with the data currently available.

While the degree of congruence is high for most phenons, there appears to be little or no overlap of genotype and phenotype for some taxa. Phenons in this category, not surprisingly, are members of the *V. splendidus* clade that include *V. splendidus* biovar I, *V. chagasii* and *V. cyclitrophicus*. These taxa have levels of phylo-phenetic agreement ranging from 0% for *V. cyclitrophicus* to 39% for *V. splendidus* biovar I. Members of this phylogenetic group are reported to have high levels of 16S rDNA sequence similarity (Le Roux *et al.*, 2004) and for some strains ambiguous DNA:DNA hybridisation affinities between species have been described (Gay *et al.*, 2004) akin to a genomovar (Ursing *et al.*, 1995). Interestingly for *V. tasmaniensis*, a member of the *V. splendidus* clade, the phylo-phenetic agreement is 100% indicating that at least one member of the clade can be clearly delineated using the polyphasic approach used in this study. The other phenon where no phylo-phenetic agreement occurred contained a single OTU, the type strain of *Ph. angustum* ATCC 25915, defined as Phenon 62a. Phenotypically and genotypically the strain has clear attributes of *V. fischeri* biovar II (Phenon 62b) associated with aquatic animals. Phylo-phenetically Phenon 62 appears as a coherent group of strains, further evidence of the validity of the polyphasic approach used in this study. The unexpected sequence identity obtained for the type strain of *Ph. angustum* points to an issue of strain provenance; re-evaluation with a new culture of the type strain is required to resolve the evident ambiguity of the findings. On this evidence, division of Phenon 62 into two subgroups is an artefact Further analysis of phenons with poor phylo-phenetic agreement is required to assess their taxonomic structure. For these taxa new 16S rDNA sequences need to be obtained to determine if incongruence is due to phenetic heterogeneity, sequencing error or inherent genotypic polymorphism.

The degree of congruence between taxonomic topologies derived from 16S rDNA sequencing and numerical taxonomies is not well described though there are an increasing number of reports comparing identifications reached using 16S rDNA sequencing and phenotypic identification schemes (O'Donnell *et al.*, 1993). The overall level of phylo-phenetic agreement obtained in this study of *Vibrionaceae* was 73% although for 52% of the clusters the intra-phenon agreement was closer to greater than 90%. The overall level of agreement is similar to that obtained for a diverse flora isolated from a mine gallery where the agreement between phenotype deduced by numerical taxonomy and sequence identity was found to be 70% (Boivin-Jahns *et al.*, 1995) and for clinical strains of *Bacteroides* species, agreement was as high as 74.5%

between phenotyping and sequence outcome (Song *et al.*, 2005). Concordance of numerical taxonomy with sequence identity has been partially explored for rhizobia isolated from woody legumes where generally good agreement between the two approaches was evident although the degree of coincidence was markedly influenced by how clusters were defined in the phenogram (Liu *et al.*, 2005). In a study of *Pseudomonas fragi* isolated from fish (García-López *et al.*, 2004), several clusters could be defined although these were differentiated by only a few tests. The overall phenotypic homogeneity was reflected in the high level of sequence similarity and despite the weakly delineated clusters, the strains formed a coherent taxonomic group of phylo-phenetic integrity. It is evident that the position of cluster boundaries can influence the extent of the overlap between phenotypic and genotypic topologies.

Phenotyping and comparison with 16S rDNA sequencing for identification of strains offers another means of assessing taxonomic congruence but needs to be approached with some caution. Invariably genomic sequencing, although a monothetic based taxonomy, is taken as the reference procedure against which a polythetic phenotypic taxonomy has been selected for comparison. Using commercial kit systems for phenotypic identification, agreements range from 0-47% (Drancourt *et al.*, 2000; Bosshard *et al.*, 2004; Hoshino *et al.*, 2005; Petti *et al.*, 2005), indicating that the phenotypes were not sufficiently descriptive of the taxa involved. While such assessments are important in determining the robustness of identification schemes, it demonstrates the deficiency of phenotypic descriptions of genospecies and the need to acquire complete and reliable data (Wayne *et al.*, 1987; Stackebrandt *et al.*, 2002). It should not be inferred that phenotyping is an inferior means of identification.

Significant advances have been made in recent years to develop a phylogenetically based taxonomic framework for the *Vibrionaceae* using both 16S rDNA sequences, DNA:DNA hybridisation and FAFLP analysis (Thompson *et al.*, 2004b). From these studies a diversity of species has emerged primarily in the form of genospeces that appear to be poorly delineated phenotypically. The species so defined do not represent a polyphasic approach to describing the species as it is recommended that the internal homogeneity of the taxon should be understood through extensive characterisation before a set of determinative phenotypic properties can be recognised (Stackebrandt *et al.*, 2002; Kämpfer & Rosselló-Mora 2004). The scope of characterisation is not specified but only makes sense where it is undertaken in the context of all the species in a genus and not from a limited range of phylogenetically similar taxa. The approach

used in this study considered the *Vibrionaceae* as a whole and developed the phenotypic dimension of the genospecies, thereby reaching a better phylo-phentic definition of species of the *Vibrionaceae*.

8.2 DEVELOP PRACTICAL, ROBUST PHENOTYPIC IDENTIFICATION SYSTEMS FOR *VIBRIO* SPECIES USING COMPUTER ASSISTED IDENTIFICATION SOFTWARE

Classification is the basis on which an understanding is obtained of taxa, their biological properties, taxonomic affiliations and place in the natural environment. Increasingly, classification of the prokaryotes is based on phylogenetic analysis using 16S rDNA sequences to establish a framework based on a natural phylogeny. The current classification of the *Vibrionaceae* has been established on phylogenetic lines, based largely on ribosomal sequences and latterly through the use of FAFLP analysis as a means of delineating species (Thompson *et al.*, 2001, Thompson *et al.*, 2004b).

A polyphasic approach to taxonomy is becoming increasingly important as a means of developing taxonomic depth to prokaryote classification. The information collected is diverse in content and includes phenotypic characters, DNA hybridisation data and chemotaxonomic markers such as fatty acids, quinones and polyamines. Integration of the data acquired from the differing taxonomic approaches has yielded the concept of the phylo-phenetic species (Rosselló-Mora & Amann, 2001), an information rich taxonomic unit in a natural classification. In the absence of phenotypic descriptions that reflect the uniqueness of a phylogenetic taxonomy, a classification cannot said to be truly achieved (Wayne *et al.*, 1987; Goodfellow & O'Donnell, 1993; Vandamme *et al.*, 1996; Stackebrandt *et al.*, 2002).

The emphasis on classification is important because identification is only possible in the context of a stable classification where species are well delineated phenotypically with unique determinative properties. Acquisition of descriptive and discriminatory attributes requires intensive and exhaustive assessment of characters, not only intra-species to determine internal diversity, but also inter-species if sufficient traits are to be identified as motifs uniquely characteristic of individual taxa. Piecemeal approaches to the description of species can make taxa appear as phenotypic islands disconnected from each other in their taxonomic neighbourhood.

Identification of *Vibrionaceae* has been undertaken or proposed by various means based on phenotypic or genotypic characters. It has been advocated recently that

phenotyping as a means of identifying *Vibrionaceae* is unreliable (Vandenberghe *et al.*, 2003; Gomez-Gil *et al.*, 2004, Thompson *et al.*, 2004b) and that molecular methods such as AFLP, 16S rRNA gene sequencing or DNA:DNA hybridisation should be used for the identification of *Vibrionaceae* (Thompson *et al.*, 2005). It has been argued that genetic based identification has significant limitations particularly where no information is available *a priori* to guide the choice of oligonucleotide probes or restriction enzymes to identify an unknown (Wilkes *et al.*, 2005). Sequencing the 16S rRNA gene is still a critical cost for diagnostic laboratories (Song *et al.*, 2005) while the capital cost of equipment for AFLP analysis is prohibitive for most laboratories which, together with the need to standardise methodologies and improve turnaround times (Janssen, 2001), means that this approach is unlikely to be adopted as a routine procedure for identification of the *Vibrionaceae*, at least in the immediate future. Despite the acknowledged accuracy of molecular tools, particularly for classification, phenotyping still represents a realistic and viable approach to identification if standardised tests are used. *Ipso facto* is the requirement for a descriptive database that does not consider just small subsets of near related species but takes a broader approach that accommodates a wide range of species using targeted differential tests. Unless a truly relational approach to construction of a database is contemplated that considers the broad panoply of species, phenotypic identification is likely to be of limited value.

Unified approaches to identification of the *Vibrionaceae* have been of two types: dichotomous keys and simultaneous tables. A widely used key is that of Alsina & Blanch (1994a; 1994b) but there are concerns that it is neither accurate (Montes *et al.*, 2003; Thompson *et al.*, 2004b) or sufficiently current as it covers only 36 of the 89 known species of *Vibrionaceae*. Keys are instinctively attractive as a rapid conclusion can be achieved and usually with only a minimal set of identification tests. For a key to be reliable however, the tests selected must represent characters that are stable and be truly positive or negative for all strains of the species. If this essential condition is not met then serious errors in identification can rapidly occur. For example, a six level key, where the probability of a correct answer is 90% for each test, the odds of achieving a correct identification at the last level of the key are 50/50, clearly an unsatisfactory outcome (Priest & Williams, 1993). Keys, because of their unreliability for bacterial identification have largely been abandoned in favour of simultaneous identification tables.

Data for identification tables are in the form of a matrix comprising tests and taxa. Depending on the quality of the tests, their separation indices and number of taxa, matrices can be small or very large. Identification may occur by undertaking all the tests and, if the matrix is small, a comparison of the profile for the unknown can be made manually to determine a match (Pankhurst, 1978). For larger data sets, a sequential process can be undertaken using subsets of the tests looking for matches using first, second and third stage tables to reach an eventual identification (Cowan, 1974). Manual inspection for anything other than small data sets is inefficient, and practicable only where most of the test data appear as positive or negative values.

Simultaneous identification, as a truly realistic proposition, is possible only with computer assistance, and has led to the development of a probabilistic approach to identifying species.

8.2.1 Probabilistic identification

Vandamme and colleagues (1996) likened bacterial species to *condensed nodes in a cloudy and confluent taxonomic space*, a graphical illustration that taxonomically, species are not necessarily discrete units but can have fuzzy, ill-defined boundaries. The concept was codified by Sneath (1974, 1978b) as the taxon-radius model, developed from the earlier ideas of Gyllenberg (1965). Taxa are considered to be hyperspheres that sit in attribute space (*a-space*) with the dimensions expressed in terms of characters. The hypersphere will have a condensed node (the centroid) comprising typical strains while less typical variants will be located further from the centre. The critical radius *r*, is set as the boundary of the species and represents a unique entity in *a-space*, Figure 8.3.

Identification of an unknown is made by calculating its position in *a-space* and its location in respect to the hyperspheres of the represented taxa. If the unknown falls within the area defined by the critical radius, an identification is reached. Alternatively, an unknown may lie on the border of a hypersphere as an outlier strain with a phenotype at variance with those near the centroid. Where an unknown occurs outside a condensed node, the strain is not identified because either the taxon to which it belongs is not represented or because it has a highly atypical phenotype of one of the taxa located in *a-space*.

Calculation of the position of an unknown in *a-space* can be undertaken in several ways but the most widely used procedure is based on probabilistic methods of which

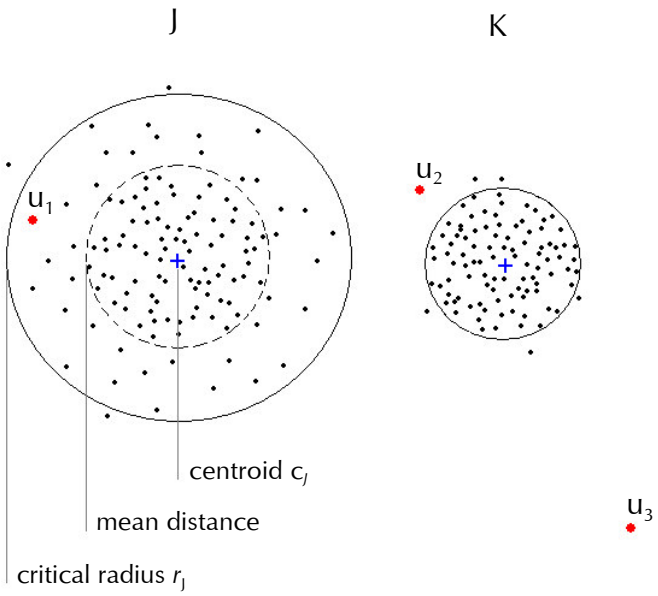

Figure 8.3 Taxon-radius model after Sneath (1974) developed from the ideas of Gyllenberg (1965). Individual strains in the two taxa J and K are shown in multidimensional attribute space in two dimensions. The boundaries of the two hyperspheres, with centroids c, are defined by the critical radius *r*. Taxon J is more diffuse, comprising strains of greater phenotypic diversity while K is a dense compact taxon that is more homogeneous. Unknown u_1 lies within the taxon radius of J and is identified as a member of this taxon; u_2 lies outside taxon K, and while having some affinity with the taxon, might be considered an intermediary of J and K; u_3 is a strain that belongs to an undefined taxon or is an aberrant strain of either J or K.

an implementation of Bayes theorem to calculate normalised probabilities is most frequently used. Development and implementation of this approach has been well described (Willcox *et al.*, 1973; Willcox *et al.*, 1980) and is the methodology used for identification by the software application, PIBWin, developed for this study of the *Vibrionaceae*. An identification is reached when the probability reaches or exceeds a nominated threshold, typically $P \geq 0.99$ or $P \geq 0.999$ which represents a likelihood of an erroneous result of 1:100 or 1:000, respectively. Situations can occur where a high Willcox probability value *P* is generated for an unknown organism even though the representative taxon is not included in the identification matrix. Such results are erroneous. Testing for such events is recommended using a qualifying statistic (Lapage *et al.*, 1973; Priest & Williams, 1993). Gyllenberg and Niemlä (1975) codified this as the *absolute affinity*, measured as the Willcox probability value, and the *relative affinity*, a measure of the goodness-of-fit of an unknown with the nominated taxon. For the identification software PIBWin *relative affinity* is determined in two ways. Firstly, the modal likelihood fraction is calculated (Dybowski & Franklin, 1968), a measure of deviance of the unknown strain against a hypothetical perfect strain of the taxon, a

metric also referred to as the R-score (Wayne *et al.*, 1980; Davis *et al.*, 1983). If both the Willcox probability score *P* and modal likelihood score exceeds set thresholds then the identification is accepted. A second qualification test is used in PIBWin lists those tests giving unexpected results, typically ones that scored positive when the expected result was negative, or the reverse, when a negative reaction occurred but a positive result was expected. Rejection of an identification would occur if at least two tests had opposite values (Bascomb *et al.*, 1973). Where expected and observed test differences are diametrically opposite, the decision to reject the identification is straightforward but where several tests disagree in the 30-70% range, it is difficult to reach an objective decision; in these circumstances the modal likelihood score provides the basis for decision.

Polythetic simultaneous methods of identification are well suited to taxa which contain divergent phenotypes that form diffuse less compact nodes (Sneath, 1974). Probabilistic identification is well justified, therefore, as a practical means of identifying species of *Vibrionaceae*, a phenotypically heterogenous taxon (Dawson & Sneath, 1985; Thompson *et al.*, 2004b).

8.2.2 Probability matrix

Test selection

Development of a probability matrix is best achieved by numerical taxonomy (Lapage *et al.*, 1973; Sneath, 1978b), which yields polythetic information that defines clusters on the basis of shared characteristics, idiosyncratic of the circumscribed taxa. This approach is particularly well suited to large and complex groups of bacteria, and has been successfully applied to slow growing mycobacteria (Wayne *et al.*, 1980, 1984), *Vibrionaceae* (Dawson & Sneath 1985; Bryant *et al.*, 1986a, 1986b) and campylobacters (On & Holmes, 1995, 1996). The clusters of a phenogram equate to the taxon-radius model where the centroid of a condensed node is the position of the cluster on the phenogram in *a-space* and the depth of a cluster branch is the scatter across the hypersphere constrained by the taxon radius (Sneath, 1978a).

The identification matrix is developed by discovery of phenotypic tests that will differentiate the taxa. It has been suggested that the number of tests required should be approximately equal to the number of taxa to be considered (Sneath & Chater, 1978). For large groups, such as the *Vibrionaceae* that currently comprise 89 species, the number of tests required to reach an identification would on this basis be unwieldy. This problem is partially solved by resorting to two stage probabilistic tables where if a

satisfactory identification is not reached with the primary table, a further round of tests is used based on a supplementary table (Bryant *et al.*, 1986b). A two or even three step approach, while making the identification process workable, still requires the use of large banks of tests, which pose considerable logistical problems for laboratories, particularly those providing diagnostic services.

The size of the VibEx7 probability matrix was kept deliberately small, even though 86 taxa were to be differentiated, so that the panel of identification tests would be manageable and economic to use. A total of 45 tests were used, comprising 39 in miniaturised format, five antibiotic sensitivity tests on a single agar plate, oxidase and agarolysis for which no specific test is required other than observation. This number of tests clearly differentiated the 86 taxa defined from this study and was almost half the anticipated number of tests required. Use of a small number of tests is justified based on the work of Matousek & Schindler (1989) who developed a 'greedy algorithm' solution using a combinatorial optimisation approach for a Minimum Set Covering Problem. For a fully discriminating set of tests, where each pair of tests is positive and negative, 100 taxa can be distinguished using only nine tests. Bacterial data sets however, are not fully discriminating as some pairs of tests will have intermediate values. It was shown that using the 'greedy algorithm' approach, the number of tests needed to identify 102 taxa of *Enterobacteriaceae* (Farmer *et al.*, 1985) could be reduced from 47 to 28.

Increasingly, the phenotype of new species of *Vibrionaceae* are being described using commercial kits such as Biolog and API but do not appear to be sufficiently descriptive or differential of the species (Vandenberghe *et al.*, 1996; Austin *et al.*, 1997; Thompson *et al.*, 2004b). Reliance on commercial phenotyping systems is constraining because of the fixed range of tests that may not be sufficiently discriminating and the risk they may be discontinued from manufacture at some time in the future (Vandamme *et al.*, 1996). It has been noted that all too often insufficient phenotypic characterisation is undertaken resulting in a narrow assessment of intra-species diversity that is of limited taxonomic value (Vandamme *et al.*, 1996; Rosselló-Mora & Amann, 2001). By comparison, numerical taxonomy has distinct advantages in establishing phenotypes as it is flexible in the range and number of tests that can be used and provides an opportunity to include new tests that may contribute to species differentiation. Discovery of phenotypic data has been achieved by numerical taxonomy for taxa that appear monolithic by conventional means. In a study of

Xanthomonas species, 295 tests were used of which only 40 had differential value (van den Mooter & Swings, 1990) but these were sufficient to differentiate the 10 taxa in the study. Use of numerical taxonomy to establish phenotypes of *Vibrionaceae* is appropriate and in particular as a means of discovery of tests that will contribute to the creation of a discriminatory identification matrix.

A matrix of high identification value will have a preponderance of tests with elevated separation values (Bascomb *et al.*, 1973), ideally fully discriminating, with as few tests as possible that have intermediate values that are only partially discriminating (Lapage *et al.*, 1973). Compact matrices comprising fewer tests can be created where tests have separation indices close to S_{MAX} and approach the theoretical ideal of a maximally discriminating matrix (Matousek & Schindler, 1989). Poorly predictive matrices tend to have more tests with low discriminatory values. The distribution of separation indices for tests of the VibEx7 matrix developed in this study compares well with Matrix 68, that is highly predictive for fermentative taxa but less so for non-fermenters since many tests for this group had low discrimination value (Lapage *et al.*, 1973). The distribution of tests for these two taxa and for VibEx7 are given as a dot plot, Figure 8.4. Most tests for the fermenters in Matrix 68 fall within the range of 30-90% of S_{MAX} while for VibEx7 most tests are at a lower level in the range of 20-60%; tests for non-fermenters in Matrix 68 were at a low level in the range 0-40%. Matrices with high proportions of tests close to S_{MAX} will be more discriminating than those with tests of low separation value. Tests which are partially discriminating should not however be considered of little or no value since in aggregate they can be sufficient to identify a taxon (Bascomb *et al.*, 1973) and may be included to weight the matrix to make it more discriminating for less well delineated taxa. For the VibEx7 matrix, tests for hydroxybutyrate and glycerol utilisation, lysine and ornithine decarboxylases and urease were included so that *Ph. damselae* ssp. *damselae, V. nereis, V. harveyi* biovar II, *V. cholerae* and *V. scophthalmi* could be identified. These tests had S_I values of 0 but at a taxon level were sufficiently discriminating to be included.

Matrix identification thresholds

Acceptance of an identification calculated by a probabilistic approach is made if the normalised probability and the modal likelihood scores reach pre-determined thresholds. The threshold value set for the Willcox probability value *P* reflects the estimates of phenotypic diversity for taxa in the matrix, the taxon radius distance for the taxa and the level of stringency required for an identification. If acceptance thresholds

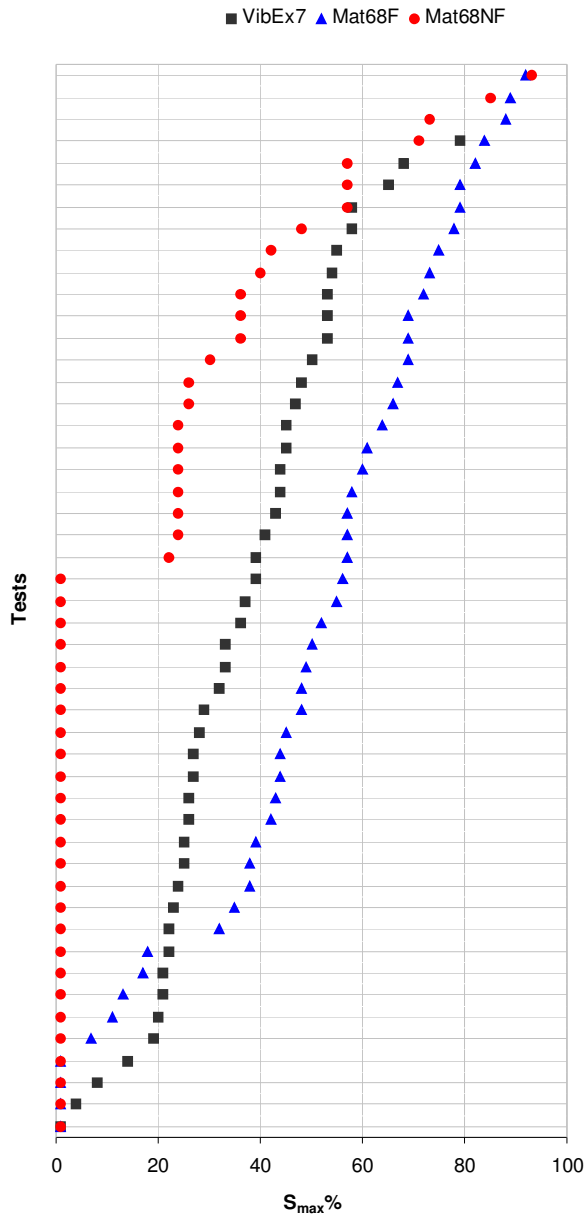

Figure 8.4 Dot plot of separation values for individual tests S_t as S_{MAX}%. Data for VibEx7 from this study, Matrix 68 (Lapage *et al.*, 1973) for fermentative strains (Mat68F) and non-fermentative strains (Mat68NF). Matrices with a high proportion of tests close to S_{MAX} will generally be more discriminating than those with tests of low S_{MAX} % values.

are set high, the error rate for an incorrect identification is reduced but then the number of identifications that meet or exceed the threshold level will be correspondingly small (Lapage *et al.*, 1973). For discrete taxa that are well separated in *a-space* and occur as dense compact nodes, a threshold of $P \geq 0.999$ is appropriate (Willcox *et al.*, 1973) but for less well delineated taxa such as the streptomycetes, a value as low as of $P \geq 0.85$ has been used (Williams *et al.*, 1983).

Some taxa in the VibEx7 matrix are clearly compact and discrete, seen as well separated clusters with deeply dividing branches such as *V. natriegens* and *V. mytili* (*Figure 7.20, Section 7.2.3 Evaluation of the probability matrix*). Other taxa are less well defined and occur as clusters with more diffuse assemblages of strains that are in close proximity to near related taxa such as *V. alginolyticus* and Phenon 10. Given the variability of taxon definitions a threshold of $P \geq 0.99$ was set for the VibEx7 matrix rather than the more stringent level of $P \geq 0.999$ recommended for well defined and discrete taxa (Willcox *et al.*, 1973). Although 85% of strains from the library of *Vibrionaceae* had Willcox probability values of $P \geq 0.999$, relaxing the threshold to $P \geq 0.99$ would increase the likelihood of an identification without increasing the risk of an erroneous result. The threshold value is consistent with that used for the only other known probability matrices for the *Vibrionaceae* (Dawson & Sneath, 1985; Bryant *et al.*, 1986b). Other probability matrices used this threshold and it was found suitable for slow growing mycobacteria (Wayne *et al.*, 1980; 1984), but for other taxa smaller values of *P* have been used: $P > 0.90$ for streptomycetes (Kämpfer & Kroppenstedt, 1991) and aeromonads (Kämpfer & Altwegg, 1992); $P \geq 0.95$ for species of *Bacillus* (Priest & Alexander, 1988) and an expanded range of aeromonads (Carson *et al.*, 2001). More stringent levels of $P \geq 0.999$ have been used for the *Enterobacteriaceae* (Holmes *et al.*, 1986b) and the *Micrococcus-Streptococcus* group of bacteria (Feltham & Sneath, 1982).

Measures of goodness-of-fit for taxa that meet an identification threshold are based either on the taxonomic distance metric of Sneath (1978b) or the modal likelihood fraction (Dybowski & Franklin, 1968). PIBWin, developed for this study, uses the modal likelihood fraction as the measure of goodness-of-fit because it can be calculated alongside the computation of Willcox probability values. A modal likelihood score or R-score of 0.01 has been used for slow growing mycobacteria (Wayne *et al.*, 1980) and marine bacteria (Davis *et al.*, 1983) but a more relaxed value of 0.0001 was used for the identification of *Acinetobacter* species (Gerner-Smidt *et al.*, 1991; Nemec *et al.*, 2000). Modal likelihood threshold scores were established from the matrix identification error determined by comparing the identification reached using the probability matrix and strain cluster designation from the numerical taxonomy used to generate data for the matrix (Wayne *et al.*, 1980). For the VibEx7 matrix a modal likelihood score of 0.001 was nominated since this did not appear to increase misidentification rates and maximised the number of correct identifications. If a more stringent modal likelihood threshold of 0.01 was used the number of acceptable

identifications was reduced to 65% although the number of strains with an identification score of $P \geq 0.99$ remained unaltered. When a threshold modal likelihood score of $P \geq 0.001$ was used, the identification rate increased to 77%. Inspection of strains with modal likelihood scores between 0.001 and 0.01 did not find instances where two tests had diametrically opposed results (0.99/0.01). Differences between expected and observed results occurred either as several tests with intermediary values or one test with a 0.99/0.01 variance and the remaining tests with intermediary values; in all cases there was agreement between the identification result and the parent cluster for these strains. There were no instances of an identification score of $P \geq 0.99$ and a modal likelihood score of ≥0.001 where two or more tests had a 0.99/0.01 difference between observed and expected results. Where two or more tests gave diametrically opposite results the modal likelihood score was always <0.001 even if $P \geq 0.99$.

Consideration was also given to the size of clusters used for defining the phenotype of taxa in the probability matrix. Over 48% of phenons in VibEx7 comprised less than four strains, which is the lower limit for the number considered sufficient to be representative of a taxon (Sneath, 1976). It is likely that clusters of these small taxa will be compact and susceptible to *type (a)* error, defined as inconsistency in the reference description of the taxon (Sneath, 1974). An effect of this on identification can be profound since only small discrepancies in observed phenotype of an unknown may place the strain outside the cluster radius (Sneath, 1974). Given the known heterogeneity of the *Vibrionaceae* and the small number of strains in some phenons, using a less stringent modal likelihood score threshold is justified. As more information is gathered for these small clusters and a better estimate of phenotypic variation obtained, the modal likelihood threshold can be made more stringent in the future.

Matrix performance

The utility of probability matrices can be evaluated sequentially in the form of theoretical and practical evaluations (Williams *et al.*, 1983). The first level assesses the matrix using strain data generated by numerical taxonomy. This is self-referential testing since the matrix has been derived from the cluster definitions of the numerical taxonomy (Bascomb *et al.*, 1973). An assessment is obtained of the efficiency, or yield, of the matrix and provides a measure of accuracy of the allocation of strains to their parent phenons. The second level of evaluation determines the practical effect of test error on identification outcome using a subset of strains from the numerical taxonomy

that have been re-characterised. The third level is a practical evaluation using wild types to determine the number of strains that meet the identification criteria for the matrix. This form of testing determines the adequacy of the estimates of phenotypic diversity obtained from the numerical taxonomy.

The VibEx7 matrix compares well with published data, collated in Table 8.4 for comparison. The yield for VibEx7 is similar to other matrices for the *Vibrionaceae*; using identification based solely on Willcox probability scores, it has the highest identification rate at 92%. The misallocation rate of strains was also low; at $P \geq 0.99$ only two strains or 0.3% were misidentified, a rate similar to the matrix of Bryant *et al.* (1986b) which had an error rate of 0.5%. Using a more stringent threshold value of $P \geq 0.999$, the yield is 85%, indicating that phenon definitions in VibEx7 are generally robust and well defined.

Table 8.4 Comparison of identification scores for probability matrices

Taxon	*P*[1] threshold	Yield	*P* + MLS[2]	Yield	Reference
Vibrionaceae VibEx7					This study
Reference data	0.99	92%	0.99 + 0.001	77%	
Retest data	0.99	77%	0.99 + 0.001	49%	
Wild type strains	0.99	79%	0.99 + 0.001	63%	
Vibrionaceae					Bryant *et al.*, 1986b
Reference data	0.99	85%	-	-	
Vibrionaceae					Dawson & Sneath, 1985
Wild type strains	0.99	79%	-	-	
Mycobacteria					Wayne *et al.*, 1980
Reference data	0.99	81%	0.99 + 0.01	67%	
Mycobacteria					Wayne *et al.*, 1984
Wild type strains	-	-	0.99 + 0.01	60%	
Micrococcus					Feltham & Sneath, 1982
Reference data	0.999	96%	-	-	
Wild type strains	0.999	93%	-	-	
Streptococcus					Feltham & Sneath, 1982
Reference data	0.999	93%	-	-	
Wild type strains	0.999	85%	-	-	
Aerobic non-fermenters					Holmes *et al.*, 1986a
Reference data	0.999	91%	-	-	
Aerobic fermenters					Holmes *et al.*, 1986b
Reference data	0.999	89%	-	-	
Motile aeromonads					Carson *et al.*, 2001
Wild type strains	0.95	80%	-	-	
Bacillus					Priest & Alexander, 1988
Wild type strains	0.95	70%	0.95 + <0.7[3]	59%	
Streptomycetes					Kämpfer & Kroppenstedt, 1991
Reference data	0.90	84%	-	-	
Wild type strains	0.90	78%	-	-	
Aeromonas					Kämpfer & Altwegg, 1992
Reference data	0.90	84%	-	-	
Wild type strains	0.90	85%	-	-	
Streptomycetes					Williams *et al.*, 1983
Wild type strains	0.85	81%	-	-	

[1]*P*: Willcox probability score; [2]Modal likelihood score; [3]SE of taxonomic distance

Where the modal likelihood score or standard error of taxonomic distance (Sneath, 1978b) is used as a qualifier of identification, the performance of matrices is reduced. For VibEx7, yield nevertheless is still good and compares favourably with matrices for slow growing mycobacteria (Wayne *et al.*, 1980; 1984). The better performance of the VibEx7 matrix can be attributed to the more relaxed threshold of 0.001 used for the modal likelihood score compared to the more stringent threshold of 0.01 used for the slow-growing mycobacteria. Raising the modal likelihood score threshold for VibEx7 did not improve accuracy of the matrix and resulted in excluding identifications because strains were not sufficiently close to the taxon centroid, though clearly were still within an acceptable radius of the parent taxon.

It should be noted that the yield for individual taxa in matrices is variable and giving aggregated performance measures can be misleading. For the VibEx7 matrix, 73% of the phenons comprised strains that all had Willcox probability scores ≥0.99. Of the remaining 27% of phenons, at least 80% of strains had identification scores ≥0.99; the lowest performing phenon was *Ph. damselae* ssp. *damselae* biovar II with a yield of 57%. Phenons which have a proportion of strains with probability levels <0.99 have less well defined phenotypes and take the form of diffuse clusters with ambiguous margins. For an acceptable identification to be achieved, a strain needs to be close to the centroid of the cluster, but if located at the margins, the strain will appear to have an aberrant phenotype even though overall it appears to have characteristics of the taxon. Incorporation of phenotypes from more strains would likely improve the definition of these taxa by establishing a more precise boundary, thereby improving the accuracy of identification (Bascomb *et al.*, 1973; Lapage *et al.*, 1973; Sneath, 1974).

Variation in efficiency has been reported with other probability matrices with yields as low as 0% for some taxa (Holmes *et al.*, 1986b). The number of taxa where the yield for a correct identification is 100% varies greatly with probability matrices. An identification yield of 100% was obtained with 74% of the taxa using a matrix for aerobic non-fermenters (Holmes *et al.*, 1986a) and is similar in efficiency to VibEx7 which had a yield of 100% with 73% of the taxa in the matrix. Performance of other matrices was consistently poorer, the highest at 62% of taxa for aerobic fermenters (Holmes *et al.*, 1986b) and as low as 39% for the streptomycetes (Kämpfer & Kroppenstedt, 1991). Where a reasonable estimate of phenotypic diversity has been obtained for a taxon, identification yield should be close to 100%, and consequently a

matrix comprising significant proportions of well defined taxa should be a reliable and efficient basis for identification.

Effect of test error on identification

Sneath (1974) describes two types of error that may influence identification outcome. *Type (a)* error is associated with the description of strains used to define a cluster while *type (b)* error can occur with the description of an unknown for identification. Simultaneous polythetic identification systems are robust to both type of error although for compact discrete taxa the effect of either *type (a)* or *type (b)* error can have a marked effect on identification outcome. For the ideal test the probability of error should be 0 or close to 0 but in practice this is unlikely to occur. From this study of *Vibrionaceae* test error *p* from the quality control assessments for the VibEx7 panel ranged from 1.0-2.9%, and is within the 4% expected intra-laboratory error range (Sneath, 1974). It is recommended that test error *p* should be kept small, ideally around 1% since as *p* increases the proportion of strains that can be identified decreases as the effective taxon radius is shortened. For compact clusters the effect is even more pronounced. In a study of *Yersinia pseudotuberculosis*, it was found that a test error of 5% would result in only 9% of strains being identified but if test error was reduced to 2%, then 81% of strains would be identified (Sneath, 1974). For this study of *Vibrionaceae*, an emphasis was made on standardisation to limit test error so that identification yield was maximised. It is important that intra-laboratory quality control is maintained so that in practical terms identification rates are not degraded.

The effect of *type (b)* error is noticeable on identification outcomes when a subset of strains from the library was retested. For the panel of 91 strains nominated for retesting, the identification rate based on phenotyping data from the numerical taxonomy was 74% using the identification thresholds of $P \geq 0.99$ and MLS≥0.001. On retesting the strains the identification rate declined to 49%. The reduction in yield was attributed primarily to test error with the effect of placing the unknown outside the critical radius of the taxon; there was no evidence however of allocation to an incorrect taxon. The reasons for the increase in test error when the subset of strains was retested is not clear and did not occur during quality control assessments where test error for the VibEx7 panel was as low as 1.0%. This reduced error rate probably resulted from greater familiarity with the identification system and further emphasises the need for careful control of tests and their interpretation. A reduction in performance may also be due to *type (a)* error but this is more difficult to correct for, as it would require redefining the

phenotypes of the clusters. There is no indication that *type (a)* error has a significant effect on cluster definitions as the overall test error for the numerical taxonomy was less than 5%, the level above which significant perturbations can occur to taxonomic structure. Polythetic approaches are more resistant to *type (a)* error, which can be accommodated if small (Sneath, 1974). Similarly, a reduction in identification scores on re-characterisation of a subset of strains was found with streptomycetes (Williams *et al.*, 1983) As with the *Vibrionaceae* retests, the main effect was a degradation of Willcox probability scores and the qualifying metric, which for the streptomycetes study was measured as taxonomic distance and its standard error.

Performance of the matrix when assessed with wild type strains is usually less compared to that based on data from the phenetic taxonomy from which the matrix was derived. Identification of wild types will be affected by *type (b)* error that degrades yield while identification outcome is determined by the accuracy of the estimates of the phenotypic diversity of the taxa in the matrix. If sufficient taxon sampling was undertaken to obtain an adequate estimate of diversity then it increases the likelihood of an unknown being located within the critical radius of the taxon assemblage. If the estimate is based on too few strains then there is a risk that the taxon hypersphere is not accurately located in *a-space* and the boundary set either too close to the centroid or too distant for meaningful identification to be achieved. The performance of the VibEx7 matrix compares well with other matrices, although there is weakness in the definition of some taxa because of inadequate sampling. For taxa that contain fewer than four strains, characterisation of new isolates is required to improve the estimates of phenotypic diversity.

A low identification score may occur where an unknown has a phenotype that spans two closely related taxa. This was noticeable for some strains that clustered either with *V. alginolyticus* or Phenon 10. The genotype of strains from Phenon 10 were consistent with *V. alginolyticus* although they had a distinct phenotype and formed a cluster more closely associated with *V. parahaemolyticus* than *V. alginolyticus*. When identifying strains either from the phenetic taxonomy or wild type strains from aquatic animals there were occurrences when an identification was not reached. In most of these cases the first and second choice was *V. alginolyticus* and Phenon 10. Where there is *a priori* knowledge of the taxa, an identification can be reached if the sum of the Willcox probability score reaches the identification threshold (Bascomb *et al.*, 1973; Willcox *et al.*, 1973). Where such an identification is reached it is to the level of a taxonomic

group. From the phenotyping data used for identification, several strains which failed to reach the identification threshold for either *V. alginolyticus* or Phenon 10 could be identified as a member of the *V. alginolyticus* complex, Table 8.5. The yield for *Ph. damselae* ssp. *damselae* biovar II was 57%, the lowest score for all the taxa, which points to a difficulty with the phenotypic description of this biovar of the species. For two of the strains however an acceptable identification was reached for *Ph. damselae* ssp. *damselae, sensu lato* although a biovar could not be determined from the phenotype. Depending on the purpose of identification this may be sufficient; a correct identification has been reached in this case other than allocation to a biovar. If a

Table 8.5 Allocation of poor scoring strains to a species complex

Cluster	Strain	1st choice	2nd choice	Sum *P*	Identification
Phenon 10	V26	Phenon 10 *P*=0.84440	V. alginolyticus *P*=0.15559	*P*=0.99999	*V. alginolyticus* complex
Phenon 10	V261	Phenon 10 *P*=0.98228	V. alginolyticus *P*=0.01772	*P*=1.00000	*V. alginolyticus* complex
V. alginolyticus	V883	V. alginolyticus *P*=0.76589	Phenon 10 *P*=0.23410	*P*=0.99999	*V. alginolyticus* complex
Ph. damselae ssp. *damselae* II	V122	*Ph. damselae* ssp. *damselae* II *P*=0.97300	*Ph. damselae* ssp. *damselae* I *P*=0.02700	*P*=1.00000	*Ph. damselae* ssp. *damselae sensu lato*
Ph. damselae ssp. *damselae* II	V798	*Ph. damselae* ssp. *damselae* II *P*=0.98130	*Ph. damselae* ssp. *damselae* I *P*=0.01870	*P*=1.00000	*Ph. damselae* ssp. *damselae sensu lato*
Ph. damselae ssp. *damselae* I	V123	*Ph. damselae* ssp. *damselae* I *P*=0.98942	*Ph. damselae* ssp. *damselae* II *P*=0.01058	*P*=1.00000	*Ph. damselae* ssp. *damselae sensu lato*

relaxed identification is used then the yield for *Ph. damselae* ssp. *damselae* biovar II is increased from 57% to 86%, representing a significant improvement. Summing identification scores, where taxonomically justified, would be applicable for other near related groups, particularly members of the *V. splendidus* complex. The estimates of identification efficiency for VibEx7 were made without using the strategy of summing Willcox probability values for taxa that might be part of a species complex. If this had been done there would have been a small improvement to the overall identification yield and marked improvements for individual taxa.

8.2.3 Identification panel

Quality control of V36 panel

Assessment of culture media and performance of batches is of particular importance when used for the purpose of identification Test failure through improper formulation can have significant effects on identification outcomes either from misidentification or degradation of the identification metrics which can make an unknown appear as a

taxon outlier, *type (b)* error. To effectively test the V36 identification panel a minimum of four species were used to ensure that a positive reaction could be observed for each test. A fifth species, *V. fluvialis* was included to provide some redundancy in test reactions, useful where some minor test variation occurs. The expected phenotypes for the five species provide a useful means of checking not only new batches of media but also enables estimates of test error to be made so that laboratories can determine the accuracy of the identification procedure. This covers not only media formulation but also operator performance from test set-up and interpretation through to data recording. As a matter of due diligence laboratories providing identification services should implement some form of internal assessment, preferably on a continuous basis, to monitor, and correct if necessary, errors of process. It has been shown from this study and elsewhere (Lapage *et al.*, 1973) that intra-laboratory error can be reduced to as little as 2% while a readily achievable level of 3% should be attainable by most laboratories with perseverance and careful control of processes.

Error rates for individual species were variable. *Vibrio mediterranei* and *V. parahaemolyticus* had the lowest errors at 1.0% and 1.2% respectively while *V. fluvialis* was the highest at 2.9%. The reasons for the differences between species is not readily apparent but probably reflects the reproducibility of tests for individual species such that for *V. mediterranei* consistency of test performance is better than for *V. fluvialis*. It is unlikely that the observed variation represents phenotypic instability within a species but is rather the result of using a particular range of tests which are less predictable than other tests. Selection of more stable tests would improve test error but would be possible only if the differential properties were not reduced as a consequence. For the V36 panel, test error appears well controlled and within an acceptable range so there is no obvious need to modify the existing panel. The effect of test variation appears minimal because for each batch of medium the correct identification for each species was obtained and the Willcox and modal likelihood thresholds achieved.

Laboratories which undertake quality control measures can use the observed and expected phenotypes for the test species in various ways. Assessment of test performance is readily undertaken by inspection to determine if expected reactions have occurred. Trend analysis derived from results of sequential batches of media should be undertaken to identify systematic failures and can also be used to calculate test error to assess intra-laboratory performance of identifications using the V36 panel.

Stability of freeze-dried panels

The MicroSys V36 panel is currently used as a wet system where concentrated media are held in 96 deep-well plates and dispensed with a multi-channel, multi-stepper pipette into micro-well plates just prior to use. The media stocks used this way have a conservative shelf-life of 6 months when stored at 2-8°C. An investigation was made into freeze-drying the media in micro-well trays as a means of improving the utility of the identification panel. From a working laboratory point of view, to be able to use an identification panel immediately rather than dispensing the panel first, is highly attractive, particularly if large numbers of strains are to be identified.

Freeze-drying the media looks a promising strategy for preparing and storing identification panels. Only a few tests failed and of those that did, most are not used for the V36 identification panel and failed after two months storage. The pooled error rate of tests over the five month evaluation period was 3.5%, which is within the range expected for intra-laboratory testing (Lapage *et al.*, 1973). The error rate for individual species was more variable and for the tests in the V36 panel ranged from 2.63% to 3.5%. Some caution needs to be applied to the assessment based on identification outcome. As has been shown, test error can degrade the results of identification as it was not possible to discern differences due to test error and failure of the medium during storage. *Vibrio metschnikovii* was the least affected of the four species tested but this may reflect identification robustness due to greater reproducibility in tests compared to the other species. Of note is that the two species which seemed most variable over time was *V. mimicus* and *V. wodanis*, both of which had an error rate of 4.0%, the highest of the species tested while *V. metschnikovii* had the lowest error rate at 2.6%. Interestingly, all four species had acceptable identifications with the panels stored after one month which indicates that while test error may account for some of the variability in identification outcome, medium degradation may also be a contributing factor. Parallel testing of freeze-dried panels with the standard wet format may help in partitioning the contribution made by test error and that due to medium changes over time. Further testing of the freeze-dried panels is required with more species and strains to determine the robustness of the format, but based on these preliminary trials, this looks a promising means of preparing and storing panels in a convenient form. Further investigation should be undertaken as to the method of drying the media and storage, in particular the use of silica gel for moisture control.

Factors affecting identification performance

Inoculum source: Development of the probability matrix VibEx7 was based on the phenetic taxonomy undertaken using the library of *Vibrionaceae*. The phenotypes of the strains were generated by preparing inocula from cultures grown on plates of JMA. All the tests require multiplication of the inoculum in the test medium and there was no direct evidence that pre-formed enzymes, either induced or constitutive, are necessary to express phenotype. From the testing undertaken using inocula prepared on SBA2, ZMA and its derivative JMA, differences in phenotype were clearly evident. The mechanism by which this might occur is not clear. The effect of medium used to prepare inocula on the subsequent expression of phenotype in test media has been reported with *Lactococcus garvieae* where inocula prepared on tryptone soy agar resulted in a suppression of phenotype in API Rapid ID 32 Strep miniaturised tests compared to inocula prepared on blood agar (Ravelo *et al.*, 2001). In contrast to these findings, it was found that phenotyping *Vibrionaceae* using blood agar reduced subsequent test activity. The observed effects with the Rapid ID 32 Strep kit and the miniaturised tests used for the *Vibrionaceae* are not entirely comparable as the tests in Rapid ID 32 Strep kit rely in large part on pre-formed enzymes unlike those of the MicroSys V36 panel which require growth *in situ*. Selection of phenotype has been found with bacteria used for sensitivity testing. Well isolated colonies used as inoculum were found to exhibit greater sensitivity to antimicrobial agents compared to those obtained from dense areas of growth (Al-Hiti & Gilbert, 1983) a reminder that bacterial cultures are not truly clonal (Spratt, 2004) and subtle phenotypic heterogeneity may exist within a population that can extend to subcultures. Based on observations between phenotypes of *Vibrionaceae* recently isolated from the natural environment and that expressed after laboratory passage (Anderson & Ordal, 1972; Grimes *et al.*, 1993), it has been suggested that strains may retain a capacity over several generations to express a phenotype based on earlier culture conditions (Ruimy *et al.*, 1994). Recent work on epigenesis has shown that bacteria possess non-heritable mechanisms, induced by environmental cues that can effect changes of phenotype in subsequent generations (Henderson *et al.*, 1999; Casadesús & D'Ari, 2002; Kussell *et al.*, 2005). If such a memory effect occurs in *Vibrionaceae*, it may account for the observations on the effect of culture medium on inocula used for tests that require subsequent growth to express phenotype.

Regardless of the mechanism that may be involved in the phenomenon, the practical effect on identification was surprisingly small, a reflection of the inherent robustness of the simultaneous polythetic method of identification and its capacity to absorb *type (b)* error. The effect on *M. viscosa, V. anguillarum* and *V. harveyi* was not apparent and for each replicate, the correct taxon was found and with an identification score of $P \geq 0.99$, irrespective of medium used for preparing the inoculum. The effect of medium on identification was less clear for *V. lentus*. The most consistent results were obtained using JMA for the inoculum but when either ZMA or SBA2 was used, the correct taxon was found but the identification scores were less than $P=0.99$. Test error may be a contributing factor to the inconsistent identification scores obtained for *V. lentus* with the three different media used for inoculum preparation. Testing was undertaken with only four species of *Vibrionaceae* and on a limited scale. The evidence obtained suggests that inoculum medium has an effect on expression of phenotype and from the assessment made any phenotypic variation associated with medium effects can be accommodated by probabilistic identification. Although the effects would appear to be marginal, test error may still have the potential to have a profound effect on identification outcome. Consequently, it is important, where possible, that causes of error should be minimised.

Inoculum concentration: Phenotyping of the *Vibrionaceae* was undertaken using a standardised inoculum concentration. Previous work (results not shown) had found that variable results occurred with the carbon source utilisation tests if the inoculum was not standardised to a density of McFarland 0.5 and diluted 1:5 prior to use. The robustness of identification to variations in inoculum density was assessed to determine the importance and degree of latitude that could be accepted in preparing an inoculum. As predicted, there was a reduction in test reaction as inoculum density decreased, with most test failures occurring with the carbon source utilisation tests. Of the three species evaluated, *V. lentus* was the most susceptible to decreases in inoculum density with fewer variations seen with *V. anguillarum* and *V. harveyi*. The effect of medium used for the preparation of the inocula also influenced test performance. The greatest differences occurred using SBA2 while the least difference occurred with JMA. Although test failure was apparent with decreasing inoculum concentration, the practical effect was not pronounced. In all instances, the three species were always correctly identified although identification scores did not always reach the threshold of $P=0.99$. Reduced identification scores most often occurred when

inocula were derived from SBA2 and used at dilute concentrations. While the effect on identification scores of medium and inoculum concentration were small for *V. anguillarum* and *V. harveyi*, dilution beyond 1:10 reduced the identification score of *V. lentus*, irrespective of the medium used for preparation of the inoculum. The results were inconsistent however and may have been the result of inherent test error. This was noticeable even for inocula prepared from JMA. At a dilution of 1:10 the identification score was *P*=0.99669; at 1:100 and 1:1,000 it was less than the threshold of *P*=0.99 but at 1:10,000, the most dilute concentration, the identification score was *P*=0.99260.

From this evaluation it would appear that standardisation of inoculum density is important, particularly for fastidious species or for taxa with compact or poorly defined clusters where *type (b)* error may result either in misidentification or identification scores below the accepted thresholds. The importance of inoculum density was seen following routine use of the MicroSys V36 identification panel (results not shown). Unexpectedly low identification scores were attributed to the manner in which inocula were prepared. More consistent results were obtained when inocula were prepared using a swab compared to a loop. While the density of the suspensions prepared by both means appeared similar, it was found that viability was different between the two preparations and for the suspension prepared with a loop, the bacterial count was always lower, presumably the result of residual heat inactivating a portion of cells. Differences in the methods used to prepare inocula invariably resulted in degradation of the modal likelihood scores, signifying a shift of the unknown away from the centroid and closer to the critical radius distance of the cluster.

The importance of inoculum concentration for tests is well recognised, particularly for assessing antibiotic susceptibilities (Syriopoulou *et al.*, 1979) or antibacterial assays (Lambert, 2000), where different inoculum concentrations may lead to different susceptibility values being determined. Expression of phenotype in identification tests can also be affected by inoculum density. Failure to standardise inocula for tolerance tests were found to give variable results when used for the phenotypic characterisation of *Campylobacter* species (On & Holmes, 1991).

Inoculum condition: During the study of *Vibrionaceae* it was observed that the phenotype of some strains immediately following revival after storage at -80°C were less reactive than that observed following several subcultures on JMA. Of the five species evaluated, changes in phenotype with subculture were observed only with

V. lentus. The identification score for *V. lentus* was less than the threshold of *P*=0.99 immediately following revival but by the second and third subcultures the identification scores had exceeded the threshold. A noticeable trend for all the species tested was the improvement in modal likelihood scores with increasing subculture, indicating that phenotypes were in closer agreement with the cluster definition. Whether the observation of improved identification metrics is another example of epigenetic regulation is not clear but evidently subculture results either in more genes being switched on or selection of clonal types that are more similar to the cluster definition of the taxon. Irrespective of the mechanism that may be involved, it demonstrates the need for a standardised approach to phenotyping and the exercise of caution when using stored material.

Test error: Performance of tests and their stability may influence identification outcomes. Test error of the MicroSys V36 panel was inferred from the phenetic taxonomic data and later from repeat testing associated with investigations of the stability of freeze-dried panels and the influence of media used for preparing inocula. A more formal assessment of error was later obtained from the quality control assessments for batches of media for the V36 identification panel. Tests of greater variability tended to be those based on chromogenic substrates where distinction between a weak positive and negative was not always clear cut. Carbon source utilisation tests were another source of error attributed mainly to difficulties in determining presence/absence of growth with poorly growing strains and substrates that were less readily utilised. It has been found that CSU tests are prone to error and can give inconsistent results (Snell & Lapage, 1973). Test variability was found to be pronounced with a few substrates and was more evident with some taxa but not others. Error rates expressed simply as cumulative percentages were as high as 17%, average 5%, but using the method of Sneath & Johnson (1972) the overall error rate was only 2.6%; the highest individual test error was 9.1%. From the phenotyping data the overall test error for CSU tests in the MicroSys V36 panel was 4.6% with the highest individual test error of 13.1% for propionate. Subsequent testing found the overall error for CSU tests ranged from 1.8% (glycerol p_I=14.6%) to 3.0% (propionate p_I=13.1%). Clearly, CSU tests have the potential to contribute a significant amount of error in the MicroSys V36 panel. The low level of CSU test error was obtained towards the end of the study and was generated from work undertaken by one operator who, over a period of 24 months, was able to reduce overall test error to 1.9%. This

demonstrates that error levels can be contained by careful technique but does require commitment and self-monitoring to maintain low levels of error, necessary to achieve accurate identification.

The simultaneous polythetic approach to identification is well suited for use with large groups of bacteria, particularly where taxa are phenotypically heterogenous. For such schemes to be of value however, every effort should be made to standardise testing so as to reduce error either in the construction of probability matrices or when characterising an unknown (Sneath, 1974). The sources of error investigated for this study of the *Vibrionaceae* point to the importance of standardisation and the need for good intra-laboratory quality control so that the utility of a probabilistic approach to identification is maximised.

8.2.4 PIBWin: probabilistic identification software

The software package PIBWin is an advanced tool for probabilistic identification. A major characteristic of the application is the emphasis on ease of use and extreme flexibility. The databases are formatted as stand alone Excel spreadsheets, which enables the user to extract information readily where required, have multiple archive files for data storage and access them across a distributed network. As the databases are 'open-format', modification to the probability matrix can be readily made by the user for the purpose of amending species descriptions or adding new species or taxa.

Since the core of PIBWin is for computing probabilistic identities, data matrices for a wide range of bacteria can be added in Excel format. This means the PIBWin has wide application in any microbiology laboratory that provides identification services and is not necessarily limited to use with *Vibrionaceae*. Where new matrices are being developed or based on published data, GBEST to identify differential tests and IDSC to determine the uniqueness of species in the matrix, can be used to assess the properties of a matrix and provide an indication of its likely performance.

PIBWin is one of the only probabilistic identification software packages currently available. Several proprietary software packages have been available but do not appear to be supported now or still available. A public access package, MATIDEN (Sneath, 1979) has been published as a program in BASIC which can be used for probabilistic identification but lacks the ease of use of PIBWin and requires a BASIC interpreter. The flexibility and straightforward, intuitive interface of PIBWin makes this a realistic tool for

probabilistic identification and is ideally suited where high-volume identification services are provided.

8.3 DEVELOP AND IMPLEMENT PCR GENE PROBES FOR THE RAPID IDENTIFICATION OF KEY BACTERIAL PATHOGENS

8.3.1 Scope of application

The purposes to which PCR primers are used are manifold and evaluation of primers must be made in regard to their intended use and the source of template DNA. In this study of *Vibrionaceae*, primer evaluation was made with the intention that they would be used with template DNA obtained from pure cultures for the purpose of confirming an identification reached by phenotyping. Identification by PCR might be used for the purpose of screening a range of isolates for a species of interest or, used to confirm the identity of an isolate which may have an aberrant phenotype or, to provide independent confirmatory identification where a finding is critical such as in the emergence of a significant pathogen.

While consideration was given to the overall specificity of primers by performing BLASTn searches, no work was undertaken to evaluate specificity of the primers when used for direct detection in fish tissues or environmental samples. Similarly, efforts were made to obtain levels of sensitivity (lower limits of detection) based on preparation of templates from pure cultures but optimisation of templates obtained from heterogenous matrices was not attempted as this was not germane to the intended purpose of this study.

8.3.2 Primer design

Varying strategies were used in the design and development of the 15 PCR systems evaluated for this study. In some cases the strategy was driven by a particular diagnostic application, such as the identification of toxin producing strains, while in other cases taxonomic relationships and accurate species differentiation or identification was the major driving factor. The purpose of the PCR has a significant bearing on how well suited it might be for use in the specific identification of the nominated species of *Vibrionaceae*.

PCR primers for species identification should target nucleotide sequences that are conserved between species and reflect existing phylogenetic relationships. The 16S rRNA gene is often used for this purpose, though if the interspecies homology of the rRNA gene is high, its usefulness for the purpose of identification may be limited. For

the *Vibrionaceae* there is only a 9% difference in sequence of 16S rDNA across the *Vibrionaceae* (Kita-Tsukamoto *et al.*, 1993; Ruimy *et al.*, 1994) and is a constraint on primer design based on this conserved gene.

The reliability of primers of toxin genes for identification is difficult to assess empirically and can only be truly evaluated by extensive testing of wild type strains. Several primers have been developed to detect toxin genes in species of the *Vibrionaceae*: *V. vulnificus* (PCRs 2, 3, 4 & 12), *V. anguillarum* (PCR 8) and *V. harveyi* (PCR 5). The primers for *V. vulnificus* were developed for the purpose of food-safety (PCRs 2, 3 and 4) or detection of acute infection in man (PCR 12). In both situations detection of the toxin gene was the purpose of the test. How well conserved this gene might be amongst different strains was not considered. For *V. anguillarum* (PCR 8; Hirono *et al.*, 1996) and *V. harveyi* (PCR 5; Conejero & Hedreyda, 2003), the respective toxin genes were considered to be potentially specific markers of the species. The primers were found to be functional for the species and able to detect small amounts of purified template. It was apparent that not all strains of the target species had a toxin gene which clearly limits the usefulness of the primers for species identification particularly if strains were obtained from a wide host or geographic range.

While primers directed to genes of known function provide some basis for assessment of their utility for the purpose of species identification, primers developed by random sequence strategy, need extensive evaluation to determine specificity and reproducibility (Priest and Austin, 1993). The target sequence *Ph. damselae* ssp. *piscicida* (PCR 6; Dalla Valle *et al.*, 2002) was obtained by identifying apparently unique species specific RAPD generated fragments while for *V. harveyi* (PCR 9; Iwamoto *et al.*, 1995), primers were based on unique *Pst*1 restriction fragments. The primers based on RAPD genomic fragments for *Ph. damselae* ssp. *piscicida* appear to be unique. When the primers were searched for sequence similarity in GenBank the only fragment with a significant match was that originally described and lodged by Dalla Valle *et al.* (2002). Based on testing with non-target species of *Vibrionaceae* in this study the primers appear to be specific but how well the construct is represented in strains of *Ph. damselae* ssp. *piscicida* can only be determined by testing an extensive library of strains from wide spread geographic and host ranges. Similar limitations apply to the primers based on the *Pst*1 restriction fragment for *V. harveyi* (formerly *V. 'trachuri'*) although this PCR was less efficient than the others that were evaluated and required a higher concentration of template DNA to obtain a yield of amplicon that was clearly

discernible. The lack of sensitivity would not be problematic where the PCR is used for identification of cultures. The usefulness of the primers remains uncertain, as, in the case of the other random sequence PCR for *Ph. damselae* ssp. *piscicida*, the distribution of the target sequence across the strains is unknown and as a consequence identification may be unreliable.

The remaining PCRs were based on either the 16S or 23S rRNA gene. The 16S rRNA gene is about 1500 nucleotides in length and is made up of highly conserved regions interspersed with hypervariable regions that are characteristic of genera and species (Barry *et al.*, 1990; Wiik *et al.* 1995; Gauger *et al.* 2002). The 23S gene is about 2900 nucleotides long with polymorphic regions clustered in five secondary-structure helices (Antón *et al.* 1999) and contains conserved species specific regions that can be used for bacterial identification (Ludwig & Schleifer, 1994; Amann & Ludwig, 2000).

Given that the level of interspecies homology of the *Vibrionaceae* is high, the design of PCR primers for species specific identification is difficult and challenging. However, where it is possible to successfully exploit the small regions of variation, there is a degree of confidence that the resulting system will reflect the well defined taxonomic framework based on the phylogeny of rRNA gene sequences.

8.3.3 BLASTn analysis of PCR primer sequences

BLASTn analysis of the primer sequences was undertaken to obtain an indication of the range of bacterial species, related or unrelated, that might be detected using the described primer sets. The BLASTn analysis did not reveal any primer pairs that were likely to fail or be completely inoperative.

In many cases the BLASTn results revealed and reflected the strategy used for the design of the various primer pairs. For example, the primers L-CTH and R-CTH of Brasher *et al.* (1998) target *Vibrio* toxin genes. Primer L-CTH is homologous with the *V. vulnificus* cytolysin and primer R-CTH is closely related to the *V. parahaemolyticus* thermolabile haemolysin gene. While this primer set would be useful for determining the presence of toxin genes, its suitability for species identification is limited as the intra-species and inter-species specificity is not known. Primer pairs targeting the 16S rRNA gene generally revealed several close matches with a range of other *Vibrio* species, reflecting the high degree of intra-species homology that exists within the *Vibrionaceae*. In general, primers that target toxin genes yielded BLASTn results which provided few if any related sequences apart from the toxin gene sequences themselves,

suggesting these primers might be highly specific though of unknown distribution. The risk of false negatives occurring intra-species using toxin primers is probably greater than the risk of false positives. In a few cases, such as the *tox*R primers for *V. harveyi* described by Conejero & Hedreyda (2003) the only bacterial sequences detected where those originally described by the designers of the PCR. During testing it was noted that some strains from different geographic areas and hosts were negative and illustrates the risk attached to using primers that are not directed to conserved regions of the genome. Other than for specialised applications, use of toxin genes for identification is of doubtful value.

8.3.4 NetPrimer analysis

NetPrimer is a Web based Java® script application, which rapidly analyses primer sequences for amplification related properties including melting temperature (T_m) and secondary structures. While the main use of this software was for optimising PCR primer design, it was considered potentially useful for revealing any fundamental underlying problems associated with the primers, such as self-complementarity leading to hairpin dimer formation, cross-dimer formation between the primer pairs, palindromic repeats and repetitive runs of repeats within primers, which might severely affect amplification.

The NetPrimer analysis was used as rapid means of revealing any primers with potentially catastrophic flaws in their design. While NetPrimer revealed that numerous primer pairs did show the potential for formation of a range of secondary structures, these were related to their likely efficiency rather than their specificity as revealed by the BLASTn analysis. All the systems were shown to produce an amplicon of the expected size in the presence of specific DNA template, with the exception of the first round of amplification with primers targeting the 23S gene for *V. vulnificus* (Arias *et al.*, 1995). The reasons for the failure could not be determined.

8.3.5 PCR Evaluation

PCR 1. Arias *et al.* (1995) – *Vibrio vulnificus* 23S rRNA gene

Background: This 2-step amplification procedure was developed for the detection of *V. vulnificus* in fish as an alternative method to culture. The design strategy centred on the use of outer primers complementary to highly conserved regions of the eubacterial 23S rRNA gene followed by the use of a species specific inner primer pair (Dvu9V-Dvu45R), which produced a 978bp amplicon. The PCR was reported to detect 1ng to 100pg, even when diluted 1:10 or 1:100 with fish DNA. It is not clear that the ratio of

target to non-target DNA represented by the 1:10 and 1:100 dilutions realistically reflects the likely level of DNA dilution that would be encountered following primary 23S amplification of a mixed infection of different bacterial species. In combination the two PCRs where reported to detect 10fg of template and be unaffected by the presence of extraneous eubacterial or eukaryotic DNA.

Assessment: While for this study it was possible to detect 5pg of purified DNA compared with the 100pg originally reported using the second round primers, the 10fg level of detection could not be achieved as it was not possible to get the first round of the nested cycle to work. The utility of the PCR is questionable, as it was not possible to optimise the PCR amplification conditions because there was significant spurious band formation with non-target species template DNA. Further investigation to optimise and improve specificity was not warranted given that other primers have been developed for *V. vulnificus*.

PCR 2. Brasher *et al.* (1998) – *Vibrio vulnificus cth* (cytolysin/haemolysin) gene

Background: The assay was developed to allow the simultaneous detection of a range of indicator organisms commonly associated with faecal contamination in water. This multiplex PCR amplification system simultaneously targets unique toxin genes of five enteric pathogens followed by a colorimetric DNA-DNA hybridisation reaction for secondary confirmation. The PCR was intended to give the already established hybridisation method greater utility by increasing the analytical sensitivity of the system. In this case the cytolysin/haemolysin gene of *V. vulnificus* was targeted and assessed as part of this study.

Assessment: The *cth* PCR assay produced clear bands after electrophoresis with an analytical threshold around 500fg when purified DNA template was used. Non-specific bands were not observed in the pooled non-target species DNA. Brasher *et al.*, (1998) looked at three annealing temperatures: 50, 55 and 60°C in an attempt to increase the analytical sensitivity of the system for detecting bacteria in the presence of tissues. They observed spurious bands at an annealing temperature of 50°C. In this study annealing temperatures ranging form 53 to 59°C were used, and while no additional bands were observed at 53°C, the decrease in yield at higher temperatures approaching 60°C was confirmed. The optimal yield was achieved with an annealing temperature of 57°C. For the assay optimised for template DNA from cultures, it was possible to detect 5pg of purified bacterial template.

Although the primers are clearly functional their utility is circumscribed because the distribution of the *cth* toxin gene across strains within *V. vulnificus* is unknown and there is a risk of false negatives if used solely for identification of the species. Conversely, it should be noted that no cross reactions were evident with any of the species in the pools of non-target organisms indicating that the primer sets are likely to be specific for *V. vulnificus*.

PCR 3. Coleman *et al.* (1996) - *Vibrio vulnificus cth* (cytolysin/haemolysin) gene

Background: The PCR was developed to detect *V. vulnificus* in oysters using as target the 1416bp cytolysin/haemolysin *cth* gene unique to *V. vulnificus* but common to both biovars 1 and 2 (Morris, 1987).

Assessment: During test development marginal improvement in the visibility of very faint bands was obtained by increasing the number of PCR cycles from 34 to 50. These results were sometimes inconsistent; suggesting the limits of performance had been reached. A lower detection limit of 50fg was obtained in this study when 50 cycles were used. Although the detection threshold could be increased by increasing cycle number, cross reactions were observed with Pools 1 and 7 of non-target species. Although the band size was slightly smaller than the target amplicon, the 24bp difference makes differentiation critical and there is a risk of false positive reactions. The observed cross-reactions together with the limitations of using a directed primer for identification makes the utility of this primer set for identification questionable.

PCR 4. Coleman & Oliver (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Background: The assay, based on the *cth* toxin gene of *V. vulnificus* (Coleman *et al.*, 1996), was optimised for the detection of viable and laboratory induced viable but non-culturable (VBNC) forms of *V. vulnificus*. It was found that 7.5mM $MgCl_2$ produced an intense electrophoretic band of 388bp and pH seemed to have less influence on overall amplification. Similar results were observed with both culturable and VBNC cells, including opaque (virulent) and translucent (avirulent) strains. They also noticed that about 1000 times the amount of DNA from VBNC cells was required to produce a band of similar intensity to DNA derived from viable cells. Although no explanation was provided they speculated that this may have been due to super coiling of DNA in the VBNC state or binding of shock proteins to the DNA as a response to the cold shock treatment used to induce the VBNC state.

Assessment: The forward sense primer in this assay was the same as that used for PCR 3, however the sense primer was modified to amplify a 340bp region of the *cth* gene, producing an amplification product of 388bp. No explanation was given for the redesign of the antisense primer used here. A clear band was obtained with only 50fg of purified DNA which is the same limit of detection as that observed with PCR 3. The value of these primers for identification however is questionable since there was marked cross reactions with all seven pools of non-target species. The reactivity of the primers is probably the result of using 7.5 mM of $MgCl_2$ in the reaction mix which, although it will increase amplicon yield has the effect of reducing specificity. The effect of lowering MgCl2 concentration to increase stringency and hence specificity was not investigated. Given the limitations of the *cth* gene as a target for identification as discussed already and the lack of specificity, the primer set is not recommended for the identification of *V vulnificus*.

PCR 5. Conejero & Hedreyda (2003) - *Vibrio harveyi tox*R gene

Background: The strategy used for this *V. harveyi* PCR was based on the *tox*R gene, which codes for a transmembrane transcription regulator. The role of *tox*R as a regulator of virulence gene expression as well as a transcription regulator of outer membrane porins was first recognised in *V. cholerae* (Miller *et al.*, 1987; Miller & Mekalanos 1988). Subsequently *tox*R was found to be present in a wide range of *Vibrionaceae* including; *G. hollisae, Photobacterium* sp. SS9*, V. alginolyticus, V. anguillarum, V. fischeri, V. fluvialis, V mimicus, V. parahaemolyticus* and *V. vulnificus* (Conejero & Hedreyda, 2003). Primers for the *tox*R gene in *V. harveyi* were designed to amplify a 390bp amplicon, with a forward primer targeting a site within the variable membrane tether region and the reverse primer targeting a site within the relatively conserved periplasmic domain.

Assessment: This system was straightforward to implement and it was possible to detect down to 5pg of purified target DNA. Multiple strain testing was not undertaken in this study but in a limited review of 10 strains from widely separated geographic regions, no amplicon was detected with two strains (Conejero & Hedreyda 2003). The absence of amplicon suggests that there is either significant sequence variation in the primer binding regions or variation in the presence of the *tox*R gene. While the PCR primers appear to be highly functional, their use as an identification tool appears limited given the structural variation of the *tox*R gene that seems to occur for strains of the species.

PCR 6. Dalla Valle *et al*. (2002) – *Photobacterium damsela*e ssp. *piscicida*, RAPD

Background: The primers for detection of *Photobacterium damselae* ssp. *piscicida* were developed using random amplified polymorphic DNA (RAPD) to generate species specific markers. *Photobacterium damselae* ssp. *piscicida* is thought to be highly homogeneous phenotypically (Magariños *et al.*, 1992; Magariños *et al.*, 1997) and genotypically. Given this apparent homogeneity, the system was developed using only eight strains of *Ph. damselae* ssp. *piscicida* and four strains of *Ph. damselae* ssp. *damselae*. The anticipated specificity of the primers was not achieved and reactions occurred with both sub-species.

Assessment: Despite careful optimisation and the use of temperature gradient PCR in this study, first and second round reactions displayed some laddering or smearing below the amplicon, even with low template concentrations. The analytical sensitivity was 5fg for both first round and nested reactions, which is similar to that previously described. A 10 fold increase in the detection limit from 20 to 2fg with nested PCR has been reported (Dalla Valle *et al.*, 2002) but given the additional care required and the risks of contamination with nested PCR, it is not obvious that the advantage gained is justified other than confirmation of the internal sequence of the primary product with the nested primers. The value of this RAPD based primer set as a tool for the identification of *Ph. damselae* ssp. *piscicida* is questionable. The primers are known to cross react with *Ph. damselae* ssp. *damselae* and the evaluation undertaken in this study and during development was undertaken with only a few strains of the species. Although a BLASTn search did not reveal matches to other DNA constructs, giving some assurance as to specificity, what is not known is the distribution of the target site through the subspecies. Since other primers based on conserved regions of DNA have been described for *Ph. damselae* ssp. *piscicida*, the RAPD derived primers are not recommended for identification purposes.

PCR 7. González ***et al*. (2003) - *Vibrio anguillarum rpoN* gene**

Background: Several primer sets have been developed for the detection of *V. anguillarum* but have targeted sites, such as the haemolysin gene (Hirono *et al.*, 1996), that may not be present in all strains, or sequences designed for colony hybridisation (Rehnstam *et al.*, 1989; Martínez-Picado *et al.*, 1994; Powell & Loutit 1994; Ito *et al.*, 1995,). The primer set evaluated for this study is based on the metabolic regulator gene *rpoN* coding for flagellum factor σ^{54}, which is also related to virulence in *V. anguillarum* (O'Toole *et al.*, 1997). The *rpoN* sequence for a number of

species of *Vibrio* has been reported and display sufficient variability on which to base the design of species specific primers.

Assessment: Using the recommended PCR conditions it was possible to detect down to 500fg of purified *V. anguillarum* DNA; no significant banding was seen with the non-target species DNA. While the primers appeared to work well, only 57 strains, including 22 *V. anguillarum* were used in the development of the system. The extent to which *rpoN* is conserved within the species is not known. The gene does not appear to be present in the non-target species of *Vibrionaceae* tested, but it is present in a wide variety of other bacterial groups (O'Toole *et al.*, 1997) and is likely to occur in other species of *Vibrionaceae* which have not been evaluated. Given the uncertainty relating to specificity, *rpoN* is unlikely to be suitable as an identification tool without *a priori* knowledge of the likely identify of the unknown.

PCR 8. Hirono *et al.* (1996) - *Vibrio anguillarum* haemolysin *vah*1 gene

Background: Primers for the haemolysin *vah*1 gene were developed for the characterisation of virulence determinants in *V. anguillarum* isolated from diseased ayu (*Plecoglossus altivelis*) (Hirono *et al.*, 1996). The PCR conditions have not been published but were provided to this study as a personal communication (I. Hirono, 2003). Specificity had been assessed using the hybridisation probe *vah*1 and was found to be specific for *V. anguillarum* and did not react with a range of *Vibrionaceae*. Of note was that the probe detected non-haemolytic strains of *V. anguillarum* including serotype O1 (formerly serotype C), the strain of major impact on farmed salmonids in Australia. This suggests that although non-functional, the haemolysin gene is present in these strains. Most of the strains of *V. anguillarum* tested were serotype O2 (formerly serotype A) but importantly not all the strains hybridised with the probe suggesting that not all strains of *V. anguillarum* may contain the haemolysin gene *vah*1.

Assessment: For the PCR implemented in this study, the threshold of detection was 50fg and no cross-reactions occurred with the panel of non-target species. While the primers appear useful as a means of assessing potential virulence in *V. anguillarum* its usefulness as a reliable tool for identification is suspect.

PCR 9. Iwamoto *et al.* (1995) - *Vibrio 'trachuri'* (*Vibrio harveyi*), *Pst*I fragment

Background: The PCR system for *Vibrio 'trachuri'*, a junior synonym of *V. harveyi* (Thompson *et al.*, 2002b), was based on a set of primers derived from a new pathogen of Japanese horse mackerel (*Trachurus japonicus*). As no sequence data was available

for *V. 'trachuri'* at the time, restriction digests of chromosomal DNA were prepared from three strains of *V. 'trachuri'* and a primer pair based on a *Pst*1 fragment was selected. Testing showed that primers amplified DNA of *V. 'trachuri'*, but not when tested with strains of *V. anguillarum, V. parahaemolyticus* or several species of *Enterobacteriaceae*. Based on serially diluted purified template the analytical sensitivity of the system was estimated to be 100fg per reaction.

Assessment: Following implementation and optimisation of the PCR the threshold of detection with purified target DNA was 50pg. This level of detection is much higher than the other PCR primers evaluated in this study which were generally in the femtogram range. BLASTn analysis failed to reveal the likely gene target for this system. It is possible that the *Pst*1 fragment is a unique construct and is a specific marker for *V. harveyi*, but in the absence of information about the target site and its distribution across strains, the use of this primer set for identification is not recommended.

PCR 10. Kim & Jeong (2001) – *Vibrio vulnificus* 16S rRNA gene, tri-primer

Background: The PCR, for the detection and differentiation of *V. vulnificus*, was developed as an alternative to routine culture methods. The *V. vulnificus* specific primers are based on the hypervariable regions (1006 to 1023 and 1278 to 1258) of the 16S rRNA gene. An additional primer for the differentiation of two rRNA genotypes of *V. vulnificus* was also described. Based on a 16S rRNA gene alignment one antisense and two sense primers were designed. The tri-primer system was intended to differentiate Type A, represented by the type strain ATCC 27562 and Type B, represented by wild type strains. Primers Vib 2 and Vib 3R were specific to both rRNA genotypes, while primer Vib 1 was specific for Type A strains (ATCC 27562) and a range of other bacteria depending on the level of 16S homology. Type A *V. vulnificus* produces two amplicons of 273bp and 825bp while a single 273bp amplicon represents Type B *V. vulnificus*. A relatively small number of strains were used to develop the system: three strains of *V. vulnificus*, five other species of *Vibrio* and four unrelated bacterial species.

Assessment: Serial dilutions of DNA suggested that the threshold of detection for this system was around 5pg when purified DNA was used as template. The cycle conditions appeared to be finely tuned as attempts to reduce the number of non-specific bands by manipulating both the annealing temperature and the Mg^{2+} concentration failed to make the amplification any clearer. A degree of smearing and faint extra bands was always present, especially at higher template concentrations. A

non-specific reaction was seen as a single band of 825bp although minor bands of other sizes were also seen with some non-target species. Interpretation of banding patterns of this tri-primer system is not straightforward. The 273bp band, diagnostic for *V. vulnificus* and specific for the Type B rRNA genotype, was clear cut. The requirement for an additional band at 825bp to confirm the Type A rRNA genotype is a potential cause of confusion since a single band at 825bp is not diagnostic of *V. vulnificus* and as other minor bands may also occur with non-target species, some care is required with interpretation.

The primers, being directed at a conserved site, have the merit of specificity within the species but as currently formatted there are clearly some potential pitfalls when using this tri-primer arrangement. If genotyping is not required, and in all probability this would be the case if used for species identification, a conventional arrangement of the primer pair Vib 2 and Vib 3R to yield a 273bp amplicon would be suitable. This has the virtue of a clean amplification product minus the potentially confusing secondary non-specific bands.

PCR 11. Kvitt *et al*. (2002) - *Photobacterium damsela*e ssp. *piscicida* 16S rRNA gene

Background: The nested PCR protocol was based on 16S rRNA gene sequence. The procedure was developed and validated using 19 strains of *Ph. damselae* ssp. *piscicida* of fish origin from a range of geographic locations, including Japan, Italy, Spain, Greece and Israel.

Two primers pairs were defined: P1/P2 produced a 570bp amplicon while P3/P4 produced a 270bp amplicon. Primer pair P1/P2 was designed with the intention of differentiating *Ph. damselae* (*sensu lato*) from *Ph. 'histaminum'*, now recognised as a junior synonym of *Ph. damselae* ssp. *damselae* (Kumura *et al.*, 2000). Using the P1/P2 primers, 350fg of purified DNA template could be detected using an annealing temperature of 53°C. The limit of detection using primers P3/P4 was around 350fg per reaction at annealing temperatures, 53 and 64°C. When an annealing temperature of 53°C was used non-specific PCR products were observed when uninfected fish tissues were tested. Some non-specific PCR products were also observed with *G. hollisae* and *Ph. leiognathi*. Raising the annealing temperature to 64°C eliminated these non specific products, but the limit of detection was affected and decreased to 3.5pg DNA.

Assessment: Implementation of the nested system was straight forward and worked well. It was possible to achieve, with an annealing temperature of 64°C, a threshold of

detection of 500fg in the first round reaction and 5fg of purified DNA detected after second round testing. The non-target species DNA did not produce non-specific bands. The system was based on the 16S rRNA gene and so there is a level of confidence that the gene target would be present in all strains. Of note is that the primers are specific for *Ph. damselae* (*sensu lato*), and will detect both subspecies: *damselae* and *piscicida*. Kvitt *et al.* (2002) reported that *Ph. 'histaminum'* was positive with primers from first round amplification, but negative with the second round primers P1-P2. This is a surprising observation given that *Ph. 'histaminum'* is a junior synonym for *Ph. damselae* ssp. *damselae* (Kimura *et al.*, 2000). An assessment of specificity with *Ph. 'histaminum'* ATCC 51805 was not made with the nested primers in this study and the previously reported findings cannot be verified. Since a more elegant and discriminatory multiplex PCR is available for *Ph. damselae* and its subspecies (Osorio *et al.*, 2000), the procedure of Kvitt *et al.* (2002) is not recommended.

PCR 12. Lee *et al.* (1998) - *Vibrio vulnificus vvh* cytolysin/haemolysin gene

Background: This study aimed to establish the optimal PCR conditions and a high yield DNA extraction method for the direct identification of *V. vulnificus* in human clinical specimens. The gene target was the cytolysin/haemolysin gene *vvh*, reported to be present in all strains of *V. vulnificus* (Morris *et al.*, 1987). Two sets of nested primers yielding a 704bp and 222bp amplicons were described.

The sensitivity of the primers, assessed by PCR with serially diluted DNA, was 100pg for P1/2 and 100fg for P3/P4. The nested protocol could detect 1fg of chromosomal DNA. The system was developed using 32 strains of bacteria including nine strains of *V. vulnificus*, 11 non-target species of *Vibrionaceae* and 12 unrelated species.

Assessment: Following implementation, the threshold of detection of this system was determined at 500fg for the first round reaction and 5fg for the second round of PCR, similar to that obtained by Lee *et al.* (1998). No significant amplification products were seen after the second round reaction when the pooled non-target species DNA were examined. As for similar PCRs where the primers are not directed at a conservative site such as the 16S rRNA gene, the value of this PCR for species identification is questionable. Similarly a nested format, while attractive as a means of improving test sensitivity for direct detection of *V. vulnificus* in clinical or environmental samples, does add another layer of complexity to the assay and increases the risk of amplicon contamination.

PCR 13. Oakey *et al.* (2003) – *Vibrio harveyi* 16S rRNA gene

Background: The PCR has been positioned as a procedure to augment the phenotypic identification of *V. harveyi*. Recognising the genetic heterogeneity and presence of mobile genetic elements in *V. harveyi*, the PCR was developed using alignments of the conserved region of the genome represented by the 16S rRNA gene. Eleven 16S rDNA sequences consisting of two partial sequences were generated from local strains and aligned with a range of other 16S rDNA sequences from GenBank, including closely related *Vibrio* species such as, *V. campbellii* and *V. alginolyticus*. Primers VH-1 and VH-2, which define a 413bp amplicon in *V. harveyi* were identified. The PCR was validated with 21 species of *V. harveyi*, 28 other non-target *Vibrionaceae* and seven unrelated species.

Specificity was problematic and an annealing temperature of 65°C was necessary to prevent non-specific bands occurring with near related species *V. campbellii, V. alginolyticus* and *V. splendidus*. All strains of *V. harveyi*, including *V. 'carchariae'*, a junior synonym of *V. harveyi*, produced a 413bp product diagnostic for the species. Cross reaction, even with an annealing temperature of 65°C, was found with some strains of *V. alginolyticus* and produced a 413bp amplicon indistinguishable from that of *V. harveyi*.

Assessment: In this study it was possible to detect down to 5fg of purified DNA with the primers even at an annealing temperature of 65°C. In the screening of the pooled non-target species DNA with primer VH-1 and VH-2, three of the seven pools showed clear amplification products similar in size to the 413bp expected for *V. harveyi*. Oakey *et al.*, (2003) also observed amplification products with a range of closely related *Vibrio* species but where able to limit this to *V. alginolyticus* by increasing the annealing temperature to 65°C. For the three pools of negative controls a total of 11 species had not been tested by Oakey *et al.* (2003) and may account for the increased number of unexpected reactions that were observed. Further attempts at optimisation to eliminate these cross reactions were not made. No amplicon was seen with Pool 3 which contained the type strain of *V. alginolyticus* ATCC 17749; this finding is consistent with the observation of Oakey *et al.* (2003) who noted that not all strains of *V. alginolyticus* produced an amplicon with the *V. harveyi* primers. The limitations in regard to specificity of the primers mean that the PCR needs to be used judiciously. Its major role will be to confirm the identity of atypical strains of *V. harveyi* that have abnormal phenotypes such as resistance to 0/129 at 150µg (S. Benedict pers. comm.). Caution

needs to be applied with this approach. Oakey *et al.* (2003) relied on the use of the Voges-Proskauer reaction in *V. alginolyticus* to differentiate this species from *V. harveyi*. In general terms this approach has merit but consideration needs to be given to *V. harveyi* biovar II (Phenon 32) where 75% of strains are Voges-Proskauer positive. For this biovar of *V. harveyi* differentiation from *V. alginolyticus* on the basis of PCR and Voges-Proskauer reaction is not possible. More useful markers to differentiate *V. alginolyticus* from both biovars of *V. harveyi* are summarised in Table 8.6.

Table 8.6 Phenotypic tests to differentiate *V. alginolyticus* and the biovars of *V. harveyi*

Species	VP[1]	PNPG[2]	Aesculin[3]	Putrescine[4]
V. alginolyticus	100%[5]	16%	10%	100%
V. harveyi biovar I	2%	95%	95%	2%
V. harveyi biovar II	75%	100%	100%	0%

[1]VP: Voges-Proskauer; [2]PNPG: hydrolysis of 2-nitrophenyl β-D-galactopyranoside;
[3]Aesculin hydrolysis; [4]Utilisation of putrescine as a sole carbon source; [5]% strains positive

PCR 14. Osorio *et al.* (1999) - *Photobacterium damselae* 16S rRNA, hemi-nested

Background: Following the reclassification of *Pasteurella piscicida* as *Ph. damselae ssp. piscicida* (Gauthier *et al.*, 1995) primers for PCR were developed as a tool for direct detection of the pathogen in fish tissue and as a means of assessing the homogeneity genotypically of 26 strains of *Ph. damselae* ssp. *piscicida* and 10 strains of *Ph. damselae* ssp. *damselae*. The optimised annealing temperature was found to be 65°C; both subspecies of *Ph. damselae* were positive and only a weak band larger than the 267bp expected amplicon occurred with *V. splendidus* biovar I. The detection limit was 10fg of purified template DNA; the second round of PCR (hemi-nested) did not improve sensitivity but did increase band intensity.

Assessment: For this study the threshold of detection was 500fg after first round PCR and down to 5fg after the nested reaction. Screening of the pooled non-target species DNA with the 16S rDNA targeted Car1 and Car2 primers produced a number of faint non-specific bands in some instances. The non-specific bands were larger than the 267bp specific amplicon and much fainter and less prominent, making them easy to differentiate from the specific positive amplification product.

The target site is the 16S rRNA, a conserved gene, suitable for identification of *Ph. damselae* (*sensu lato*) but not the two subspecies which are indistinguishable using the primers Car1 and Car2. The PCR was designed with an objective of direct detection of *Ph. damselae* ssp. *piscicida* in fish tissue. The hemi-nested implementation of the PCR to increase sensitivity is not warranted where the source of template DNA is

bacterial cultures. A revised design of the PCR in multiplex format to differentiate both subspecies has been developed and is a superior and elegant implementation (Osorio *et al.*, 2000); see PCR 15.

PCR 15. Osorio *et al.* (2000) - *Photobacterium damselae* ssp. *damselae* and *piscicida*, *ureC* and 16S genes, multiplex format

Background: The purpose of this PCR is to identify and differentiate the two subspecies of *Ph. damselae*: ssp. *damselae* and ssp. *piscicida*. The test format is multiplex using two primer pairs, one directed against the 16S rRNA gene for *Ph. damselae* and the other primer pair for the urease *ureC* gene. Using this strategy *Ph. damselae* is identified by the 267bp amplicon derived from the 16S rRNA gene and the two subspecies differentiated by the *ureC* gene which is present in *Ph. damselae ssp. damselae* as a 448bp amplicon but absent from *Ph. damselae* ssp. *piscicida*.

In the *Vibrionaceae* the 16S rRNA gene may be represented in multiple operons within a single genome whereas *ureC* appears to be a single copy gene. The differences in copy number of the two target genes required manipulation of primer concentrations to ensure that amplicon intensity was similar.

The multiplex PCR was evaluated with 25 strains of *Ph. damselae ssp. piscicida* and 15 strains of *Ph. damselae ssp. damselae*; all strains gave the anticipated pattern of reactions.

Assessment: Following implementation the threshold of detection was found to be 500fg for the urease primers and 50fg for the *Ph. damselae* specific 16S rRNA primers. While a number of faint non-specific bands were observed in some pools of non-target DNA, these were easy to differentiate from the specific positive amplification products. No further modification of the PCR was attempted to suppress these occasional weak bands. The format appeared robust and was found useful for verifying cluster integrity for Phenon 63, *Ph. damselae* ssp. *damselae* biovar I, Phenon 75, *Ph. damselae* ssp. *damselae* biovar II and Phenon 76, *Ph. damselae* ssp. *piscicida*.

While the PCR format appears very suitable for identification of pure cultures, primers for the *ureC* gene may not make the assay suitable for direct detection in fish tissue. Failure to detect a 448bp band may be the result of copy number imbalance between the 16S rRNA and urease genes where the target organism in a sample is close to the threshold of detection. Absence of a band corresponding to the urease gene may simply be the result of the copy number for the urease gene being below the

threshold of detection. A second limitation may arise where urease positive non-target organisms are present alongside *Ph. damselae* ssp. *piscicida*. There is a risk in such circumstances of concluding that only *Ph. damselae* ssp. *damselae* is present in the infected tissue. Given the importance of *Ph. damselae* ssp. *piscicida,* failure to detect the pathogen may have serious consequences.

8.4 Determine the range of *Vibrio* species associated with farmed fish, shellfish and crustacea in Australia

The diversity of species associated with aquatic animals in Australia as identified from this project is surprisingly small. To some extent this may reflect the basis on which strains were obtained. In nearly all cases, isolates were sent from veterinary laboratories that provide diagnostic and disease investigation services. It would be common practice to retain in a culture collection strains associated with a disease condition or likely to cause disease or could be unambiguously identified. As a result the catalogue of species identified represents an understandable bias. Strains from striped trumpeter for example, are well represented as they were from a library developed to study early stages of bacterial colonisation of larvae (Battaglene *et al.*, 2006). Nevertheless, the range of named species is instructive if only to confirm what was previously known and to establish a base line of enzootic strains.

There were a limited number of unusual findings. Of note was the isolation of *V. penaeicida* from phyllosoma of southern rock lobster and more recently the isolation of *Ph. damselae* ssp. *piscicida* as an incidental finding from a health survey of southern bluefin tuna. Although not part of this study, identification was made using the MicroSys V36 panel and confirmed by multiplex PCR (Osorio *et al.*, 2000) that had been evaluated and implemented as described in this report (pers. comm., M. Higgins, T. Wilson, B. Nowak). Both species prior to this project were considered exotic, and while the evidence suggests that they represent an uncommon finding, knowledge that they do occur in the Australian aquatic environment is of value. *Vibrio penaeicida* is the cause of vibriosis in Kuruma prawns (de la Peña *et al.*, 1993; Ishimaru *et al.*, 1995) while *Ph. damselae* ssp. *piscicida* is a pathogen of a diverse range of farmed fish (Romalde 2002) including bluefin tuna in Europe (Mladineo *et al.*, 2006). *Vibrio tubiashii* is a species of interest in that it appears to be a taxon that is commonly misidentified. Of a total of 14 strains submitted as *V. tubiashii* only one strain had characteristics of the taxon. The remaining strains were identified as a range of other named species or

Phenons. The reasons for the evident misidentification are not apparent but these findings indicate the species occurs only rarely and is not as abundant as commonly believed. Only three strains were identified as *V. tubiashii*; one was the cause of bacillary necrosis in Pacific oyster larvae and is consistent with the known pathogenicity of the species (Hada *et al.*, 1984) while the other two strains were isolated from two species of marine turtle.

Three species have wide host ranges: *V. harveyi* biovar I, *Ph. damselae* ssp. *damselae* and *V. alginolyticus*. No information was provided from submitters on the condition of the host so it is not appropriate to infer much about the capacity of strains to cause disease. All three species are known to be agents of disease in aquatic animals and the diversity of hosts points to the importance of these species as causes of infection. Host range, however, can be misleading as an indicator of strain significance. Of greater importance is the frequency of isolation from hosts, which provides a better assessment of impact. The two most commonly occurring species were *V. anguillarum* and *V. harveyi* biovar I followed by *V. splendidus* biovar I. The number of strains of the species represented in the library suffers to an extent from regional and collection bias. *Vibrio anguillarum* is a temperate water pathogen that has had a significant impact on sea-farming of salmonids in Tasmania over many years and a large number of strains have been acquired over a 20 year period. Clearly this species is over-represented but is an indication of the significance of the pathogen for farmed aquatic animals.

Table 8.7 Hosts with unique protospecies yet to be classified

Host	Taxon	No. strains in taxon
Abalone	Phenon 15	8
Molluscs: abalone & mussels	Phenon 24	4
Striped trumpeter	Phenon 29	17
Leafy sea dragon	Phenon 42	4
Abalone	Phenon 46	6
Rainbow trout	Phenon 58	4
Pacific oyster	Phenon 69	5

Many hosts were the source not only of species that could be identified but also yielded a range of taxa that could not be allocated to known species. The distribution across hosts generally appeared to be host independent such as Phenon 83, which included Atlantic salmon, leafy sea dragon as teleost fish and southern rock lobster, a crustacean. Other phenons appeared to be host specific as listed in Table 8.7. The number of strains for a given phenon is generally small, but in the case of Phenon 15 and Phenon 29, they each contain several strains and points to the potential

importance as taxa that have a close, and what appears to be a unique relationship with a specific host. The identity of the phenons is unknown other than they appear to represent groups with high levels of phenotypic similarity and in some cases sequence homogeneity as well. Based on partial sequences, Phenon 24 has a high level of sequence similarity to *V. pomeroyi* and it is of note that of the four strains originally described, two were from molluscan bivalve larvae, *Nodipecten nodosus*, the lion's paw scallop (Thompson *et al.*, 2003d). Strains of Phenon 46 all come from abalone at a single farm which experienced recurrent disease episodes. The isolates formed a tight and apparently unique cluster with high phenotypic and genotypic similarity; this protospecies would appear to have significance as a pathogen of abalone.

A characteristic of importance for aquatic animal health is the pathogenicity, if any that these un-named phenons may have for their hosts; similarly other than some of the named species, the pathogenicity for their natural hosts is not always apparent. For the named species at least an indication of pathogenic potential, in the absence of direct evidence, can be inferred on the basis of pathogenicity in other hosts. Species of uncertain pathogenicity would include *V. diazotrophicus* isolated from mullet or *V. nereis* from flatback turtles or yellowtail. Neither bacterial species has been reported to be a pathogen of aquatic animals, so it is more likely that they represent normal flora. The role of other bacterial species is less clear when they have been isolated from hosts different to those described originally. In particular, *V. scophthalmi*, which was first isolated from the gut of juvenile turbot (Cerdà-Cuéllar *et al.*, 1997), has been isolated from Atlantic salmon with signs of disease, while *V. ichthyoenteri* was isolated from barramundi, mahi mahi and Atlantic salmon. The species was first isolated from the gut of diseased juvenile Japanese flounder (Ishimaru *et al.*, 1996) and subsequently from diseased barramundi in Indonesia (Anon, 2001). On the evidence presented it is likely that both *V. scophthalmi* and *V. ichthyoenteri* may act as pathogens in some species of aquatic animals in Australia. Assigning significance to the newer species of *Vibrionaceae* is problematic. Initial assessments of pathogenicity have been made with a range of species using rainbow trout as a model (Austin *et al.*, 2005). Many of the recently described species appeared non-pathogenic except for strains of *V. brasiliensis, V. coralliilyticus, V. ezurae, V. fortis, V. kanaloae, V. neptunius, V. rotiferianus* and *V. tubiashii* which all exhibited various levels of pathogenicity in both rainbow trout and *Artemia* nauplii. For the un-named phenons recovered from this study of *Vibrionaceae* and species such as *V. ichthyoenteri* and *V. scophthalmi*, assessment of pathogenic

potential needs to be made to determine the significance of these taxa as agents of disease. Isolation of strains assigned to *A. sobria sensu stricto* is interesting as they were all obtained from apparently healthy normal salmonids at freshwater sites. The strains were unusual as they were sensitive to the vibriostat 0/129. The species is of interest as it has been reported to be a significant pathogen of freshwater perch (*Perca fluviatilis*) (Wahil *et al.*, 2005) and is a potential pathogen of other exotic and native fish species.

The survey of *Vibrionaceae* from aquatic animals also confirms the absence of several important pathogens of farmed fish and shellfish. Species that do not appear to have been encountered by laboratories in Australia include: *M. viscosa, V. ordalii, V. salmonicida, V. vulnificus* bv II, *V. wodanis, V. tapetis* and *V. pectenicida*, pathogens of varying degrees of severity for finfish and shellfish. It would appear that Australia is free of these pathogens. It has been reported previously that *V. ordalii* has been isolated from rainbow trout in Tasmania (Cameron *et al.*, 1988) and mainland Australia (Austin *et al.*, 1997). From the extensive library of strains used in this study, which included strains of *Vibrionaceae* from Tasmania collected over a 20 year period, none were identified as *V. ordalii*. No strains of *V. ordalii* isolated in 1988 are extant (C. Garland, pers. comm.) and the original findings cannot be confirmed. Austin and colleagues (1997) received strains of *V. ordalii* from Australia but were mis-attributed to this country, coming originally from New Zealand (A. Thomas pers. com.) where the pathogen is known to occur (Wards *et al.*, 1991). On the evidence available, *V. ordalii* should be considered exotic to Australia. Strains from abalone formed a proportion of the library, most derived from farmed stock in Tasmania. None of the strains however were identified as *V. superstes* which was first isolated from the gastrointestinal tract of

Table 8.8 Zoonotic species of *Vibrionaceae* isolated from aquatic animals in Australia with known disease conditions in man (after Pavia *et al.*, 1989, Daniels & Shafaie, 2000)

Species	Gastroenteritis	Wound infection	Primary septicaemia	Ear infection
Ph. damselae ssp. *damselae*		++		
Vibrio alginolyticus	+	++		+
Vibrio cholerae non-O1	++	+	+	(+)
Vibrio cincinnatiensis				(+)
Vibrio fluvialis	++	(+)	(+)	
Vibrio furnissii	++			
Vibrio harveyi		(+)		
Vibrio mimicus	++	(+)	(+)	(+)
Vibrio parahaemolyticus	++	+	(+)	(+)
Vibrio vulnificus	+	++	++	

++ common presentation; + less common presentation; (+) rare presentation

species of abalone off the coast of Victoria (Hayahi *et al.*, 2003). The absence of this species from the library probably reflects the discrete distribution in the gut of abalone and the specialised niche which it occupies. Subsequent testing of the type strain of *V. superstes* with the MicroSys V36 panel and the VibEx7 database (data not shown) found that there was no match with any of the protospecies designated as numbered phenons, further evidence that the species was not represented in the library of strains.

Several species isolated from aquatic animals are recognised as pathogens of man and should be considered as zoonotic agents. Species recognised as pathogens of man (Pavia *et al.*, 1989; Daniels & Shafaie, 2000) are given in Table 8.8 and other than *Grimontia hollisae, Vibrio cholerae* O1 and *Vibrio metschnikovii*, all the species listed were isolated from aquatic animals in Australia.

Table 8.9 Zoonotic *Vibrionaceae* isolated from farmed aquatic animals

Species	Host
Ph. damselae ssp. *damselae*	Atlantic salmon Rainbow trout Southern bluefin tuna
Vibrio alginolyticus	Yellowtail
Vibrio furnissii	Atlantic salmon
Vibrio mimicus	Marron Yabby
Vibrio parahaemolyticus	Mussels Rainbow trout
Vibrio vulnificus	Barramundi

Some species were isolated from food production animals and represent potential public health risks from either consumption or through handling of infected stock. Zoonotic agents associated with food production animals are listed in Table 8.9. There is no evidence that disease in man has resulted from farmed aquatic animals in Australia but the presence of these species is an indication of the potential for zoonotic infections to occur.

8.5 TECHNOLOGY TRANSFER WORKSHOP FOR STATE VETERINARY DIAGNOSTIC MICROBIOLOGISTS

Although the level of participation at the workshop was low, it did provide an opportunity to assess the robustness of the probabilistic identification system and PCR assays when used by veterinary microbiologists not familiar with the processes. Both molecular and phenotypic identification systems performed well, indicating that both methods are accessible and can be readily implemented by laboratories. As with all new procedures the transition to a different process was variable across participants. Of note was that correct identifications were obtained by all participants, with

differences observed in the modal likelihood scores for each identification reached. Several identifications had Willcox probability scores of $P<0.99$ or had modal likelihood scores <0.001. This invariably was the result of reading tests incorrectly or participants not being sufficiently familiar with some tests and not being able to discriminate weak reactions when they occurred. Given that this was the first time the participants had encountered the format, the results obtained were encouraging. It did emphasise the need to obtain familiarity with the system and to use control organisms with stable phenotypes as a means of internal quality control.

8.6 Develop an ANZSDP for the Identification of *Vibrio* species in aquatic animals

The preponderance of Australian aquaculture is maritime based and consequently the *Vibrionaceae* are the pre-eminent group of bacteria associated with aquatic animals. For veterinary laboratories providing diagnostic services, identification of *Vibrionaceae* represents not only a commonly occurring group of bacteria of some importance but also a taxonomic challenge because of the diversity and number of species to be considered. Difficulties in providing an accurate identification are compounded by the diversity of possible procedures to choose from and the absence of reliable commercial kits.

The identification approach developed from this study is intended to establish a minimum set of criteria using standardised tests that can be used to identify a range of *Vibrionaceae* associated with aquatic animals in Australia. Probabilistic identification was selected as the basis for taxon matching since this has been shown to be the most expedient and reliable means of identification. It is particularly suited for large groups of bacteria comprising many species such as the *Vibrionaceae*. The phenotypic diversity of the *Vibrionaceae* imposes some logistical constraints on identification because the panel of tests required is relatively large, which means that implementation for routine use will be resource intensive when used with conventionally formatted tests. A commercially available kit, MicroSys® V36, uses miniaturised tests that are compact in format and can be readily integrated for routine use by diagnostic or research laboratories.

The identification matrix developed for the *Vibrionaceae* covers 54 formally described species and a further 25 protospecies defined as phenons. Missing from the matrix are 35 newer species which were proposed after the numerical taxonomic study was nearing completion. On the evidence available from 16S rDNA sequencing it

would appear that few if any of these newer species are represented by the 25 protospecies. The matrix, while incomplete taxonomically, is representative of the diversity of *Vibrionaceae* associated with aquatic animals in Australia and its utility should not be compromised by the absence of what clearly appear to be exotic species.

While it is anticipated that phenotyping will remain the principal means of identification for routine diagnostic purposes, molecular methods based on PCR will have specific application. Selection of species for evaluation was made on the basis of pathogens known to be of significance to aquatic animals and to a lesser extent as zoonotic agents. Immediate application of PCR is as a confirmatory test for an identification reached by phenotyping, particularly for suspect outliers or an independent means of confirming an unusual finding in a new host or geographic location. Validation of the primer sets was not undertaken in fish tissues and no confidence can be ascribed to their use for direct detection based on the work undertaken in this study. If in the future there is a need to use the primers with samples derived from heterogenous matrices, the optimisation and assessment that was undertaken here provides a sound basis for further work if the PCR primers are to be used for direct detection.

Taxonomies are not static but dynamic with a clearly evident cycle of continuous classification and identification (Rypka, 1975). New information is obtained either from the recognition of new taxa or from refinements in existing classifications leading to new understandings of the relationships between existing taxa or from improved phenotypic descriptions. For identification matrices to remain useful, feedback and readjustment is required either by correction of phenon definitions or fusion or splitting of existing groups to better describe taxa in the identification matrix (Gyllenberg & Niemlä, 1975). The probability matrix VibEx7 cannot be considered complete; it would be imprudent to think otherwise. The virtue of the probability matrix and the phenetic taxonomy from which it was derived is that it provides a platform from which improvements can be made in the future. New species will be described or existing ones, thought to be exotic to Australia, will emerge. These can be readily incorporated into the identification matrix so that it remains relevant and functional well into the future.

9. FUTURE PROSPECTS

Identification of the *Vibrionaceae* is likely to remain a complex issue for which complimentary and competing approaches will be used. A clear understanding of the purpose of identification needs to be reached to determine the most appropriate strategy. For taxonomists, particularly those developing a classification or resolving some ambiguity or anomaly, identification by molecular methods is likely to be the most effective and accurate methodology. The needs of the taxonomist are somewhat different from those of the investigative microbiologist who is faced with the prospect of having no precognition of the species to be identified. In a diagnostic setting the identification strategy, in some practical sense, must be able to accommodate a range of identification possibilities. For this process to be efficient a unified and integrated approach must be used that can accommodate the known range of species.

Phenotyping represents a practical approach to identification provided good estimates of phenotypic diversity have been obtained and reliable tests determined that can clearly differentiate the species. On the basis of the study reported here, there is good evidence to show that a phenotypic approach to identification of the *Vibrionaceae* is achievable. To realise the phenotypic approach it is evident that an elaborate programme of phenotypic discovery must be used, a requirement that could be perceived as discouraging to future expansion and refinements. This need not be the case. Although the VibEx7 data matrix and V36 panel of miniaturised tests cover the range of taxa associated with aquatic animals in Australia, the identification matrix is by no means complete and some 30 or so species are not included. Adding them to the matrix should be straightforward since the framework for phenotyping has been established through this study.

A challenge for future studies to expand and harden the VibEx7 data matrix, will be the collection of sufficient strains for each species so that a reasonable estimate of phenotypic diversity is obtained. Since many new species are classified using molecular techniques, there is an alarming trend toward using very few strains to propose a new species. In many instances only one strain is used. The need for several strains to define a species has been recommended (Christensen *et al.*, 2001) and is particularly pertinent with regards to phenotypic descriptions and obtaining an estimate of diversity.

While phenotyping is likely to remain the pre-eminent means of identification for the *Vibrionaceae* cognisance of the rapid development in molecular techniques needs to be made. The high level of 16S rDNA sequence similarity amongst species of the

Vibrionaceae has meant that this particular construct is unlikely to contain sufficient differences for it to be used for the generation of unique oligonucleotides capable of differentiating all the species of the *Vibrionaceae*. Recent work on multilocus sequence typing (Thompson *et al.*, 2005) is likely to yield a useful range of conserved constructs, sufficient to identify species of the *Vibrionaceae*. This will open the way for development of microarray chip based identification (Marsh & Cardy, 2004). The use of microarrays for the identification of *Vibrionaceae* shows considerable promise and has been used already for the human pathogens *V. cholerae, V. mimicus, V. parahaemolyticus,* and *V. vulnificus* (Panicker *et al.*, 2004; Vora *et al.*, 2005) and of note for the detection of the fish pathogens *Ph. damselae* ssp. *damselae* and *V. anguillarum* (González *et al.*, 2004). While equipment costs for microarray analysis is expensive, this approach, perhaps in the not too near distant future, may become the standard means of identifying the *Vibrionaceae*.

10. BENEFITS

Accurate identification of the *Vibrionaceae* and the means to do so has been a major problem not only for veterinary diagnostic laboratories, but also fish farmers, health care professionals and regulators. The principal outcome of this project has been the development of a practical system by which this important group of bacteria can be identified. The phenetic probabilistic identification approach was supplemented by the evaluation and implementation of PCR primers for confirming the identity of several species of *Vibrionaceae*, significant pathogens of farmed aquatic animals.

The phenetic based identification system that has been developed is underpinned by a numerical taxonomy of the *Vibrionaceae*. This approach lays the foundation on which new species, as they emerge or gain importance, can be evaluated and incorporated into the existing probabilistic identification data matrix. In development of the probabilistic identification method it was necessary to acquire an extensive library of bacterial strains, extant for most states in Australia and representative of the diversity of floras from aquatic animals in both temperate and tropical climates. The assembled library provides a reasonable estimate of the diversity of *Vibrionaceae* from aquatic animals in Australia and should prove useful as an inventory and benchmark of species.

The basis for accurate identification of *Vibrionaceae* is supported by the development of a standard diagnostic procedure in a form that can be adopted by laboratories providing diagnostic services. Much background information relating to probabilistic identification and the factors influencing this approach are provided in this report together with extensive supporting data. This information will be of direct benefit to microbiologists in aquatic animal health particularly in relation to quality control and the standardisation of identification tests.

To undertake the numerical taxonomy it was necessary to obtain a diversity of type and reference strains of the *Vibrionaceae*. This material is a valuable laboratory resource for both quality control and familiarisation purposes. As a result of the project, Biosecurity Australia (Department of Agriculture, Fisheries & Forestry) undertook a risk assessment and determined that government laboratories with PC2 facilities may hold species of the *Vibrionaceae*. As a result of this decision, veterinary diagnostic laboratories in Tasmania, Western Australia and Queensland now hold type and reference strains of *Vibrionaceae* used in this project.

The ability to identify *Vibrionaceae* with greater certainty will enable the cause of disease in aquatic animals to be more clearly described and provides the basis for more informed decisions for disease treatment and management. An ability to accurately identify disease agents underpins the development of vaccines. Establishing the cause of disease is an essential first stage of vaccine development as is a capacity to determine phenotypic strain variation. Realisation of a vaccine is only possible once the diversity within the species has been assessed. Phenotypic characterisation using the V36 identification panel can be used not only for identification purposes but also as a means of assessing diversity.

Use of the V36 panel of tests for probabilistic identification of the *Vibrionaceae* has already been adopted by Berrimah Veterinary Laboratories, Department of Primary Industry, Fisheries and Mines, Northern Territory; Primary Industries Research, Victoria; and Fish Health Unit, Department of Primary Industries & Water, Tasmania. The panel of tests has also been used at the School of Aquaculture, University of Tasmania for several research projects. At an international level the identification software PIBWin has found wide acceptance with almost 3,000 downloads of the software made in the past two years.

11. FURTHER DEVELOPMENT

While the probabilistic identification matrix reflects the known range of *Vibrionaceae* in Australia further improvements and enhancements are required. Several of the phenons in the matrix are based on four or less strains. The estimates of diversity for these phenons in particular is marginal and further strains need to be acquired to obtain more robust descriptions of phenotype. During the period of this study many new species were formally proposed. While it would appear that most if not all of these species are exotic to Australia, it would be prudent nevertheless to ensure that at some future date full descriptions are obtained and incorporated into the VibEx7 probability data matrix.

From this study several phenotypically well defined taxa were recognised and on the basis of 16S rRNA gene sequencing appear to represent new species of the *Vibrionaceae*. These phenons require additional taxonomic analysis to determine their status. In particular DNA:DNA hybridisation analysis with near related species needs to be undertaken. Some of these taxa were isolated almost exclusively from particular hosts and consideration needs to be given to their significance as pathogens. Some form of pathogenicity evaluation is warranted for these taxa as a means of assessing their importance and role as potential pathogens.

Molecular taxonomic tools in the form of 16S rRNA gene sequencing was used as a means of determining cluster homogeneity based on BLASTn searches. Further work is warranted to determine the congruence between phenetic clusters and sequence analysis. In particular phylogenetic analysis needs to be undertaken, including multiple alignment of sequences of strains from individual clusters. Multilocus assessment of strains using other gene sequences such as *gyrB*, *rpoA*, *recA* and *pyrH* (Thompson *et al.*, 2005) may prove useful for determining cluster homogeneity. An estimate of congruence can be determined by calculating the correlation coefficient between similarity values of the molecular and phenetic taxonomic approaches (Sneath, 1978a) and would provide more information about the quality of the taxonomic structure and confidence that clusters defined by phenotype are valid constructs.

Consideration needs to be given to presenting the miniaturised V36 identification panel in a freeze-dried format to simplify its use and encourage uptake of the panel by diagnostic laboratories.

12. PLANNED OUTCOMES

The primary goal of the project was to develop a system for the identification of *Vibrionaceae* using both phenetic and genetic approaches. Both methodologies have been successfully implemented. Tangible outcomes are development of the V36 identification panel of tests and corresponding probability matrix; creation of the software package PIBWin for probabilistic identification; implementation and evaluation of PCR primers for the rapid identification of major pathogens; exploration of numerical methods for the identification of bacteria; and development of miniaturised tests in the MicroSys V36 format.

An anticipated outcome will be the twin goals of a simplified approach to identification of the *Vibrionaceae* and an improved level of accuracy. The framework established by the project should enable additions to be readily made to the identification data matrix to accommodate new species as they emerge in the future.

An important outcome for the project will be the adoption of the identification methodologies by veterinary diagnostic laboratories. This will be achieved in some measure by codifying the probabilistic identification approach and use of PCR as a proposed Australian-New Zealand Standard Diagnostic Procedure (Appendix 5). Approval of the ANZSDP will contribute to laboratories using the procedure.

Identification of *Vibrionaceae* based on phenotype has received little attention in the past 20 years with current views on identification entrenched in the use of molecular procedures. It is intended that the findings from this study are prepared for publication as peer-reviewed papers.

13. CONCLUSIONS

Objective 1.

Undertake a definitive numerical taxonomic analysis to describe and characterise the phenotypes of aquatic animal Vibrios in Australia

A library of 823 strains of *Vibrionaceae* from aquatic animals in Australia, including 56 named species and biovars, was assembled for numerical taxonomic analysis. The data was analysed by cluster analysis using the Jaccard similarity coefficient and Ward's method to identify clusters. On the basis of 107 phenotypic tests, 86 unique phenons could be defined representing the 56 named species and biovars together with 25 unnamed protospecies and four new biovars of existing species; one phenon contained strains of *Aeromonas sobria*. Homogeneity of clusters was assessed independently from 16S rRNA gene partial sequences to determine strain identity based on BLASTn matches. Overall agreement was high with many phenons showing complete agreement between phenotyping and identity reached by BLASTn matching. For some phenons there was only partial agreement. The lack of congruence in these cases was not immediately apparent but may be related to ambiguities arising from using only partial sequences. Nearly complete 16S rRNA gene sequences were obtained for the HMOs of the 25 protospecies. Three of the phenons were tentatively identified as *V. coralliilyticus, V. pomeroyi* and *V. xuii* but the remaining phenons appeared to represent potentially new species. On the basis of the evidence obtained, the numerical taxonomy appears generally to have achieved a good phenotypic description of the library of strains obtained from aquatic animals in Australia.

Objective 2.

Develop practical, robust phenotypic identification systems for Vibrio *species using computer assisted identification software*

A software package, PIBWin, was developed for the probabilistic identification of bacteria. Included in the program were various tools that were used for the development and assessment of the probability identification matrix. A panel of 46 tests was identified which could differentiate all 86 species, biovars and protospecies defined by the numerical taxonomy. In miniaturised format an identification can be reached after 48hr at 25°C or 5d at 15°C. The matrix, VibEx7 had a high level of performance. Using an identification threshold of $P \geq 0.99$, the theoretical yield of the matrix was 93% with a corresponding error rate of 0.3%. Performance of the matrix assessed by practical and prospective testing was found to be 77% and 79%, yields

considered comparatively high. Overall, the matrix appeared to be robust to test error, which had the effect of eroding modal likelihood scores, the metric to assess goodness-of-fit, but did not lead to an incorrect identification.

Objective 3.

Develop and implement PCR gene probes for the rapid identification of key bacterial pathogens

The performance of PCR primers for several significant pathogens of aquatic animals was undertaken to determine their utility and to establish parameters by which they could be used for rapid identification. All the constructs were found to be functional but some proved unwieldy in use while the amplicon in others was ambiguous in appearance. Primers based on conserved genes such as 16S rRNA and 23S rRNA are likely to have wider application compared to those targeting genes that may be less conserved and not necessarily present in all strains of a species. Nevertheless primers for these targets are useful if not for species identification then for testing particular characteristics, for example the presence of pathogenicity factors such as haemolysin, cytolysin or flagellum virulence factors. On the basis of the testing undertaken and the narrow range of conserved targets available, PCR should be limited to use with pure cultures and is a tool best suited to confirming a tentative identification reached with phenotyping.

Objective 4.

Determine the range of Vibrio *species associated with farmed fish, shellfish and crustacea in Australia*

The library of *Vibrionaceae* is based on a diversity of farmed and wild aquatic animals in Australia, principally from Northern Territory, Western Australia, Tasmania and Queensland. Both temperate and tropical climates are represented. Of the species represented, there were 31 types of finfish, nine crustacea, five molluscs, five reptiles, four marine mammals, two cephalopods and a cnidarian. Several protospecies had a unique host range, most noticeably Phenon 15 and 46 associated with abalone and Phenon 29 from striped trumpeter. No exotic pathogens were detected other than an isolation of *Ph. damselae* ssp. *piscicida* as normal flora from a southern bluefin tuna and *V. penaeicida* from rock lobster phyllosoma; there was no evidence of either the salmonid pathogens *M. viscosa, V. wodanis, V. ordalii* and *V. salmonicida* or the molluscan pathogens *V. tapetis* and *V. pectenicida*. The pathogen of greatest host range was *Ph. damselae* ssp. *damselae*. This species is a zoonotic agent of disease and together with several other species of *Vibrionaceae*, was isolated from a diversity of

aquatic animals. While there was no evidence of disease transmission with these species, it reinforces the fact that aquatic animals may in certain circumstances pose a health risk to some groups in the community.

Objective 5.

Technology transfer workshop for state veterinary diagnostic microbiologists

A training workshop was conducted for diagnostic laboratories in the identification of *Vibrionaceae* using the V36 panel of tests and probabilistic software PIBWin. Training was also given in the use of PCR for identification and how best this would be used in a diagnostic setting. Responses were good from participants using the miniaturised format of identification tests, which is now used routinely by four veterinary diagnostic laboratories.

Objective 6.

Develop an ANZSDP for the Identification of Vibrio *species in aquatic animals*

The proposed standard is intended to provide a firm basis on which identification of *Vibrionaceae* can be undertaken using phenotyping for probabilistic identification with PCR available for key pathogens as a means for rapid identification and confirmation of unusual identifications where required. The identification database covers all the currently known range of *Vibrionaceae* that occur in Australia. The approach taken in developing the identification system will allow the addition of data to accommodate new species of aquatic animals in Australia as they emerge in the future.

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15. APPENDICES

APPENDIX 1: INTELLECTUAL PROPERTY

The intellectual property arising from this research is as listed:

1. Copyright in this report.
2. Media formulations for miniaturised tests developed during this study.
3. VibEx7 database for the identification of *Vibrionaceae*.
4. PIBWin software for probabilistic identification.

APPENDIX 2: STAFF

Staff engaged on the project:

Dr Jeremy Carson	Fish Health Unit, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, Launceston, Tasmania
Melissa Higgins	Fish Health Unit, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, Launceston, Tasmania
Dr Teresa Wilson	Fish Health Unit, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, Launceston, Tasmania
Nick Gudkovs	Australian Fish Disease Laboratory, CSIRO Australian Animal Health Laboratory, Geelong, Victoria
Dr Trevor Bryant	Medical Statistics & Computing, University of Southampton, Hampshire, United Kingdom

APPENDIX 3: FORMULAE OF MEDIA

Cryopreservative (Ward & Watt, 1972)

Proteose peptone no.3 (Difco)	1.0g
Glycerol	8.0ml
Distilled water	92.0ml

Aliquot as 5 ml volumes and autoclave at 121°C for 15 minutes.

Johnson's Marine Agar (Johnson, 1968)

Peptone (Oxoid LP0037)	5.0g
Yeast extract	1.0g
Ferrous (II) sulphate ($FeSO_4 \cdot 7H_2O$)	0.2g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3g
Agar	12.0g
Aged seawater	900ml
Distilled water	100ml
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

Seawater Agar NCIMB 252

Lab-Lemco (Oxoid LP0029)	10g
Neutralised bacteriological peptone (Oxoid LP0037)	10g
Filtered, aged seawater	750ml
Distilled water	250ml
Agar	13.0g
pH	7.3

Autoclave at 121°C for 15 minutes

Cool the medium to 55°C and aseptically add 100ml of sterile foetal calf serum. Mix gently and pour as plates. A broth version of the medium can be made by omitting the agar.

Sheep Blood Agar with 2% NaCl

Blood agar base No. 2 (Oxoid, CM0271)	40g
NaCl	15g
Distilled water	1000ml
pH	7.4±0.2

Autoclave at 121°C for 15 minutes and cool to 50°C; aseptically add 70ml of defibrinated sheep's blood. Mix gently and pour as plates.

***Vibrio* Recovery Medium**

Peptone (Oxoid LP0037)	5.0g
Yeast extract	1.0g
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.2g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3g
Sodium pyruvate	1.0g
Bacteriological charcoal	2.0g
Aged seawater	900ml
Distilled water	100ml
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

ZoBell's Marine Agar 2216E (Oppenheimer & ZoBell, 1952)

Peptone (Oxoid LP0037)	5.0g
Yeast extract (Oxoid LP0021)	1.0g
Ferric (III) phosphate ($FePO_4.4H_2O$)	0.1g
Aged seawater	900ml
Distilled water	100ml
Agar	12.0g
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

A commercial form of the medium was used in this study, supplied by Amyl Media (Melbourne) formulated with artificial seawater based on the inorganic ions specified by Lyman & Fleming (1940) and found suitable for the growth of marine bacteria (MacLeod *et al.*, 1954).

APPENDIX 4: SUPPORTING AND SUPPLEMENTARY MATERIAL

A4.1 Phenotypic data for phenons defined by cluster analysis

Data as % strains positive		*A. sobria* HG7	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leiognathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
Oxidase		+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Swarming		-	-	-	-	-	-	-	-	-	-	N	-	14	46	-	-
Amino acid accumulation		50	50	-	63	-	93	71	80	-	75	N	67	80	-	11	-
Gluconate oxidation		86	-	-	13	-	-	-	-	-	-	N	-	-	6	68	-
Indole		86	75	-	-	-	3	-	-	-	-	-	+	-	+	93	+
Nitrate reduction		+	+	+	+	+	+	+	-	+	75	N	+	+	96	+	+
Phenylalanine deamination		86	-	-	13	-	24	-	-	-	25	N	-	57	96	26	+
TTC reduction		14	-	+	-	-	51	60	60	+	50	N	-	-	83	81	-
Voges Proskauer (Acetoin)		86	-	-	-	-	+	+	+	+	+	+	-	14	+	94	-
Arginine dihydrolase		86	-	-	57	-	+	+	+	+	75	+	+	-	3	96	+
Lysine decarboxylase		+	-	-	38	+	62	57	-	+	-	-	-	-	+	-	25
Ornithine decarboxylase		29	-	-	-	-	-	-	-	-	-	-	-	-	66	-	-
Citrulline deamination		43	-	-	63	-	96	14	-	-	-	N	-	29	73	-	25
Leucine deamination		-	-	-	38	-	3	-	-	-	-	N	-	-	3	-	-
Acid:	Glucose	+	+	+	+	+	+	+	+	+	+	N	+	+	+	+	+
	Amygdalin	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
	L-arabinose	14	+	-	-	-	-	-	-	+	-	N	-	-	-	75	-
	Arbutin	14	-	-	-	-	-	-	-	-	-	-	-	14	3	-	67
	Cellobiose	86	-	-	-	+	82	40	-	+	-	N	+	+	96	91	75
	Galactose	+	+	-	13	+	+	+	+	+	+	N	+	+	3	+	+
	Gentiobiose	14	-	-	-	+	-	-	-	-	-	-	-	+	-	-	75
	Inositol	-	-	-	-	-	-	-	-	-	-	N	-	-	-	20	-
	Mannitol	+	-	-	-	+	-	-	-	33	-	-	+	+	+	98	50
	Mannose	+	+	-	-	+	+	+	80	+	+	N	+	14	60	88	+
	Raffinose	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
	Rhamnose	14	-	-	-	-	-	-	-	-	-	N	-	71	-	16	-
	Salicin	14	-	-	-	-	-	-	-	-	-	-	-	57	20	-	75
	Sorbitol	14	-	-	-	-	17	-	-	33	25	N	-	-	-	93	25
	Sucrose	57	-	-	-	-	-	-	-	33	50	-	+	-	+	98	75
Resistance:	0/129 10µg	+	-	+	38	-	-	14	-	-	-	-	-	-	70	3	-
	0/129 150µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
	Ampicillin 10µg	57	-	-	-	+	65	14	20	-	75	-	-	-	+	91	-
	Carbenicillin 100µg	17	-	-	-	+	89	42	20	33	75	-	33	-	+	90	-
	Novobiocin 5µg	86	25	-	-	N	27	14	20	33	50	-	67	14	90	11	-
	Polymyxin B 50iu	-	-	-	-	-	-	-	-	-	-	N	-	-	3	33	-
Growth:	0% NaCl	71	-	-	13	-	-	-	-	-	25	N	-	-	6	8	-
	1% NaCl	+	+	-	+	+	+	+	+	+	+	N	+	+	+	+	+
	4% NaCl	57	+	+	63	+	+	+	+	+	+	N	+	86	+	+	+
	7% NaCl	-	+	+	-	+	86	+	-	+	50	+	67	29	+	93	25

Data as % strains positive		*A. sobria* HG7	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leignathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
	10% NaCl	-	25	+	-	-	-	-	-	-	-	-	-	-	+	3	-
	bile 0.8%	+	+	-	13	+	+	+	+	+	+	N	67	86	93	71	+
	$CoCl_2$ 0.0008%	+	+	-	63	+	+	+	+	+	+	N	+	14	96	98	75
IXP alkaline phosphatase		86	50	-	13	+	+	85	20	33	25	+	+	43	+	88	50
LGN γ-glutamyl transpeptidase		71	-	+	+	-	48	14	20	-	75	-	-	29	96	15	50
LPN prolyl aminopeptidase		+	-	+	+	-	82	71	-	+	+	N	-	86	+	25	+
NPS sulphatase		14	-	+	-	+	-	-	-	-	25	-	-	14	40	1	-
ONPG β-D-galactosidase		86	25	+	-	-	3	-	20	33	25	N	67	86	10	+	+
PNPG α-D-galactosidase		29	-	+	-	-	3	-	20	-	-	+	+	+	16	53	+
PNPP acid phosphatase		+	75	+	+	+	+	+	+	+	+	N	+	+	96	71	75
Hydrolysis:	Aesculin	71	-	-	20	N	31	-	-	-	25	-	33	+	10	63	+
	Agarolysis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	Amylase	+	-	-	+	-	27	14	20	+	-	-	+	43	+	96	+
	Gelatinase	71	-	-	+	-	24	33	20	-	25	-	67	-	+	77	50
	Urease	14	-	-	-	+	+	+	-	-	25	-	-	-	-	-	-
Utilisation:	1-propanol	-	+	-	13	-	-	-	-	+	-	N	-	-	86	-	-
	Acetamide	14	50	+	50	-	20	-	-	-	-	N	-	-	10	6	50
	Acetate	29	+	+	88	+	93	-	60	+	50	-	67	43	96	51	+
	Adenine	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	25
	Alanine	-	+	+	25	-	10	-	+	-	-	-	+	14	+	76	+
	Allantoin	-	-	-	-	-	6	-	60	-	-	N	-	-	-	6	-
	Amygdalin	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
	Arabitol	-	33	-	13	-	-	14	-	67	25	N	-	-	-	-	-
	Arbutin	29	-	-	-	-	3	-	-	-	-	N	-	-	3	-	-
	Aspartate	-	-	-	75	-	65	14	20	-	-	N	33	-	33	23	+
	Butyramide	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
	Cellobiose	57	-	-	-	+	6	-	20	+	-	N	+	86	3	91	50
	Citrate	43	+	-	-	+	-	-	-	+	-	-	+	-	96	95	+
	Citrulline	-	-	-	38	-	-	-	-	-	-	-	-	-	20	16	+
	DL-3-hydroxybutyrate	-	-	-	-	-	-	-	-	-	25	-	-	-	-	-	+
	DL-lactate	14	+	+	+	-	79	-	-	-	+	+	+	29	+	91	+
	DL-malate	-	-	-	-	+	3	-	20	-	50	N	33	-	3	-	-
	Ethanol	-	+	-	-	-	-	-	-	-	-	N	-	-	36	-	-
	Galactose	57	+	-	-	+	93	28	60	+	25	+	+	71	13	71	+
	Galacturonate	14	50	-	-	-	3	-	-	33	-	N	-	-	6	-	25
	Gluconate	+	75	+	-	-	3	14	20	+	+	-	+	14	+	96	75
	Glucosamine	+	25	-	50	+	+	42	60	+	+	+	+	86	+	95	+
	Glucose	+	+	+	+	+	96	85	80	+	+	N	+	+	+	98	+
	Glucuronate	-	-	-	-	-	-	-	-	33	-	-	-	-	-	1	-
	Glycerol	+	75	+	+	+	+	42	60	+	75	+	+	-	+	95	+
	Histidine	29	50	-	88	-	-	-	-	-	-	-	-	-	+	93	50
	Hydroxyproline	-	-	-	-	+	-	-	-	-	-	-	-	-	+	1	75

Data as % strains positive		*A. sobria* HG7	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leiognathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
Inulin		-	-	-	-	-	3	14	-	-	-	N	-	-	-	-	-
Lactose		-	-	-	-	-	-	-	20	-	-	+	-	-	-	1	-
L-arabinose		29	+	-	-	-	-	14	-	-	-	N	-	14	-	80	-
Leucine		-	-	-	-	-	-	-	-	-	-	N	-	-	+	1	-
Mannose		+	25	-	-	+	96	+	80	+	+	N	+	14	20	88	+
Melezitose		-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
Melibiose		29	-	-	13	-	3	-	20	-	-	N	-	-	-	-	50
Propionate		14	+	-	-	-	-	-	-	-	-	-	67	-	+	6	+
Putrescine		14	-	-	-	-	-	-	-	+	-	-	-	-	+	-	50
Serine		71	75	+	75	-	86	57	40	-	50	N	67	29	+	81	+
Sorbitol		-	-	-	-	-	-	-	-	+	-	N	-	-	-	88	25
Succinate		86	75	+	+	+	89	42	40	+	75	+	+	29	+	93	75
Sucrose		57	-	-	-	-	-	-	-	33	50	-	+	-	+	96	75
Urocanate		14	25	-	-	-	-	-	20	-	-	N	-	14	-	3	-
Valerate		-	-	-	-	-	-	-	-	-	-	N	-	-	43	-	-
Xylose		-	75	-	13	-	-	-	-	-	25	N	-	71	10	-	-
α-ketoglutarate		14	50	+	-	+	3	-	20	-	-	+	67	-	+	36	25

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	*No. strains*	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
Oxidase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swarming		-	-	-	-	-	-	-	-	22	40	-	-	6	-	-	33
Amino acid accumulation		-	19	17	+	-	25	60	60	22	10	+	67	5	22	83	67
Gluconate oxidation		-	-	33	-	-	-	-	-	-	-	-	-	3	4	-	33
Indole		+	95	+	-	85	+	20	-	+	+	-	33	97	+	-	-
Nitrate reduction		+	+	+	66	+	+	80	+	+	+	+	+	98	+	+	+
Phenylalanine deamination		+	95	67	-	85	+	-	10	33	30	-	-	79	96	83	-
TTC reduction		-	5	42	-	14	-	40	10	11	10	50	-	5	4	-	-
Voges Proskauer (Acetoin)		-	5	58	33	-	-	-	-	11	-	+	-	2	75	-	-
Arginine dihydrolase		-	95	-	-	+	+	-	-	+	+	-	-	15	-	-	-

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	*No. strains*	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
Lysine decarboxylase		50	5	+	-	28	-	20	55	-	-	-	-	98	+	-	-
Ornithine decarboxylase		-	-	+	-	28	-	20	33	11	-	-	-	+	-	-	-
Citrulline deamination		50	-	+	-	28	-	20	20	-	-	-	-	+	-	-	-
Leucine deamination		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid:	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Amygdalin	-	-	-	33	-	67	-	-	44	-	33	-	24	-	-	-
	L-arabinose	-	-	-	66	-	+	-	-	+	+	+	67	3	-	-	-
	Arbutin	-	-	-	66	-	+	-	-	+	-	+	-	29	-	-	-
	Cellobiose	+	+	-	+	+	+	80	90	78	-	66	-	+	+	33	-
	Galactose	-	+	+	66	+	+	+	+	+	+	-	+	98	+	-	+
	Gentiobiose	50	-	-	33	14	-	60	90	11	-	-	-	95	9	-	-
	Inositol	-	-	-	33	-	-	-	-	-	-	-	-	-	-	-	-
	Mannitol	50	+	+	+	85	+	40	87	+	+	33	+	94	83	60	+
	Mannose	50	+	83	66	+	-	80	+	+	90	66	67	+	+	+	+
	Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
	Rhamnose	-	-	-	-	-	+	-	-	-	20	-	-	-	-	-	-
	Salicin	75	5	-	66	-	+	40	40	+	-	+	-	79	4	-	-
	Sorbitol	-	-	-	-	-	-	-	20	11	-	33	-	31	39	-	-
	Sucrose	-	43	+	66	+	+	20	-	+	+	+	67	65	-	66	+
Resistance:	0/129 10µg	25	19	8	66	28	25	-	-	44	60	+	-	82	91	16	67
	0/129 150µg	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-
	Ampicillin 10µg	+	+	25	-	-	-	60	80	33	80	-	33	97	-	33	-
	Carbenicillin 100µg	+	+	33	-	-	-	+	80	11	80	-	33	95	-	33	-
	Novobiocin 5µg	+	19	17	50	71	75	-	10	+	+	+	33	94	74	66	-
	Polymyxin B 50iu	-	-	9	-	-	-	-	-	22	-	-	-	10	-	-	-
Growth:	0% NaCl	-	-	92	66	-	25	-	-	89	90	-	-	2	-	-	33
	1% NaCl	+	+	+	66	+	+	+	+	+	+	+	33	+	+	+	+
	4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7% NaCl	+	95	58	+	+	+	-	70	+	+	+	67	+	+	33	67
	10% NaCl	-	10	-	66	-	50	-	-	56	90	66	33	5	4	16	-
	bile 0.8%	+	+	+	-	+	+	50	+	78	90	33	33	+	+	+	+
	$CoCl_2$ 0.0008%	+	+	92	+	71	+	-	50	+	+	+	-	97	91	-	+
IXP alkaline phosphatase		+	+	92	+	85	+	80	+	+	+	50	33	+	+	+	+
LGN γ-glutamyl transpeptidase		+	86	+	-	71	+	-	50	+	+	-	-	94	87	-	-
LPN prolyl aminopeptidase		+	+	33	33	+	75	40	80	+	+	-	+	97	+	66	+
NPS sulphatase		-	+	8	-	71	25	60	+	11	-	-	-	60	61	16	33
ONPG β-D-galactosidase		-	+	+	+	+	+	40	10	+	+	+	67	55	9	16	-
PNPG α-D-galactosidase		-	76	75	+	+	+	-	20	33	44	+	+	95	+	-	-
PNPP acid phosphatase		+	+	75	66	+	+	+	+	89	+	33	33	+	+	+	+
Hydrolysis:	Aesculin	+	+	22	+	+	+	80	+	88	10	+	33	95	+	66	-
	Agarolysis	-	-	-	33	-	-	-	-	-	-	-	-	-	-	-	-
	Amylase	75	+	75	66	+	25	40	55	+	70	+	-	+	+	-	-
	Gelatinase	+	+	67	33	+	25	20	-	44	30	+	33	97	83	16	-
	Urease	-	10	-	-	-	-	+	+	-	-	-	-	63	-	-	-

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	*No. strains*	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
Utilisation:	1-propanol	-	-	-	-	-	-	-	-	+	80	-	-	29	4	-	-
	Acetamide	25	19	8	-	14	-	-	-	-	-	-	33	18	4	-	+
	Acetate	25	+	+	33	+	+	80	-	+	90	+	33	89	+	83	+
	Adenine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Alanine	50	+	67	-	+	+	20	-	+	+	33	-	+	+	33	-
	Allantoin	25	-	-	-	-	-	20	-	-	10	-	-	3	-	-	-
	Amygdalin	-	-	-	33	-	25	-	-	-	-	-	-	13	-	-	-
	Arabitol	-	-	-	-	-	-	-	-	+	+	33	-	-	-	16	-
	Arbutin	-	-	-	33	-	+	-	-	78	-	+	-	23	-	-	-
	Aspartate	25	+	-	33	+	-	-	-	78	70	66	33	58	4	-	-
	Butyramide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cellobiose	+	+	-	+	+	+	20	90	67	-	+	-	97	+	-	-
	Citrate	+	95	92	+	+	+	-	60	+	+	66	-	95	+	16	-
	Citrulline	25	62	-	-	85	-	-	-	56	10	-	-	6	-	-	-
	DL-3-hydroxybutyrate	-	-	-	-	14	-	40	-	78	80	-	-	-	-	-	-
	DL-lactate	75	+	+	66	+	+	-	-	+	+	66	+	+	+	66	+
	DL-malate	-	-	-	-	-	-	80	-	-	-	-	-	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	89	80	-	-	7	-	-	-
	Galactose	-	+	83	66	85	+	40	+	+	+	-	+	73	74	-	-
	Galacturonate	-	-	-	-	-	+	-	-	89	+	-	-	7	4	-	-
	Gluconate	-	67	+	66	+	+	-	20	+	+	33	-	+	+	+	-
	Glucosamine	-	+	+	+	+	75	20	+	+	+	33	+	84	96	+	+
	Glucose	+	+	+	+	+	+	80	+	+	+	+	+	+	+	+	+
	Glucuronate	-	14	25	-	-	25	-	-	78	10	+	-	61	13	+	-
	Glycerol	+	+	+	+	+	-	40	90	+	+	+	-	85	83	-	-
	Histidine	-	10	67	-	71	+	-	-	89	+	-	-	8	-	-	-
	Hydroxyproline	-	10	-	-	71	-	-	-	11	-	-	-	50	-	-	-
	Inulin	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-
	Lactose	-	-	-	-	28	50	-	-	-	-	-	-	-	-	-	-
	L-arabinose	-	-	-	+	-	75	-	-	+	+	+	-	3	-	-	-
	Leucine	-	-	-	-	28	-	-	-	-	-	-	-	39	-	-	-
	Mannose	50	+	83	33	+	-	40	+	+	90	+	-	+	+	+	+
	Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Melibiose	-	-	-	33	-	-	20	-	-	-	-	-	3	9	-	-
	Propionate	-	95	58	-	+	75	-	-	+	+	66	-	94	78	16	-
	Putrescine	-	5	-	-	-	+	-	-	33	90	-	-	2	-	-	-
	Serine	+	+	42	33	+	25	20	50	+	+	33	-	98	+	16	-
	Sorbitol	-	-	-	-	-	-	-	-	11	-	33	-	26	-	-	-
	Succinate	+	+	75	+	+	75	40	90	+	90	+	-	97	+	-	+
	Sucrose	-	43	+	66	+	+	60	-	89	+	+	67	65	4	66	+
	Urocanate	-	-	-	-	14	50	-	-	-	-	-	-	2	4	-	-
	Valerate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Xylose	-	5	-	33	14	+	-	-	-	-	+	-	2	-	-	-
	α-ketoglutarate	+	+	+	-	+	-	-	-	+	+	-	-	97	-	-	-

Data as % strains positive		*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test	*No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Oxidase		+	+	+	-	+	80	+	+	+	+	+	+	+	+	+	+
Swarming		-	-	-	-	-	-	-	-	-	-	-	-	11	-	-	-
Amino acid accumulation		88	50	5	40	29	-	17	-	17	33	33	-	-	50	20	-
Gluconate oxidation		-	-	-	40	-	-	-	-	-	-	50	17	6	-	-	-
Indole		63	-	95	60	+	-	27	+	83	67	-	+	+	-	-	+
Nitrate reduction		50	+	95	-	+	+	+	+	+	+	67	+	94	+	+	+
Phenylalanine deamination		75	-	95	60	43	+	+	+	+	-	-	+	+	-	+	+
TTC reduction		13	-	5	60	14	-	-	-	50	-	-	17	-	-	-	-
Voges Proskauer (Acetoin)		-	-	-	+	-	-	-	-	17	-	-	-	-	-	-	-
Arginine dihydrolase		+	-	84	+	-	+	-	-	+	-	-	+	6	+	-	-
Lysine decarboxylase		-	+	22	60	+	-	-	-	-	-	-	-	+	-	-	-
Ornithine decarboxylase		13	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Citrulline deamination		13	50	-	-	+	-	-	-	33	-	-	-	+	-	20	+
Leucine deamination		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid:	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Amygdalin	-	-	89	-	-	+	83	-	-	-	-	-	-	-	-	-
	L-arabinose	13	-	11	-	-	+	+	-	67	-	-	17	94	-	-	-
	Arbutin	-	-	-	-	-	25	83	75	-	-	-	-	-	-	-	-
	Cellobiose	71	50	+	-	86	+	83	+	67	+	-	+	94	-	70	+
	Galactose	88	+	+	80	+	+	+	33	50	+	-	+	+	-	+	+
	Gentiobiose	-	-	21	-	-	+	+	25	-	50	-	-	-	-	-	40
	Inositol	-	-	71	60	-	-	-	-	-	-	-	-	-	-	-	-
	Mannitol	75	+	+	+	86	+	+	+	67	-	-	+	+	-	+	+
	Mannose	71	+	+	+	+	-	42	50	67	+	-	+	+	-	30	+
	Raffinose	-	-	-	-	-	-	50	-	-	-	-	-	-	-	-	-
	Rhamnose	-	-	26	-	-	20	+	25	-	-	-	-	-	-	-	-
	Salicin	-	-	80	-	-	+	92	50	-	+	-	17	18	-	-	-
	Sorbitol	38	-	95	-	-	-	8	-	-	-	-	-	6	-	-	20
	Sucrose	13	-	95	+	-	+	+	+	+	-	+	+	11	-	+	80
Resistance:	0/129 10µg	+	-	-	-	-	+	+	25	33	-	-	-	61	-	60	-
	0/129 150µg	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-
	Ampicillin 10µg	13	+	-	40	14	-	8	50	-	-	50	-	94	50	10	-
	Carbenicillin 100µg	25	+	42	40	43	-	8	75	-	-	17	-	94	+	20	-
	Novobiocin 5µg	-	-	11	40	57	60	+	75	80	-	-	17	94	-	70	80
	Polymyxin B 50iu	-	-	84	-	-	-	-	50	-	+	-	-	-	-	-	-
Growth:	0% NaCl	-	-	-	80	86	-	8	25	17	-	-	-	6	-	-	-
	1% NaCl	50	+	+	+	+	+	+	+	+	+	83	+	+	+	+	+
	4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7% NaCl	38	-	74	80	86	+	+	+	+	-	-	67	+	-	80	80
	10% NaCl	-	-	5	40	14	+	75	-	67	-	-	-	89	-	-	-
	bile 0.8%	+	50	+	40	71	+	+	+	67	-	60	+	+	-	90	+
	$CoCl_2$ 0.0008%	+	-	+	60	+	+	+	+	+	33	83	50	+	-	80	+
IXP alkaline phosphatase		75	+	89	40	86	+	75	+	67	67	40	83	+	+	+	+
LGN γ-glutamyl transpeptidase		38	+	+	-	+	60	67	50	+	67	+	83	+	-	80	+
LPN prolyl aminopeptidase		38	+	+	+	29	+	92	50	+	+	83	83	+	50	+	+

Data as % strains positive		*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test	*No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
NPS sulphatase		25	+	79	-	33	-	58	-	-	-	-	-	56	-	70	60
ONPG β-D-galactosidase		+	-	+	80	+	+	67	50	17	+	-	33	44	-	80	+
PNPG α-D-galactosidase		50	-	+	+	43	75	75	-	-	+	-	67	17	-	+	+
PNPP acid phosphatase		+	+	95	20	+	80	+	+	+	+	+	+	+	+	+	+
Hydrolysis:	Aesculin	71	+	95	+	-	+	+	75	-	50	-	20	11	-	+	+
	Agarolysis	-	-	5	-	-	-	8	-	-	-	-	-	-	-	-	-
	Amylase	38	-	84	+	-	+	92	+	67	67	50	+	+	+	20	80
	Gelatinase	63	-	32	60	71	-	42	75	17	+	+	67	+	-	20	80
	Urease	-	-	-	-	-	-	-	-	-	-	-	-	17	-	-	-
Utilisation:	1-propanol	-	-	-	-	-	-	67	-	83	-	-	-	61	-	-	-
	Acetamide	-	-	5	-	-	-	33	-	17	-	-	-	22	-	-	-
	Acetate	88	50	+	60	+	+	92	75	+	67	-	67	+	-	90	80
	Adenine	-	-	32	-	-	-	-	-	-	-	-	67	-	-	-	-
	Alanine	75	-	+	60	+	+	+	75	+	+	67	+	+	+	+	+
	Allantoin	25	-	-	-	14	-	-	-	-	-	17	-	-	-	-	-
	Amygdalin	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-
	Arabitol	-	-	5	-	-	-	75	-	-	-	-	-	17	-	-	-
	Arbutin	-	-	-	-	-	-	75	25	-	-	-	-	-	-	-	-
	Aspartate	25	50	58	-	-	-	58	-	+	67	83	+	39	+	70	+
	Butyramide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cellobiose	25	+	+	-	-	+	42	+	17	+	-	+	-	-	-	-
	Citrate	13	+	+	40	+	+	92	+	+	+	+	+	94	-	80	+
	Citrulline	-	-	11	-	-	20	83	-	83	-	-	-	17	-	60	+
	DL-3-hydroxybutyrate	-	-	16	-	-	-	83	-	+	+	-	50	6	-	20	-
	DL-lactate	63	-	+	40	+	+	+	+	+	+	17	+	+	+	+	+
	DL-malate	25	50	-	-	14	-	83	-	-	-	17	-	-	-	-	-
	Ethanol	-	-	5	-	-	-	33	-	67	-	-	-	61	-	-	-
	Galactose	25	+	+	40	86	80	83	-	33	+	-	67	89	-	+	+
	Galacturonate	-	-	-	-	-	-	50	-	-	-	-	-	33	-	-	-
	Gluconate	25	+	5	+	+	+	92	+	+	33	-	+	+	-	+	+
	Glucosamine	13	+	+	20	+	+	+	+	+	+	50	83	+	50	70	+
	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Glucuronate	-	-	58	-	86	-	33	25	-	33	-	-	17	-	-	-
	Glycerol	38	+	+	+	+	+	92	+	+	+	33	83	+	+	+	+
	Histidine	-	-	89	-	+	+	+	+	+	+	33	17	+	-	80	-
	Hydroxyproline	-	-	-	-	-	-	25	-	-	-	-	83	83	-	-	-
	Inulin	-	-	5	-	-	-	-	-	-	-	-	-	-	-	-	-
	Lactose	-	-	+	40	-	20	8	-	-	+	-	-	-	-	10	20
	L-arabinose	-	-	-	-	-	60	+	-	50	-	-	-	89	-	-	-
	Leucine	-	-	5	-	-	-	58	-	67	-	-	17	+	-	-	-
	Mannose	38	+	+	80	+	-	42	25	67	+	17	+	94	-	30	+
	Melezitose	-	-	-	-	-	+	8	50	-	-	-	-	-	-	-	-
	Melibiose	-	-	74	-	-	20	42	-	-	+	-	-	11	-	10	-
	Propionate	13	-	+	-	50	+	92	+	+	67	-	67	89	-	60	80
	Putrescine	13	-	+	-	-	-	83	-	83	-	-	83	89	-	+	+
	Serine	63	+	95	20	29	+	+	+	+	+	-	+	89	+	+	+

Data as % strains positive		*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test	*No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Sorbitol		-	-	89	-	14	-	8	-	-	67	-	-	6	-	-	-
Succinate		50	+	95	60	+	80	+	+	83	+	-	+	89	50	+	+
Sucrose		-	-	+	+	-	+	+	+	+	-	83	+	11	-	+	80
Urocanate		-	-	16	-	-	-	-	-	-	+	-	-	11	+	-	-
Valerate		-	-	-	-	-	-	42	-	33	-	-	-	28	-	-	-
Xylose		-	-	5	-	-	+	33	-	-	-	-	-	11	-	-	-
α-ketoglutarate		50	+	37	-	+	20	67	+	+	+	17	-	89	-	20	20

Data as % strains positive		*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. salmonicida*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	*V. wodanis*	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	4	12	3	1	11	37	3	1	8	7	7	5	1	7	7	6
Oxidase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swarming		-	67	-	-	-	-	-	-	-	-	-	-	-	14	-	83
Amino acid accumulation		25	-	+	-	55	38	-	+	63	-	-	-	-	-	29	-
Gluconate oxidation		-	42	-	-	-	8	-	-	-	-	-	-	-	-	14	17
Indole		-	75	-	-	-	95	66	-	63	+	+	-	+	+	86	83
Nitrate reduction		+	+	+	-	91	95	+	+	+	+	71	+	+	86	+	+
Phenylalanine deamination		-	8	-	-	91	87	66	-	+	+	85	+	-	+	+	+
TTC reduction		-	+	33	-	9	5	33	-	-	29	-	-	-	14	-	33
Voges Proskauer (Acetoin)		-	+	-	-	-	-	-	-	-	-	-	-	-	14	-	67
Arginine dihydrolase		-	+	+	-	9	+	+	-	+	+	-	-	-	+	+	17
Lysine decarboxylase		-	8	33	-	-	-	-	-	-	-	+	60	-	29	-	83
Ornithine decarboxylase		-	-	-	-	-	-	-	-	-	-	+	-	-	29	-	+
Citrulline deamination		-	-	-	-	-	-	-	-	-	-	+	20	-	29	-	67
Leucine deamination		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid:	Glucose	+	+	67	+	+	97	+	+	+	+	+	+	+	+	+	+
	Amygdalin	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-
	L-arabinose	-	-	33	-	-	5	33	-	-	-	-	-	-	86	+	17
	Arbutin	-	-	-	-	-	-	-	-	-	-	71	+	-	+	-	-
	Cellobiose	+	75	33	-	-	89	+	-	+	+	71	+	-	50	-	+
	Galactose	33	-	33	-	18	95	33	+	-	+	+	+	+	29	+	+
	Gentiobiose	-	-	-	-	-	-	-	-	-	+	28	40	-	40	-	-
	Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	-
	Mannitol	-	+	33	+	10	97	+	-	71	+	71	-	-	+	+	+
	Mannose	+	+	+	+	91	97	33	+	+	+	+	80	+	+	-	+
	Raffinose	-	-	-	-	-	-	-	-	-	-	14	-	-	-	-	-
	Rhamnose	-	-	-	-	18	-	-	-	-	-	-	-	-	-	-	-
	Salicin	-	-	-	-	-	8	-	-	25	+	57	+	-	+	-	33

Data as % strains positive		*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. salmonicida*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	*V. wodanis*	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	4	12	3	1	11	37	3	1	8	7	7	5	1	7	7	6
	Sorbitol	-	+	-	-	-	8	33	-	-	14	-	-	-	-	14	-
	Sucrose	-	-	+	-	+	8	-	-	25	+	-	-	+	86	+	83
Resistance:	0/129 10µg	25	67	-	-	9	29	+	-	63	14	-	-	-	-	57	+
	0/129 150µg	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-
	Ampicillin 10µg	-	58	-	+	-	3	+	-	-	-	-	-	-	-	57	+
	Carbenicillin 100µg	-	-	33	+	-	18	+	-	-	-	-	-	-	-	71	+
	Novobiocin 5µg	-	+	-	-	27	3	33	-	-	57	57	20	-	+	57	+
	Polymyxin B 50iu	33	8	-	-	-	-	-	+	-	-	33	-	-	-	-	17
Growth:	0% NaCl	-	8	-	-	-	5	-	-	-	-	-	-	-	-	-	-
	1% NaCl	+	+	+	+	+	97	+	-	+	+	+	+	+	+	+	+
	4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7% NaCl	-	+	67	+	36	71	33	-	75	57	85	20	-	+	+	+
	10% NaCl	-	+	33	-	-	11	-	-	-	-	-	-	-	+	+	83
	bile 0.8%	-	+	33	-	+	+	+	-	+	+	+	+	-	+	+	83
	$CoCl_2$ 0.0008%	50	+	67	+	60	76	+	-	50	86	+	+	-	+	71	+
IXP alkaline phosphatase		50	92	+	-	82	92	+	-	+	86	85	+	-	+	+	+
LGN γ-glutamyl transpeptidase		-	75	+	-	18	50	+	-	13	+	-	-	-	+	86	67
LPN prolyl aminopeptidase		+	+	+	-	+	92	+	-	88	+	+	40	+	+	+	+
NPS sulphatase		-	-	-	-	-	79	-	-	+	-	-	-	-	86	86	50
ONPG β-D-galactosidase		+	-	+	+	64	87	66	-	63	+	+	40	-	14	29	50
PNPG α-D-galactosidase		+	-	67	-	9	92	-	-	-	+	+	80	-	14	29	50
PNPP acid phosphatase		50	+	+	+	91	97	+	+	88	71	+	+	+	+	+	+
Hydrolysis:	Aesculin	-	-	33	-	+	97	+	-	+	+	42	25	-	+	-	83
	Agarolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Amylase	33	+	67	-	-	84	+	+	13	+	+	+	+	+	71	83
	Gelatinase	-	+	67	-	-	89	+	-	13	+	+	80	-	14	+	+
	Urease	-	-	33	-	-	-	-	-	-	-	-	-	-	14	-	-
Utilisation:	1-propanol	-	-	-	+	-	3	-	-	-	-	-	-	-	+	-	+
	Acetamide	-	17	-	-	-	5	-	-	-	-	-	-	-	14	-	-
	Acetate	+	+	67	+	45	76	+	-	+	86	85	+	+	+	+	+
	Adenine	-	25	-	-	-	-	-	-	-	57	-	-	-	-	-	17
	Alanine	+	+	+	-	-	97	+	-	+	+	+	+	-	+	+	+
	Allantoin	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-	-
	Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	29	-	-
	Arabitol	-	-	33	-	9	-	-	-	-	-	-	-	-	29	-	33
	Arbutin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Aspartate	75	25	+	-	36	89	33	-	+	+	57	60	-	86	57	67
	Butyramide	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Cellobiose	+	-	+	+	-	89	+	-	75	+	71	+	-	29	-	33
	Citrate	+	+	+	+	91	97	+	+	+	+	+	+	+	+	+	+
	Citrulline	-	25	-	-	-	24	-	-	-	+	-	-	-	71	14	17
	DL-3-hydroxybutyrate	25	-	-	+	18	-	-	-	-	+	-	-	-	+	-	-
	DL-lactate	+	+	67	-	+	97	66	+	+	+	+	+	-	+	+	+
	DL-malate	25	-	-	-	-	3	-	-	-	-	-	60	-	-	-	-
	Ethanol	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	67
	Galactose	+	-	+	+	36	92	-	-	-	+	+	+	-	-	43	67

Data as % strains positive		*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. salmonicida*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	*V. wodanis*	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	4	12	3	1	11	37	3	1	8	7	7	5	1	7	7	6
Galacturonate		-	-	33	-	-	-	-	-	-	-	-	-	-	43	14	50
Gluconate		+	+	67	+	82	84	-	-	+	+	+	+	-	+	+	+
Glucosamine		+	+	+	-	+	89	66	+	+	+	+	80	-	+	86	+
Glucose		+	+	+	+	+	97	+	+	+	+	+	+	+	+	+	+
Glucuronate		25	-	-	-	64	21	-	-	-	86	85	80	-	-	-	17
Glycerol		+	+	+	+	-	+	+	+	+	+	85	+	+	+	+	+
Histidine		+	+	33	-	-	3	-	-	-	+	28	-	-	86	+	83
Hydroxyproline		-	+	-	-	-	-	-	-	-	86	-	-	-	-	-	+
Inulin		-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Lactose		+	-	33	-	-	21	-	-	-	+	-	-	-	-	-	-
L-arabinose		-	-	33	+	-	3	-	-	-	-	14	-	-	86	86	17
Leucine		-	17	-	-	-	-	-	-	-	43	-	-	-	14	14	83
Mannose		+	+	+	+	+	97	33	+	+	+	+	80	+	+	-	+
Melezitose		-	-	33	-	-	-	-	-	-	-	-	-	-	-	-	17
Melibiose		+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Propionate		75	+	33	-	18	39	33	-	13	86	+	80	-	+	86	+
Putrescine		-	+	-	+	-	5	-	-	-	29	-	-	-	+	14	17
Serine		75	+	+	-	10	97	+	-	+	+	85	60	-	+	+	+
Sorbitol		+	92	-	+	-	5	-	-	-	-	-	-	-	-	29	-
Succinate		+	+	+	+	91	+	+	+	+	86	+	+	+	+	86	+
Sucrose		-	8	+	+	+	3	33	-	25	+	-	-	+	86	+	83
Urocanate		-	8	-	-	18	-	-	-	13	-	-	-	-	-	14	-
Valerate		-	-	-	-	-	-	-	-	-	-	-	-	-	71	-	67
Xylose		-	-	33	-	-	-	-	-	-	-	-	-	-	-	-	33
α-ketoglutarate		+	+	67	-	9	+	+	+	38	-	+	80	-	+	+	+

Data as % strains positive		Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46	Phenon 52
Test	*No. strains*	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6	3
Oxidase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Swarming		-	-	-	-	-	-	-	6	-	-	-	50	9	-	-	-
Amino acid accumulation		88	-	17	-	50	43	12	18	41	50	17	+	9	50	+	+
Gluconate oxidation		-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	67
Indole		+	+	+	88	-	93	+	+	+	50	33	-	91	25	+	-
Nitrate reduction		+	+	83	+	+	+	+	+	+	+	+	75	+	+	+	67
Phenylalanine deamination		+	75	+	+	+	+	93	+	+	+	+	-	+	+	+	-
TTC reduction		-	25	-	13	-	-	6	-	-	-	33	+	9	-	-	67
Voges Proskauer (Acetoin)		25	-	-	-	-	-	-	-	-	-	17	+	9	-	-	67
Arginine dihydrolase		+	+	83	88	+	+	+	+	+	50	+	+	+	+	+	33
Lysine decarboxylase		25	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-
Ornithine decarboxylase		-	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-

Data as % strains positive		Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46	Phenon 52
Test	*No. strains*	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6	3
Citrulline deamination		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine deamination		-	-	-	-	-	-	-	-	-	-	-	-	-	25	-	-
Acid:	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	33
	Amygdalin	-	-	-	75	-	-	-	-	-	-	-	-	-	-	-	-
	L-arabinose	13	-	-	-	25	-	6	-	-	-	-	-	-	-	-	-
	Arbutin	+	-	83	+	-	21	-	-	-	-	-	-	9	-	-	-
	Cellobiose	+	+	50	+	+	+	+	+	+	17	-	-	+	-	60	33
	Galactose	+	+	83	+	+	+	+	+	+	60	+	-	9	50	67	-
	Gentiobiose	86	-	83	+	25	57	-	-	-	-	-	-	9	-	-	-
	Inositol	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Mannitol	+	+	67	+	+	77	+	+	88	50	-	50	91	25	+	-
	Mannose	+	+	83	+	+	+	+	+	+	+	+	+	91	+	-	-
	Raffinose	-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	-
	Rhamnose	38	-	-	13	-	-	-	6	-	-	-	-	-	-	-	-
	Salicin	-	-	+	+	-	57	-	-	+	-	-	-	27	-	-	-
	Sorbitol	75	25	-	13	-	7	6	6	-	-	-	-	-	25	-	-
	Sucrose	+	+	+	+	+	+	+	+	12	-	+	+	82	25	-	-
Resistance:	0/129 10µg	-	-	-	-	-	14	-	12	-	-	-	-	-	-	33	67
	0/129 150µg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ampicillin 10µg	-	-	-	-	-	-	-	-	-	-	+	75	9	25	-	33
	Carbenicillin 100µg	-	-	-	13	-	21	6	-	65	-	+	+	9	25	-	-
	Novobiocin 5µg	-	50	17	-	-	-	18	-	-	-	67	+	18	-	-	+
	Polymyxin B 50iu	88	-	83	13	-	-	-	-	-	20	-	-	-	-	-	-
Growth:	0% NaCl	-	-	-	-	-	7	-	6	-	-	-	-	9	-	-	-
	1% NaCl	+	+	+	+	+	+	+	+	+	83	+	+	91	+	-	67
	4% NaCl	+	75	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	7% NaCl	63	25	83	+	+	43	+	88	88	50	83	75	91	25	+	+
	10% NaCl	-	-	-	-	-	-	31	-	-	17	-	-	55	-	-	67
	bile 0.8%	+	+	+	+	50	+	93	+	+	33	+	75	82	+	50	-
	$CoCl_2$ 0.0008%	38	+	33	+	75	+	+	94	+	83	+	75	82	50	-	67
IXP alkaline phosphatase		+	25	83	+	+	+	+	+	+	-	83	+	+	+	+	67
LGN γ-glutamyl transpeptidase		50	+	50	+	-	14	+	82	94	67	+	+	55	25	17	-
LPN prolyl aminopeptidase		88	50	+	+	+	+	+	+	+	83	+	+	+	+	67	-
NPS sulphatase		88	-	17	88	+	93	+	+	+	-	-	-	55	50	50	-
ONPG β-D-galactosidase		+	+	17	+	+	+	+	+	+	+	17	-	36	-	+	67
PNPG α-D-galactosidase		+	+	17	+	+	+	+	88	+	+	+	-	-	-	-	33
PNPP acid phosphatase		+	50	83	+	+	+	+	+	+	50	+	+	91	+	+	+
Hydrolysis:	Aesculin	+	+	67	+	-	+	+	+	+	40	50	-	+	-	+	+
	Agarolysis	13	-	-	-	-	-	-	-	6	-	-	-	9	-	-	-
	Amylase	63	+	+	+	+	+	93	94	+	67	+	+	73	50	+	67
	Gelatinase	13	-	+	+	75	+	+	+	94	-	67	50	55	25	+	+
	Urease	-	-	50	-	-	-	6	-	-	-	-	-	-	-	-	-
Utilisation:	1-propanol	-	-	-	-	-	-	6	-	-	17	-	-	9	-	-	33
	Acetamide	-	-	-	-	-	-	6	-	-	-	-	-	18	25	60	33
	Acetate	38	75	+	88	+	57	+	88	+	+	+	50	73	+	+	67
	Adenine	-	-	-	-	-	-	-	-	-	-	17	-	-	-	-	-
	Alanine	13	+	83	+	50	+	+	94	+	83	+	+	+	+	-	33
	Allantoin	-	-	-	-	-	14	-	-	-	-	-	-	-	-	-	-
	Amygdalin	-	-	-	-	-	-	6	6	-	-	-	-	-	-	-	-

Data as % strains positive		Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46	Phenon 52
Test	*No. strains*	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6	3
Arabitol		-	-	-	-	-	-	-	-	-	-	-	25	-	-	-	-
Arbutin		-	-	-	38	-	-	-	-	-	-	-	-	-	-	-	-
Aspartate		38	+	+	+	+	+	+	+	+	67	+	+	+	+	+	33
Butyramide		-	-	-	13	-	-	-	-	-	-	-	-	-	-	-	-
Cellobiose		+	+	+	+	25	+	+	+	+	17	-	25	+	-	+	33
Citrate		88	+	83	+	50	+	+	+	94	+	67	75	91	+	-	33
Citrulline		-	25	40	88	-	-	+	-	+	50	67	25	55	-	-	-
DL-3-hydroxybutyrate		-	75	67	-	-	-	-	6	6	33	-	-	-	-	-	33
DL-lactate		+	+	+	+	25	+	+	94	+	+	+	+	+	75	+	33
DL-malate		13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol		-	-	-	-	-	-	-	-	-	17	-	-	-	-	-	33
Galactose		+	+	83	+	+	+	+	88	+	+	+	-	9	50	+	-
Galacturonate		-	-	-	-	-	-	-	12	-	-	-	-	-	-	-	-
Gluconate		50	-	83	+	+	79	+	94	+	33	83	25	+	75	+	33
Glucosamine		+	+	+	+	+	+	+	94	+	+	-	+	+	+	+	67
Glucose		+	+	+	+	+	+	+	94	+	+	+	+	+	+	+	+
Glucuronate		13	-	-	-	+	-	6	53	+	17	-	-	-	-	-	-
Glycerol		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	67
Histidine		-	-	33	13	-	-	18	12	-	67	17	-	9	-	-	+
Hydroxyproline		-	-	+	-	-	-	-	-	-	-	83	-	18	-	-	33
Inulin		-	-	-	-	-	-	-	-	-	-	-	-	-	25	-	-
Lactose		-	25	-	-	-	-	-	6	6	-	-	-	-	-	-	-
L-arabinose		-	-	-	-	-	-	6	-	-	-	-	-	-	-	-	33
Leucine		-	-	-	-	-	-	-	-	-	-	17	-	-	-	-	-
Mannose		+	+	83	+	+	+	+	94	+	+	+	+	+	+	+	67
Melezitose		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melibiose		-	25	-	-	-	-	-	-	6	83	-	-	-	-	-	-
Propionate		-	+	67	+	-	7	68	88	94	83	+	+	91	75	-	67
Putrescine		+	-	17	-	-	-	6	-	-	33	-	-	9	-	-	-
Serine		38	75	+	+	50	+	+	94	+	33	+	+	91	+	50	+
Sorbitol		88	-	-	-	-	7	-	-	-	17	-	-	-	25	-	33
Succinate		+	+	83	+	+	93	+	+	+	+	+	+	91	+	+	-
Sucrose		+	+	+	+	+	+	+	+	18	17	+	+	82	25	-	+
Urocanate		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Valerate		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	33
Xylose		63	50	-	-	-	-	-	-	6	-	-	-	-	-	-	-
α-ketoglutarate		38	-	+	+	25	+	+	69	+	+	-	-	9	25	+	-

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Oxidase		+	+	+	+	+	+
Swarming		-	25	-	-	-	-
Amino acid accumulation		22	25	+	36	25	66
Gluconate oxidation		-	-	-	-	-	-

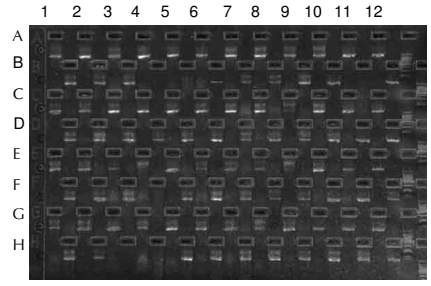
Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Indole		+	75	-	7	+	-
Nitrate reduction		+	+	+	+	+	+
Phenylalanine deamination		+	75	50	21	-	-
TTC reduction		-	-	-	-	+	16
Voges Proskauer (Acetoin)		-	-	-	-	+	-
Arginine dihydrolase		11	+	25	-	80	+
Lysine decarboxylase		-	+	+	93	-	-
Ornithine decarboxylase		-	-	-	-	-	-
Citrulline deamination		11	50	25	-	-	+
Leucine deamination		-	25	-	-	-	-
Acid:	Glucose	+	+	+	+	+	+
	Amygdalin	-	-	-	-	-	-
	L-arabinose	-	-	-	-	60	-
	Arbutin	-	-	-	-	-	-
	Cellobiose	50	25	-	+	+	50
	Galactose	-	+	+	+	+	+
	Gentiobiose	-	25	-	93	-	-
	Inositol	-	-	-	-	-	-
	Mannitol	+	+	+	-	+	-
	Mannose	+	+	+	+	+	+
	Raffinose	-	-	-	-	-	-
	Rhamnose	-	-	-	-	-	-
	Salicin	13	-	-	7	20	-
	Sorbitol	11	-	-	-	+	16
	Sucrose	+	-	-	14	+	-
Resistance:	0/129 10µg	89	-	-	-	40	-
	0/129 150µg	-	-	-	-	-	-
	Ampicillin 10µg	11	75	50	14	-	-
	Carbenicillin 100µg	11	+	+	71	-	-
	Novobiocin 5µg	89	-	-	-	50	-
	Polymyxin B 50iu	-	-	-	-	25	66
Growth:	0% NaCl	-	-	-	7	+	-
	1% NaCl	+	+	+	+	+	+
	4% NaCl	+	+	+	+	80	+
	7% NaCl	89	75	25	21	20	-
	10% NaCl	11	-	-	-	-	-
	bile 0.8%	+	+	50	71	20	+
	$CoCl_2$ 0.0008%	89	75	50	50	80	66
IXP alkaline phosphatase		+	+	+	93	-	33
LGN γ-glutamyl transpeptidase		33	-	-	-	-	66
LPN prolyl aminopeptidase		+	+	+	93	-	+
NPS sulphatase		44	+	50	+	20	16
ONPG β-D-galactosidase		-	+	+	+	+	33
PNPG α-D-galactosidase		-	+	+	+	80	+
PNPP acid phosphatase		+	+	+	+	+	+
Hydrolysis:	Aesculin	56	50	-	93	-	-
	Agarolysis	-	-	-	-	-	-
	Amylase	-	+	-	-	80	+
	Gelatinase	11	75	-	21	-	+
	Urease	-	75	+	+	80	-

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Utilisation:	1-propanol	-	25	-	-	80	-
	Acetamide	33	25	25	7	-	33
	Acetate	+	50	50	7	+	+
	Adenine	-	-	-	-	-	-
	Alanine	-	+	-	-	80	-
	Allantoin	-	-	-	-	-	-
	Amygdalin	-	-	-	-	-	-
	Arabitol	-	25	-	-	-	-
	Arbutin	-	-	-	-	-	-
	Aspartate	11	50	25	-	40	83
	Butyramide	-	-	-	-	-	-
	Cellobiose	33	50	-	+	+	-
	Citrate	89	75	+	+	+	50
	Citrulline	-	-	-	-	-	-
	DL-3-hydroxybutyrate	89	-	-	-	60	-
	DL-lactate	+	75	-	-	60	+
	DL-malate	-	-	-	-	-	-
	Ethanol	-	25	-	-	20	-
	Galactose	-	+	+	+	60	83
	Galacturonate	-	25	-	-	-	-
	Gluconate	-	75	25	7	+	-
	Glucosamine	+	+	+	+	20	+
	Glucose	+	+	+	+	+	+
	Glucuronate	44	75	-	-	-	-
	Glycerol	44	+	+	+	+	+
	Histidine	-	-	-	-	60	50
	Hydroxyproline	11	-	-	-	-	-
	Inulin	-	-	-	-	-	-
	Lactose	-	75	+	+	20	-
	L-arabinose	-	-	-	-	60	-
	Leucine	22	-	-	-	20	-
	Mannose	+	+	+	+	+	+
	Melezitose	-	-	-	-	-	-
	Melibiose	-	-	-	-	20	33
	Propionate	+	-	-	-	40	-
	Putrescine	-	-	-	-	+	-
	Serine	89	75	-	-	40	+
	Sorbitol	11	-	-	7	80	-
	Succinate	78	+	+	93	+	+
	Sucrose	+	-	-	7	80	-
	Urocanate	-	-	-	-	-	16
	Valerate	22	-	-	-	-	-
	Xylose	-	-	-	-	-	-
	α-ketoglutarate	-	25	-	-	80	33

A4.2 Gel electrophoresis of purified 16S rDNA PCR amplification products with the Invitrogen E-Gel® 96 Gel System

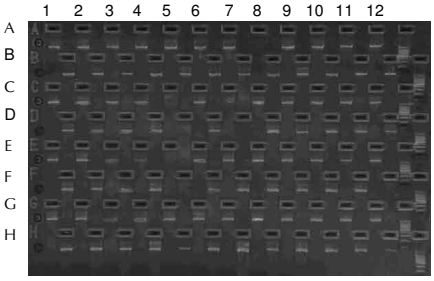
Tables are of strain numbers corresponding to positions on the gel.

Tray 1



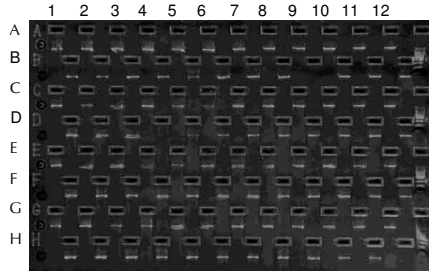
	1	2	3	4	5	6	7	8	9	10	11	12
A	V1	V9	V17	V769	V771	V778	V49	V57	V65	V73	V783	V786
B	V2	V10	V18	V26	V34	V42	V50	V58	V66	V74	V82	V787
C	V3	V11	V19	V770	V773	V43	V51	V59	V67	V75	V784	V788
D	V4	V12	V20	V28	V774	V779	V52	V60	V68	V76	V84	V92
E	V5	V13	V21	V29	V775	V45	V53	V61	V69	V77	V85	V93
F	V6	V14	V22	V30	V38	V46	V54	V62	V70	V78	V785	V94
G	V7	V15	V23	V31	V776	V47	V781	V63	V71	V782	V87	V95
H	V8	V16	V24	V32	V777	V48	V56	V64	V72	V80	V88	C

Tray 2



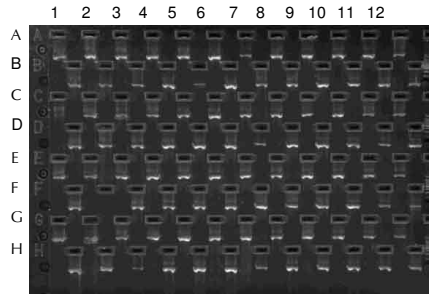
	1	2	3	4	5	6	7	8	9	10	11	12
A	V97	V105	V113	V121	V129	V794	V145	V898	V161	V169	V177	V185
B	V98	V106	V114	V122	V130	V138	V146	V154	V162	V170	V798	V186
C	V99	V107	V115	V123	V131	V139	V147	V155	V163	V797	V179	V187
D	V790	V108	V792	V124	V132	V140	V897	V156	V164	V172	V180	V188
E	V101	V109	V117	V125	V133	V141	V795	V157	V165	V173	V181	V189
F	V102	V110	V118	V126	V134	V142	V150	V158	V166	V174	V182	V190
G	V103	V791	V119	V127	V135	V143	V151	V159	V796	V175	V183	V191
H	V104	V112	V120	V128	V136	V144	V152	V160	V168	V176	V184	V799

Tray 3



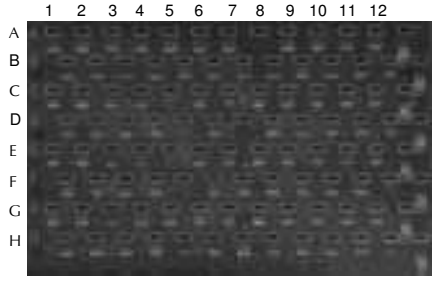
	1	2	3	4	5	6	7	8	9	10	11	12
A	V193	V201	V209	V217	V225	V233	V241	V249	V257	V265	V273	V281
B	V194	V202	V210	V218	V226	V807	V242	V250	V258	V266	V274	V818
C	V800	V203	V211	V219	V227	V808	V243	V251	V259	V267	V275	V283
D	V196	V801	V212	V220	V804	V236	V244	V252	V260	V268	V816	V284
E	V197	V205	V213	V221	V805	V237	V810	V812	V261	V269	V277	V285
F	V198	V206	V214	V222	V230	V809	V811	V813	V262	V270	V278	V286
G	V199	V802	V215	V223	V231	V239	V247	V255	V263	V271	V279	V819
H	V200	V803	V216	V224	V232	V240	V248	V814	V264	V272	V817	V288

Tray 4



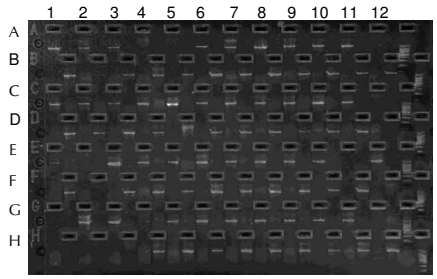
	1	2	3	4	5	6	7	8	9	10	11	12
A	V289	V821	V305	V826	V321	V329	V337	V828	V353	V361	V369	V377
B	V290	V298	V824	V314	V322	V330	V338	V346	V354	V362	V370	V834
C	V291	V299	V307	V315	V323	V331	V339	V829	V831	V363	V371	V379
D	V292	V300	V308	V316	V324	V332	V340	V830	V356	V364	V372	V380
E	V293	V301	V309	V317	V325	V333	V341	V349	V357	V365	V373	V381
F	V294	V822	V310	V318	V326	V334	V827	V350	V832	V366	V374	V382
G	V295	V303	V311	V319	V327	V335	V343	V351	V359	V367	V833	V383
H	V820	V823	V825	V320	V328	V336	V344	V352	V360	V368	V376	V384

Tray 5



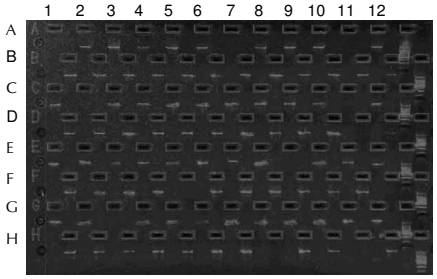
	1	2	3	4	5	6	7	8	9	10	11	12
A	V385	V393	V401	V409	V417	V425	V433	V441	V449	V457	V465	V473
B	V386	V394	V402	V410	V418	V426	V434	V442	V450	V458	V466	V474
C	V387	V395	V403	V411	V419	V427	V435	V443	V451	V459	V467	V475
D	V388	V396	V404	V412	V420	V428	V436	V444	V452	V460	V468	V476
E	V389	V397	V405	V413	V421	V429	V437	V445	V453	V461	V469	V477
F	V390	V398	V406	V414	V422	V430	V438	V446	V454	V462	V470	V478
G	V391	V399	V407	V415	V423	V431	V439	V447	V455	V463	V471	V479
H	V392	V400	V408	V416	V424	V432	V440	V448	V456	V464	V472	V480

Tray 6



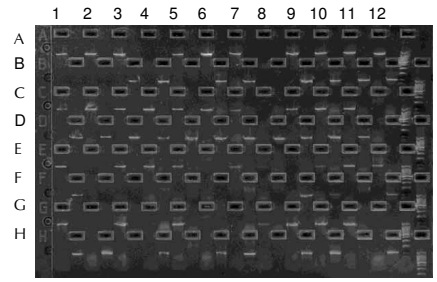
	1	2	3	4	5	6	7	8	9	10	11	12
A	V481	V489	V497	V505	V513	V521	V529	V837	V545	V553	V561	V569
B	V482	V490	V498	V506	V514	V522	V530	V538	V546	V554	V562	V570
C	V483	V491	V499	V507	V515	V835	V531	V539	V547	V555	V563	V571
D	V484	V492	V500	V508	V516	V836	V532	V540	V840	V556	V564	V572
E	V485	V493	V501	V509	V517	V525	V533	V541	V549	V557	V565	V573
F	V486	V494	V502	V510	V518	V526	V534	V542	V550	V558	V566	V574
G	V487	V495	V503	V511	V519	V527	V535	V839	V551	V559	V567	V575
H	V488	V496	V504	V512	V520	V528	V536	V544	V552	V560	V568	V576

Tray 7



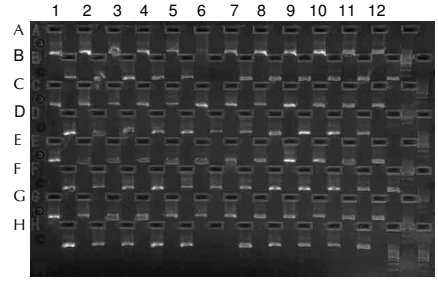
	1	2	3	4	5	6	7	8	9	10	11	12
A	V577	V585	V593	V601	V609	V617	V625	V633	V641	V649	V845	V665
B	V578	V586	V594	V602	V610	V618	V626	V634	V843	V650	V658	V666
C	V579	V587	V595	V603	V611	V619	V627	V635	V643	V651	V659	V667
D	V580	V588	V596	V604	V612	V620	V628	V636	V644	V652	V660	V668
E	V581	V589	V597	V841	V613	V621	V629	V637	V645	V653	V661	V669
F	V582	V590	V598	V606	V614	V622	V630	V638	V646	V654	V662	V870
G	V583	V591	V599	V607	V615	V623	V631	V639	V647	V655	V847	V881
H	V584	V592	V600	V608	V616	V624	V632	V842	V648	V844	V664	V672

Tray 8



	1	2	3	4	5	6	7	8	9	10	11	12
A	V673	V681	V689	V697	V705	V713	V891	V903	V737	V745	V753	V761
B	V674	V682	V690	V698	V706	V714	V892	V904	V738	V746	V754	V762
C	V675	V683	V893	V699	V707	V884	V894	V905	V910	V747	V755	V763
D	V676	V684	V692	V700	V882	V885	V895	V906	V911	V748	V756	V764
E	V677	V685	V693	V701	V709	V887	V896	V907	V912	V749	V757	V765
F	V886	V686	V694	V880	V883	V888	V899	V908	V742	V750	V758	V766
G	V679	V687	V879	V703	V711	V889	V900	V909	V743	V751	V759	V767
H	V680	V688	V696	V704	V712	V890	V901	V736	V744	V752	V760	V768

Tray 9



	1	2	3	4	5	6	7	8	9	10	11	12
A	V101	V125	V132	V164	V203	V283	V335	V4	V431	V468	V5	V525
B	V102	V126	V133	V166	V220	V284	V385	V401	V432	V485	V50	V53
C	V110	V127	V134	V180	V223	V291	V387	V404	V433	V487	V504	V533
D	V117	V128	V135	V187	V24	V295	V388	V406	V434	V488	V505	V54
E	V118	V129	V138	V188	V259	V298	V389	V414	V435	V493	V51	V541
F	V12	V13	V146	V191	V26	V30	V390	V415	V437	V494	V513	V550
G	V120	V130	V160	V201	V261	V32	V391	V416	V447	V495	V518	V551
H	V124	V131	V161	V202	V281	V320	V392	V424	V449	V496	V519	V552

A4.3 Single line summary of BLASTn results, arranged by phenon

V = Vibrio Library Number; Strain = Isolate submitted as; R = BLASTn rank in first 10 results; N = sequence does not match phenon identity, highest similarity shown; * prefix indicates a type strain; r prefix indicates named species from a culture collection

Phenon No.	V	Strain	R	BLAST results
Phenon 1 V. natriegens	303	303 V diazotrophicus	N	AY174868 AY174868 Vibrio sp. QY102 16S ribosomal RNA gene, p... 1138 0.0
Phenon 1 V. natriegens	330	330 V natriegens	N	AY332217 AY332217 Tenacibaculum sp. GWS-TZ-H201 16S ribosoma... 1219 0.0
Phenon 1 V. natriegens	335	335 V natriegens		No sequence
Phenon 1 V. natriegens	341	341 V diazotrophicus	N	VT16SRRNA X74725 V.tubiashi (ATCC 19109T) gene for 16S ribos... 1160 0.0
Phenon 1 V. natriegens	485	485 V natriegens	N	VPE16SRRA X74722 V.pelagius (ATCC 25916T) gene for 16S ribos... 1203 0.0
Phenon 1 V. natriegens	711	711 V natriegens	1	VN16SRRNA X74714 V.natriegens (ATCC 14048T) gene for 16S rib... 1253 0.0
Phenon 1 V. natriegens	750	750 V natriegens	N	VPE16SRRA X74722 V.pelagius (ATCC 25916T) gene for 16S ribos... 1201 0.0
Phenon 1 V. natriegens	882	882 V sp.	N	VPE16SRRA X74722 V.pelagius (ATCC 25916T) gene for 16S ribos... 1191 0.0
Phenon 1 V. natriegens	885	885 V sp.	9	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 876 0.0
Phenon 1 V. natriegens	458	* V natriegens 458	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1292 0.0
Phenon 1 V. natriegens	705	* V natriegens 705	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1287 0.0
Phenon 1 V. natriegens	827	r V natriegens 827	2	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1195 0.0
Phenon 2 V. diazotrophicus	305	305 V diazotrophicus	3	VDZ16SRRA X74701 V. diazotrophicus (ATCC 33466T) gene for 16S... 1193 0.0
Phenon 2 V. diazotrophicus	483	483 V mediterranei	N	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1239 0.0
Phenon 2 V. diazotrophicus	484	* V diazotrophicus 484	1	VDZ16SRRA X74701 V. diazotrophicus (ATCC 33466T) gene for 16S... 1249 0.0
Phenon 2 V. diazotrophicus	714	* V diazotrophicus 714	1	VDZ16SRRA X74701 V. diazotrophicus (ATCC 33466T) gene for 16S... 1265 0.0
Phenon 3 V. mytili	752	752 V mytili	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1261 0.0
Phenon 3 V. mytili	482	* V mytili 482	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1269 0.0
Phenon 3 V. mytili	693	* V mytili 693	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1271 0.0
Phenon 3 V. mytili	446	r V mytili 446	N	VMT16RRNA X74712 V. metschnikovii (NCTC 11170) gene for 16S r... 1197 0.0
Phenon 3 V. mytili	447	r V mytili 447	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1251 0.0
Phenon 4 V. furnissii	239	239 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1235 0.0
Phenon 4 V. furnissii	240	240 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1219 0.0
Phenon 4 V. furnissii	241	241 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1291 0.0
Phenon 4 V. furnissii	242	242 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1289 0.0
Phenon 4 V. furnissii	243	243 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1308 0.0
Phenon 4 V. furnissii	277	277 V fluvialis	3	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1271 0.0
Phenon 4 V. furnissii	291	291 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1296 0.0
Phenon 4 V. furnissii	646	646 V furnissii	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1285 0.0
Phenon 4 V. furnissii	244	* V furnissii 244	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1300 0.0
Phenon 4 V. furnissii	292	* V furnissii 292	1	VFUR16SRA X74704 V. furnisii (ATCC 35016T) gene for 16S ribos... 1275 0.0
Phenon 5 V. fluvialis	273	273 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1298 0.0
Phenon 5 V. fluvialis	274	274 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1261 0.0
Phenon 5 V. fluvialis	275	275 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1247 0.0
Phenon 5 V. fluvialis	278	278 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1267 0.0
Phenon 5 V. fluvialis	766	766 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1275 0.0
Phenon 5 V. fluvialis	777	777 V fluvialis	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1273 0.0
Phenon 5 V. fluvialis	778	778 V furnissii	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1273 0.0
Phenon 5 V. fluvialis	476	* V fluvialis 476	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1068 0.0
Phenon 5 V. fluvialis	664	* V fluvialis 664	2	VFLU16SRA X74703 V. fluvialis (ATCC 33809T) gene for 16S ribo... 1283 0.0
Phenon 6	307	307 V campbellii	1	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 1215 0.0
Phenon 6	309	309 V campbellii	1	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 1225 0.0
Phenon 6	324	324 V mediterranei	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1292 0.0
Phenon 6	353	353 V tubiashii	1	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 846 0.0
Phenon 6	356	356 V campbellii	1	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 1227 0.0
Phenon 6	357	357 V campbellii	1	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 1275 0.0
Phenon 6	87	87 V sp.	1	MSP429499 AJ429499 Marinobacter sp. 2sq31 partial 16S rRNA g... 1166 0.0
Phenon 7 V. nereis	183	183 V sp.	2	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1187 0.0
Phenon 7 V. nereis	189	189 V fluvialis	1	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1187 0.0
Phenon 7 V. nereis	299	299 V sp.	3	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1199 0.0
Phenon 7 V. nereis	737	* V nereis 737	1	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1247 0.0
Phenon 7 V. nereis	904	* V nereis 904	2	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1245 0.0
Phenon 7 V. nereis	840	r V nereis 840	1	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1285 0.0
Phenon 8	107	107 V sp.	1	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1241 0.0
Phenon 8	199	199 V tubiashii	1	HSP551094 AJ551094 Hyphomicrobium sp. wp13 partial 16S rRNA ... 967 0.0
Phenon 8	200	200 V sp.	1	AY292936 AY292936 Vibrio lentus strain Sat201 16S ribosomal ... 1269 0.0
Phenon 8	201	201 V sp.	1	AY292936 AY292936 Vibrio lentus strain Sat201 16S ribosomal ... 1269 0.0
Phenon 8	580	580 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1154 0.0
Phenon 8	598	598 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1271 0.0
Phenon 8	661	661 V nereis	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1255 0.0
Phenon 9 V. alginolyticus	181	181 V alginolyticus	8	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1174 0.0
Phenon 9 V. alginolyticus	182	182 V alginolyticus	6	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1233 0.0
Phenon 9 V. alginolyticus	186	186 V alginolyticus	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1231 0.0
Phenon 9 V. alginolyticus	194	194 V alginolyticus	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1259 0.0
Phenon 9 V. alginolyticus	203	203 V alginolyticus	5	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1251 0.0
Phenon 9 V. alginolyticus	210	210 V alginolyticus	8	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1146 0.0
Phenon 9 V. alginolyticus	214	214 V alginolyticus	2	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1269 0.0
Phenon 9 V. alginolyticus	264	264 V alginolyticus	1	AY357910 AY357910 Vibrio alginolyticus strain NRL-SS41 16S r... 468 e-129
Phenon 9 V. alginolyticus	265	265 V alginolyticus	5	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1130 0.0
Phenon 9 V. alginolyticus	266	266 V alginolyticus	9	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1217 0.0
Phenon 9 V. alginolyticus	267	267 V alginolyticus	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1265 0.0
Phenon 9 V. alginolyticus	28	28 V sp.	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1241 0.0
Phenon 9 V. alginolyticus	288	288 V mediterranei	4	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1116 0.0
Phenon 9 V. alginolyticus	30	30 V sp.	2	AY373027 AY373027 Vibrio alginolyticus 16S ribosomal RNA gen... 1241 0.0
Phenon 9 V. alginolyticus	34	34 V sp.	7	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1118 0.0
Phenon 9 V. alginolyticus	42	42 V alginolyticus	3	AY373027 AY373027 Vibrio alginolyticus 16S ribosomal RNA gen... 1227 0.0
Phenon 9 V. alginolyticus	498	498 V alginolyticus	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1294 0.0
Phenon 9 V. alginolyticus	643	643 V mediterranei	2	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1294 0.0
Phenon 9 V. alginolyticus	770	770 V alginolyticus	5	AY264938 AY264938 Vibrio alginolyticus strain UQM 2770 16S r... 1207 0.0
Phenon 9 V. alginolyticus	771	771 V alginolyticus	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1287 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 9 V. alginolyticus	849	849 V alginolyticus		No sequence
Phenon 9 V. alginolyticus	858	858 V alginolyticus		No sequence
Phenon 9 V. alginolyticus	860	860 V alginolyticus		No sequence
Phenon 9 V. alginolyticus	873	873 V alginolyticus		No sequence
Phenon 9 V. alginolyticus	878	878 V alginolyticus		No sequence
Phenon 9 V. alginolyticus	881	881 V alginolyticus	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1269 0.0
Phenon 9 V. alginolyticus	883	883 V alginolyticus	N	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1104 0.0
Phenon 9 V. alginolyticus	886	886 V sp.	3	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1275 0.0
Phenon 9 V. alginolyticus	898	898 V ordalii	N	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1261 0.0
Phenon 9 V. alginolyticus	473	* V alginolyticus 473	7	AY264938 AY264938 Vibrio alginolyticus strain UQM 2770 16S r... 1235 0.0
Phenon 9 V. alginolyticus	564	* V alginolyticus 564	6	VALG16SRR X74691 V. alginolyticus (CIP 70.65) gene for 16S ri... 1249 0.0
Phenon 10	202	202 V tubiashii	2	VALG16SRR X74691 V.alginolyticus (CIP 70.65) gene for 16S ri... 1275 0.0
Phenon 10	26	26 V sp.	5	AY373027 AY373027 Vibrio alginolyticus 16S ribosomal RNA gen... 1235 0.0
Phenon 10	261	261 V alginolyticus	9	AY373027 AY373027 Vibrio alginolyticus 16S ribosomal RNA gen... 1237 0.0
Phenon 10	262	262 V alginolyticus	1	AY357910 AY357910 Vibrio alginolyticus strain NRL-SS41 16S r... 464 e-128
Phenon 10	263	263 V alginolyticus	5	AY373027 AY373027 Vibrio alginolyticus 16S ribosomal RNA gen... 1187 0.0
Phenon 10	673	673 V harveyi	N	VCH16SRRA X74693 V.carchiariae (ATCC 35084T) gene for 16S ri... 1302 0.0
Phenon 11 V. parahaemolyticus	247	247 V parahaemolyticus	2	AP005084 AP005084 Vibrio parahaemolyticus DNA, chromosome 2,... 1296 0.0
Phenon 11 V. parahaemolyticus	248	248 V parahaemolyticus	2	AP005084 AP005084 Vibrio parahaemolyticus DNA, chromosome 2,... 1304 0.0
Phenon 11 V. parahaemolyticus	249	249 V parahaemolyticus	1	AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri... 1178 0.0
Phenon 11 V. parahaemolyticus	301	301 V sp.	2	AP005083 AP005083 Vibrio parahaemolyticus DNA, chromosome 1,... 1241 0.0
Phenon 11 V. parahaemolyticus	314	314 V sp.	1	AP005083 AP005083 Vibrio parahaemolyticus DNA, chromosome 1,... 1265 0.0
Phenon 11 V. parahaemolyticus	666	666 V parahaemolyticus	1	VPA16SRRA X74721 V. parahaemolyticus (CIP 73.30) gene for 16S... 1279 0.0
Phenon 11 V. parahaemolyticus	761	761 V parahaemolyticus	1	AP005084 AP005084 Vibrio parahaemolyticus DNA, chromosome 2,... 1271 0.0
Phenon 11 V. parahaemolyticus	787	787 V parahaemolyticus	1	AP005083 AP005083 Vibrio parahaemolyticus DNA, chromosome 1,... 1176 0.0
Phenon 11 V. parahaemolyticus	850	850 V parahaemolyticus		No sequence
Phenon 11 V. parahaemolyticus	853	853 V parahaemolyticus		No sequence
Phenon 11 V. parahaemolyticus	861	861 V parahaemolyticus		No sequence
Phenon 11 V. parahaemolyticus	874	874 V parahaemolyticus		No sequence
Phenon 11 V. parahaemolyticus	427	* V parahaemolyticus 427	1	AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri... 1269 0.0
Phenon 11 V. parahaemolyticus	697	* V parahaemolyticus 697	1	AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri... 1283 0.0
Phenon 11 V. parahaemolyticus	423	r V parahaemolyticus 423	N	AB089204 AB089204 Vibrio sp. No.6 gene for 16S rRNA, partial... 1237 0.0
Phenon 11 V. parahaemolyticus	424	r V parahaemolyticus 424		No sequence
Phenon 11 V. parahaemolyticus	425	r V parahaemolyticus 425	1	VPA16SRRA X74721 V. parahaemolyticus (CIP 73.30) gene for 16S... 1271 0.0
Phenon 11 V. parahaemolyticus	436	r V parahaemolyticus 436	2	AP005083 AP005083 Vibrio parahaemolyticus DNA, chromosome 1,... 1219 0.0
Phenon 12 V. proteolyticus	188	188 V proteolyticus	N	AJ784137 AJ784137 Vibrio sp. K3-11 partial 16S rRNA gene. 8/... 1289 0.0
Phenon 12 V. proteolyticus	32	32 V proteolyticus	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1257 0.0
Phenon 12 V. proteolyticus	387	387 V vulnificus		No sequence
Phenon 12 V. proteolyticus	388	388 Ph damselae piscicida	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1259 0.0
Phenon 12 V. proteolyticus	390	390 Ph damselae piscicida	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1257 0.0
Phenon 12 V. proteolyticus	391	391 Ph damselae piscicida	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1257 0.0
Phenon 12 V. proteolyticus	392	392 V fluvialis	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1257 0.0
Phenon 12 V. proteolyticus	393	393 V vulnificus	N	AF388393 AF388393 Vibrio sp. 3d clone 3d7 16S ribosomal RNA ... 1255 0.0
Phenon 12 V. proteolyticus	394	394 V hollisae	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1277 0.0
Phenon 12 V. proteolyticus	760	760 V proteolyticus	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1277 0.0
Phenon 12 V. proteolyticus	788	788 V proteolyticus	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1253 0.0
Phenon 12 V. proteolyticus	679	* V proteolyticus 679	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1281 0.0
Phenon 13 V. harveyi bv I	173	173 V alginolyticus	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1176 0.0
Phenon 13 V. harveyi bv I	174	174 V sp.	N	AF388387 AF388387 Vibrio parahaemolyticus clone Vp16 16S rib... 1148 0.0
Phenon 13 V. harveyi bv I	177	177 V sp.	N	AY357902 AY357902 Vibrio sp. NRL-TS19 16S ribosomal RNA gene... 436 0.0
Phenon 13 V. harveyi bv I	185	185 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1164 0.0
Phenon 13 V. harveyi bv I	191	191 V harveyi	1	VHAR16SRA X74706 V. harveyi (ATCC 14126T) gene for 16S riboso... 1132 0.0
Phenon 13 V. harveyi bv I	192	192 V sp.		No sequence
Phenon 13 V. harveyi bv I	212	212 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1176 0.0
Phenon 13 V. harveyi bv I	213	213 V parahaemolyticus	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 936 0.0
Phenon 13 V. harveyi bv I	218	218 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1235 0.0
Phenon 13 V. harveyi bv I	219	219 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1201 0.0
Phenon 13 V. harveyi bv I	234	234 V harveyi		No sequence
Phenon 13 V. harveyi bv I	236	236 V harveyi	9	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1229 0.0
Phenon 13 V. harveyi bv I	268	268 V furnissii	N	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1271 0.0
Phenon 13 V. harveyi bv I	269	269 V harveyi	N	VH16SRRNA X74707 V. hollisae(ATCC 33564T) gene for 16S riboso... 1243 0.0
Phenon 13 V. harveyi bv I	270	270 V harveyi	N	AY129278 AY129278 Vibrio tapetis strain LP2 16S ribosomal RN... 1247 0.0
Phenon 13 V. harveyi bv I	271	271 V harveyi	N	VT16SRRNA X74725 V. tubiashi (ATCC 19109T) gene for 16S ribos... 1221 0.0
Phenon 13 V. harveyi bv I	272	272 V harveyi	N	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1245 0.0
Phenon 13 V. harveyi bv I	308	308 V campbellii	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1291 0.0
Phenon 13 V. harveyi bv I	316	316 V sp.	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1259 0.0
Phenon 13 V. harveyi bv I	318	318 V sp.	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1257 0.0
Phenon 13 V. harveyi bv I	337	337 V sp.	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1285 0.0
Phenon 13 V. harveyi bv I	350	350 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1261 0.0
Phenon 13 V. harveyi bv I	351	351 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1201 0.0
Phenon 13 V. harveyi bv I	352	352 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1243 0.0
Phenon 13 V. harveyi bv I	354	354 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1257 0.0
Phenon 13 V. harveyi bv I	380	380 V alginolyticus	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1263 0.0
Phenon 13 V. harveyi bv I	381	381 V alginolyticus	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1269 0.0
Phenon 13 V. harveyi bv I	429	429 V 'carchariae'	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1292 0.0
Phenon 13 V. harveyi bv I	475	475 V harveyi	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1197 0.0
Phenon 13 V. harveyi bv I	493	493 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1298 0.0
Phenon 13 V. harveyi bv I	501	501 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1241 0.0
Phenon 13 V. harveyi bv I	502	502 V harveyi	N	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1310 0.0
Phenon 13 V. harveyi bv I	503	503 V harveyi	N	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1285 0.0
Phenon 13 V. harveyi bv I	521	521 V harveyi	N	AB018210 AB018210 Enterococcus faecium gene for 16S rRNA, pa... 1308 0.0
Phenon 13 V. harveyi bv I	525	525 V harveyi		No sequence
Phenon 13 V. harveyi bv I	526	526 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1189 0.0
Phenon 13 V. harveyi bv I	527	527 V harveyi	N	VCA16SRRA X74692 V.campbelli (ATCC 25920T) gene for 16S ribo... 1294 0.0
Phenon 13 V. harveyi bv I	588	588 V sp.	N	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1261 0.0
Phenon 13 V. harveyi bv I	594	594 V sp.	N	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1134 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 13 V. harveyi bv I	595	595 V sp.	N	VN16SRRNA X74714 V.natriegens (ATCC 14048T) gene for 16S rib... 1241 0.0
Phenon 13 V. harveyi bv I	608	608 V sp.	N	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1031 0.0
Phenon 13 V. harveyi bv I	648	648 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1289 0.0
Phenon 13 V. harveyi bv I	649	649 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1146 0.0
Phenon 13 V. harveyi bv I	650	650 V harveyi	N	AF500207 AF500207 Vibrio sp. CJ11052 16S ribosomal RNA gene,... 1243 0.0
Phenon 13 V. harveyi bv I	651	651 V harveyi	5	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1166 0.0
Phenon 13 V. harveyi bv I	654	654 V harveyi	5	VCH16SRRA X74693 V.carchiariae (ATCC 35084T) gene for 16S ri... 1255 0.0
Phenon 13 V. harveyi bv I	836	836 V harveyi	N	AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri... 1235 0.0
Phenon 13 V. harveyi bv I	846	846 V harveyi		No sequence
Phenon 13 V. harveyi bv I	848	848 V harveyi		No sequence
Phenon 13 V. harveyi bv I	85	85 V harveyi	N	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1201 0.0
Phenon 13 V. harveyi bv I	851	851 V nigripulchritudo		No sequence
Phenon 13 V. harveyi bv I	866	866 V harveyi		No sequence
Phenon 13 V. harveyi bv I	868	868 V harveyi		No sequence
Phenon 13 V. harveyi bv I	871	871 V harveyi		No sequence
Phenon 13 V. harveyi bv I	872	872 V harveyi		No sequence
Phenon 13 V. harveyi bv I	884	884 V sp.	N	VCA16SRRA X74692 V.campbelli (ATCC 25920T) gene for 16S ribo... 1263 0.0
Phenon 13 V. harveyi bv I	890	890 V sp.	5	VCH16SRRA X74693 V.carchiariae (ATCC 35084T) gene for 16S ri... 1243 0.0
Phenon 13 V. harveyi bv I	92	92 V sp.	N	AY520560 AY520560 Kangiella koreensis 16S ribosomal RNA gene... 666 0.0
Phenon 13 V. harveyi bv I	486	* V harveyi 486	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1243 0.0
Phenon 13 V. harveyi bv I	699	* V harveyi 699	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1310 0.0
Phenon 13 V. harveyi bv I	237	r V harveyi 237	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1247 0.0
Phenon 13 V. harveyi bv I	692	r V harveyi 692	2	VCH16SRRA X74693 V.carchiariae (ATCC 35084T) gene for 16S ri... 1170 0.0
Phenon 14 V. mediterranei	431	431 V mediterranei		No sequence
Phenon 14 V. mediterranei	488	488 V sp.		No sequence
Phenon 14 V. mediterranei	489	489 V sp.	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1225 0.0
Phenon 14 V. mediterranei	500	500 V mediterranei	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1271 0.0
Phenon 14 V. mediterranei	514	514 V sp.	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1269 0.0
Phenon 14 V. mediterranei	518	518 V sp.		No sequence
Phenon 14 V. mediterranei	519	519 V sp.		No sequence
Phenon 14 V. mediterranei	600	600 V sp.	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1027 0.0
Phenon 14 V. mediterranei	653	653 V sp.	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1154 0.0
Phenon 14 V. mediterranei	662	662 V mediterranei	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1279 0.0
Phenon 14 V. mediterranei	785	785 V mediterranei	2	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1045 0.0
Phenon 14 V. mediterranei	786	786 V mediterranei	N	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1080 0.0
Phenon 14 V. mediterranei	897	897 V splendidus	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1261 0.0
Phenon 14 V. mediterranei	450	* V mediterranei 450	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1261 0.0
Phenon 14 V. mediterranei	689	* V mediterranei 689	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1144 0.0
Phenon 14 V. mediterranei	369	* V shilonii 369	1	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1263 0.0
Phenon 14 V. mediterranei	448	r V mediterranei 448	N	AY345403 AY345403 Bacterium K2-74 16S ribosomal RNA gene, pa... 1255 0.0
Phenon 14 V. mediterranei	449	r V mediterranei 449		No sequence
Phenon 14 V. mediterranei	451	r V mediterranei 451	N	AB089204 AB089204 Vibrio sp. No.6 gene for 16S rRNA, partial... 1253 0.0
Phenon 15	138	138 V sp.	1	VSP345064 AJ345064 Vibrio sp. LMG 20363 partial 16S rRNA gen... 1063 0.0
Phenon 15	139	139 V sp.	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1037 0.0
Phenon 15	217	217 V sp.	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 977 0.0
Phenon 15	658	658 V splendidus I		No sequence
Phenon 15	743	743 V aestuarianus	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1067 0.0
Phenon 15	759	759 V aestuarianus	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1057 0.0
Phenon 15	769	769 V aestuarianus	1	VSP345064 AJ345064 Vibrio sp. LMG 20363 partial 16S rRNA gen... 1068 0.0
Phenon 15	927	927 V navarrensis		No sequence
Phenon 16 V. calviensis	504	504 V aestuarianus	1	VSP316172 AJ316172 Vibrio sp. LMG 20546 16S rRNA gene, strai... 1245 0.0
Phenon 16 V. calviensis	852	852 V nigripulchritudo		No sequence
Phenon 16 V. calviensis	855	855 V nigripulchritudo		No sequence
Phenon 16 V. calviensis	420	* V calviensis 420	1	AF118021 AF118021 Vibrio calviensis strain RE35F/12 16S ribo... 1221 0.0
Phenon 17 V. tubiashii	286	286 V splendidus I	10	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 892 0.0
Phenon 17 V. tubiashii	287	287 V splendidus I		No sequence
Phenon 17 V. tubiashii	614	614 V sp.		No sequence
Phenon 17 V. tubiashii	792	792 V tubiashii	10	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 1180 0.0
Phenon 17 V. tubiashii	298	* V tubiashii 298	1	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 1277 0.0
Phenon 17 V. tubiashii	479	* V tubiashii 479	1	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 1158 0.0
Phenon 17 V. tubiashii	669	* V tubiashii 669	1	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 1257 0.0
Phenon 18 V. orientalis	295	295 V tubiashii	6	VO16SRRNA X74719 V. orientalis (ATCC 33934T) gene for 16S rib... 1181 0.0
Phenon 18 V. orientalis	516	516 V sp.	7	VO16SRRNA X74719 V. orientalis (ATCC 33934T) gene for 16S rib... 1037 0.0
Phenon 18 V. orientalis	712	* V orientalis 712	1	VO16SRRNA X74719 V. orientalis (ATCC 33934T) gene for 16S rib... 1275 0.0
Phenon 18 V. orientalis	922	* V orientalis 922		No sequence
Phenon 18 V. orientalis	829	r V orientalis 829	1	VO16SRRNA X74719 V. orientalis (ATCC 33934T) gene for 16S rib... 1185 0.0
Phenon 18 V. orientalis	830	r V orientalis 830	1	VO16SRRNA X74719 V. orientalis (ATCC 33934T) gene for 16S rib... 1287 0.0
Phenon 19	2	2 V sp.	1	VT16SRRNA X74725 V. tubiashii (ATCC 19109T) gene for 16S ribos... 1080 0.0
Phenon 19	51	51 V sp.	1	AY254040 AY254040 Vibrio hispanicus strain LMG 13240 clone 2... 1176 0.0
Phenon 19	619	619 V sp.	1	VT16SRRNA X74725 V. tubiashi (ATCC 19109T) gene for 16S ribos... 1094 0.0
Phenon 19	862	862 V ichthyoenteri		No sequence
Phenon 20	184	184 V tubiashii	1	VSP514914 AJ514914 Vibrio fortis partial 16S rRNA gene, stra... 1110 0.0
Phenon 20	198	198 V sp.	1	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1187 0.0
Phenon 20	285	285 V splendidus II	1	AY034144 AY034144 Vibrio sp. C33 16S ribosomal RNA gene, par... 967 0.0
Phenon 20	289	289 V mediterranei	1	VSP345064 AJ345064 Vibrio sp. LMG 20363 partial 16S rRNA gen... 1259 0.0
Phenon 20	322	322 V tubiashii	1	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1277 0.0
Phenon 20	856	856 V nigripulchritudo		No sequence
Phenon 21	103	103 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1132 0.0
Phenon 21	132	132 V sp.	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1283 0.0
Phenon 21	19	19 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1180 0.0
Phenon 21	20	20 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 987 0.0
Phenon 21	56	56 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1261 0.0
Phenon 21	57	57 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1158 0.0
Phenon 21	573	573 V navarrensis	1	AY345418 AY345418 Bacterium K2-38 16S ribosomal RNA gene, pa... 1178 0.0
Phenon 21	59	59 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1170 0.0
Phenon 22 V. rumoiensis	161	161 V sp.	N	AY620964 AY620964 Vibrio tasmaniensis strain 562 16S ribosom... 1245 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 22 V. rumoiensis	45	45 V sp.	N	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 934 0.0
Phenon 22 V. rumoiensis	914	* V rumoiensis 914	1	AB013297 AB013297 Vibrio rumoence gene for 16S rRNA, complet... 1251 0.0
Phenon 23 V. aestuarianus	320	320 V sp.	1	VAE16SRRN X74689 V. aestuarianus (ATCC 35048T) gene for 16S r... 1281 0.0
Phenon 23 V. aestuarianus	472	* V aestuarianus 472	1	VAE16SRRN X74689 V. aestuarianus (ATCC 35048T) gene for 16S r... 1269 0.0
Phenon 23 V. aestuarianus	920	* V aestuarianus 920	1	VAE16SRRN X74689 V. aestuarianus (ATCC 35048T) gene for 16S r... 1281 0.0
Phenon 24	140	140 V sp.	1	VPO491290 AJ491290 Vibrio pomeroyi partial 16S rRNA gene, ty... 1235 0.0
Phenon 24	141	141 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1259 0.0
Phenon 24	145	145 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1219 0.0
Phenon 24	152	152 V sp.	1	AY620972 AY620972 Vibrio splendidus strain 636 16S ribosomal... 1281 0.0
Phenon 25	1	1 V sp.	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1259 0.0
Phenon 25	14	14 V sp.	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1255 0.0
Phenon 25	362	362 V sp.	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1300 0.0
Phenon 25	363	363 V sp.	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1249 0.0
Phenon 25	365	365 V sp.	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1124 0.0
Phenon 25	453	453 V splendidus I	1	AY129278 AY129278 Vibrio tapetis strain LP2 16S ribosomal RN... 1285 0.0
Phenon 25	576	576 V sp.	1	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1269 0.0
Phenon 25	61	61 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1215 0.0
Phenon 25	62	62 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1219 0.0
Phenon 25	63	63 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1237 0.0
Phenon 25	67	67 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1213 0.0
Phenon 25	69	69 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1217 0.0
Phenon 25	73	73 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1223 0.0
Phenon 25	845	845 V sp.	1	AY620964 AY620964 Vibrio tasmaniensis strain 562 16S ribosom... 1209 0.0
Phenon 26a V. cyclitrophicus	127	127 V sp.	N	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1285 0.0
Phenon 26a V. cyclitrophicus	128	128 V sp.	N	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1285 0.0
Phenon 26a V. cyclitrophicus	215	215 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1259 0.0
Phenon 26a V. cyclitrophicus	620	620 V sp.		No sequence
Phenon 26a V. cyclitrophicus	758	758 V tubiashii	N	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1283 0.0
Phenon 26a V. cyclitrophicus	684	* V cyclitrophicus 684	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1140 0.0
Phenon 26a V. cyclitrophicus	209	209 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1138 0.0
Phenon 26	102	102 V sp.	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1285 0.0
Phenon 26	105	105 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1255 0.0
Phenon 26	106	106 V sp.	1	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1259 0.0
Phenon 26	109	109 V sp.	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1199 0.0
Phenon 26	126	126 V sp.	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1285 0.0
Phenon 26	129	129 V sp.	1	MBAJ2566 AJ002566 Marine bacterium (isolate DPT1.1) 16S rRNA... 1263 0.0
Phenon 26	159	159 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1257 0.0
Phenon 26	163	163 V sp.		No sequence
Phenon 26	164	164 V sp.	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1283 0.0
Phenon 26	176	176 V tubiashii	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1154 0.0
Phenon 26	18	18 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1207 0.0
Phenon 26	21	21 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1237 0.0
Phenon 26	226	226 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1144 0.0
Phenon 26	587	587 V sp.	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1178 0.0
Phenon 26	919	919 V navarrensis		No sequence
Phenon 26	98	98 V sp.	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1271 0.0
Phenon 27	142	142 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1185 0.0
Phenon 27	143	143 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1229 0.0
Phenon 27	147	147 V sp.	1	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1199 0.0
Phenon 27	15	15 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1178 0.0
Phenon 27	154	154 V sp.	1	AB055792 AB055792 Vibrio sp. Cdeep-1 gene for 16S rRNA. 10/2002 993 0.0
Phenon 27	155	155 V sp.	1	MBAJ2567 AJ002567 Marine bacterium (isolate DPT1.2) 16S rRNA... 1249 0.0
Phenon 27	223	223 V sp.	1	VSP560649 AJ560649 Vibrio pomeroyi partial 16S rRNA gene. 5/... 1277 0.0
Phenon 27	224	224 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 922 0.0
Phenon 27	225	225 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1251 0.0
Phenon 27	227	227 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1199 0.0
Phenon 27	230	230 V sp.	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1251 0.0
Phenon 27	455	455 V morestus	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1229 0.0
Phenon 27	456	456 V morestus	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1178 0.0
Phenon 27	599	599 V sp.	1	AB038025 AB038025 Vibrio sp. TK327 gene for 16S rRNA, partia... 1197 0.0
Phenon 27	622	622 V sp.	1	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1283 0.0
Phenon 27	624	624 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1231 0.0
Phenon 27	657	657 V splendidus I		No sequence
Phenon 28 V. chagasii	101	101 V sp.	N	VSP560649 AJ560649 Vibrio pomeroyi partial 16S rRNA gene. 5/... 1289 0.0
Phenon 28 V. chagasii	108	108 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1217 0.0
Phenon 28 V. chagasii	144	144 V sp.	N	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1253 0.0
Phenon 28 V. chagasii	160	160 V sp.	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1259 0.0
Phenon 28 V. chagasii	175	175 V sp.	N	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1221 0.0
Phenon 28 V. chagasii	179	179 V harveyi	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1128 0.0
Phenon 28 V. chagasii	190	190 V sp.	2	VSP490157 AJ490157 Vibrio chagasii partial 16S rRNA gene, st... 1047 0.0
Phenon 28 V. chagasii	195	195 V sp.		No sequence
Phenon 28 V. chagasii	196	196 V sp.	N	AY292936 AY292936 Vibrio lentus strain Sat201 16S ribosomal ... 1249 0.0
Phenon 28 V. chagasii	197	197 V sp.	N	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1251 0.0
Phenon 28 V. chagasii	321	321 V tubiashii	N	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1308 0.0
Phenon 28 V. chagasii	452	452 V baumanii	N	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1185 0.0
Phenon 28 V. chagasii	461	461 V baumanii	N	AB038025 AB038025 Vibrio sp. TK327 gene for 16S rRNA, partia... 1233 0.0
Phenon 28 V. chagasii	469	469 V splendidus I	2	VSP490157 AJ490157 Vibrio chagasii partial 16S rRNA gene, st... 1247 0.0
Phenon 28 V. chagasii	470	470 V baumanii	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1215 0.0
Phenon 28 V. chagasii	471	471 V baumanii	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1269 0.0
Phenon 28 V. chagasii	49	49 V sp.	N	VSU64024 U64024 Vibrio sp. 16S ribosomal RNA gene, partial s... 448 0.0
Phenon 28 V. chagasii	675	675 V splendidus I	N	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1263 0.0
Phenon 28 V. chagasii	676	676 V splendidus I	N	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1241 0.0
Phenon 28 V. chagasii	814	814 Ph damselae damselae	N	AB038025 AB038025 Vibrio sp. TK327 gene for 16S rRNA, partia... 1162 0.0
Phenon 28 V. chagasii	95	95 V sp.	N	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1273 0.0
Phenon 29	10	10 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1176 0.0
Phenon 29	11	11 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1065 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 29	12	12 V sp.	1	MBAJ2566 AJ002566 Marine bacterium (isolate DPT1.1) 16S rRNA... 1255 0.0
Phenon 29	13	13 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1249 0.0
Phenon 29	3	3 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1205 0.0
Phenon 29	4	4 V sp.		No sequence
Phenon 29	46	46 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1168 0.0
Phenon 29	5	5 V sp.		No sequence
Phenon 29	64	64 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1207 0.0
Phenon 29	65	65 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1191 0.0
Phenon 29	66	66 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1164 0.0
Phenon 29	7	7 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1181 0.0
Phenon 29	72	72 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1205 0.0
Phenon 29	74	74 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1164 0.0
Phenon 29	75	75 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1106 0.0
Phenon 29	76	76 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1051 0.0
Phenon 29	8	8 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1187 0.0
Phenon 31 V. splendidus bv I	104	104 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1164 0.0
Phenon 31 V. splendidus bv I	130	130 V sp.	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1237 0.0
Phenon 31 V. splendidus bv I	131	131 V sp.	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1259 0.0
Phenon 31 V. splendidus bv I	133	133 V sp.	N	AF388393 AF388393 Vibrio sp. 3d clone 3d7 16S ribosomal RNA ... 1217 0.0
Phenon 31 V. splendidus bv I	134	134 V sp.	2	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1277 0.0
Phenon 31 V. splendidus bv I	148	148 V sp.		No sequence
Phenon 31 V. splendidus bv I	151	151 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1112 0.0
Phenon 31 V. splendidus bv I	156	156 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1203 0.0
Phenon 31 V. splendidus bv I	158	158 V splendidus I	6	VSP16SRRA X74724 V. splendidus (ATCC 33125T) gene for 16S rib... 1205 0.0
Phenon 31 V. splendidus bv I	162	162 V sp.		No sequence
Phenon 31 V. splendidus bv I	231	231 V pelagius II	2	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1265 0.0
Phenon 31 V. splendidus bv I	294	294 V tubiashii	4	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1233 0.0
Phenon 31 V. splendidus bv I	360	360 V sp.	N	AF388393 AF388393 Vibrio sp. 3d clone 3d7 16S ribosomal RNA ... 1245 0.0
Phenon 31 V. splendidus bv I	361	361 V sp.	N	VVU16SRRA X74727 V. vulnificus (ATCC 29307) gene for 16S ribo... 1275 0.0
Phenon 31 V. splendidus bv I	364	364 V sp.	N	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1183 0.0
Phenon 31 V. splendidus bv I	366	366 V sp.	N	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1255 0.0
Phenon 31 V. splendidus bv I	402	402 V lentus	8	VSP515227 AJ515227 Vibrio splendidus partial 16S rRNA gene, ... 948 0.0
Phenon 31 V. splendidus bv I	457	457 V splendidus I	N	VME16SRRA X74710 V. mediterranei (CIP 103203T) gene for 16S r... 1148 0.0
Phenon 31 V. splendidus bv I	460	460 V morestus	N	AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r... 1294 0.0
Phenon 31 V. splendidus bv I	463	463 V lentus	4	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1227 0.0
Phenon 31 V. splendidus bv I	464	464 V lentus	3	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1233 0.0
Phenon 31 V. splendidus bv I	466	466 V lentus	1	VN16SRRNA X74714 V. natriegens (ATCC 14048T) gene for 16S rib... 1320 0.0
Phenon 31 V. splendidus bv I	468	468 V splendidus I	2	AY620974 AY620974 Vibrio splendidus strain 630 16S ribosomal... 1255 0.0
Phenon 31 V. splendidus bv I	575	575 V sp.	N	AF388393 AF388393 Vibrio sp. 3d clone 3d7 16S ribosomal RNA ... 1247 0.0
Phenon 31 V. splendidus bv I	577	577 V sp.	N	AB038026 AB038026 Vibrio sp. OC25 gene for 16S rRNA, partial... 1229 0.0
Phenon 31 V. splendidus bv I	578	578 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1170 0.0
Phenon 31 V. splendidus bv I	584	584 V sp.	N	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1263 0.0
Phenon 31 V. splendidus bv I	585	585 V sp.	2	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1162 0.0
Phenon 31 V. splendidus bv I	592	592 V sp.	5	VSP16SRRA X74724 V. splendidus (ATCC 33125T) gene for 16S rib... 1197 0.0
Phenon 31 V. splendidus bv I	60	60 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1199 0.0
Phenon 31 V. splendidus bv I	71	71 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1154 0.0
Phenon 31 V. splendidus bv I	763	763 V pelagius II	3	AY620974 AY620974 Vibrio splendidus strain 630 16S ribosomal... 1259 0.0
Phenon 31 V. splendidus bv I	790	790 V splendidus I	4	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1217 0.0
Phenon 31 V. splendidus bv I	9	9 V sp.	N	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1203 0.0
Phenon 31 V. splendidus bv I	94	94 V campbellii	N	MBAJ2566 AJ002566 Marine bacterium (isolate DPT1.1) 16S rRNA... 1205 0.0
Phenon 31 V. splendidus bv I	96	96 V sp.	N	AF069667 AF069667 North Sea bacterium H120 16S ribosomal RNA... 1223 0.0
Phenon 31 V. splendidus bv I	97	97 V sp.	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1245 0.0
Phenon 31 V. splendidus bv I	935	* V splendidus I 935	2	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1273 0.0
Phenon 32 V. harveyi bv II	522	522 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1269 0.0
Phenon 32 V. harveyi bv II	639	639 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1294 0.0
Phenon 32 V. harveyi bv II	742	742 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1229 0.0
Phenon 32 V. harveyi bv II	744	744 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1136 0.0
Phenon 32 V. harveyi bv II	745	745 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1296 0.0
Phenon 32 V. harveyi bv II	747	747 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1142 0.0
Phenon 32 V. harveyi bv II	753	753 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1267 0.0
Phenon 32 V. harveyi bv II	754	754 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1300 0.0
Phenon 32 V. harveyi bv II	755	755 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1285 0.0
Phenon 32 V. harveyi bv II	756	756 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1211 0.0
Phenon 32 V. harveyi bv II	757	757 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1285 0.0
Phenon 32 V. harveyi bv II	768	768 V mimicus	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1300 0.0
Phenon 32 V. harveyi bv II	779	779 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1271 0.0
Phenon 32 V. harveyi bv II	782	782 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1294 0.0
Phenon 32 V. harveyi bv II	783	783 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1112 0.0
Phenon 32 V. harveyi bv II	784	784 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 955 0.0
Phenon 32 V. harveyi bv II	832	832 V harveyi	N	AF462458 AF462458 Vibrio ruber 16S ribosomal RNA gene, parti... 1065 0.0
Phenon 32 V. harveyi bv II	833	833 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1253 0.0
Phenon 32 V. harveyi bv II	834	834 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1205 0.0
Phenon 32 V. harveyi bv II	835	835 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1292 0.0
Phenon 32 V. harveyi bv II	837	837 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1300 0.0
Phenon 32 V. harveyi bv II	839	839 V harveyi	2	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1156 0.0
Phenon 32 V. harveyi bv II	847	847 V harveyi	1	VCH16SRRA X74693 V. carchiariae (ATCC 35084T) gene for 16S ri... 1221 0.0
Phenon 33 V. cholerae	206	206 V cholerae	2	VCOL16SRA X74694 V. cholerae (ATCC 14033) gene for 16S riboso... 1120 0.0
Phenon 33 V. cholerae	331	331 V cholerae	N	VSP514917 AJ514917 Vibrio fortis partial 16S rRNA gene, stra... 1187 0.0
Phenon 33 V. cholerae	332	332 V cholerae	N	VSP316181 AJ316181 Vibrio sp. R-15052 16S rRNA gene, strain ... 1130 0.0
Phenon 33 V. cholerae	333	333 V cholerae	N	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1277 0.0
Phenon 33 V. cholerae	334	334 V cholerae	N	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1187 0.0
Phenon 33 V. cholerae	497	497 V cholerae	2	VCOL16SRA X74694 V. cholerae (ATCC 14033) gene for 16S riboso... 1257 0.0
Phenon 33 V. cholerae	610	610 V cholerae	1	AY292952 AY292952 Vibrio cholerae 16S ribosomal RNA gene, co... 1267 0.0
Phenon 33 V. cholerae	672	672 V cholerae	2	VCOL16SRA X74694 V. cholerae (ATCC 14033) gene for 16S riboso... 1257 0.0
Phenon 33 V. cholerae	674	674 V cholerae	1	AE004157 AE004157 Vibrio cholerae O1 biovar eltor str. N1696... 997 0.0
Phenon 33 V. cholerae	772	772 V cholerae		No sequence

Phenon No.	V	Strain	R	BLAST results
Phenon 33 V. cholerae	773	773 V cholerae	2	VCOL16SRA X74694 V. cholerae (ATCC 14033) gene for 16S riboso... 1275 0.0
Phenon 33 V. cholerae	774	774 V cholerae	2	VCOL16SRA X74694 V. cholerae (ATCC 14033) gene for 16S riboso... 1243 0.0
Phenon 34 V. mimicus	172	172 V mimicus	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1128 0.0
Phenon 34 V. mimicus	220?	220 V mimicus	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1237 0.0
Phenon 34 V. mimicus	344	344 V cholerae	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1233 0.0
Phenon 34 V. mimicus	492	492 V mimicus	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1162 0.0
Phenon 34 V. mimicus	696	* V mimicus 696	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1245 0.0
Phenon 34 V. mimicus	825	r V mimicus 825	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1265 0.0
Phenon 34 V. mimicus	826	r V mimicus 826	1	VMI16SRRA X74713 V. mimicus (ATCC 33653T) gene for 16S riboso... 1265 0.0
Phenon 35a V. vulnificus bv I	250	250 V parahaemolyticus	1	VVU16SRRA X74727 V. vulnificus (ATCC 29307) gene for 16S ribo... 1164 0.0
Phenon 35a V. vulnificus bv I	251	251 V parahaemolyticus	1	VVU16SRRA X74727 V. vulnificus (ATCC 29307) gene for 16S ribo... 1249 0.0
Phenon 35a V. vulnificus bv I	311	311 V mimicus	1	VVU16SRRA X74727 V. vulnificus (ATCC 29307) gene for 16S ribo... 1239 0.0
Phenon 35a V. vulnificus bv I	329	329 V vulnificus	N	AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r... 1237 0.0
Phenon 35a V. vulnificus bv I	437	437 V mimicus	1	AP005343 AP005343 Vibrio vulnificus YJ016 DNA, chromosome I,... 1277 0.0
Phenon 35a V. vulnificus bv I	438	438 V mimicus	1	VVU16SRRA X74727 V. vulnificus (ATCC 29307) gene for 16S ribo... 1292 0.0
Phenon 35a V. vulnificus bv I	700	* V vulnificus I 700	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1287 0.0
Phenon 35b V. vulnificus bv II	386	386 V vulnificus II	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1283 0.0
Phenon 35b V. vulnificus bv II	694	r V vulnificus II 694	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1269 0.0
Phenon 35b V. vulnificus bv II	923	r V vulnificus II 923	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1053 0.0
Phenon 35b V. vulnificus bv II	924	r V vulnificus II 924	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1271 0.0
Phenon 35b V. vulnificus bv II	925	r V vulnificus II 925	1	VV16SRRNA X74726 V. vulnificus (ATCC 27562T) gene for 16S rib... 1273 0.0
Phenon 36	22	22 V sp.	1	AY136119 AY136119 Vibrio sp. MED103 16S rRNA gene, partial s... 934 0.0
Phenon 36	31	31 V sp.	1	AY136119 AY136119 Vibrio sp. MED103 16S rRNA gene, partial s... 1055 0.0
Phenon 36	445	445 V sp.	1	AY129278 AY129278 Vibrio tapetis strain LP2 16S ribosomal RN... 1061 0.0
Phenon 36	596	596 V sp.	1	AB038023 AB038023 Vibrio sp. EN276 gene for 16S rRNA, partia... 1110 0.0
Phenon 36	813	813 Ph damselae damselae	1	AY129278 AY129278 Vibrio tapetis strain LP2 16S ribosomal RN... 1187 0.0
Phenon 36	583	r V tasmaniensis 583	1	AB038023 AB038023 Vibrio sp. EN276 gene for 16S rRNA, partia... 1176 0.0
Phenon 37 V. nigripulchritudo	487	487 V nigripulchritudo	N	No sequence
Phenon 37 V. nigripulchritudo	680	* V nigripulchritudo 680	1	VNI16SRRA X74717 V. nigripulchritudo (ATCC 27043T) gene for 1... 761 0.0
Phenon 37 V. nigripulchritudo	844	r V nigripulchritudo 844	1	VNI16SRRA X74717 V. nigripulchritudo (ATCC 27043T) gene for 1... 1108 0.0
Phenon 38 V. penaeicida	565	565 V sp.	1	VPE421444 AJ421444 Vibrio penaeicida 16S rRNA gene, strain D... 1279 0.0
Phenon 38 V. penaeicida	396	* V penaeicida 396	N	AY129277 AY129277 Vibrio splendidus strain LP1 16S ribosomal... 1233 0.0
Phenon 38 V. penaeicida	682	* V penaeicida 682	1	VPE421444 AJ421444 Vibrio penaeicida 16S rRNA gene, strain D... 1267 0.0
Phenon 38 V. penaeicida	926	* V penaeicida 926	1	VPE421444 AJ421444 Vibrio penaeicida 16S rRNA gene, strain D... 1259 0.0
Phenon 39 V. pelagius bv II	187	187 V sp.	2	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1273 0.0
Phenon 39 V. pelagius bv II	323	323 V mediterranei	N	AF108137 AF108137 Uncultured Vibrio sp. 'Artemia' 16S riboso... 1277 0.0
Phenon 39 V. pelagius bv II	652	652 V sp.	6	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1150 0.0
Phenon 39 V. pelagius bv II	842	r V pelagius II 842	6	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1261 0.0
Phenon 39 V. pelagius bv II	870	r V pelagius II 870	6	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1265 0.0
Phenon 40 V. pelagius bv I	23	23 V sp.	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1209 0.0
Phenon 40 V. pelagius bv I	24	24 V pelagius I	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1265 0.0
Phenon 40 V. pelagius bv I	310	310 V pelagius I		No sequence
Phenon 40 V. pelagius bv I	563	563 V pelagius I	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1257 0.0
Phenon 40 V. pelagius bv I	762	762 V pelagius II	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1273 0.0
Phenon 40 V. pelagius bv I	459	* V pelagius I 459	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1277 0.0
Phenon 40 V. pelagius bv I	704	* V pelagius I 704	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1285 0.0
Phenon 40 V. pelagius bv I	911	* V pelagius I 911	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1296 0.0
Phenon 40 V. pelagius bv I	916	* V pelagius I 916	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1285 0.0
Phenon 40 V. pelagius bv I	841	r V pelagius I 841	1	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1296 0.0
Phenon 41	495	495 V ichthyoenteri		No sequence
Phenon 41	512	512 V sp.	1	VSP316168 AJ316168 Vibrio sp. R-14968 16S rRNA gene, strain ... 1130 0.0
Phenon 41	513	513 V sp.		No sequence
Phenon 41	515	515 V sp.	1	VSP316168 AJ316168 Vibrio sp. R-14968 16S rRNA gene, strain ... 868 0.0
Phenon 41	517	517 V sp.	1	VSP440005 AJ440005 Vibrio corallilyticus partial 16S rRNA ge... 1237 0.0
Phenon 41	520	520 V sp.	1	VSP316168 AJ316168 Vibrio sp. R-14968 16S rRNA gene, strain ... 1219 0.0
Phenon 42	208	208 V sp.		No sequence
Phenon 42	35	35 Ph damselae damselae		No sequence
Phenon 42	43	43 V sp.	1	AY034144 AY034144 Vibrio sp. C33 16S ribosomal RNA gene, par... 1104 0.0
Phenon 42	794	794 Ph phosphoreum	1	AY034144 AY034144 Vibrio sp. C33 16S ribosomal RNA gene, par... 1136 0.0
Phenon 43	150	150 V sp.	1	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1098 0.0
Phenon 43	252	252 V parahaemolyticus	1	VT16SRRNA X74725 V. tubiashi (ATCC 19109T) gene for 16S ribos... 1150 0.0
Phenon 43	29	29 V sp.	1	VSP316172 AJ316172 Vibrio sp. LMG 20546 16S rRNA gene, strai... 1124 0.0
Phenon 43	38	38 V sp.	1	VM16SRNA X99761 V. mytili 16S ribosomal RNA. 6/2003 1158 0.0
Phenon 43	659	659 V sp.	1	AF500076 AF500076 Shewanella pacifica KMM 3590 16S ribosomal... 1130 0.0
Phenon 43	660	660 V tubiashii	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1283 0.0
Phenon 43	677	677 V splendidus I	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1237 0.0
Phenon 43	781	781 V harveyi	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1277 0.0
Phenon 43	791	791 V splendidus I	1	AY620969 AY620969 Vibrio tasmaniensis strain 236.10 16S ribo... 1277 0.0
Phenon 43	99	99 V sp.	1	BPS277984 AJ277984 Bacillus psychrodurans 16S rRNA gene, str... 1225 0.0
Phenon 43	590	590 V sp.	1	VSP316194 AJ316194 Vibrio sp. LMG 19999 16S rRNA gene, strai... 1241 0.0
Phenon 44 V. tasmaniensis	505	505 V sp.		No sequence
Phenon 44 V. tasmaniensis	574	574 V sp.	1	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1269 0.0
Phenon 44 V. tasmaniensis	589	589 V sp.	1	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1257 0.0
Phenon 44 V. tasmaniensis	887	887 V sp.	1	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1265 0.0
Phenon 44 V. tasmaniensis	889	889 V sp.	1	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1263 0.0
Phenon 44 V. tasmaniensis	591	r V tasmaniensis 591	3	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1257 0.0
Phenon 44 V. tasmaniensis	581	* V tasmaniensis 581	3	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1150 0.0
Phenon 44 V. tasmaniensis	232	232 V sp.	3	VTA514912 AJ514912 Vibrio tasmaniensis partial 16S rRNA gene... 1257 0.0
Phenon 45	810	810 Ph damselae damselae	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1255 0.0
Phenon 45	812	812 Ph damselae damselae	1	AF293974 AF293974 Unidentified bacterium 4c 16S ribosomal RN... 1140 0.0
Phenon 45	88	88 V sp.	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1207 0.0
Phenon 45	899	899 V ordalii	1	VSP316203 AJ316203 Vibrio sp. R-1556 16S rRNA gene, strain R... 1104 0.0
Phenon 46	630	630 V splendidus I	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1251 0.0
Phenon 46	631	631 V splendidus I	1	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1247 0.0
Phenon 46	632	632 V splendidus I	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1277 0.0
Phenon 46	633	633 V splendidus I	1	AY620964 AY620964 Vibrio tasmaniensis strain 562 16S ribosom... 1259 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 46	634	634 V splendidus I	1	AY262019 AY262019 Vibrio sp. YWA 16S ribosomal RNA gene, par... 1150 0.0
Phenon 46	635	635 V splendidus I	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1247 0.0
Phenon 47 V. navarrensis	496	496 V tubiashii	1	No sequence
Phenon 47 V. navarrensis	709	709 V campbellii	3	VNA16SRNA X74715 V. navarrensis (CIP 103381T) gene for 16S ri... 1180 0.0
Phenon 47 V. navarrensis	765	765 V navarrensis	4	VNA16SRNA X74715 V. navarrensis (CIP 103381T) gene for 16S ri... 1185 0.0
Phenon 47 V. navarrensis	641	* V navarrensis 641	1	VNA16SRNA X74715 V. navarrensis (CIP 103381T) gene for 16S ri... 1229 0.0
Phenon 48a V. campbellii	474	* V campbellii 474	1	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1287 0.0
Phenon 48a V. campbellii	703	* V campbellii 703	1	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1180 0.0
Phenon 48a V. campbellii	690	r V campbellii 690	1	VCA16SRRA X74692 V. campbelli (ATCC 25920T) gene for 16S ribo... 1170 0.0
Phenon 48a V. campbellii	821	r V campbellii 821	N	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1271 0.0
Phenon 48b V. splendidus. bv II	698	r V splendidus II 698	1	AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r... 1275 0.0
Phenon 48b V. splendidus. bv II	736	r V splendidus II 736	1	AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r... 1281 0.0
Phenon 48b V. splendidus. bv II	843	r V splendidus II 843	2	AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r... 1041 0.0
Phenon 48b V. splendidus. bv II	627	627 V navarrensis	N	AF022409 AF022409 Vibrio sp. ANG.218 16S ribosomal RNA gene,... 977 0.0
Phenon 49 V. lentus	403	403 V lentus	6	VLE278881 AJ278881 Vibrio lentus 16S rRNA gene, strain 40M4T... 1076 0.0
Phenon 49 V. lentus	404	404 V lentus	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1225 0.0
Phenon 49 V. lentus	405	405 V lentus	9	VLE278881 AJ278881 Vibrio lentus 16S rRNA gene, strain 40M4T... 882 0.0
Phenon 49 V. lentus	407	407 V lentus	3	VLE278881 AJ278881 Vibrio lentus 16S rRNA gene, strain 40M4T... 1116 0.0
Phenon 49 V. lentus	408	408 V lentus	2	VLE278881 AJ278881 Vibrio lentus 16S rRNA gene, strain 40M4T... 1084 0.0
Phenon 49 V. lentus	613	613 V sp.	N	AF500080 AF500080 Shewanella affinis KMM 3586 16S ribosomal ... 1112 0.0
Phenon 49 V. lentus	462	* V lentus 462	4	VLE278881 AJ278881 Vibrio lentus 16S rRNA gene, strain 40M4T... 1209 0.0
Phenon 49 V. lentus	418	r V lentus 418	N	VNI16SRRA X74717 V. nigripulchritudo (ATCC 27043T) gene for 1... 1086 0.0
Phenon 50 - M. marina	880	* M marina 880	1	VMRRNA16S X82142 V. marinus 16S rRNA gene (NCIMB 1144T). 7/1998 753 0.0
Phenon 52	205	205 V sp.	1	AY305857 AY305857 Pseudoalteromonas sp. SM9913 16S ribosomal... 1160 0.0
Phenon 52	343	343 V sp.		No sequence
Phenon 52	888	888 V sp.	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1273 0.0
Phenon 53	290	290 V mediterranei	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1076 0.0
Phenon 53	315	315 V sp.	1	AY345409 AY345409 Bacterium K2-61 16S ribosomal RNA gene, pa... 1082 0.0
Phenon 53	317	317 V sp.	1	AF388394 AF388394 Vibrio sp. 3d clone 3d8 16S ribosomal RNA ... 1031 0.0
Phenon 53	338	338 V sp.	1	VSP345064 AJ345064 Vibrio sp. LMG 20363 partial 16S rRNA gen... 1035 0.0
Phenon 53	655	655 V splendidus I	1	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1261 0.0
Phenon 53	857	857 V ichthyoenteri		No sequence
Phenon 53	859	859 V ichthyoenteri		No sequence
Phenon 53	864	864 V ichthyoenteri		No sequence
Phenon 53	875	875 V ichthyoenteri		No sequence
Phenon 54 V. scophthalmi	384	384 V fluvialis	3	VSU46579 U46579 Vibrio scophthalmi 16S rRNA gene. 7/1997 1080 0.0
Phenon 54 V. scophthalmi	442	442 V ordalii	N	AY174869 AY174869 Vibrio sp. QY101 16S ribosomal RNA gene, p... 1271 0.0
Phenon 54 V. scophthalmi	443	443 V sp.	N	VAG310648 AJ310648 Vibrio agarivorans 16S rRNA gene, strain ... 1166 0.0
Phenon 54 V. scophthalmi	511	511 V ichthyoenteri	3	VSU46579 U46579 Vibrio scophthalmi 16S rRNA gene. 7/1997 892 0.0
Phenon 54 V. scophthalmi	410	* V scophthalmi 410	2	VSU46579 U46579 Vibrio scophthalmi 16S rRNA gene. 7/1997 1227 0.0
Phenon 54 V. scophthalmi	411	r V scophthalmi 411	8	AF493805 AF493805 Vibrio scophthalmi 16S ribosomal RNA gene,... 1043 0.0
Phenon 54 V. scophthalmi	412	r V scophthalmi 412	N	VPE421444 AJ421444 Vibrio penaeicida 16S rRNA gene, strain D... 1275 0.0
Phenon 54 V. scophthalmi	413	r V scophthalmi 413	N	AF124055 AF124055 Vibrio aerogenes 16S ribosomal RNA gene, p... 1009 0.0
Phenon 54 V. scophthalmi	414	r V scophthalmi 414	4	VSU46579 U46579 Vibrio scophthalmi 16S rRNA gene. 7/1997 1164 0.0
Phenon 54 V. scophthalmi	415	r V scophthalmi 415	4	VSU46579 U46579 Vibrio scophthalmi 16S rRNA gene. 7/1997 1170 0.0
Phenon 54 V. scophthalmi	582	r V tasmaniensis 582	N	AF118021 AF118021 Vibrio calviensis strain RE35F/12 16S ribo... 912 0.0
Phenon 55 V. ichthyoenteri bv I	193	193 V ichthyoenteri	4	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1162 0.0
Phenon 55 V. ichthyoenteri bv I	319?	319 V ichthyoenteri?	N	AF423297 AF423297 Uncultured soil bacterium clone 889-1 16S ... 1124 0.0
Phenon 55 V. ichthyoenteri bv I	623	623 V sp.	N	AF500076 AF500076 Shewanella pacifica KMM 3590 16S ribosomal... 1059 0.0
Phenon 55 V. ichthyoenteri bv I	748	748 V ichthyoenteri	1	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1088 0.0
Phenon 55 V. ichthyoenteri bv I	749	749 V ichthyoenteri	1	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 963 0.0
Phenon 55 V. ichthyoenteri bv I	395	* V ichthyoenteri 395	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1253 0.0
Phenon 55 V. ichthyoenteri bv I	910	* V ichthyoenteri 910	N	VPE293802 AJ293802 Vibrio pelagius 16S rRNA gene, strain CEC... 1257 0.0
Phenon 56 V. anguillarum	114	114 Ph damselae damselae	N	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1261 0.0
Phenon 56 V. anguillarum	157?	157 v sp.		No sequence
Phenon 56 V. anguillarum	165	165 V sp.	N	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1261 0.0
Phenon 56 V. anguillarum	216	216 V sp.	N	VNER16SRA X74716 V. nereis (ATCC 25917T) gene for 16S ribosom... 1217 0.0
Phenon 56 V. anguillarum	293	293 V tubiashii	N	VSP515222 AJ515222 Vibrio splendidus partial 16S rRNA gene, ... 660 0.0
Phenon 56 V. anguillarum	325	325 V anguillarum	N	AF094702 AF094702 Vibrio sp. Lu8 16S ribosomal RNA gene, par... 1130 0.0
Phenon 56 V. anguillarum	327	327 V anguillarum	N	AF094702 AF094702 Vibrio sp. Lu8 16S ribosomal RNA gene, par... 902 0.0
Phenon 56 V. anguillarum	328	328 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1247 0.0
Phenon 56 V. anguillarum	374	374 V piscium	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	382	382 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1259 0.0
Phenon 56 V. anguillarum	383	383 V anguillarum		No sequence
Phenon 56 V. anguillarum	385	385 V fluvialis	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1304 0.0
Phenon 56 V. anguillarum	430	430 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1273 0.0
Phenon 56 V. anguillarum	506	506 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1259 0.0
Phenon 56 V. anguillarum	507	507 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1239 0.0
Phenon 56 V. anguillarum	508	508 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1273 0.0
Phenon 56 V. anguillarum	509	509 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1259 0.0
Phenon 56 V. anguillarum	510	510 V anguillarum		No sequence
Phenon 56 V. anguillarum	528	528 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1273 0.0
Phenon 56 V. anguillarum	529	529 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1289 0.0
Phenon 56 V. anguillarum	530	530 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	531	531 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1211 0.0
Phenon 56 V. anguillarum	532	532 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1253 0.0
Phenon 56 V. anguillarum	533	533 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1168 0.0
Phenon 56 V. anguillarum	534	534 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	535	535 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1287 0.0
Phenon 56 V. anguillarum	536	536 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	538	538 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1243 0.0
Phenon 56 V. anguillarum	539	539 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	540	540 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1231 0.0
Phenon 56 V. anguillarum	541	541 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1132 0.0
Phenon 56 V. anguillarum	542	542 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1273 0.0
Phenon 56 V. anguillarum	544	544 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 56 V. anguillarum	545	545 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1257 0.0
Phenon 56 V. anguillarum	546	546 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1164 0.0
Phenon 56 V. anguillarum	547	547 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1201 0.0
Phenon 56 V. anguillarum	549	549 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1269 0.0
Phenon 56 V. anguillarum	550	550 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1277 0.0
Phenon 56 V. anguillarum	551	551 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1269 0.0
Phenon 56 V. anguillarum	552	552 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1195 0.0
Phenon 56 V. anguillarum	553	553 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1168 0.0
Phenon 56 V. anguillarum	554	554 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1227 0.0
Phenon 56 V. anguillarum	555	555 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1251 0.0
Phenon 56 V. anguillarum	556	556 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1281 0.0
Phenon 56 V. anguillarum	557	557 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1267 0.0
Phenon 56 V. anguillarum	558	558 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1265 0.0
Phenon 56 V. anguillarum	559	559 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1237 0.0
Phenon 56 V. anguillarum	560	560 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1176 0.0
Phenon 56 V. anguillarum	562	562 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1237 0.0
Phenon 56 V. anguillarum	567	567 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1269 0.0
Phenon 56 V. anguillarum	568	568 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1269 0.0
Phenon 56 V. anguillarum	569	569 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1118 0.0
Phenon 56 V. anguillarum	570	570 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1269 0.0
Phenon 56 V. anguillarum	571	571 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1255 0.0
Phenon 56 V. anguillarum	572	572 V anguillarum	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1168 0.0
Phenon 56 V. anguillarum	586	586 V sp.	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1174 0.0
Phenon 56 V. anguillarum	597	597 V sp.	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1296 0.0
Phenon 56 V. anguillarum	647	647 V anguillarum	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1007 0.0
Phenon 56 V. anguillarum	326	* V anguillarum 326	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1253 0.0
Phenon 56 V. anguillarum	480	* V anguillarum 480		No sequence
Phenon 56 V. anguillarum	908	r V anguillarum 908		No sequence
Phenon 57	16	16 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1215 0.0
Phenon 57	180	180 V sp.	1	VSP560649 AJ560649 Vibrio pomeroyi partial 16S rRNA gene. 5/... 1296 0.0
Phenon 57	68	68 V sp.	1	VPR16SRRA X74723 V. proteolyticus (ATCC 15338T) gene for 16S ... 1199 0.0
Phenon 57	767	767 V logei	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1017 0.0
Phenon 58	221	221 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1183 0.0
Phenon 58	222	222 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1203 0.0
Phenon 58	764	764 V fischeri	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1253 0.0
Phenon 58	776	776 V fischeri	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1249 0.0
Phenon 59	153	153 V sp.		No sequence
Phenon 59	17	17 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1219 0.0
Phenon 59	47	47 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1025 0.0
Phenon 59	48	48 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1205 0.0
Phenon 59	50	50 V sp.		No sequence
Phenon 59	52	52 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1094 0.0
Phenon 59	53	53 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1217 0.0
Phenon 59	54	54 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1235 0.0
Phenon 59	55	55 V sp.		No sequence
Phenon 59	58	58 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1180 0.0
Phenon 59	6	6 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1181 0.0
Phenon 59	70	70 V sp.	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1128 0.0
Phenon 59	775	775 V fischeri	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1136 0.0
Phenon 59	811	811 Ph damselae damselae	1	AY494613 AY494613 Uncultured Photobacterium sp. clone GAMMA5... 1249 0.0
Phenon 60 V. logei	907	* V logei 907	2	AY292934 AY292934 Vibrio logei strain SR181 16S ribosomal RN... 1132 0.0
Phenon 60 V. logei	934	* V logei 934	2	AY292933 AY292933 Vibrio logei strain 35077 16S ribosomal RN... 1255 0.0
Phenon 61 Ph phosphoreum	932	* Ph phosphoreum 940	1	No sequence
Phenon 62a Ph. angustum	929	* Ph angustum 929	N	AY292920 AY292920 Vibrio fischeri isolate ESPaiko 16S riboso... 1291 0.0
Phenon 62b V. fischeri bv II	146	146 V sp.	1	AY292946 AY292946 Vibrio fischeri strain MJ101 16S ribosomal... 1289 0.0
Phenon 62b V. fischeri bv II	211	211 V sp.	1	AY292926 AY292926 Vibrio fischeri strain SR5 16S ribosomal R... 1273 0.0
Phenon 62b V. fischeri bv II	579	579 V sp.	1	AY292934 AY292934 Vibrio logei strain SR181 16S ribosomal RN... 1112 0.0
Phenon 62b V. fischeri bv II	611	611 V fischeri	1	AY292922 AY292922 Vibrio fischeri isolate EM17 16S ribosomal... 1181 0.0
Phenon 62b V. fischeri bv II	628	628 V harveyi	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1180 0.0
Phenon 62b V. fischeri bv II	636	636 V fischeri	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1267 0.0
Phenon 62b V. fischeri bv II	637	637 V fischeri	1	AY292920 AY292920 Vibrio fischeri isolate ESPaiko 16S riboso... 1287 0.0
Phenon 62b V. fischeri bv II	638	638 V fischeri	1	AY292920 AY292920 Vibrio fischeri isolate ESPaiko 16S riboso... 1302 0.0
Phenon 62b V. fischeri bv II	644	644 V fischeri	1	AY292920 AY292920 Vibrio fischeri isolate ESPaiko 16S riboso... 1283 0.0
Phenon 62b V. fischeri bv II	77	77 V sp.	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1136 0.0
Phenon 63 - Ph d ssp dam bv I	113	113 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1195 0.0
Phenon 63 - Ph d ssp dam bv I	115	115 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1267 0.0
Phenon 63 - Ph d ssp dam bv I	118	118 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1285 0.0
Phenon 63 - Ph d ssp dam bv I	123	123 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1277 0.0
Phenon 63 - Ph d ssp dam bv I	255	255 Ph damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1239 0.0
Phenon 63 - Ph d ssp dam bv I	257	257 Ph damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1168 0.0
Phenon 63 - Ph d ssp dam bv I	259	259 Ph damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1283 0.0
Phenon 63 - Ph d ssp dam bv I	260	260 Ph damselae	1	AY163243 AY163243 Photobacterium damselae ssp. piscicida s... 456 0.0
Phenon 63 - Ph d ssp dam bv I	284	284 V splendidus II	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1291 0.0
Phenon 63 - Ph d ssp dam bv I	300	300 V sp.	1	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1237 0.0
Phenon 63 - Ph d ssp dam bv I	336	336 V sp.	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1277 0.0
Phenon 63 - Ph d ssp dam bv I	340	340 V sp.	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1265 0.0
Phenon 63 - Ph d ssp dam bv I	499	499 Ph damselae damselae	1	AB032015 AB032015 Photobacterium damselae ssp. damselae ge... 1265 0.0
Phenon 63 - Ph d ssp dam bv I	78	78 Ph damselae damselae	8	VD16SRRNA X74700 V. damsela (ATCC 33539T) gene for 16S riboso... 1249 0.0
Phenon 63 - Ph d ssp dam bv I	793	793 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1316 0.0
Phenon 63 - Ph d ssp dam bv I	800	800 Ph damselae damselae	1	AY191126 AY191126 Photobacterium damselae ssp. damselae st... 1029 0.0
Phenon 63 - Ph d ssp dam bv I	801	801 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1235 0.0
Phenon 63 - Ph d ssp dam bv I	802	802 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1261 0.0
Phenon 63 - Ph d ssp dam bv I	803	803 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1306 0.0
Phenon 63 - Ph d ssp dam bv I	804	804 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1291 0.0
Phenon 63 - Ph d ssp dam bv I	817	817 Ph damselae damselae	1	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1253 0.0
Phenon 63 - Ph d ssp dam bv I	854	854 Ph damselae	1	AY147859 AY147859 Photobacterium damselae ssp. piscicida s... 1253 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 63 - Ph d ssp dam bv I	863	863 Ph damselae	1	AY191126 AY191126 Photobacterium damselae ssp. damselae st... 1221 0.0
Phenon 63 - Ph d ssp dam bv I	876	876 Ph damselae	1	AY191125 AY191125 Photobacterium damselae ssp. damselae st... 1219 0.0
Phenon 63 - Ph d ssp dam bv I	877	877 Ph damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1265 0.0
Phenon 63 - Ph d ssp dam bv I	807	* Ph dam. ssp. damselae 807	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1279 0.0
Phenon 63 - Ph d ssp dam bv I	805	r Ph dam. ssp. damselae 805	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1237 0.0
Phenon 63 - Ph d ssp dam bv I	808	r Ph dam. ssp. damselae 808		No sequence
Phenon 64 - A. sobria HG7	601	601 V sp.	1	ASO16SRRA X74683 A.sobria (ATCC 43979T) gene for 16S ribosom... 1092 0.0
Phenon 64 - A. sobria HG7	602	602 V sp.	1	ASO16SRRA X74683 A.sobria (ATCC 43979T) gene for 16S ribosom... 1138 0.0
Phenon 64 - A. sobria HG7	603	603 V sp.	1	ASO16SRRA X74683 A.sobria (ATCC 43979T) gene for 16S ribosom... 1013 0.0
Phenon 64 - A. sobria HG7	604	604 V sp.	1	AB038023 AB038023 Vibrio sp. EN276 gene for 16S rRNA, partia... 1059 0.0
Phenon 64 - A. sobria HG7	606	606 V sp.	1	ASO16SRRA X74683 A.sobria (ATCC 43979T) gene for 16S ribosom... 1057 0.0
Phenon 64 - A. sobria HG7	607	607 V sp.	1	ASO16SRRA X74683 A.sobria (ATCC 43979T) gene for 16S ribosom... 1294 0.0
Phenon 64 - A. sobria HG7	609	609 V sp.	N	AY345498 AY345498 Unidentified bacterium clone W4-B32 16S ri... 1211 0.0
Phenon 65 V. metschnikovii	279	279 V metschnikovii	1	VMT16SRRA X74711 V. metschnikovii (CIP 69.14T) gene for 16S r... 1302 0.0
Phenon 65 V. metschnikovii	346	346 V metschnikovii	1	VMT16SRRA X74711 V. metschnikovii (CIP 69.14T) gene for 16S r... 1306 0.0
Phenon 65 V. metschnikovii	349	349 V sp.	1	VMT16SRRA X74711 V. metschnikovii (CIP 69.14T) gene for 16S r... 1296 0.0
Phenon 65 V. metschnikovii	665	* V metschnikovii 665	1	VMT16SRRA X74711 V. metschnikovii (CIP 69.14T) gene for 16S r... 1294 0.0
Phenon 65 V. metschnikovii	281	r V metschnikovii 281	1	VMT16RRNA X74712 V. metschnikovii (NCTC 11170) gene for 16S r... 1261 0.0
Phenon 65 V. metschnikovii	738	r V metschnikovii 738	1	VMT16RRNA X74712 V. metschnikovii (NCTC 11170) gene for 16S r... 1223 0.0
Phenon 67a V. gazogenes	481	* V gazogenes 481	1	VGA16SRRA X74705 V. gazogenes (ATCC 29988T) gene for 16S ribo... 1283 0.0
Phenon 67a V. gazogenes	707	* V gazogenes 707	7	VGA16SRRA X74705 V. gazogenes (ATCC 29988T) gene for 16S ribo... 664 0.0
Phenon 67a V. gazogenes	828	r V gazogenes 828	1	VGA16SRRA X74705 V. gazogenes (ATCC 29988T) gene for 16S ribo... 1102 0.0
Phenon 67b V. cincinnatiensis	136	136 V sp.	N	AB076561 AB076561 Agarivorans albus gene for 16S rRNA, parti... 1096 0.0
Phenon 67b V. cincinnatiensis	494	494 Ph damselae piscicida	N	AY548949 AY548949 Bacillus pumilus strain 8N-4 16S ribosomal... 1156 0.0
Phenon 67b V. cincinnatiensis	612	* V cincinnatiensis 612	1	VCIN16SRA X74698 V. cincinnatiensis (ATCC 35912T) gene for 16... 1144 0.0
Phenon 68 - Ph. iliopiscarium	119	119 Ph iliopiscarium	1	HSP551094 AJ551094 Hyphomicrobium sp. wp13 partial 16S rRNA ... 967 0.0
Phenon 68 - Ph. iliopiscarium	822	* Ph iliopiscarium 822	1	AB000278 AB000278 Vibrio iliopiscarius gene for 16S ribosoma... 1027 0.0
Phenon 68 - Ph. iliopiscarium	823	r Ph iliopiscarium 823	4	AB000278 AB000278 Vibrio iliopiscarius gene for 16S ribosoma... 1197 0.0
Phenon 69	375	375 V penaeicida		No sequence
Phenon 69	379	379 Ph damselae piscicida	1	VSP316200 AJ316200 Vibrio sp. R-3884 16S rRNA gene, strain R... 1207 0.0
Phenon 69	615	615 V sp.	1	OCERRDA M22365 O.linum 16S small subunit ribosomal RNA. 4/1993 880 0.0
Phenon 69	616	616 V sp.	1	AY549002 AY549002 Uncultured bacterium clone sacC13A2 16S ri... 942 0.0
Phenon 69	869	869 V parahaemolyticus		No sequence
Phenon 70 V. salmonicida	879	* V salmonicida 879	8	VSNC16S X70643 V. salmonicida gamma subclass (NCMB 2262) gene... 1203 0.0
Phenon 71 - G. hollisae	477	* V hollisae 477	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1174 0.0
Phenon 71 - G. hollisae	681	* V hollisae 681	1	VH16SRRNA X74707 V. hollisae(ATCC 33564T) gene for 16S riboso... 1166 0.0
Phenon 71 - G. hollisae	566	r G hollisae 566	N	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1158 0.0
Phenon 71 - G. hollisae	683	r G hollisae 683	1	VH16SRRNA X74707 V. hollisae(ATCC 33564T) gene for 16S riboso... 1265 0.0
Phenon 73 - Ph. leiognathi	751	751 V ichthyoenteri	N	AB089204 AB089204 Vibrio sp. No.6 gene for 16S rRNA, partial... 957 0.0
Phenon 73 - Ph. leiognathi	819	819 Ph iliopiscarium	1	PL16SRRNA X74686 Photobacterium leiognathi (ATCC 25521T) gen... 1181 0.0
Phenon 73 - Ph. leiognathi	931	* Ph leiognathi 931	1	PL16SRRNA X74686 Photobacterium leiognathi (ATCC 25521T) gen... 1291 0.0
Phenon 73 - Ph. leiognathi	939	r V fischeri 939	1	AY292944 AY292944 Photobacterium leiognathi strain LN101 16S... 1241 0.0
Phenon 75 - Ph d ssp dam bv II	110	110 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1292 0.0
Phenon 75 - Ph d ssp dam bv II	120	120 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1304 0.0
Phenon 75 - Ph d ssp dam bv II	122	122 Ph sp.	N	AB095446 AB095446 Photobacterium phosphoreum gene for 16S rR... 944 0.0
Phenon 75 - Ph d ssp dam bv II	371	371 V piscailia	N	PPH16SRRA X74687 Photobacterium phosphoreum (ATCC 11040T) ge... 1199 0.0
Phenon 75 - Ph d ssp dam bv II	796	796 Ph damselae damselae	1	AY191125 AY191125 Photobacterium damselae ssp. damselae st... 898 0.0
Phenon 75 - Ph d ssp dam bv II	798	798 Ph damselae damselae	1	AY147861 AY147861 Photobacterium damselae ssp. damselae st... 1302 0.0
Phenon 76 - Ph dam ssp piscicida	112	112 Ph damselae damselae	N	AB095446 AB095446 Photobacterium phosphoreum gene for 16S rR... 1223 0.0
Phenon 76 - Ph dam ssp piscicida	372	372 Ph damselae piscicida	1	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1269 0.0
Phenon 76 - Ph dam ssp piscicida	373	373 Ph damselae piscicida	1	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1241 0.0
Phenon 76 - Ph dam ssp piscicida	376	376 V piscailia	N	PPH16SRRA X74687 Photobacterium phosphoreum (ATCC 11040T) ge... 1193 0.0
Phenon 76 - Ph dam ssp piscicida	816	816 Ph damselae damselae	4	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1263 0.0
Phenon 76 - Ph dam ssp piscicida	865	865 Ph damselae		No sequence
Phenon 76 - Ph dam ssp piscicida	903	903 Ph damselae piscicida	4	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1283 0.0
Phenon 76 - Ph dam ssp piscicida	370	* Ph dam. ssp. piscicida 370	4	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1300 0.0
Phenon 76 - Ph dam ssp piscicida	906	* Ph dam. ssp. piscicida 906	1	AY147860 AY147860 Photobacterium damselae ssp. piscicida s... 1279 0.0
Phenon 76 - Ph dam ssp piscicida	824	r V ichthyoenteri 824	N	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1221 0.0
Phenon 77 V. ichthyoenteri bv II	166	166 V sp.	1	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1205 0.0
Phenon 77 V. ichthyoenteri bv II	168	168 V sp.	1	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1207 0.0
Phenon 77 V. ichthyoenteri bv II	170	170 V sp.	1	VIC421445 AJ421445 Vibrio ichthyoenteri 16S rRNA gene, strai... 1088 0.0
Phenon 78 V. halioticoli	435	435 V halioticoli	1	AB000390 AB000390 Vibrio halioticoli gene for 16S rRNA, part... 1243 0.0
Phenon 78 V. halioticoli	359	* V halioticoli 359	1	AB000390 AB000390 Vibrio halioticoli gene for 16S rRNA, part... 1209 0.0
Phenon 78 V. halioticoli	685	r V halioticoli 685	1	AY426979 AY426979 Vibrio halioticoli ssp. discus strain HD... 797 0.0
Phenon 79 V. fischeri bv I	701	* V fischeri 701	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1283 0.0
Phenon 79 V. fischeri bv I	913	* V fischeri 913	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1292 0.0
Phenon 79 V. fischeri bv I	930	r Ph damselae damselae 930	1	VF16SRRNA X74702 V. fisheri (ATCC 7744T) gene for 16S ribosom... 1287 0.0
Phenon 79 V. fischeri bv I	936	r V fischeri 936	1	AY292920 AY292920 Vibrio fischeri isolate ESPaiko 16S riboso... 1302 0.0
Phenon 79 V. fischeri bv I	937	r V fischeri 937	7	AY292949 AY292949 Vibrio fischeri strain SI1E 16S ribosomal ... 1197 0.0
Phenon 79 V. fischeri bv I	938	r V fischeri 938	7	AY292949 AY292949 Vibrio fischeri strain SI1E 16S ribosomal ... 1197 0.0
Phenon 79 V. fischeri bv I	687	r V logei 687	5	AY292949 AY292949 Vibrio fischeri strain SI1E 16S ribosomal ... 1227 0.0
Phenon 80 V. wodanis	667	* V wodanis 667	1	VWO132227 AJ132227 Vibrio wodanis 16S rRNA gene, strain NVI ... 1249 0.0
Phenon 81 V. tapetis	688	* V tapetis 688	1	VT16SRR Y08430 V. tapetis 16S rRNA gene. 10/1996 1263 0.0
Phenon 82 V. agarivorans	398	398 V agarivorans	N	AF388392 AF388392 Vibrio sp. 3d clone 3d4 16S ribosomal RNA ... 1253 0.0
Phenon 82 V. agarivorans	399	399 V agarivorans	N	VSC16SR Y13830 Vibrio pectenicida 16S rRNA gene. 7/1998 1271 0.0
Phenon 82 V. agarivorans	400	400 V agarivorans	N	VSC16SR Y13830 Vibrio pectenicida 16S rRNA gene. 7/1998 1277 0.0
Phenon 82 V. agarivorans	401	401 V agarivorans	1	VAG310648 AJ310648 Vibrio agarivorans 16S rRNA gene, strain ... 1279 0.0
Phenon 82 V. agarivorans	406	406 V agarivorans	1	VAG310648 AJ310648 Vibrio agarivorans 16S rRNA gene, strain ... 1283 0.0
Phenon 82 V. agarivorans	416	* V agarivorans 416	2	VAG310648 AJ310648 Vibrio agarivorans 16S rRNA gene, strain ... 731 0.0
Phenon 82 V. agarivorans	419	r V agarivorans 419	N	AB089204 AB089204 Vibrio sp. No.6 gene for 16S rRNA, partial... 1223 0.0
Phenon 83	124	124 Ph damselae damselae	1	HSP551094 AJ551094 Hyphomicrobium sp. wp13 partial 16S rRNA ... 1063 0.0
Phenon 83	125	125 Ph damselae damselae	1	HSP551094 AJ551094 Hyphomicrobium sp. wp13 partial 16S rRNA ... 1055 0.0
Phenon 83	339	339 V sp.	1	HSP551094 AJ551094 Hyphomicrobium sp. wp13 partial 16S rRNA ... 1084 0.0
Phenon 83	629	629 V splendidus I	1	AB038032 AB038032 Photobacterium sp. HAR72 gene for 16S rRNA... 1088 0.0
Phenon 83	799	799 Ph damselae damselae	1	AY435161 AY435161 Photobacterium phosphoreum strain RHE-10 1... 682 0.0
Phenon 83	809	809 Ph damselae damselae	1	AY435161 AY435161 Photobacterium phosphoreum strain RHE-10 1... 757 0.0

Phenon No.	V	Strain	R	BLAST results
Phenon 83	818	818 Ph damselae damselae	1	AB038032 AB038032 Photobacterium sp. HAR72 gene for 16S rRNA... 817 0.0
Phenon 84 - M. viscosa	377	377 M viscosa	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1265 0.0
Phenon 84 - M. viscosa	891	891 M viscosa	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1187 0.0
Phenon 84 - M. viscosa	892	892 M viscosa	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1255 0.0
Phenon 84 - M. viscosa	893	893 M viscosa	2	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1223 0.0
Phenon 84 - M. viscosa	894	894 M viscosa	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1241 0.0
Phenon 84 - M. viscosa	918	* M viscosa 918	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1271 0.0
Phenon 84 - M. viscosa	820	r M viscosa 820	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1223 0.0
Phenon 84 - M. viscosa	895	895 M viscosa	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1158 0.0
Phenon 84 - M. viscosa	917	r M viscosa 917	1	VVI132226 AJ132226 Vibrio viscosus 16S rRNA gene, strain NVI... 1271 0.0
Phenon 85 V. pectenicida	367	* V pectenicida 367	2	VSC16SR Y13830 Vibrio pectenicida 16S rRNA gene. 7/1998 910 0.0
Phenon 85 V. pectenicida	368	r V pectenicida 368	1	VSC16SR Y13830 Vibrio pectenicida 16S rRNA gene. 7/1998 1080 0.0
Phenon 86 V. ordalii	439	439 V ordalii	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1304 0.0
Phenon 86 V. ordalii	440	440 V ordalii	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 718 0.0
Phenon 86 V. ordalii	441	441 V ordalii	N	AF388391 AF388391 Vibrio sp. 3d clone 3d2 16S ribosomal RNA ... 1229 0.0
Phenon 86 V. ordalii	706	* V ordalii 706	1	AY530930 AY530930 Vibrio ordalii strain 2003/09/511-2063 16S... 1205 0.0
Phenon 86 V. ordalii	921	*V ordalii 921	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1259 0.0
Phenon 86 V. ordalii	668	r V ordalii 668	1	VO16SRRA X74718 V. ordalii (ATCC 33509T) gene for 16S ribosom... 1289 0.0

A4.4 Summary of BLASTn results for 16S rDNA sequence data of Hypothetical Median Organisms and exemplars.

Phenon 6 (Strain: V357) - 1489 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|7262513|dbj|AB039966.1| Marine gliding bacterium UWA-1 g...	2637	0.0
gi|9971313|dbj|AB032504.1| Tenacibaculum mesophilum gene fo...	2512	0.0
gi|9971312|dbj|AB032503.1| Tenacibaculum mesophilum gene fo...	2512	0.0
gi|9971310|dbj|AB032501.1| Tenacibaculum mesophilum gene fo...	2504	0.0
gi|50313356|gb|AY661694.1| Tenacibaculum marilutum strain T...	2502	0.0
gi|50313355|gb|AY661693.1| Tenacibaculum marilutum strain T...	2494	0.0
gi|50313354|gb|AY661692.1| Tenacibaculum marilutum strain T...	2494	0.0
gi|50313353|gb|AY661691.1| Tenacibaculum marilutum strain T...	2494	0.0
gi|21832087|dbj|AB078044.1| Flexibacter aurantiacus ssp. ...	2440	0.0
gi|18766572|gb|AF469612.1| Tenacibaculum cellulophagum stra...	2357	0.0

Phenon 8 (Strain: V201) - 1518 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|34100973|gb|AY292936.1| Vibrio lentus strain Sat201 16S ...	2942	0.0
gi|34100972|gb|AY292935.1| Vibrio lentus strain Sat101 16S ...	2942	0.0
gi|21693016|emb|AJ316193.1|VSP316193 Vibrio sp. LMG 20539 1...	2914	0.0
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2809	0.0
gi|27819281|gb|AY129277.1| Vibrio splendidus strain LP1 16S...	2801	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2791	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2791	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2787	0.0
gi|21685378|emb|AJ491290.1|VPO491290 Vibrio pomeroyi partia...	2787	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2775	0.0

Phenon 10 (Strain: V261) - 1421 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|4691430|dbj|AB026194.1| Alpha proteobacterium MBIC1876 g...	2454	0.0
gi|50261374|gb|AY654762.1| Mucus bacterium 107 16S ribosoma...	2452	0.0
gi|50261366|gb|AY654754.1| Mucus bacterium 116 16S ribosoma...	2424	0.0
gi|50261386|gb|AY654774.1| Mucus bacterium 31 16S ribosomal...	2420	0.0
gi|50261358|gb|AY654746.1| Mucus bacterium 103 16S ribosoma...	2381	0.0
gi|11137582|emb|AJ391197.1|RSP391197 Ruegeria sp. AS-36, pa...	2381	0.0
gi|1737206|gb|U77644.1|SLU77644 Silicibacter lacuscaerulens...	2365	0.0
gi|19924256|gb|AF098491.2| Silicibacter pomeroyi strain DSS...	2355	0.0
gi|50261409|gb|AY654797.1| Mucus bacterium 64 16S ribosomal...	2345	0.0
gi|3492834|dbj|D88527.1| Agrobacterium meteori gene for 16S...	2345	0.0

Phenon 15 (Strain: V759) - 1517 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|7007472|dbj|AB038026.1| Vibrio sp. OC25 gene for 16S rRN...	2621	0.0
gi|22799128|emb|AJ316194.1|VSP316194 Vibrio sp. LMG 19999 1...	2607	0.0
gi|2253387|gb|AF007115.1| Vibrio shiloi 16S ribosomal RNA g...	2583	0.0
gi|7007470|dbj|AB038024.1| Vibrio sp. EN280 gene for 16S rR...	2565	0.0
gi|1633036|emb|Y08430.1|VT16SRR V.tapetis 16S rRNA gene	2549	0.0
gi|27819281|gb|AY129277.1| Vibrio splendidus strain LP1 16S...	2547	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2541	0.0
gi|50261391|gb|AY654779.1| Mucus bacterium 49 16S ribosomal...	2539	0.0
gi|14669624|gb|AF388394.1| Vibrio sp. 3d clone 3d8 16S ribo...	2535	0.0
gi|22218067|emb|AJ244774.1|VSP244774 Vibrio sp. V4.BP.07 16...	2535	0.0

Phenon 19 (Strain: V51) – 1518 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|30315101|gb|AF500207.1| Vibrio sp. CJ11052 16S ribosomal...	2696	0.0
gi|47118310|dbj|BA000031.2| Vibrio parahaemolyticus DNA, ch...	2694	0.0
gi|33392080|gb|AY345403.1| Bacterium K2-74 16S ribosomal RN...	2692	0.0
gi|8571418|gb|AF246980.1|AF246980 Vibrio sp. 98CJ11027 16S ...	2692	0.0
gi|47118311|dbj|BA000032.2| Vibrio parahaemolyticus DNA, ch...	2686	0.0
gi|14669617|gb|AF388387.1| Vibrio parahaemolyticus clone Vp...	2668	0.0
gi|14669619|gb|AF388389.1| Vibrio parahaemolyticus clone Vp...	2660	0.0
gi|38091113|emb|AJ514915.1|VSP514915 Vibrio fortis partial ...	2648	0.0
gi|18034133|gb|AF410779.1|AF410779 Vibrio sp. NLEP97-1599 1...	2648	0.0
gi|38091114|emb|AJ514917.1|VSP514917 Vibrio fortis partial ...	2637	0.0

Phenon 20 (Strain: V322) - 1514 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|21262136|emb|AJ316181.1|VSP316181 Vibrio sp. R-15052 16S...	2841	0.0
gi|45773744|emb|AJ440009.1|VSP440009 Vibrio gallicus partia...	2841	0.0
gi|34493876|emb|AJ345063.1|VSP345063 Vibrio probioticus par...	2807	0.0
gi|38091112|emb|AJ514914.1|VSP514914 Vibrio fortis partial ...	2797	0.0
gi|21262135|emb|AJ316172.1|VSP316172 Vibrio sp. LMG 20546 1...	2750	0.0
gi|14669616|gb|AF388386.1| Vibrio parahaemolyticus clone Vp...	2728	0.0
gi|14669618|gb|AF388388.1| Vibrio parahaemolyticus clone F4...	2720	0.0
gi|30315101|gb|AF500207.1| Vibrio sp. CJ11052 16S ribosomal...	2710	0.0
gi|22799106|emb|AJ316168.1|VSP316168 Vibrio sp. R-14968 16S...	2700	0.0
gi|22799107|emb|AJ316169.1|VSP316169 Vibrio sp. LMG 19270 1...	2688	0.0

Phenon 21 (Strain: V20) – 1519 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2993	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2956	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2940	0.0
gi|30387171|gb|AF539781.1| Pseudoalteromonas sp. RE1-12a 16...	2926	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2924	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2924	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2920	0.0
gi|30387172|gb|AF539782.1| Vibrio sp. RE1-3 16S ribosomal R...	2914	0.0
gi|30387169|gb|AF539779.1| Pseudoalteromonas sp. R12 16S ri...	2914	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2872	0.0

Phenon 24 (Strain: V140) - 1519 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|21685378|emb|AJ491290.1|VPO491290 Vibrio pomeroyi partia...	2932	0.0
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2890	0.0
gi|11321102|gb|AF293974.1|AF293974 Unidentified bacterium 4...	2878	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2868	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2868	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2868	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2861	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2857	0.0
gi|12964681|dbj|AB055792.1| Vibrio sp. Cdeep-1 gene for 16S...	2847	0.0
gi|30387171|gb|AF539781.1| Pseudoalteromonas sp. RE1-12a 16...	2839	0.0

Phenon 25 (Strain: V69) - 1522 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2950	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2916	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2912	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2896	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2896	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2896	0.0
gi|18034663|gb|AF456338.1|AF456338 Vibrio sp. LT21 16S ribo...	2872	0.0
gi|30387171|gb|AF539781.1| Pseudoalteromonas sp. RE1-12a 16...	2866	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2861	0.0
gi|30387172|gb|AF539782.1| Vibrio sp. RE1-3 16S ribosomal R...	2855	0.0

Phenon 29 (Strain: V4) – 1519 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|22799133|emb|AJ316200.1|VSP316200 Vibrio sp. R-3884 16S ...	2912	0.0
gi|14669621|gb|AF388391.1| Vibrio sp. 3d clone 3d2 16S ribo...	2892	0.0
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2890	0.0
gi|2832420|emb|AJ002566.1|MBAJ2566 Marine bacterium (isolat...	2890	0.0
gi|7007472|dbj|AB038026.1| Vibrio sp. OC25 gene for 16S rRN...	2872	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2864	0.0
gi|9968801|emb|AJ278881.1|VLE278881 Vibrio lentus 16S rRNA ...	2864	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2861	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2853	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2845	0.0

Phenon 32 (Strain: V639) - 1516 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|53830772|gb|AY750578.1| Vibrio harveyi strain S35 16S ri...	2944	0.0
gi|53830771|gb|AY750577.1| Vibrio harveyi strain S20 16S ri...	2942	0.0
gi|13183633|gb|AF319768.1|AF319768 Vibrio sp. BB4 16S ribos...	2898	0.0
gi|400496|emb|X74693.1|VCH16SRRA V.carchiariae (ATCC 35084T...	2870	0.0
gi|22859705|emb|AJ312382.1|VTR312382 Vibrio trachuri 16S rR...	2857	0.0
gi|53830769|gb|AY750575.1| Vibrio harveyi strain NCIMB1280T...	2835	0.0
gi|53830770|gb|AY750576.1| Vibrio harveyi strain LMG4404T 1...	2825	0.0
gi|15777743|gb|AY046956.1| Vibrio harveyi strain M4 16S rib...	2789	0.0
gi|400530|emb|X74723.1|VPR16SRRA V.proteolyticus (ATCC 1533...	2755	0.0
gi|21262135|emb|AJ316172.1|VSP316172 Vibrio sp. LMG 20546 1...	2740	0.0

Phenon 36 (Strain: V31) – 1523 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|38091109|emb|AJ514911.1|VHO514911 Vibrio hollisae partia...	2333	0.0
gi|38091107|emb|AJ514909.1|VHO514909 Vibrio hollisae partia...	2305	0.0
gi|400510|emb|X74707.1|VH16SRRNA V.hollisae(ATCC 33564T) ge...	2194	0.0
gi|7007478|dbj|AB038032.1| Photobacterium sp. HAR72 gene fo...	2190	0.0
gi|46911992|emb|CR378664.1| Photobacterium profundum SS9; s...	2183	0.0
gi|7007477|dbj|AB038031.1| Photobacterium sp. HAR23 gene fo...	2181	0.0
gi|47169774|dbj|AB179540.1| Photobacterium phosphoreum gene...	2175	0.0
gi|34100988|gb|AY292951.1| Photobacterium leiognathi strain...	2175	0.0
gi|54873690|gb|AY785249.1| Uncultured Vibrio sp. clone 2 16...	2171	0.0

Phenon 41 (Strain: V515) - 1517 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|20387077|emb|AJ316167.1|VSP316167 Vibrio sp. LMG 10953 1...	2829	0.0
gi|22799108|emb|AJ316170.1|VSP316170 Vibrio sp. LMG 20548 1...	2793	0.0
gi|20387081|emb|AJ440005.1|VSP440005 Vibrio corallilyticus ...	2777	0.0
gi|20387080|emb|AJ440004.1|VSP440004 Vibrio corallilyticus ...	2765	0.0
gi|21262134|emb|AJ316171.1|VSP316171 Vibrio sp. LMG 20536 1...	2748	0.0
gi|21667909|gb|AF513461.1| Vibrio sp. PH1 16S ribosomal RNA...	2720	0.0
gi|21307628|gb|AY034144.1| Vibrio sp. C33 16S ribosomal RNA...	2674	0.0
gi|14669619|gb|AF388389.1| Vibrio parahaemolyticus clone Vp...	2629	0.0
gi|2253387|gb|AF007115.1| Vibrio shiloi 16S ribosomal RNA g...	2577	0.0
gi|7007476|dbj|AB038030.1| Vibrio splendidus biovar II gene...	2553	0.0

Phenon 42 (Strain: V794) - 1505 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|22799107|emb|AJ316169.1|VSP316169 Vibrio sp. LMG 19270 1...	2791	0.0
gi|20387080|emb|AJ440004.1|VSP440004 Vibrio corallilyticus ...	2771	0.0
gi|38091112|emb|AJ514914.1|VSP514914 Vibrio fortis partial ...	2769	0.0
gi|22799106|emb|AJ316168.1|VSP316168 Vibrio sp. R-14968 16S...	2769	0.0
gi|22799108|emb|AJ316170.1|VSP316170 Vibrio sp. LMG 20548 1...	2759	0.0
gi|38091113|emb|AJ514915.1|VSP514915 Vibrio fortis partial ...	2757	0.0
gi|30315101|gb|AF500207.1| Vibrio sp. CJ11052 16S ribosomal...	2750	0.0
gi|38091111|emb|AJ514913.1|VSP514913 Vibrio fortis partial ...	2750	0.0
gi|47118310|dbj|BA000031.2| Vibrio parahaemolyticus DNA, ch...	2748	0.0
gi|38091114|emb|AJ514917.1|VSP514917 Vibrio fortis partial ...	2746	0.0

Phenon 43 (Strain: V781) - 1516 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|37359192|gb|AY147861.1| Photobacterium damselae ssp. d...	2983	0.0
gi|7077163|dbj|AB032015.1| Photobacterium damselae ssp. d...	2983	0.0
gi|7077161|dbj|AB032014.1| Photobacterium histaminum gene f...	2983	0.0
gi|37359191|gb|AY147860.1| Photobacterium damselae ssp. p...	2976	0.0
gi|37359190|gb|AY147859.1| Photobacterium damselae ssp. p...	2976	0.0
gi|37359189|gb|AY147858.1| Photobacterium damselae ssp. p...	2976	0.0
gi|37359188|gb|AY147857.1| Photobacterium damselae ssp. p...	2976	0.0
gi|37359187|gb|AY147856.1| Photobacterium damselae ssp. p...	2976	0.0
gi|5524748|emb|Y18496.1|PDA18496 Photobacterium damselae 16...	2938	0.0
gi|51971255|dbj|AB166849.1| Photobacterium damselae gene fo...	2926	0.0

Phenon 45 (Strain: V810) - 1520 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|14669624|gb|AF388394.1| Vibrio sp. 3d clone 3d8 16S ribo...	2904	0.0
gi|14669622|gb|AF388392.1| Vibrio sp. 3d clone 3d4 16S ribo...	2904	0.0
gi|27819281|gb|AY129277.1| Vibrio splendidus strain LP1 16S...	2902	0.0
gi|7331263|gb|AF242271.1|AF242271 Vibrio sp. Da2 16S riboso...	2894	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2876	0.0
gi|7007470|dbj|AB038024.1| Vibrio sp. EN280 gene for 16S rR...	2876	0.0
gi|22218067|emb|AJ244774.1|VSP244774 Vibrio sp. V4.BP.07 16...	2866	0.0
gi|9622367|gb|AF172840.1|AF172840 Vibrio aestuarianus 16S r...	2864	0.0
gi|11321102|gb|AF293974.1|AF293974 Unidentified bacterium 4...	2863	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2861	0.0

Phenon 46 (Strain: V632) - 1522 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2938	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2900	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2896	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2884	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2884	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2880	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2876	0.0
gi|7331266|gb|AF242274.1|AF242274 Vibrio sp. Sk1 16S riboso...	2872	0.0
gi|7331265|gb|AF242273.1|AF242273 Vibrio sp. Sr3 16S riboso...	2872	0.0
gi|30387171|gb|AF539781.1| Pseudoalteromonas sp. RE1-12a 16...	2855	0.0

Phenon 52 (Strain: V205) - 1501 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|32141043|gb|AY305857.1| Pseudoalteromonas sp. SM9913 16S...	2948	0.0
gi|7007482|dbj|AB038036.1| Pseudoalteromonas sp. SUR560 gen...	2934	0.0
gi|54303735|gb|AY745828.1| Pseudoalteromonas sp. NPO-JL-58 ...	2922	0.0
gi|5052003|gb|AF155038.1|AF155038 Pseudoalteromonas sp. ER7...	2922	0.0
gi|54303732|gb|AY745825.1| Pseudoalteromonas sp. JL-54 16S ...	2914	0.0
gi|11036396|dbj|AB000389.1| Pseudoalteromonas elyakovii gen...	2912	0.0
gi|45826479|gb|AY573035.1| Pseudoalteromonas sp. ARCTIC-P16...	2910	0.0
gi|13537359|dbj|AB051165.1| Pseudoalteromonas sp. KM-Y92-00...	2906	0.0
gi|10505262|gb|AF295034.1|AF295034 Pseudoalteromonas sp. EH...	2902	0.0
gi|50355672|dbj|AB183513.1| Pseudoalteromonas sp. T5304 gen...	2894	0.0

Phenon 53 (Strain: V859) - 1516 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|531551|emb|X76336.1|VF16SRR2 V.furnissii (ATCC 35016 T) ...	2666	0.0
gi|33392086|gb|AY345409.1| Bacterium K2-61 16S ribosomal RN...	2660	0.0
gi|29169866|emb|AJ550855.1|AEN550855 Allomonas enterica par...	2575	0.0
gi|400508|emb|X74704.1|VFUR16SRA V.furnisii (ATCC 35016T) g...	2545	0.0
gi|34493876|emb|AJ345063.1|VSP345063 Vibrio probioticus par...	2537	0.0
gi|14669616|gb|AF388386.1| Vibrio parahaemolyticus clone Vp...	2533	0.0
gi|14669617|gb|AF388387.1| Vibrio parahaemolyticus clone Vp...	2520	0.0
gi|14669618|gb|AF388388.1| Vibrio parahaemolyticus clone F4...	2518	0.0
gi|18034132|gb|AF410778.1|AF410778 Vibrio sp. NLEP97-1598 1...	2512	0.0
gi|18034133|gb|AF410779.1|AF410779 Vibrio sp. NLEP97-1599 1...	2510	0.0

Phenon 57 (Strain: V68) - 1,1510 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|21667911|gb|AF513463.1| Vibrio proteolyticus 16S ribosom...	2831	0.0
gi|400530|emb|X74723.1|VPR16SRRA V.proteolyticus (ATCC 1533...	2813	0.0
gi|23237775|dbj|AB081772.1| Vibrio sp. 442 gene for 16S rRN...	2795	0.0
gi|47118310|dbj|BA000031.2| Vibrio parahaemolyticus DNA, ch...	2773	0.0
gi|33392080|gb|AY345403.1| Bacterium K2-74 16S ribosomal RN...	2765	0.0
gi|47118311|dbj|BA000032.2| Vibrio parahaemolyticus DNA, ch...	2765	0.0
gi|30315101|gb|AF500207.1| Vibrio sp. CJ11052 16S ribosomal...	2759	0.0
gi|53830772|gb|AY750578.1| Vibrio harveyi strain S35 16S ri...	2742	0.0
gi|53830771|gb|AY750577.1| Vibrio harveyi strain S20 16S ri...	2740	0.0
gi|52696503|gb|AY738129.1| Vibrio campbellii strain 90-69B3...	2712	0.0

Phenon 58 (Strain: V776) - 1508 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|34100961|gb|AY292924.1| Vibrio fischeri strain SA1G 16S ...	2684	0.0
gi|34100985|gb|AY292948.1| Vibrio fischeri strain SI1D 16S ...	2676	0.0
gi|34100976|gb|AY292939.1| Vibrio fischeri strain CG101 16S...	2676	0.0
gi|34100987|gb|AY292950.1| Vibrio fischeri strain SL518 16S...	2668	0.0
gi|34100980|gb|AY292943.1| Vibrio fischeri strain ET401 16S...	2668	0.0
gi|34100979|gb|AY292942.1| Vibrio fischeri strain ET301 16S...	2668	0.0
gi|34100960|gb|AY292923.1| Vibrio fischeri strain ET101 16S...	2668	0.0
gi|34100983|gb|AY292946.1| Vibrio fischeri strain MJ101 16S...	2660	0.0
gi|34100982|gb|AY292945.1| Vibrio fischeri strain MDR7 16S ...	2660	0.0
gi|34100956|gb|AY292919.1| Vibrio fischeri 16S ribosomal RN...	2660	0.0

Phenon 59 (Strain: V48) – 1509 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|34100969|gb|AY292932.1| Vibrio logei strain 15382 16S ri...	2676	0.0
gi|34100980|gb|AY292943.1| Vibrio fischeri strain ET401 16S...	2648	0.0
gi|34100979|gb|AY292942.1| Vibrio fischeri strain ET301 16S...	2648	0.0
gi|34100961|gb|AY292924.1| Vibrio fischeri strain SA1G 16S ...	2648	0.0
gi|34100960|gb|AY292923.1| Vibrio fischeri strain ET101 16S...	2648	0.0
gi|34100985|gb|AY292948.1| Vibrio fischeri strain SI1D 16S ...	2640	0.0
gi|34100983|gb|AY292946.1| Vibrio fischeri strain MJ101 16S...	2640	0.0
gi|34100982|gb|AY292945.1| Vibrio fischeri strain MDR7 16S ...	2640	0.0
gi|34100976|gb|AY292939.1| Vibrio fischeri strain CG101 16S...	2640	0.0
gi|34100956|gb|AY292919.1| Vibrio fischeri 16S ribosomal RN...	2640	0.0
gi|34100987|gb|AY292950.1| Vibrio fischeri strain SL518 16S...	2633	0.0

Phenon 64 (Strain: V606) - 1509 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|39005|emb|X60412.1|AS16SRDNA Aeromonas sobria 16S rRNA g...	2956	0.0
gi|39007|emb|X60407.1|AS16SRDNC Aeromonas salmonicida ssp...	2900	0.0
gi|33392182|gb|AY345498.1| Unidentified bacterium clone W4-...	2896	0.0
gi|3646019|emb|AJ009860.1|HPI009860 Haemophilus piscium 16S...	2896	0.0
gi|39009|emb|X60417.1|AS16SRDNE Aeromonas sp. 16S rRNA gene...	2892	0.0
gi|39546471|gb|AY374113.1| Rainbow trout intestinal bacteri...	2886	0.0
gi|46399240|gb|AY532688.1| Aeromonas molluscorum strain 93M...	2884	0.0
gi|39004|emb|X60405.1|AS16SRDN Aeromonas salmonicida ssp....	2884	0.0
gi|39006|emb|X60406.1|AS16SRDNB Aeromonas bestiarum 16S rRN...	2884	0.0
gi|46399242|gb|AY532690.1| Aeromonas molluscorum strain 848...	2872	0.0

Phenon 69 (Strain: V615) - 1517 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|30315101|gb|AF500207.1| Vibrio sp. CJ11052 16S ribosomal...	2948	0.0
gi|47118310|dbj|BA000031.2| Vibrio parahaemolyticus DNA, ch...	2930	0.0
gi|18034133|gb|AF410779.1|AF410779 Vibrio sp. NLEP97-1599 1...	2924	0.0
gi|47118311|dbj|BA000032.2| Vibrio parahaemolyticus DNA, ch...	2922	0.0
gi|33392080|gb|AY345403.1| Bacterium K2-74 16S ribosomal RN...	2912	0.0
gi|3372817|gb|AF064637.1|AF064637 Vibrio sp. NAP-4 16S ribo...	2910	0.0
gi|14669616|gb|AF388386.1| Vibrio parahaemolyticus clone Vp...	2896	0.0
gi|14669618|gb|AF388388.1| Vibrio parahaemolyticus clone F4...	2888	0.0
gi|13183634|gb|AF319769.1| Vibrio sp. Ex25 16S ribosomal RN...	2861	0.0
gi|18034132|gb|AF410778.1|AF410778 Vibrio sp. NLEP97-1598 1...	2861	0.0

Phenon 75 (Strain: V798) - 1518 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|27752559|gb|AY174869.1| Vibrio sp. QY101 16S ribosomal R...	2977	0.0
gi|38091110|emb|AJ514912.1|VTA514912 Vibrio tasmaniensis pa...	2944	0.0
gi|22799127|emb|AJ316192.1|VSP316192 Vibrio sp. LMG 20012 1...	2940	0.0
gi|30027209|gb|AY262020.1| Vibrio sp. WYB 16S ribosomal RNA...	2924	0.0
gi|30027208|gb|AY262019.1| Vibrio sp. YWA 16S ribosomal RNA...	2924	0.0
gi|22799136|emb|AJ316203.1|VSP316203 Vibrio sp. R-1556 16S ...	2924	0.0
gi|18034663|gb|AF456338.1|AF456338 Vibrio sp. LT21 16S ribo...	2900	0.0
gi|30387171|gb|AF539781.1| Pseudoalteromonas sp. RE1-12a 16...	2894	0.0
gi|7007471|dbj|AB038025.1| Vibrio sp. TK327 gene for 16S rR...	2888	0.0
gi|30387172|gb|AF539782.1| Vibrio sp. RE1-3 16S ribosomal R...	2882	0.0

Phenon 77 (Strain: V170) - 1513 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|20338705|emb|AJ437192.1|VIC437192 Vibrio ichthyoenteri p...	2811	0.0
gi|1184961|gb|U46579.1|VSU46579 Vibrio scophthalmi 16S rRNA...	2805	0.0
gi|53913368|emb|AJ845014.1| Vibrio aestuarianus partial 16S...	2746	0.0
gi|22799115|emb|AJ316179.1|VSP316179 Vibrio sp. LMG 20023 1...	2742	0.0
gi|22090463|dbj|AB089204.1| Vibrio sp. No.6 gene for 16S rR...	2728	0.0
gi|53913369|emb|AJ845015.1| Vibrio aestuarianus partial 16S...	2726	0.0
gi|53913375|emb|AJ845021.1| Vibrio aestuarianus partial 16S...	2720	0.0
gi|53913367|emb|AJ845013.1| Vibrio aestuarianus partial 16S...	2714	0.0
gi|53913376|emb|AJ845022.1| Vibrio aestuarianus partial 16S...	2698	0.0
gi|53913374|emb|AJ845020.1| Vibrio aestuarianus partial 16S...	2676	0.0

Phenon 83 (Strain: V125) - 1507 nucleotides

Sequences producing significant alignments:	Score (bits)	E Value
gi|7007478|dbj|AB038032.1| Photobacterium sp. HAR72 gene fo...	2625	0.0
gi|34100984|gb|AY292947.1| Photobacterium leiognathi strain...	2605	0.0
gi|47169774|dbj|AB179540.1| Photobacterium phosphoreum gene...	2597	0.0
gi|34525809|emb|AJ551094.1|HSP551094 Hyphomicrobium sp. wp1...	2597	0.0
gi|54019681|emb|AJ746357.1| Photobacterium phosphoreum 16S ...	2559	0.0
gi|51971253|dbj|AB166847.1| Photobacterium phosphoreum gene...	2541	0.0
gi|7007477|dbj|AB038031.1| Photobacterium sp. HAR23 gene fo...	2537	0.0
gi|37719720|gb|AY341438.1| Photobacterium phosphoreum strai...	2527	0.0
gi|37719721|gb|AY341439.1| Photobacterium phosphoreum strai...	2512	0.0
gi|45664|emb|Z19107.1|PP16SRNAC Photobacterium phosphoeum 1...	2512	0.0

A4.5 VibEx7 probability matrix for the identification of *Vibrionaceae*

Data as % strains positive		*A. sobria HG7*	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leignathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
Arginine dihydrolase		86	1	1	57	1	99	99	99	99	75	99	99	1	3	96	99
Acid:	Arbutin	14	1	1	1	1	1	1	1	1	1	1	1	14	3	1	67
	Mannitol	99	1	1	1	99	1	1	1	33	1	1	99	99	99	98	50
	Salicin	14	1	1	1	1	1	1	1	1	1	1	1	57	20	1	75
	Sucrose	57	1	1	1	1	1	1	1	33	50	1	99	1	99	98	75
	Gentiobiose	14	1	1	1	99	1	1	1	1	1	1	1	99	1	1	75
Growth:	7% NaCl	1	99	99	1	99	86	99	1	99	50	99	67	29	99	93	25
	10% NaCl	1	25	99	1	1	1	1	1	1	1	1	1	1	99	3	1
Amylase		99	1	1	99	1	27	14	20	99	1	1	99	43	99	96	99
Voges Proskauer (Acetoin)		86	1	1	1	1	99	99	99	99	99	99	1	14	99	94	1
Gelatinase		71	1	1	99	1	24	33	20	1	25	1	67	1	99	77	50
Indole		86	75	1	1	1	3	1	1	1	1	1	99	1	99	93	99
IXP alkaline phosphatase		86	50	1	13	99	99	85	20	33	25	99	99	43	99	88	50
PNPG α-D-galactosidase		29	1	99	1	1	3	1	20	1	1	99	99	99	16	53	99
LGN γ-glutamyl transpeptidase		71	1	99	99	1	48	14	20	1	75	1	1	29	96	15	50
NPS sulphatase		14	1	99	1	99	1	1	1	1	25	1	1	14	40	1	1
Aesculin hydrolysis		71	1	1	20	50	31	1	1	1	25	1	33	99	10	63	99
Utilisation:	α-ketoglutarate	14	50	99	1	99	3	1	20	1	1	99	67	1	99	36	25
	Acetate	29	99	99	88	99	93	1	60	99	50	1	67	43	96	51	99
	Alanine	1	99	99	25	1	10	1	99	1	1	1	99	14	99	76	99
	Citrate	43	99	1	1	99	1	1	1	99	1	1	99	1	96	95	99
	Citrulline	1	1	1	38	1	1	1	1	1	1	1	1	1	20	16	99
	Galactose	57	99	1	1	99	93	28	60	99	25	99	99	71	13	71	99
	Gluconate	99	75	99	1	1	3	14	20	99	99	1	99	14	99	96	75
	Glucosamine	99	25	1	50	99	99	42	60	99	99	99	99	86	99	95	99
	Glucuronate	1	1	1	1	1	1	1	1	33	1	1	1	1	1	1	1
	Glycerol	99	75	99	99	99	99	42	60	99	75	99	99	1	99	95	99
	Histidine	29	50	1	88	1	1	1	1	1	1	1	1	1	99	93	50
	DL-3-hydroxybutyrate	1	1	1	1	1	1	1	1	1	25	1	1	1	1	1	99
	Hydroxyproline	1	1	1	1	99	1	1	1	1	1	1	1	1	99	1	75
	DL-lactate	14	99	99	99	1	79	1	1	1	99	99	99	29	99	91	99
	Lactose	1	1	1	1	1	1	1	20	1	1	99	1	1	1	1	1
	Propionate	14	99	1	1	1	1	1	1	1	1	1	67	1	99	6	99
	Putrescine	14	1	1	1	1	1	1	1	99	1	1	1	1	99	1	50
	Succinate	86	75	99	99	99	89	42	40	99	75	99	99	29	99	93	75
	Sucrose	57	1	1	1	1	1	1	1	33	50	1	99	1	99	96	75
Oxidase		99	99	99	99	99	99	99	99	99	99	1	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	99	1	1	1
Resistance:	0/129 10µg	99	1	99	38	1	1	14	1	1	1	1	1	1	70	3	1
	0/129 150µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Data as % strains positive		*A. sobria HG7*	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leiognathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
Ampicillin 10µg		57	1	1	1	99	65	14	20	1	75	1	1	1	99	91	1
Novobiocin 5µg		86	25	1	1	50	27	14	20	33	50	1	67	14	90	11	1
Carbenicillin 100µg		17	1	1	1	99	89	42	20	33	75	1	33	1	99	90	1
Lysine decarboxylase		99	1	1	38	99	62	57	1	99	1	1	1	1	99	1	25
Ornithine decarboxylase		29	1	1	1	1	1	1	1	1	1	1	1	1	66	1	1
Urease		14	1	1	1	99	99	99	1	1	25	1	1	1	1	1	1

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	No. strains	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
Arginine dihydrolase		1	95	1	1	99	99	1	1	99	99	1	1	15	1	1	1
Acid:	Arbutin	1	1	1	66	1	99	1	1	99	1	99	1	29	1	1	1
	Mannitol	50	99	99	99	85	99	40	87	99	99	33	99	94	83	60	99
	Salicin	75	5	1	66	1	99	40	40	99	1	99	1	79	4	1	1
	Sucrose	1	43	99	66	99	99	20	1	99	99	99	67	65	1	66	99
	Gentiobiose	50	1	1	33	14	1	60	90	11	1	1	1	95	9	1	1
Growth:	7% NaCl	99	95	58	99	99	99	1	70	99	99	99	67	99	99	33	67
	10% NaCl	1	10	1	66	1	50	1	1	56	90	66	33	5	4	16	1
Amylase		75	99	75	66	99	25	40	55	99	70	99	1	99	99	1	1
Voges Proskauer (Acetoin)		1	5	58	33	1	1	1	1	11	1	99	1	2	75	1	1
Gelatinase		99	99	67	33	99	25	20	1	44	30	99	33	97	83	16	1
Indole		99	95	99	1	85	99	20	1	99	99	1	33	97	99	1	1
IXP alkaline phosphatase		99	99	92	99	85	99	80	99	99	99	50	33	99	99	99	99
PNPG α-D-galactosidase		1	76	75	99	99	99	1	20	33	44	99	99	95	99	1	1
LGN γ-glutamyl transpeptidase		99	86	99	1	71	99	1	50	99	99	1	1	94	87	1	1
NPS sulphatase		1	99	8	1	71	25	60	99	11	1	1	1	60	61	16	33
Aesculin hydrolysis		99	99	22	99	99	99	80	99	88	10	99	33	95	99	66	1
Utilisation: α-ketoglutarate		99	99	99	1	99	1	1	1	99	99	1	1	97	1	1	1
	Acetate	25	99	99	33	99	99	80	1	99	90	99	33	89	99	83	99
	Alanine	50	99	67	1	99	99	20	1	99	99	33	1	99	99	33	1
	Citrate	99	95	92	99	99	99	1	60	99	99	66	1	95	99	16	1
	Citrulline	25	62	1	1	85	1	1	1	56	10	1	1	6	1	1	1

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	No. strains	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
	Galactose	1	99	83	66	85	99	40	99	99	99	1	99	73	74	1	1
	Gluconate	1	67	99	66	99	99	1	20	99	99	33	1	99	99	99	1
	Glucosamine	1	99	99	99	99	75	20	99	99	99	33	99	84	96	99	99
	Glucuronate	1	14	25	1	1	25	1	1	78	10	99	1	61	13	99	1
	Glycerol	99	99	99	99	99	1	40	90	99	99	99	1	85	83	1	1
	Histidine	1	10	67	1	71	99	1	1	89	99	1	1	8	1	1	1
	DL-3-hydroxybutyrate	1	1	1	1	14	1	40	1	78	80	1	1	1	1	1	1
	Hydroxyproline	1	10	1	1	71	1	1	1	11	1	1	1	50	1	1	1
	DL-lactate	75	99	99	66	99	99	1	1	99	99	66	99	99	99	66	99
	Lactose	1	1	1	1	28	50	1	1	1	1	1	1	1	1	1	1
	Propionate	1	95	58	1	99	75	1	1	99	99	66	1	94	78	16	1
	Putrescine	1	5	1	1	1	99	1	1	33	90	1	1	2	1	1	1
	Succinate	99	99	75	99	99	75	40	90	99	90	99	1	97	99	1	99
	Sucrose	1	43	99	66	99	99	60	1	89	99	99	67	65	4	66	99
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	33	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	25	19	8	66	28	25	1	1	44	60	99	1	82	91	16	67
	0/129 150µg	1	1	1	1	1	1	1	1	1	10	1	1	1	1	1	1
	Ampicillin 10µg	99	99	25	1	1	1	60	80	33	80	1	33	97	1	33	1
	Novobiocin 5µg	99	19	17	50	71	75	1	10	99	99	99	33	94	74	66	1
	Carbenicillin 100µg	99	99	33	1	1	1	99	80	11	80	1	33	95	1	33	1
Lysine decarboxylase		50	5	99	1	28	1	20	55	1	1	1	1	98	99	1	1
Ornithine decarboxylase		1	1	99	1	28	1	20	33	11	1	1	1	99	1	1	1
Urease		1	10	1	1	1	1	99	99	1	1	1	1	63	1	1	1

Data as % strains positive		*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test	*No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Arginine dihydrolase		99	1	84	99	1	99	1	1	99	1	1	99	6	99	1	1
Acid:	Arbutin	1	1	1	1	1	25	83	75	1	1	1	1	1	1	1	1
	Mannitol	75	99	99	99	86	99	99	99	67	1	1	99	99	1	99	99
	Salicin	1	1	80	1	1	99	92	50	1	99	1	17	18	1	1	1
	Sucrose	13	1	95	99	1	99	99	99	99	1	99	99	11	1	99	80
	Gentiobiose	1	1	21	1	1	99	99	25	1	50	1	1	1	1	1	40
Growth:	7% NaCl	38	1	74	80	86	99	99	99	99	1	1	67	99	1	80	80
	10% NaCl	1	1	5	40	14	99	75	1	67	1	1	1	89	1	1	1
Amylase		38	1	84	99	1	99	92	99	67	67	50	99	99	99	20	80

Data as % strains positive	*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test *No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Voges Proskauer (Acetoin)	1	1	1	99	1	1	1	1	17	1	1	1	1	1	1	1
Gelatinase	63	1	32	60	71	1	42	75	17	99	99	67	99	1	20	80
Indole	63	1	95	60	99	1	27	99	83	67	1	99	99	1	1	99
IXP alkaline phosphatase	75	99	89	40	86	99	75	99	67	67	40	83	99	99	99	99
PNPG α-D-galactosidase	50	1	99	99	43	75	75	1	1	99	1	67	17	1	99	99
LGN γ-glutamyl transpeptidase	38	99	99	1	99	60	67	50	99	67	99	83	99	1	80	99
NPS sulphatase	25	99	79	1	33	1	58	1	1	1	1	1	56	1	70	60
Aesculin hydrolysis	71	99	95	99	1	99	99	75	1	50	1	20	11	1	99	99
Utilisation: α-ketoglutarate	50	99	37	1	99	20	67	99	99	99	17	1	89	1	20	20
Acetate	88	50	99	60	99	99	92	75	99	67	1	67	99	1	90	80
Alanine	75	1	99	60	99	99	99	75	99	99	67	99	99	99	99	99
Citrate	13	99	99	40	99	99	92	99	99	99	99	99	94	1	80	99
Citrulline	1	1	11	1	1	20	83	1	83	1	1	1	17	1	60	99
Galactose	25	99	99	40	86	80	83	1	33	99	1	67	89	1	99	99
Gluconate	25	99	5	99	99	99	92	99	99	33	1	99	99	1	99	99
Glucosamine	13	99	99	20	99	99	99	99	99	99	50	83	99	50	70	99
Glucuronate	1	1	58	1	86	1	33	25	1	33	1	1	17	1	1	1
Glycerol	38	99	99	99	99	99	92	99	99	99	33	83	99	99	99	99
Histidine	1	1	89	1	99	99	99	99	99	99	33	17	99	1	80	1
DL-3-hydroxybutyrate	1	1	16	1	1	1	83	1	99	99	1	50	6	1	20	1
Hydroxyproline	1	1	1	1	1	1	25	1	1	1	1	83	83	1	1	1
DL-lactate	63	1	99	40	99	99	99	99	99	99	17	99	99	99	99	99
Lactose	1	1	99	40	1	20	8	1	1	99	1	1	1	1	10	20
Propionate	13	1	99	1	50	99	92	99	99	67	1	67	89	1	60	80
Putrescine	13	1	99	1	1	1	83	1	83	1	1	83	89	1	99	99
Succinate	50	99	95	60	99	80	99	99	83	99	1	99	89	50	99	99
Sucrose	1	1	99	99	1	99	99	99	99	1	83	99	11	1	99	80
Oxidase	99	99	99	1	99	80	99	99	99	99	99	99	99	99	99	99
Agarolysis	1	1	5	1	1	1	8	1	1	1	1	1	1	1	1	1
Resistance: 0/129 10µg	99	1	1	1	1	99	99	25	33	1	1	1	61	1	60	1
0/129 150µg	1	1	1	1	1	1	1	1	1	1	1	1	6	1	1	1
Ampicillin 10µg	13	99	1	40	14	1	8	50	1	1	50	1	94	50	10	1
Novobiocin 5µg	1	1	11	40	57	60	99	75	80	1	1	17	94	1	70	80
Carbenicillin 100µg	25	99	42	40	43	1	8	75	1	1	17	1	94	99	20	1
Lysine decarboxylase	1	99	22	60	99	1	1	1	1	1	1	1	99	1	1	1
Ornithine decarboxylase	13	99	1	1	99	1	1	1	1	1	1	1	99	1	1	1
Urease	1	1	1	1	1	1	1	1	1	1	1	1	17	1	1	1

Data as % strains positive		*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. salmonicida*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	*V. wodanis*	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	4	12	3	1	11	37	3	1	8	7	7	5	1	7	7	6
Arginine dihydrolase		1	99	99	1	9	99	99	1	99	99	1	1	1	99	99	17
Acid:	Arbutin	1	1	1	1	1	1	1	1	1	1	71	99	1	99	1	1
	Mannitol	1	99	33	99	10	97	99	1	71	99	71	1	1	99	99	99
	Salicin	1	1	1	1	1	8	1	1	25	99	57	99	1	99	1	33
	Sucrose	1	1	99	1	99	8	1	1	25	99	1	1	99	86	99	83
	Gentiobiose	1	1	1	1	1	1	1	1	1	99	28	40	1	40	1	1
Growth:	7% NaCl	1	99	67	99	36	71	33	1	75	57	85	20	1	99	99	99
	10% NaCl	1	99	33	1	1	11	1	1	1	1	1	1	1	99	99	83
Amylase		33	99	67	1	1	84	99	99	13	99	99	99	99	99	71	83
Voges Proskauer (Acetoin)		1	99	1	1	1	1	1	1	1	1	1	1	1	14	1	67
Gelatinase		1	99	67	1	1	89	99	1	13	99	99	80	1	14	99	99
Indole		1	75	1	1	1	95	66	1	63	99	99	1	99	99	86	83
IXP alkaline phosphatase		50	92	99	1	82	92	99	1	99	86	85	99	1	99	99	99
PNPG α-D-galactosidase		99	1	67	1	9	92	1	1	1	99	99	80	1	14	29	50
LGN γ-glutamyl transpeptidase		1	75	99	1	18	50	99	1	13	99	1	1	1	99	86	67
NPS sulphatase		1	1	1	1	1	79	1	1	99	1	1	1	1	86	86	50
Aesculin hydrolysis		1	1	33	1	99	97	99	1	99	99	42	25	1	99	1	83
Utilisation: α-ketoglutarate		99	99	67	1	9	99	99	99	38	1	99	80	1	99	99	99
	Acetate	99	99	67	99	45	76	99	1	99	86	85	99	99	99	99	99
	Alanine	99	99	99	1	1	97	99	1	99	99	99	99	1	99	99	99
	Citrate	99	99	99	99	91	97	99	99	99	99	99	99	99	99	99	99
	Citrulline	1	25	1	1	1	24	1	1	1	99	1	1	1	71	14	17
	Galactose	99	1	99	99	36	92	1	1	1	99	99	99	1	1	43	67
	Gluconate	99	99	67	99	82	84	1	1	99	99	99	99	1	99	99	99
	Glucosamine	99	99	99	1	99	89	66	99	99	99	99	80	1	99	86	99
	Glucuronate	25	1	1	1	64	21	1	1	1	86	85	80	1	1	1	17
	Glycerol	99	99	99	99	1	99	99	99	99	99	85	99	99	99	99	99
	Histidine	99	99	33	1	1	3	1	1	1	99	28	1	1	86	99	83
	DL-3-hydroxybutyrate	25	1	1	99	18	1	1	1	1	99	1	1	1	99	1	1
	Hydroxyproline	1	99	1	1	1	1	1	1	1	86	1	1	1	1	1	99
	DL-lactate	99	99	67	1	99	97	66	99	99	99	99	99	1	99	99	99
	Lactose	99	1	33	1	1	21	1	1	1	99	1	1	1	1	1	1
	Propionate	75	99	33	1	18	39	33	1	13	86	99	80	1	99	86	99
	Putrescine	1	99	1	99	1	5	1	1	1	29	1	1	1	99	14	17
	Succinate	99	99	99	99	91	99	99	99	99	86	99	99	99	99	86	99
	Sucrose	1	8	99	99	99	3	33	1	25	99	1	1	99	86	99	83
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	25	67	1	1	9	29	99	1	63	14	1	1	1	1	57	99
	0/129 150µg	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10µg	1	58	1	99	1	3	99	1	1	1	1	1	1	1	57	99
	Novobiocin 5µg	1	99	1	1	27	3	33	1	1	57	57	20	1	99	57	99
	Carbenicillin 100µg	1	1	33	99	1	18	99	1	1	1	1	1	1	1	71	99
Lysine decarboxylase		1	8	33	1	1	1	1	1	1	1	99	60	1	29	1	83
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	99	1	1	29	1	99
Urease		1	1	33	1	1	1	1	1	1	1	1	1	1	14	1	1

Data as % strains positive		Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46	Phenon 52
Test	*No. strains*	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6	3
Arginine dihydrolase		99	99	83	88	99	99	99	99	99	50	99	99	99	99	99	33
Acid:	Arbutin	99	1	83	99	1	21	1	1	1	1	1	1	9	1	1	1
	Mannitol	99	99	67	99	99	77	99	99	88	50	1	50	91	25	99	1
	Salicin	1	1	99	99	1	57	1	1	99	1	1	1	27	1	1	1
	Sucrose	99	99	99	99	99	99	99	99	12	1	99	99	82	25	1	1
	Gentiobiose	86	1	83	99	25	57	1	1	1	1	1	1	9	1	1	1
Growth:	7% NaCl	63	25	83	99	99	43	99	88	88	50	83	75	91	25	99	99
	10% NaCl	1	1	1	1	1	1	31	1	1	17	1	1	55	1	1	67
Amylase		63	99	99	99	99	99	93	94	99	67	99	99	73	50	99	67
Voges Proskauer (Acetoin)		25	1	1	1	1	1	1	1	1	1	17	99	9	1	1	67
Gelatinase		13	1	99	99	75	99	99	99	94	1	67	50	55	25	99	99
Indole		99	99	99	88	1	93	99	99	99	50	33	1	91	25	99	1
IXP alkaline phosphatase		99	25	83	99	99	99	99	99	99	1	83	99	99	99	99	67
PNPG α-D-galactosidase		99	99	17	99	99	99	99	88	99	99	99	1	1	1	1	33
LGN γ-glutamyl transpeptidase		50	99	50	99	1	14	99	82	94	67	99	99	55	25	17	1
NPS sulphatase		88	1	17	88	99	93	99	99	99	1	1	1	55	50	50	1
Aesculin hydrolysis		99	99	67	99	1	99	99	99	99	40	50	1	99	1	99	99
Utilisation: α-ketoglutarate		38	1	99	99	25	99	99	69	99	99	1	1	9	25	99	1
	Acetate	38	75	99	88	99	57	99	88	99	99	99	50	73	99	99	67
	Alanine	13	99	83	99	50	99	99	94	99	83	99	99	99	99	1	33
	Citrate	88	99	83	99	50	99	99	99	94	99	67	75	91	99	1	33
	Citrulline	1	25	40	88	1	1	99	1	99	50	67	25	55	1	1	1
	Galactose	99	99	83	99	99	99	99	88	99	99	99	1	9	50	99	1
	Gluconate	50	1	83	99	99	79	99	94	99	33	83	25	99	75	99	33
	Glucosamine	99	99	99	99	99	99	99	94	99	99	1	99	99	99	99	67
	Glucuronate	13	1	1	1	99	1	6	53	99	17	1	1	1	1	1	1
	Glycerol	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	67
	Histidine	1	1	33	13	1	1	18	12	1	67	17	1	9	1	1	99
	DL-3-hydroxybutyrate	1	75	67	1	1	1	1	6	6	33	1	1	1	1	1	33
	Hydroxyproline	1	1	99	1	1	1	1	1	1	1	83	1	18	1	1	33
	DL-lactate	99	99	99	99	25	99	99	94	99	99	99	99	99	75	99	33
	Lactose	1	25	1	1	1	1	1	6	6	1	1	1	1	1	1	1
	Propionate	1	99	67	99	1	7	68	88	94	83	99	99	91	75	1	67
	Putrescine	99	1	17	1	1	1	6	1	1	33	1	1	9	1	1	1
	Succinate	99	99	83	99	99	93	99	99	99	99	99	99	91	99	99	1
	Sucrose	99	99	99	99	99	99	99	99	18	17	99	99	82	25	1	99
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		13	1	1	1	1	1	1	1	6	1	1	1	9	1	1	1
Resistance:	0/129 10μg	1	1	1	1	1	14	1	12	1	1	1	1	1	1	33	67
	0/129 150μg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10μg	1	1	1	1	1	1	1	1	1	1	99	75	9	25	1	33
	Novobiocin 5μg	1	50	17	1	1	1	18	1	1	1	67	99	18	1	1	99
	Carbenicillin 100μg	1	1	1	13	1	21	6	1	65	1	99	99	9	25	1	1
Lysine decarboxylase		25	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1
Urease		1	1	50	1	1	1	6	1	1	1	1	1	1	1	1	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Arginine dihydrolase		11	99	25	1	80	99
Acid:	Arbutin	1	1	1	1	1	1
	Mannitol	99	99	99	1	99	1
	Salicin	13	1	1	7	20	1
	Sucrose	99	1	1	14	99	1
	Gentiobiose	1	25	1	93	1	1
Growth:	7% NaCl	89	75	25	21	20	1
	10% NaCl	11	1	1	1	1	1
Amylase		1	99	1	1	80	99
Voges Proskauer (Acetoin)		1	1	1	1	99	1
Gelatinase		11	75	1	21	1	99
Indole		99	75	1	7	99	1
IXP alkaline phosphatase		99	99	99	93	1	33
PNPG α-D-galactosidase		1	99	99	99	80	99
LGN γ-glutamyl transpeptidase		33	1	1	1	1	66
NPS sulphatase		44	99	50	99	20	16
Aesculin hydrolysis		56	50	1	93	1	1
Utilisation:	α-ketoglutarate	1	25	1	1	80	33
	Acetate	99	50	50	7	99	99
	Alanine	1	99	1	1	80	1
	Citrate	89	75	99	99	99	50
	Citrulline	1	1	1	1	1	1
	Galactose	1	99	99	99	60	83
	Gluconate	1	75	25	7	99	1
	Glucosamine	99	99	99	99	20	99
	Glucuronate	44	75	1	1	1	1
	Glycerol	44	99	99	99	99	99
	Histidine	1	1	1	1	60	50
	DL-3-hydroxybutyrate	89	1	1	1	60	1
	Hydroxyproline	11	1	1	1	1	1
	DL-lactate	99	75	1	1	60	99
	Lactose	1	75	99	99	20	1
	Propionate	99	1	1	1	40	1
	Putrescine	1	1	1	1	99	1
	Succinate	78	99	99	93	99	99
	Sucrose	99	1	1	7	80	1
Oxidase		99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1
Resistance:	0/129 10µg	89	1	1	1	40	1
	0/129 150µg	1	1	1	1	1	1
	Ampicillin 10µg	11	75	50	14	1	1
	Novobiocin 5µg	89	1	1	1	50	1
	Carbenicillin 100µg	11	99	99	71	1	1
Lysine decarboxylase		1	99	99	93	1	1
Ornithine decarboxylase		1	1	1	1	1	1
Urease		1	75	99	99	80	1

A4.6 Summary of PCR Systems Evaluated

Summary of the gene target, primers, reaction mixture, cycle conditions and expected amplicon size for 15 PCR systems evaluated as originally described.

No.	Source
1	Arias *et al.* (1995) – 23S rRNA nested *Vibrio vulnificus*
2	Brasher *et al.* (1998) – Shellfish multiplex, including *cth* gene of *Vibrio vulnificus*
3	Coleman *et al.* (1996) PCR of cytolysin/haemolysin gene of *Vibrio vulnificus* biotypes 1 & 2
4	Coleman & Oliver (1996) PCR optimisation of *cth* cytolysin/haemolysin gene of *V. vulnificus*
5	Conejero & Hedreyda (2003) *Vibrio harveyi tox*R gene
6	Dalla Valle *et al.* (2002) *Photobacterium damselae* ssp. *piscicida*, unique RAPD
7	González *et al.* (2003) *Vibrio anguillarum rpoN* gene
8	Hirono *et al.* (1996) *Vibrio anguillarum vah*1 haemolysin gene
9	Iwamoto *et al.* (1995) *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment
10	Kim & Jeong (2001) *Vibrio vulnificus* 16S rRNA gene, tri-primer
11	Kvitt *et al.* (2002) *Photobacterium damselae ssp. piscicida* 16S rRNA gene
12	Lee *et al.* (1998) *Vibrio vulnificus vvh* cytolysin/haemolysin gene
13	Oakey *et al.* (2003) *Vibrio harveyi* 16S rRNA gene
14	Osorio *et al.* (1999) *Photobacterium damselae* 16S rRNA, hemi-nested
15	Osorio *et al.* (2000) *Ph. damselae* subspecies, multiplex PCR for *ureC* and 16S rRNA

Arias *et al.* (1995) – *Vibrio vulnificus* 23S rRNA gene

Primers	
118V	CCGAATGGGGAAACCCA
1037R	CGACAAGGAATTTCGCTAC
Dvu9V	GACCGAATACGGTCACC
Dvu45R	AAGATACTTGTAACCCATC

First Round – Universal 23S primers	
118V	3′-CCGAATGGGGAAACCCA-5′ 17mer 112 to 130 (sense primer)
1037R	3′-CGACAAGGAATTTCGCTAC-5′ 19mer 1930 to 1948 (antisense primer)
1828bp product	
Second Round – *V. vulnificus* specific	
Dvu9V	3′-GACCGAATACGGTCACC-5′ 17mer 129 to 147 *E. coli* 23S (sense primer)
Dvu45R	3′-AAGATACTTGTAACCCATC-5′ 19mer 1169 to 1190 *E. coli* 23S (antisense primer)
978bp product	

Reaction mix	
Volume:	50µl
Primer:	5µM
Polymerase:	0.03U Taq (Promega)
Buffer:	5µl
25mM $MgCl_2$:	5µl
dNTPs:	0.2mM each
Template DNA:	20ng

Cycle parameters	
94°C	1min.
35 cycles	
94°C	20sec.
52°C	30sec. 52°C first round (23S) 52 to 54°C second round
72°C	45sec. with 15sec. increment with each repeat

Brasher *et al.* (1998) – *Vibrio vulnificus cth* cytolysin/haemolysin gene

Primers	
L-CTH	TTCCAACTTCAAACCGAACTATGAC
R-CTH	GCTACTTTCTAGCATTTTCTCTGC

Reaction mix:	
Volume:	100µl
Primer:	1µM each
Polymerase:	2.5U AmpliTaq (PE)
Buffer:	1X reaction buffer to give: 50mM Tris-HCl, pH8.9
KCl:	50mM
$MgCl_2$:	2.5mM
dNTPs:	200µM each
Template DNA:	1µg DNA

Cycle parameters	
94°C	3min.
30 cycles	
94°C	1min.
55°C	2min.
72°C	3min.
72°C	10min. final extension

Coleman *et al.* (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Primers	
Vv-Primer 1	CGCCGCTCACTGGGGCAGTGGCTG
Vv- Primer 2	GCGGGTGGTTCGGTTAACGGCTGG

Primer characteristics	
Primer 1	5′-CGCCGCTCACTGGGGCAGTGGCTG-3′ 24mer
Primer 2	5′-GCGGGTGGTTCGGTTAACGGCTGG-3′ 24mer
344bp product	

Reaction mix	
Volume:	40µl
Primer:	0.45mM each primer
Polymerase:	1.25U AmpliTaq (PE)
Buffer:	10X Buffer II (PE)
$MgCl_2$:	6.4 or 2.4µl 25mM – biotype 1 4mM and biotype 2 1.5mM-2mM
dNTPs:	250mM each
Template DNA:	either 5µl sample of DNA extracted from oyster or eel

Cycle parameters	
34 cycles for eel and 50 cycles for oyster	
94°C	30sec.
64.5°C	30sec.
1 cycle	
94°C	30sec.
64.5°C	10min.

Coleman & Oliver (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Primers	
VvPCR1	CGCCGCTCACTGGGGCAGTGGCTG
VvPCR2	CCAGCCGTTAACCGAACCACCCGC

Primer characteristics
VvPCR1 5′-CGCCGCTCACTGGGGCAGTGGCTG-3′ 24mer
VvPCR2 5′-CCAGCCGTTAACCGAACCACCCGC –3′ 24mer
388bp product

Reaction mix:	
Volume:	50µl
Primer:	0.36µM each primer
Polymerase:	1.25U AmpliTaq (Perkin-Elmer)
Buffer:	10µl of 5X buffer
	7.5mM $MgCl_2$, pH9.0 (best for culture)
	12.5mM $MgCl_2$, pH 9.0 (best for non-culture)
dNTPs:	250µM each
Template DNA:	5µl bacterial cells

Cycle parameters:	
34 cycles	
94°C	30sec.
65°C	30sec.
1 cycle	
94°C	30sec.
65°C	10min.

Conejero & Hedreyda (2003) - *Vibrio harveyi toxR* gene

Primers	
Vh-toxR-F	TTCTGAAGCAGCACTCAC
Vh-toxR-R	TCGACTGGTGAAGACTCA

Primer characteristics			
Vh-toxR-F	5′-TTCTGAAGCAGCACTCAC-3′	18mer	159-172 *toxR* gene
Vh-toxR-R	5′-TCGACTGGTGAAGACTCA-3′	18mer	549-533 *toxR* gene
390bp product			

Reaction mix (pers. comm. C.T. Hedreyda)	
Volume:	25 µl
Primer:	0.5 uM each
Polymerase:	0.025 U/µl Taq DNA polymerase (Invitrogen)
Buffer:	"1x PCR buffer"
$MgCl_2$:	3 mM
dNTPs:	200 µM each
Template DNA:	50-100µg genomic purified DNA

Cycle parameters
94ºC for 5 min
30 cycles
94ºC 1min
63ºC 1min
72ºC 1min
72°C 5min. final extension

Dalla Valle *et al.* (2002) – *Photobacterium damsela*e ssp. *piscicida*, unique RAPD

Primers	
PdP1	CCCCTAATAGGCCCCATTCTT
PdP2	CCGTCCCCTTGATGGTGGCACGA
PdP5	TTGCGATGACGACAGCTATG
PdP6	TCTGGCGACCAACAAACGTA

First Round	
PDP1	5′-CCCCTAATAGGCCCCATTCTT-3′
PDP2	5′-CCGTCCCCTTGATGGTGGCACGA-3′
474bp product	
Second Round	
PDP5	5′-TTGCGATGACGACAGCTATG-3′
PDP6	5′-TCTGGCGACCAACAAACGTA-3′
323bp product	

Reaction mix	
Volume:	50µl
Primer:	0.25µM each primer
Polymerase:	0.75U Taq
Buffer:	10mM Tris-HCl, pH 8.3
KCl:	50mM
$MgCl_2$:	1.2mM
Triton X-100:	0.1% (v/v)
dNTPs:	100µM each
Template DNA:	Either 5µl sample or 100ng (Bead Beater, 50µl 1:10 tissue homogenate with QIAGEN DNeasy)

Cycle parameters:	
40 cycles	
94°C	45sec.
60°C	45sec.
72°C	45sec. PdP1/2 (First round) or 30sec. PdP5/6 (Second round)

González *et al.* (2003) - *Vibrio anguillarum rpoN* gene

Primers	
rpoN-ang5'	GTTCATAGCATCAATGAGGAG
rpoN-ang3'	GAGCAGACAATATGTTGGATG

Primer characteristics
PCR - factor σ^{54} *rpoN* gene
rpoN-ang5' 5′-GTTCATAGCATCAATGAGGAG-3′ 21mer Position 181-201 in *V. anguillarum rpoN* gene
rpoN-ang3' 5′-GAGCAGACAATATGTTGGATG-3′ 21mer 693-714 in *V. anguillarum rpoN* gene
519bp product

Reaction mix: Ready-To-Go-Beads	
Volume:	25µl
Primer:	150pM each
Polymerase:	2 to 2.5U pure Taq DNA polymerase
Buffer:	10mM Tris-HCl pH 9.0
$MgCl_2$:	1.5mM
dNTPs:	200µM each
KCl:	50mM
Template DNA:	

Cycle parameters	
95°C	3min.
30 cycles	
95°C	1min.
62°C	1min.
72°C	40sec.
72°C	5min. final extension

Hirono *et al.* (1996) - *Vibrio anguillarum vah*1 haemolysin gene

Primers	
VaH1-P1	ACCGATGCCATCGCTCAAGA
VaH1-P2	GGATATTGACCGAAGAGTCA

Primer characteristics	
VAH1-P1	5′-ACCGATGCCATCGCTCAAGA-3′
VAH1-P2	5′-GGATATTGACCGAAGAGTCA-3′
~500bp product	

Reaction mix	
Volume:	
Primer:	
Polymerase:	Data not published
Buffer:	
dNTPs:	
Template DNA:	10ng -10pg; product from 10pg can be seen on gel

Cycle parameters (pers. comm. I. Hirono)	
30 cycles	
94°C	30sec.
55°C	30sec.
72°C	60sec.

Iwamoto *et al.* (1995) - *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment

Primers	
Pst I-1a	TGCGCTGACGTGTCTGAATT
Pst I-1b	AAGCAGCGATGACAAGCAGT

Primer Characteristics	
Pst I-1a	5′-TGCGCTGACGTGTCTGAATT-3′
Pst I-1b	5′-AAGCAGCGATGACAAGCAGT-3′
417 bp product	

Reaction mix:	
Volume:	50 µl
Primer:	1.25µl (20µM stock solution)
Polymerase:	0.25ul AmpliTaq (5U/µl)
Buffer:	10X Buffer: 100mM Tris.HCl, pH 8.3, 500mM KCl, 15mM $MgCl_2$, 0.01% gelatin
$MgCl_2$:	1.5mM
dNTPs:	4µl dNTP mixture 2.5µM each in 50µl
Template DNA:	5µl pre-treated at 97°C for 7 minutes.

Cycle parameters	
97°C	7 min.
35 cycles	
94°C	30sec.
60°C	30sec.
72°C	60sec.
72°C	5min. final extension

Kim & Jeong (2001) – *Vibrio vulnificus* 16S rRNA gene, tri-primer

Primers	
TriVib 1	GTGGTAGTGTTAATAGCACT
TriVib 2	TCTAGCGGAGACGCTGGA
TriVib 3R	GCTCACTTTCGCAAGTTGGCC

Primer Characteristics
Vib 1 5′-GTGGTAGTGTTAATAGCACT-3′ 454 TO 473 *E. coli* (sense primer)
Vib 2 5′-TCTAGCGGAGACGCTGGA-3′ 1006 to 1023 *E. coli* (sense primer)
Vib 3R 5′-GCTCACTTTCGCAAGTTGGCC-3′ 1278 TO 1258 *E. coli* (antisense primer)
273bp and 825bp products with *V. vulnificus* Type A DNA
273bp product with *V. vulnificus* Type B DNA
825bp product from other bacteria (test negative)

Reaction mix	
Volume:	50µl
Primer:	1µM each
Polymerase:	1.25U AmpliTaq (Perkin Elmer)
Buffer:	10mM Tris-HCl, pH 8.3
KCl:	50mM
$MgCl_2$:	1.5mM
Gelatin:	0.001% (w/v)
Tween-20:	0.5%
dNTPs:	200µM each
Template DNA:	100ng DNA

Cycle parameters	
35 cycles	
94°C	30sec.
55°C	30sec.
72°C	30sec.

Kvitt *et al.* (2002) - *Photobacterium damsela*e ssp. *piscicida* 16S rRNA gene

Primers	
Pdp-P1f	TAGTGTAGTTAACACCTGCAC
Pdp-P2r	ACACTCGAATCTCTTCAAGT
Pdp-P3f	AAGCTTGAAGAGATTCGAGTG
Pdp-P4r	CCACCTCGCGGTCTTGCTGC

Primer characteristics	
P1f	5′-TAGTGTAGTTAACACCTGCAC-3′ 444-452
P2r	5′-ACACTCGAATCTCTTCAAGT-3′ 982-1001
570bp product	

Primer characteristics	
P3f	5′-AAGCTTGAAGAGATTCGAGTG-3′ 980-1000
P4r	5′-CCACCTCGCGGTCTTGCTGC-3′ 1231-1250
270bp product	

Reaction mix	
Volume:	50µl
Primer:	0.25µM each
Polymerase:	1U Taq
Buffer:	10mM Tris-HCl, pH 8.8
KCl:	50mM
$MgCl_2$:	1.5mM
Triton X-100:	0.1% (v/v)
dNTPs:	0.2mM each
Template DNA:	50-100ng

Cycle parameters	
94°C	3min.
30 cycles	
94°C	1min.
64°C	1min.
72°C	90sec.
72°C	5min. final extension

Lee *et al.* (1998) - *Vibrio vulnificus vvh* cytolysin/haemolysin gene

Primers	
Vv-P1	GACTATCGCATCAACAACCG
Vv-P2	AGGTAGCGAGTATTACTGCC
Vv-P3	GCTATTTCACCGCCGCTCAC
Vv-P4	CCGCAGAGCCGTAAACCGAA

Primer characteristics	
Vv-P1	5′-GACTATCGCATCAACAACCG-3′ sense
Vv-P2	5′-AGGTAGCGAGTATTACTGCC-3′ antisense
704bp product	
Vv-P3	5′-GCTATTTCACCGCCGCTCAC-3′ sense
Vv-P4	5′-CCGCAGAGCCGTAAACCGAA-3′ antisense
222bp product	

Reaction mix (nested):	
Volume:	50µl
Primer:	0.5µM each
Polymerase:	2U Taq (Boehringer)
Buffer:	none specified
$MgCl_2$:	3mM external primers 4mM internal primers
dNTPs:	250µM each
Template DNA:	5µl first round added to second round

Cycle parameters	
95°C	3min.
50 cycles	
95°C	30sec.
57°C	30sec. external 59°C internal
72°C	1min.
72°C	10min. final extension

Oakey *et al.* (2003) – *Vibrio harveyi* 16S rRNA gene

Primers	
VH-1	AACGAGTTATCTGAACCTTC
VH-2	GCAGCTATTAACTACACTACC

Primer characteristics
VH-1 5′-AACGAGTTATCTGAACCTTC-3′ Bases 59-87 *E. coli* J01859 numbering
VH-2 5′-GCAGCTATTAACTACACTACC-3′ Bases 453-473 *E. coli* J01859 numbering
413 bp product

Reaction mix:	
Volume:	50µl
Primer:	10 pmol each primer
$MgCl_2$:	3mM
Polymerase:	1U (MBI Fermentas)
Buffer:	10X buffer supplied (MBI Fermentas)
dNTPs:	200µM each
Template DNA:	50 to 250 ng

Cycle parameters
94°C 2min.
40 cycles
94°C 1min.
65°C 1min.
72°C 2 min.
72°C 5min. final extension

Osorio *et al.* (1999) - *Photobacterium damsela*e 16S rRNA gene, hemi-nested

Primers	
Car1	GCTTGAAGAGATTCGAGT
Car2	CACCTCGCGGTCTTGCTG
Nest-car1	GGTCTTGCTGCCCTCTG
CARSOND	TACAATGGCATATACAGA

Primer characteristics
First round
Car1 5′-GCTTGAAGAGATTCGAGT-3′ 18mer 1016-1033 *E. coli*
Car2 5′-CACCTCGCGGTCTTGCTG-3′ 18mer 1266-1283 *E. coli*
267bp product
Second round
Car1 as above
Nest-car1 5′-GGTCTTGCTGCCCTCTG-3′ 17mer 1259-1275 *E. coli*
259bp product
Probe
CARSOND 5′-TACAATGGCATATACAGA-3′ 18mer 1245-1262 *E. coli*

Reaction mix (hemi-nested):	
Volume:	100µl
Primer:	0.5µg each
Polymerase:	2U Taq (PE)
Buffer:	10µl 10X Taq polymerase buffer (Perkin Elmer)
$MgCl_2$:	4µl of 50mM
dNTPs:	200µM each
Template DNA:	0.5µl first round added to second round

Cycle parameters	
95°C	4min.
30 cycles	
95°C	1min.
55-65°C	1min.
72°C	20sec.
72°C	5min. final extension

Osorio *et al*. (2000) - *Photobacterium damsela*e ssp. *damsela*e and *piscicida*, *ur*eC and 16S rRNA genes, multiplex format

Primers	
Ure-5′	TCCGGAATAGGTAAAGCGGG
Ure-3′	CTTGAATATCCATCTCATCTGC
Car1	GCTTGAAGAGATTCGAGT
Car2	CACCTCGCGGTCTTGCTG

Primer characteristics	
UreC PCR	
Ure-5′	5′-TCCGGAATAGGTAAAGCGGG-3′ 20mer
Ure-3′	5′-CTTGAATATCCATCTCATCTGC-3′ 22mer
448bp product	
16S PCR	
Car1	5′-GCTTGAAGAGATTCGAGT-3′ 18mer 1016-1033 *E. coli* as above
Car2	5′-CACCTCGCGGTCTTGCTG-3′ 18mer 1266-1283 *E. coli* as above
267bp product	

Reaction mix (multiplex):	
Volume:	100µl
Primer:	160pM each
Polymerase:	2U Taq (PE)
Buffer:	1X Taq polymerase buffer
$MgCl_2$:	2mM
dNTPs:	200µM each
Template DNA:	100ng

Cycle parameters	
95°C	4min.
30 cycles	
95°C	1min.
60°C	1min.
72°C	40sec.
72°C	5min. final extension

A4.7 Summary of BLASTn Analysis of PCR Primer Sequences

The primer name and sequence in 5′ to 3′ orientation are shown followed by a summary of the first 10 matches of the BLASTn sequence similarity search. The bit score S, followed by the E values are shown at the end of each line.

Arias *et al.* (1995) – *Vibrio vulnificus* 23S rRNA gene

Primer 118Vc
TGGGTTTCCCCATTCGG

28848563	AF142677.4	Bacillus megaterium plasmid pBM400, c	34.2	1.5
32263857	AY312056.1	Paracoccus sp. Ol18 plasmid pOL18 rrn	34.2	1.5
31789387	AY281353.1	Uncultured Acidobacteria bacterium cl	34.2	1.5
31789363	AY281352.1	Uncultured Acidobacteria bacterium cl	34.2	1.5
9657351	AE004341.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9657344	AE004340.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9657074	AE004319.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9655148	AE004157.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9654802	AE004126.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9654718	AE004119.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9654561	AE004106.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
9654454	AE004097.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
27359304	AE016813.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
27360771	AE016801.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
27360459	AE016800.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5

Primer 1037Rc
GTAGCGAAATTCCTTGTCG

28848563	AF142677.4	Bacillus megaterium plasmid pBM400, c	38.2	0.096
32263857	AY312056.1	Paracoccus sp. Ol18 plasmid pOL18 rrn	38.2	0.096
38374131	M62806.2 FBCRRL	Flexibacter flexilis large sub...	38.2	0.096
37912923	AY372454.1	Uncultured marine gamma proteobact...	38.2	0.096
31790346	AY281358.1	AY281357S2 Uncultured Acidobacteria ba	38.2	0.096
31789464	AY281356.1	Uncultured Acidobacteria bacterium cl	38.2	0.096
31789410	AY281354.1	Uncultured Acidobacteria bacterium cl	38.2	0.096
31789387	AY281353.1	Uncultured Acidobacteria bacterium cl	38.2	0.096
31789363	AY281352.1	Uncultured Acidobacteria bacterium cl	38.2	0.096
34105231	AE016924.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
34104646	AE016922.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
34104365	AE016921.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
34102743	AE016915.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
34102185	AE016913.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
34101612	AE016911.1	Chromobacterium violaceum ATCC 124...	38.2	0.096
9657351	AE004341.1	Vibrio cholerae O1 biovar eltor str...	38.2	0.096
9657344	AE004340.1	Vibrio cholerae O1 biovar eltor str...	38.2	0.096
9657074	AE004319.1	Vibrio cholerae O1 biovar eltor str...	38.2	0.096

Primer Dvu9V
CCACTGGCATAAGCCAG

9655054	AE004148.1	Vibrio cholerae O1 biovar eltor str..	34.2	1.5
27359304	AE016813.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
27360771	AE016801.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
27360459	AE016800.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
27359902	AE016798.1	Vibrio vulnificus CMCP6 chromosome...	34.2	1.5
11863362	AL138960.16	Human DNA sequence from clone RP...	34.2	1.5
58801792	AC131033.7	Mus musculus chromosome 15, clone ...	34.2	1.5
37509038	BA000038.2	Vibrio vulnificus YJ016 DNA, chromos	34.2	1.5
37509034	BA000037.2	Vibrio vulnificus YJ016 DNA, chromos	34.2	1.5
2244695	X87294.1 VV23S2138	V.vulnificus 23S rRNA gene, bi	34.2	1.5
2244696	X87293.1 VV23S2756	V.vulnificus 23S rRNA gene, bi	34.2	1.5
606868	U10951.1 VVU10951	Vibrio vulnificus ATCC 27562 23S	34.2	1.5
57863189	AC112935.24	Mus musculus chromosome 15, clone RP	34.2	1.5
40217708	AC117662.13	Mus musculus chromosome 16, clone...	32.2	5.9

Primer Dvu45R
CTACCCAATGTTCATAGAA

2244695	X87294.1 VV23S2138	V.vulnificus 23S rRNA gene, bi	38.2	0.096
2244696	X87293.1 VV23S2756	V.vulnificus 23S rRNA gene, bi	38.2	0.096
71404871	DQ099432.2	Human adenovirus B strain Guangzhou01	36.2	0.38
71382236	DQ105654.2	Human adenovirus B strain Guangzhou02	36.2	0.38
40457576	AF321310.2	Human adenovirus type 7 strain 200...	36.2	0.38
40457571	AF321305.2	Human adenovirus type 7 strain ATC...	36.2	0.38
40737116	AY509991.1	Human adenovirus type 16 strain AT...	36.2	0.38
40737115	AY509990.1	Human adenovirus type 3+7 strain T...	36.2	0.38

```
16972748   AL136085.12  Human DNA sequence from clone RP...   36.2    0.38
762955     Z48954.1 MAV7HE3  Human adenovirus type 7 E3 reg.  36.2    0.38
22657462   AC131237.3  Homo sapiens 3 BAC RP11-47L11 (Ros...  36.2    0.38
16519423   AB073632.1  Human adenovirus type 16 genes fo...   36.2    0.38
16303269   AB073222.1  Human adenovirus type 21 genes fo...   36.2    0.38
```

Brasher *et al.* (1998) – *Vibrio vulnificus cth* cytolysin/haemolysin gene

Primer L-CTH
TTCCAACTTCAAACCgAACTATgAC

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    50  7e-05
AY046900 AY046900 Vibrio vulnificus cytolysin (vvhA) gene, p...    50  7e-05
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    50  7e-05
AF376033 AF376033 Vibrio vulnificus isolate IF Vv11 cytolysi...    50  7e-05
AF376032 AF376032 Vibrio vulnificus isolate IF Vv10 cytolysi...    50  7e-05
AF376031 AF376031 Vibrio vulnificus isolate IF Vv24 cytolysi...    50  7e-05
AF376029 AF376029 Vibrio vulnificus isolate CNRVC 970121 cyt...    50  7e-05
AF376028 AF376028 Vibrio vulnificus isolate CNRVC 970120 cyt...    50  7e-05
AF376027 AF376027 Vibrio vulnificus strain CIP 75.4T cytolys...    50  7e-05
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    50  7e-05
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    50  7e-05
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    50  7e-05
AF376030 AF376030 Vibrio vulnificus isolate IF Vv18 cytolysi...    42  0.016
AK025098 AK025098 Homo sapiens cDNA FLJ21445 fis, A-COL04457...    34  3.9
AC092544 AC092544 Homo sapiens BAC clone RP11-317F4 from 4, ...    34  3.9
U41547 U41547 Caenorhabditis elegans cosmid F22A3, complete ...    34  3.9
U29380 U29380 Caenorhabditis elegans cosmid ZK546, complete ...    34  3.9
```

Primer R-CTH
gCTACTTTCTAgCATTTTCTCTgC

```
VIBHEMOX M36437 V.parahaemolyticus thermolabile hemolysin (T...    48  2e-04
AY289609 AY289609 Vibrio parahaemolyticus thermolabile hemol...    48  2e-04
AP005084 AP005084 Vibrio parahaemolyticus DNA, chromosome 2,...    48  2e-04
AB012596 AB012596 Vibrio parahaemolyticus gene for LDH, comp...    48  2e-04
AP005084 AP005084 Vibrio parahaemolyticus DNA, chromosome 2,...    48  2e-04
AL928905 AL928905 Mouse DNA sequence from clone RP23-70E24 o...    40  0.054
AL663082 AL663082 Mouse DNA sequence from clone RP23-327G1 o...    38  0.21
AL445123 AL445123 Human DNA sequence from clone RP11-242F11 ...    38  0.21
AC009303 AC009303 Homo sapiens BAC clone RP11-98C1 from 2, c...    38  0.21
AC092853 AC092853 Mus musculus clone mgs1-271d8 map X strain...    36  0.84
AF149786 AF149786 Homo sapiens chromosome 8 clone GS1-190e2 ...    34  3.3
AP004837 AP004837 Oryza sativa (japonica cultivar-group) gen...    34  3.3
```

Coleman *et al.* (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Primer Vv-Primer1
CgCCgCTCACTggggCAgTggCTg

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    40  0.009
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AF492764 AF492764 Drosophila melanogaster transposon rover, ...    32  2.2
AF195879 AF195879 Drosophila melanogaster chromosome 9 unkno...    32  2.2
AE003680 AE003680 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003604 AE003604 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003497 AE003497 Drosophila melanogaster chromosome X, sect...    32  2.2
BX927148 BX927148 Corynebacterium glutamicum ATCC 13032, IS ...    32  2.2
AP005274 AP005274 Corynebacterium glutamicum ATCC 13032 DNA,...    32  2.2
AE003680 AE003680 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003604 AE003604 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003497 AE003497 Drosophila melanogaster chromosome X, sect...    32  2.2
```

Primer Vv-Primer2
gCgggTggTTCggTTAACggCTgg

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
MUSPPPPA M81475 Murine phosphoprotein phosphatase mRNA, comp...    34  0.55
BC046617 BC046617 Mus musculus protein phosphatase 3, cataly...    34  0.55
AC025669 AC025669 Mus musculus chromosome 14, clone RP23-5O1...    34  0.55
AK015002 AK015002 Mus musculus adult male testis cDNA, RIKEN...    34  0.55
BX005231 BX005231 Mouse DNA sequence from clone RP23-392C6 o...    32  2.2
AL645570 AL645570 Mouse DNA sequence from clone RP23-377H12 ...    32  2.2
AL356111 AL356111 Human DNA sequence from clone RP11-501G6 o...    32  2.2
AC132516 AC132516 Homo sapiens 3 BAC RP11-264A3 (Roswell Par...    32  2.2
AC116301 AC116301 Homo sapiens 3 BAC RP11-625D20 (Roswell Pa...    32  2.2
AC060788 AC060788 Homo sapiens chromosome 8, clone CTD-2008O...    32  2.2
```

```
AE001025 AE001025 Archaeoglobus fulgidus DSM 4304 section 82...    32  2.2
AP001680 AP001680 Homo sapiens genomic DNA, chromosome 21q, ...    30  8.7
AP000942 AP000942 Homo sapiens genomic DNA, chromosome 11 cl...    30  8.7
AF205888 AF205888 Homo sapiens AXIN2 (AXIN2) mRNA, complete ...    30  8.7
AC113168 AC113168 Homo sapiens chromosome 3 clone RP11-268B2...    30  8.7
AC023825 AC023825 Homo sapiens chromosome 16 clone RP11-322D...    30  8.7
CNS09ZLG BX832686 Arabidopsis thaliana Full-length cDNA Comp...    30  8.7
```

Coleman and Oliver (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene

Query= VvPCR1
CgCCgCTCACTggggCAgTggCTg

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    48  2e-04
AF376033 AF376033 Vibrio vulnificus isolate IF Vv11 cytolysi...    48  2e-04
AF376032 AF376032 Vibrio vulnificus isolate IF Vv10 cytolysi...    48  2e-04
AF376031 AF376031 Vibrio vulnificus isolate IF Vv24 cytolysi...    48  2e-04
AF376029 AF376029 Vibrio vulnificus isolate CNRVC 970121 cyt...    48  2e-04
AF376028 AF376028 Vibrio vulnificus isolate CNRVC 970120 cyt...    48  2e-04
AF376027 AF376027 Vibrio vulnificus strain CIP 75.4T cytolys...    48  2e-04
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    48  2e-04
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    48  2e-04
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    42  0.014
AF376030 AF376030 Vibrio vulnificus isolate IF Vv18 cytolysi...    42  0.014
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    42  0.014
AC115108 AC115108 Homo sapiens PAC clone RP5-1188A4 from 7, ...    38  0.21
AC115107 AC115107 Homo sapiens PAC clone RP5-1111K7 from 7, ...    38  0.21
AC115106 AC115106 Homo sapiens PAC clone RP5-864I6 from 7, c...    38  0.21
AC105418 AC105418 Homo sapiens BAC clone RP11-450O3 from 7, ...    38  0.21
AC006014 AC006014 Homo sapiens PAC clone RP5-953A4 from 7q11...    38  0.21
AC005488 AC005488 Homo sapiens BAC clone RP11-313P13 from 7,...    38  0.21
AC005049 AC005049 Homo sapiens BAC clone CTB-23I15 from 7, c...    38  0.21
AC022161 AC022161 Homo sapiens chromosome 16 clone RP11-273P...    34  3.3
```

Query= VvPCR2
CCAgCCgTTAACCgAACCACCCgC

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    48  2e-04
AY046900 AY046900 Vibrio vulnificus cytolysin (vvhA) gene, p...    48  2e-04
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    48  2e-04
AF376033 AF376033 Vibrio vulnificus isolate IF Vv11 cytolysi...    48  2e-04
AF376032 AF376032 Vibrio vulnificus isolate IF Vv10 cytolysi...    48  2e-04
AF376031 AF376031 Vibrio vulnificus isolate IF Vv24 cytolysi...    48  2e-04
AF376030 AF376030 Vibrio vulnificus isolate IF Vv18 cytolysi...    48  2e-04
AF376029 AF376029 Vibrio vulnificus isolate CNRVC 970121 cyt...    48  2e-04
AF376028 AF376028 Vibrio vulnificus isolate CNRVC 970120 cyt...    48  2e-04
AF376027 AF376027 Vibrio vulnificus strain CIP 75.4T cytolys...    48  2e-04
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    48  2e-04
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    48  2e-04
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    48  2e-04
AF007807 AF007807 Daucus carota Met1-type cytosine DNA-methy...    34  3.3
```

Conejero & Hedreyda (2003) - *Vibrio harveyi toxR* gene

Primer Vh-toxR-F
TTCTgAAgCAgCACTCAC

```
AC011373 AC011373 Homo sapiens chromosome 5 clone CTB-108O6,...    36  0.14
AC008701 AC008701 Homo sapiens chromosome 5 clone CTB-75N16,...    36  0.14
AY247418 AY247418 Vibrio harveyi ToxR (toxR) gene, partial c...    36  0.14
AL669896 AL669896 Mouse DNA sequence from clone RP23-477F4 o...    34  0.55
HS1033H22 AL109613 Human DNA sequence from clone RP5-1033H22...    34  0.55
AC022240 AC022240 Homo sapiens chromosome 11, clone RP11-231...    34  0.55
AP003595 AP003595 Nostoc sp. PCC 7120 DNA, complete genome, ...    32  2.2
AL133321 AL133321 Human DNA sequence from clone RP13-17E1 on...    30  8.7
AC087354 AC087354 Homo sapiens chromosome 8, clone RP11-398H...    30  8.7
AC004072 AC004072 Human Chromosome X clone bWXD342, complete...    30  8.7
BX649428 BX649428 Zebrafish DNA sequence from clone CH211-51...    30  8.7
```

Primer Vh-toxR-R
TCgACTggTgAAgACTCA

```
AY247418 AY247418 Vibrio harveyi ToxR (toxR) gene, partial c...    36  0.14
AL138691 AL138691 Human DNA sequence from clone RP11-310D8 o...    32  2.2
AC117475 AC117475 Homo sapiens 3 BAC RP11-742C10 (Roswell Pa...    32  2.2
RNU32372 U32372 Rattus norvegicus tyrosine-ester sulfotransf...    30  8.7
AL928841 AL928841 Mouse DNA sequence from clone RP23-95M4 on...    30  8.7
YSCSEQCAT M95580 Yeast ORF1 gene sequence. 4/1993                  30  8.7
SCYBR149W Z36018 S.cerevisiae chromosome II reading frame OR...    30  8.7
AC127376 AC127376 Mus musculus BAC clone RP23-49A4 from chro...    30  8.7
AC125325 AC125325 Mus musculus BAC clone RP23-296K22 from ch...    30  8.7
AC107992 AC107992 Homo sapiens chromosome 15, clone RP11-150...    30  8.7
AC147026 AC147026 Mus musculus chromosome 19 clone RP23-249A...    30  8.7
```

Dalla Valle *et al.* (2002) – *Photobacterium damselae* ssp. *piscicida*, RAPD

Primer PdP1
CCCCTAATAggCCCCATTCTT

```
PDA306693 AJ306693 Photobacterium damselae ssp. piscicida ...      42  0.007
AC120194 AC120194 Homo sapiens chromosome 8, clone RP11-110M...    34  1.7
AC022730 AC022730 Homo sapiens chromosome 8, clone RP11-333A...    34  1.7
AE003758 AE003758 Drosophila melanogaster chromosome 3R, sec...    34  1.7
AC009981 AC009981 Drosophila melanogaster, chromosome 3R, re...    34  1.7
AE003758 AE003758 Drosophila melanogaster chromosome 3R, sec...    34  1.7
AC139938 AC139938 Mus musculus chromosome 6, clone RP23-10C7...    32  6.6
AC121805 AC121805 Mus musculus BAC clone RP23-443L2 from chr...    32  6.6
BS000153 BS000153 Pan troglodytes chromosome 22 clone:RP43-0...    32  6.6
AP002371 AP002371 Homo sapiens genomic DNA, chromosome 11q c...    32  6.6
AL513315 AL513315 Human DNA sequence from clone RP11-262N9 o...    32  6.6
AF418271 AF418271 Homo sapiens interleukin 10 (IL10) gene, c...    32  6.6
AC103805 AC103805 Homo sapiens chromosome 18, clone RP11-106...    32  6.6
AC092799 AC092799 Homo sapiens chromosome 1 clone RP11-5F19,...    32  6.6
AC091691 AC091691 Homo sapiens chromosome 18, clone RP11-47G...    32  6.6
AC123048 AC123048 Mus musculus chromosome 18 clone RP24-394D...    32  6.6
```

Primer PdP2
CCgTCCCCTTgATggTggCACgA

```
PDA306693 AJ306693 Photobacterium damselae ssp. piscicida ...    46  7e-04
AL359636 AL359636 Human DNA sequence from clone RP11-542K23 ...    34  2.8
AC096588 AC096588 Homo sapiens BAC clone RP11-725D20 from 4,...    34  2.8
AC084277 AC084277 Homo sapiens BAC clone RP11-68O1 from 4, c...    34  2.8
```

Primer PdP5
TTgCgATgACgACAgCTATg

```
PDA306694 AJ306694 Photobacterium damselae ssp. piscicida ...    40  0.009
AK070289 AK070289 Oryza sativa (japonica cultivar-group) cDN...    32  2.2
AC133216 AC133216 Oryza sativa (japonica cultivar-group) chr...    32  2.2
AC084317 AC084317 Leishmania major chromosome 2 clone B2015 ...    32  2.2
AE017160 AE017160 Chlamydophila pneumoniae TW-183, section 4...    30  8.7
```

Primer PdP6
TCTggCgACCAACAAACgTA

```
PDA306694 AJ306694 Photobacterium damselae ssp. piscicida ...    40  0.009
VCH249205 AJ249205 Vibrio cholerae ret, abpA and abpB genes ...    34  0.55
AE004309 AE004309 Vibrio cholerae O1 biovar eltor str. N1696...    34  0.55
AL390866 AL390866 Human DNA sequence from clone RP11-218D6 o...    32  2.2
OSJN00097 AL606650 Oryza sativa genomic DNA, chromosome 4, B...    30  8.7
AY089617 AY089617 Drosophila melanogaster RE32881 full inser...    30  8.7
AY228750 AY228750 Uncultured ascomycete clone H1 18S small s...    30  8.7
AY144994 AY144994 Saccharomyces kluyveri clone P2191 BAP2 ge...    30  8.7
AP003936 AP003936 Oryza sativa (japonica cultivar-group) gen...    30  8.7
AY089617 AY089617 Drosophila melanogaster RE32881 full inser...    30  8.7
AE003488 AE003488 Drosophila melanogaster chromosome X, sect...    30  8.7
AC105351 AC105351 Drosophila melanogaster X BAC RP98-34B12 (...    30  8.7
AC023690 AC023690 Drosophila melanogaster X BAC RP98-6M20 (R...    30  8.7
CNS0970A BX045158 Single read from an extremity of a full-le...    30  8.7
OSJN00097 AL606650 Oryza sativa genomic DNA, chromosome 4, B...    30  8.7
AP003936 AP003936 Oryza sativa (japonica cultivar-group) gen...    30  8.7
AE003488 AE003488 Drosophila melanogaster chromosome X, sect...    30  8.7
```

González *et al.* (2003) - *Vibrio anguillarum rpoN* gene

Primer rpoN-ang5
gTTCATAgCATCAATgAggAg

```
VAU86585 U86585 Vibrio anguillarum RNA polymerase sigma-54 s...    42  0.007
AC125783 AC125783 Oryza sativa (japonica cultivar-group) chr...    38  0.11
AC139886 AC139886 Mus musculus BAC clone RP23-120M10 from ch...    34  1.7
AP006292 AP006292 Homo sapiens genomic DNA, chromosome 9 clo...    34  1.7
AB020869 AB020869 Homo sapiens genomic DNA of 9q32 anti-onco...    34  1.7
AF016662 AF016662 Caenorhabditis elegans cosmid C33C12, comp...    34  1.7
AL627229 AL627229 Mouse DNA sequence from clone RP23-354L19 ...    32  6.6
AL353744 AL353744 Human DNA sequence from clone RP13-100A9 o...    32  6.6
AP002033 AP002033 Arabidopsis thaliana genomic DNA, chromoso...    32  6.6
BX085195 BX085195 Zebrafish DNA sequence from clone DKEY-226...    32  6.6
AP002033 AP002033 Arabidopsis thaliana genomic DNA, chromoso...    32  6.6
```

Primer rpoN-ang5
gTTCATAgCATCAATgAggAg

```
VAU86585 U86585 Vibrio anguillarum RNA polymerase sigma-54 s...    42  0.007
```

```
AC125783 AC125783 Oryza sativa (japonica cultivar-group) chr...    38  0.11
AC139886 AC139886 Mus musculus BAC clone RP23-120M10 from ch...    34  1.7
AP006292 AP006292 Homo sapiens genomic DNA, chromosome 9 clo...    34  1.7
AB020869 AB020869 Homo sapiens genomic DNA of 9q32 anti-onco...    34  1.7
AF016662 AF016662 Caenorhabditis elegans cosmid C33C12, comp...    34  1.7
AL627229 AL627229 Mouse DNA sequence from clone RP23-354L19 ...    32  6.6
AL353744 AL353744 Human DNA sequence from clone RP13-100A9 o...    32  6.6
AP002033 AP002033 Arabidopsis thaliana genomic DNA, chromoso...    32  6.6
BX085195 BX085195 Zebrafish DNA sequence from clone DKEY-226...    32  6.6
AP002033 AP002033 Arabidopsis thaliana genomic DNA, chromoso...    32  6.6
```

Hirono *et al.* (1996) - *Vibrio anguillarum vah*1 haemolysin gene

Primer VaH1-P1
ACCgATgCCATCgCTCAAgA

```
S83534 S83534 VAH1=hemolysin/AHH1 and ASH4 homolog [Vibrio a...    40  0.009
AY122158 AY122158 Drosophila melanogaster LD11783 full inser...    34  0.55
BC050577 BC050577 Homo sapiens chromosome 20 open reading fr...    34  0.55
AK095322 AK095322 Homo sapiens cDNA FLJ38003 fis, clone CTON...    34  0.55
AB046810 AB046810 Homo sapiens mRNA for KIAA1590 protein, pa...    34  0.55
DROSADH02 AE003408 Drosophila melanogaster, chromosome 2L, r...    34  0.55
BT003538 BT003538 Drosophila melanogaster RE07016 full inser...    34  0.55
AY122158 AY122158 Drosophila melanogaster LD11783 full inser...    34  0.55
AF254741 AF254741 Drosophila melanogaster Centaurin Gamma 1A...    34  0.55
AE003641 AE003641 Drosophila melanogaster chromosome 2L, sec...    34  0.55
AC092223 AC092223 Drosophila melanogaster, chromosome 2L, re...    34  0.55
AP006573 AP006573 Gloeobacter violaceus PCC 7421 DNA, comple...    34  0.55
AF346500 AF346500 Photorhabdus luminescens strain W14 toxin ...    34  0.55
HSM807718 BX647572 Homo sapiens mRNA; cDNA DKFZp313B0719 (fr...    34  0.55
HSDJ777L9 AL049794 Human DNA sequence from clone RP4-777L9 o...    34  0.55
AK095322 AK095322 Homo sapiens cDNA FLJ38003 fis, clone CTON...    34  0.55
BC050577 BC050577 Homo sapiens chromosome 20 open reading fr...    34  0.55
AB177647 AB177647 Daucus carota DcPA 1 mRNA for plasma membr...    34  0.55
AE003641 AE003641 Drosophila melanogaster chromosome 2L, sec...    34  0.55
AE008311 AE008311 Agrobacterium tumefaciens str. C58 linear ...    32  2.2
```

Primer VaH1-P2
ggATATTgACCgAAgAgTCA

```
S83534 S83534 VAH1=hemolysin/AHH1 and ASH4 homolog [Vibrio a...    40  0.009
AP000991 AP000991 Thermoplasma volcanium genomic DNA, comple...    40  0.009
VMU68271 U68271 Vibrio mimicus heat-labile hemolysin (vmhA) ...    38  0.036
VIBHLYB M36855 V.cholerae hemolysin (hlyA) gene, complete cd...    38  0.036
VIBHEMO D58374 Vibrio cholerae gene for hemolysin, complete ...    38  0.036
VCHLYAH X51746 Vibrio cholerae hlyA gene for haemolysin. 9/1994    38  0.036
VCHLYA Y00557 Vibrio cholerae DNA for hlyA, hlyB, lipA, lipB...    38  0.036
VCH576090 AJ576090 Vibrio cholerae partial hlyA gene for hem...    38  0.036
AF194418 AF194418 Vibrio cholerae hemolysin precursor (hlyA)...    38  0.036
AE004362 AE004362 Vibrio cholerae O1 biovar eltor str. N1696...    38  0.036
AY427780 AY427780 Vibrio cholerae non-O1/non-O139 el tor hem...    38  0.036
HVGNIRE X57845 H.vulgare gene for nitrate reductase. 5/1995        32  2.2
AY013246 AY013246 Hordeum vulgare chromosome 5 BAC 635P2, co...    32  2.2
AF427791 AF427791 Hordeum vulgare Mla locus, complete sequen...    32  2.2
SAG766852 AL766852 Streptococcus agalactiae NEM316 complete ...    32  2.2
AY485643 AY485643 Hordeum vulgare ssp. vulgare BAC 615K1, ...    32  2.2
HVU279072 AJ279072 Hordeum vulgare BARE-2 and partial BAGY-2...    30  8.7
AF474982 AF474982 Hordeum vulgare clone BAC 011009, complete...    30  8.7
AF474373 AF474373 Hordeum vulgare BAC 259I16, complete seque...    30  8.7
```

Iwamoto *et al.* (1995) - *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment

Primer Pst-I-1a
TgCgCTgACgTgTCTgAATT

```
S83260 S83260 {pathogenic Vibrio trachuri-specific sequence}...    40  0.009
T8M17 AF296835 Arabidopsis thaliana BAC T8M17. 8/2000              32  2.2
LMFL7758 AL352980 Leishmania major Friedlin chromosome 5 cos...    32  2.2
CEU49263 U49263 Caenorhabditis elegans non-muscle myosin hea...    32  2.2
CET22C1 Z75550 Caenorhabditis elegans cosmid T22C1, complete...    32  2.2
AC024756 AC024756 Caenorhabditis elegans cosmid Y34D9A, comp...    32  2.2
AY263990 AY263990 Corynebacterium jeikeium plasmid pA505, co...    32  2.2
CET22C1 Z75550 Caenorhabditis elegans cosmid T22C1, complete...    32  2.2
CNS05TC9 AL355075 Human chromosome 14 DNA sequence BAC R-203...    30  8.7
BC009377 BC009377 Homo sapiens UDP-N-acteylglucosamine pyrop...    30  8.7
AC005878 AC005878 citb_255_f_20, complete sequence. 11/1999        30  8.7
AC007109 AC007109 Arabidopsis thaliana chromosome 2 clone T1...    30  8.7
AC113579 AC113579 Tetraodon nigroviridis clone GSTNA-27O10, ...    30  8.7
BOV39KD J04204 Bos taurus 32 kd acessory protein mRNA, compl...    30  8.7
AF092099 AF092099 Trypanosoma cruzi CL Brener surface antige...    30  8.7
AE003682 AE003682 Drosophila melanogaster chromosome 3R, sec...    30  8.7
AE009791 AE009791 Pyrobaculum aerophilum strain IM2 section ...    30  8.7
```

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AE003682 AE003682 Drosophila melanogaster chromosome 3R, sec...    30  8.7
```

Primer Pst-I-1b
AAgCAgCgATgACAAgCAgT

```
S83260 S83260 {pathogenic Vibrio trachuri-specific sequence}...    40  0.009
AC114574 AC114574 Mus musculus chromosome 6, clone RP23-151F...    34  0.55
AL357503 AL357503 Human DNA sequence from clone RP5-899C14 o...    32  2.2
AY205154 AY205154 Vibrio vulnificus phosphomannomutase (pmm)...    32  2.2
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    32  2.2
AE016810 AE016810 Vibrio vulnificus CMCP6 chromosome II sect...    32  2.2
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    32  2.2
AC091206 AC091206 Drosophila melanogaster 3L BAC RP98-19M9 (...    30  8.7
AC010018 AC010018 Drosophila melanogaster 3L BAC RP98-17M19 ...    30  8.7
```

Kim and Jeong (2001) – *Vibrio vulnificus* 16S rRNA gene, tri-primer

Primer TriVib-1
gTggTAgTgTTAATAgCACT

```
VV16SRRNX Z22992 V.vulnificus (ATCC 33147) gene for 16S ribo...    40  0.009
VV16SRRNA X74726 V.vulnificus (ATCC 27562T) gene for 16S rib...    40  0.009
VV16SRRN X56582 V.vulnificus 16S ribosomal RNA. 6/2003          40  0.009
VV16SRR X76333 V.vulnificus (ATCC 27562 T) 16S rRNA gene. 8/...    40  0.009
VSP580582 AJ580582 Vibrio sp. CH-291 partial 16S rRNA gene. ...    40  0.009
AY298794 AY298794 Vibrio parahaemolyticus clone c27 16S ribo...    40  0.009
AY298793 AY298793 Vibrio parahaemolyticus clone c20 16S ribo...    40  0.009
AY298792 AY298792 Vibrio parahaemolyticus clone c45 16S ribo...    40  0.009
AY298791 AY298791 Vibrio parahaemolyticus clone c53 16S ribo...    40  0.009
AY298790 AY298790 Vibrio parahaemolyticus clone c54 16S ribo...    40  0.009
AY298789 AY298789 Vibrio parahaemolyticus clone c52 16S ribo...    40  0.009
AY264936 AY264936 Vibrio vulnificus 16S ribosomal RNA gene, ...    40  0.009
AY241424 AY241424 Photobacterium sp. UST020129-014 16S ribos...    40  0.009
AY038544 AY038544 Uncultured gamma proteobacterium clone CD4...    40  0.009
AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri...    40  0.009
AF388387 AF388387 Vibrio parahaemolyticus clone Vp16 16S rib...    40  0.009
AF343948 AF343948 Vibrio sp. NBF27 16S ribosomal RNA gene, p...    40  0.009
AF319770 AF319770 Vibrio sp. Ex97 16S ribosomal RNA gene, pa...    40  0.009
AF055832 AF055832 Marine proteobacterium 'Tomales Bay wh' 16...    40  0.009
AY038544 AY038544 Uncultured gamma proteobacterium clone CD4...    40  0.009
```

Primer TriVib-2
TCTAgCggAgACgCTggA

```
VVU10862 U10862 Vibrio vulnificus ATCC 27562 16S small subun...    36  0.14
VV16SRRNA X74726 V.vulnificus (ATCC 27562T) gene for 16S rib...    36  0.14
VV16SRR X76333 V.vulnificus (ATCC 27562 T) 16S rRNA gene. 8/...    36  0.14
VMI16SRRA X74713 V.mimicus (ATCC 33653T) gene for 16S riboso...    36  0.14
VCU10955 U10955 Vibrio cholerae ATCC 14035 16S rRNA gene, pa...    36  0.14
VCOL16SRA X74694 V.cholerae (ATCC 14033) gene for 16S riboso...    36  0.14
VCL16SRRA X74696 V.cholerae (ATCC 14731) gene for 16S riboso...    36  0.14
VCL16SRR1 X74697 V.cholerae (ATCC 14731) gene for 16S riboso...    36  0.14
VCHO16SRA X74695 V.cholerae (ATCC 14035T) gene for 16S ribos...    36  0.14
VC16SRRNA X76337 V.cholerae (CECT 514 T) 16S rRNA gene. 8/1994     36  0.14
AY494843 AY494843 Vibrio cholerae strain TP 16S ribosomal RN...    36  0.14
AY494842 AY494842 Vibrio cholerae strain SIO 16S ribosomal R...    36  0.14
AY345423 AY345423 Bacterium K23 16S ribosomal RNA gene, part...    36  0.14
AY264936 AY264936 Vibrio vulnificus 16S ribosomal RNA gene, ...    36  0.14
AP005343 AP005343 Vibrio vulnificus YJ016 DNA, chromosome I,...    36  0.14
AF493802 AF493802 Vibrio sp. KB81 16S ribosomal RNA gene, pa...    36  0.14
AE016801 AE016801 Vibrio vulnificus CMCP6 chromosome I secti...    36  0.14
AE004157 AE004157 Vibrio cholerae O1 biovar eltor str. N1696...    36  0.14
AY513501 AY513501 Vibrio cholerae strain VC13-Janaba 16S rib...    36  0.14
AY513500 AY513500 Vibrio cholerae strain VC12-Ogawa 16S ribo...    36  0.14
```

Primer TriVib-3R
gCTCACTTTCgCAAgTTggCC

```
VVU16SRRA X74727 V.vulnificus (ATCC 29307) gene for 16S ribo...    42  0.007
VSP514914 AJ514914 Vibrio fortis partial 16S rRNA gene, stra...    42  0.007
VSP316172 AJ316172 Vibrio sp. LMG 20546 16S rRNA gene, strai...    42  0.007
VIB16SRR10 D11315 Vibrio proteolyticus 16S rRNA. 11/1999        42  0.007
VIB16SR05 D11310 Vibrio nereis 16S rRNA. 11/1999                42  0.007
AY456924 AY456924 Vibrio parahaemolyticus strain EcGS020801 ...    42  0.007
AY345462 AY345462 Bacterium K59 16S ribosomal RNA gene, part...    42  0.007
AY345446 AY345446 Bacterium KA62 16S ribosomal RNA gene, par...    42  0.007
AY345386 AY345386 Bacterium JB13 16S ribosomal RNA gene, par...    42  0.007
AY345375 AY345375 Bacterium JB8 16S ribosomal RNA gene, part...    42  0.007
AY332566 AY332566 Vibrio alginolyticus strain EcGS021001 16S...    42  0.007
AY217772 AY217772 Vibrio sp. BLI-41 16S ribosomal RNA gene, ...    42  0.007
AP005342 AP005342 Vibrio vulnificus YJ016 DNA, chromosome I,...    42  0.007
AP005332 AP005332 Vibrio vulnificus YJ016 DNA, chromosome I,...    42  0.007
AF540018 AF540018 Vibrio parahaemolyticus strain RLUH-1 16S ...    42  0.007
AF284229 AF284229 Vibrio sp. 01 16S ribosomal RNA gene, part...    42  0.007
AF199438 AF199438 Vibrio sp. DS40M5 16S ribosomal RNA gene, ...    42  0.007
```

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AF064637 AF064637 Vibrio sp. NAP-4 16S ribosomal RNA gene, p...    42  0.007
AE016800 AE016800 Vibrio vulnificus CMCP6 chromosome I secti...    42  0.007
AE016798 AE016798 Vibrio vulnificus CMCP6 chromosome I secti...    42  0.007
```

Kvitt *et al.* (2002) - *Photobacterium damsela*e ssp. *piscicida* 16S rRNA gene

Primer Pdp-P1f
TAgTgTAgTTAACACCTgCAC

```
PP16SRRW X78106 P.damselea (wild isolate) 16S rRNA gene. 2/1995    42  0.007
PP16SRR X78105 P.damsela (NCIMB 2058) 16S rRNA gene. 1/1995        42  0.007
PDA18496 Y18496 Photobacterium damselae 16S rRNA gene. 7/1999      42  0.007
AY191124 AY191124 Photobacterium damselae ssp. piscicida s...    42  0.007
AY191123 AY191123 Photobacterium damselae ssp. piscicida s...    42  0.007
AY191122 AY191122 Photobacterium damselae ssp. piscicida s...    42  0.007
AY163244 AY163244 Photobacterium damselae ssp. piscicida s...    42  0.007
AY163243 AY163243 Photobacterium damselae ssp. piscicida s...    42  0.007
AY163242 AY163242 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    42  0.007
PHR16SRD2 D25308 Photobacterium histaminum 16S rDNA gene. 2/...    40  0.027
AY191125 AY191125 Photobacterium damselae ssp. damselae st...    40  0.027
AY163246 AY163246 Photobacterium damselae ssp. damselae st...    40  0.027
AY163245 AY163245 Photobacterium damselae ssp. piscicida s...    40  0.027
AB032015 AB032015 Photobacterium damselae ssp. damselae ge...    40  0.027
AB032014 AB032014 Photobacterium histaminum gene for 16S rRN...    40  0.027
```

Primer Pdp-P2r
ACACTCgAATCTCTTCAAgT

```
VO16SR275 X71834 V.ordalii (ssp. MSC 275) gene for 16S ribos...    38  0.036
VD16SRRNA X74700 V.damsela (ATCC 33539T) gene for 16S riboso...    38  0.036
PP16SRRW X78106 P.damselea (wild isolate) 16S rRNA gene. 2/1995    38  0.036
PHR16SRD2 D25308 Photobacterium histaminum 16S rDNA gene. 2/...    38  0.036
PDA18496 Y18496 Photobacterium damselae 16S rRNA gene. 7/1999      38  0.036
AY198105 AY198105 Uncultured bacterium clone ARKMP10 16S rib...    38  0.036
AY147861 AY147861 Photobacterium damselae ssp. damselae st...    38  0.036
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    38  0.036
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    38  0.036
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    38  0.036
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    38  0.036
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    38  0.036
AF468347 AF468347 Arctic sea ice bacterium ARK10170 16S ribo...    38  0.036
AF468346 AF468346 Arctic sea ice bacterium ARK10162 16S ribo...    38  0.036
AF468344 AF468344 Arctic sea ice bacterium ARK10150 16S ribo...    38  0.036
AF468323 AF468323 Uncultured bacterium clone ARKMP-22 16S ri...    38  0.036
AF468241 AF468241 Uncultured bacterium clone ARKDMS-28 16S r...    38  0.036
AB032015 AB032015 Photobacterium damselae ssp. damselae ge...    38  0.036
AB032014 AB032014 Photobacterium histaminum gene for 16S rRN...    38  0.036
AB026844 AB026844 Photobacterium damselae ssp. piscicida g...    38  0.036
```

Primer Pdp-P3f
AAgCTTgAAgAgATTCgAgTg

```
VD16SRRNA X74700 V.damsela (ATCC 33539T) gene for 16S riboso...    42  0.007
PP16SRR X78105 P.damsela (NCIMB 2058) 16S rRNA gene. 1/1995        42  0.007
PHR16SRD2 D25308 Photobacterium histaminum 16S rDNA gene. 2/...    42  0.007
AY227004 AY227004 Arctic sea ice bacterium ARK10167 isolate ...    42  0.007
AY198105 AY198105 Uncultured bacterium clone ARKMP10 16S rib...    42  0.007
AY147861 AY147861 Photobacterium damselae ssp. damselae st...    42  0.007
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    42  0.007
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    42  0.007
AF468350 AF468350 Arctic sea ice bacterium ARK10299 16S ribo...    42  0.007
AF468349 AF468349 Arctic sea ice bacterium ARK10284 16S ribo...    42  0.007
AF468348 AF468348 Arctic sea ice bacterium ARK10172 16S ribo...    42  0.007
AF468347 AF468347 Arctic sea ice bacterium ARK10170 16S ribo...    42  0.007
AF468346 AF468346 Arctic sea ice bacterium ARK10162 16S ribo...    42  0.007
AF468323 AF468323 Uncultured bacterium clone ARKMP-22 16S ri...    42  0.007
AF468241 AF468241 Uncultured bacterium clone ARKDMS-28 16S r...    42  0.007
AF441962 AF441962 Uncultured gamma proteobacterium clone CD1...    42  0.007
AB032015 AB032015 Photobacterium damselae ssp. damselae ge...    42  0.007
AB032014 AB032014 Photobacterium histaminum gene for 16S rRN...    42  0.007
```

Primer Pdp-P4r
CCACCTCgCggTCTTgCTgC

```
VD16SRRNA X74700 V.damsela (ATCC 33539T) gene for 16S riboso...    40  0.009
UPL241008 AJ241008 uncultured Planctomycetes Sva0500 16S rRN...    40  0.009
UEU81675 U81675 Unidentified eubacterium clone vadinBC32 16S...    40  0.009
UEQ408208 AJ408208 Uncultured equine intestinal eubacterium ...    40  0.009
```

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UEAJ9501 AJ009501 uncultured bacterium SJA-171 16S rRNA gene...    40  0.009
PP16SRRW X78106 P.damselea (wild isolate) 16S rRNA gene. 2/1995    40  0.009
CDI582245 AJ582245 Uncultured candidate division OP8 bacteri...    40  0.009
AY508290 AY508290 Uncultured bacterium clone HH41_4 16S ribo...    40  0.009
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    40  0.009
AF323775 AF323775 Uncultured bacterium clone BA059 16S ribos...    40  0.009
AF317742 AF317742 Unidentified bacterium wb1_A09 small subun...    40  0.009
AF050585 AF050585 Uncultured eubacterium WCHB1-71 16S riboso...    40  0.009
AB032015 AB032015 Photobacterium damselae ssp. damselae ge...    40  0.009
AB032014 AB032014 Photobacterium histaminum gene for 16S rRN...    40  0.009
AB026844 AB026844 Photobacterium damselae ssp. piscicida g...    40  0.009
AY540494 AY540494 Uncultured bacterium clone A3 16S ribosoma...    40  0.009
UEU81675 U81675 Unidentified eubacterium clone vadinBC32 16S...    40  0.009
AF050585 AF050585 Uncultured eubacterium WCHB1-71 16S riboso...    40  0.009
AY571810 AY571810 Uncultured Rubrobacter sp. clone 347E 16S ...    40  0.009
AE015936 AE015936 Clostridium tetani E88, section 1 of 10 of...    34  0.55
```

Lee *et al.* (1998) - *Vibrio vulnificus vvh* cytolysin/haemolysin gene

Primer Vv-P1
gACTATCgCATCAACAACCg

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    40  0.009
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AF492764 AF492764 Drosophila melanogaster transposon rover, ...    32  2.2
AF195879 AF195879 Drosophila melanogaster chromosome 9 unkno...    32  2.2
AE003680 AE003680 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003604 AE003604 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003497 AE003497 Drosophila melanogaster chromosome X, sect...    32  2.2
BX927148 BX927148 Corynebacterium glutamicum ATCC 13032, IS ...    32  2.2
AP005274 AP005274 Corynebacterium glutamicum ATCC 13032 DNA,...    32  2.2
AE003680 AE003680 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003604 AE003604 Drosophila melanogaster chromosome 3R, sec...    32  2.2
AE003497 AE003497 Drosophila melanogaster chromosome X, sect...    32  2.2
```

Primer Vv-P2
AggTAgCgAgTATTACTgCC

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
MUSPPPPA M81475 Murine phosphoprotein phosphatase mRNA, comp...    34  0.55
BC046617 BC046617 Mus musculus protein phosphatase 3, cataly...    34  0.55
AC025669 AC025669 Mus musculus chromosome 14, clone RP23-5O1...    34  0.55
AK015002 AK015002 Mus musculus adult male testis cDNA, RIKEN...    34  0.55
BX005231 BX005231 Mouse DNA sequence from clone RP23-392C6 o...    32  2.2
AL645570 AL645570 Mouse DNA sequence from clone RP23-377H12 ...    32  2.2
AL356111 AL356111 Human DNA sequence from clone RP11-501G6 o...    32  2.2
AC132516 AC132516 Homo sapiens 3 BAC RP11-264A3 (Roswell Par...    32  2.2
AC116301 AC116301 Homo sapiens 3 BAC RP11-625D20 (Roswell Pa...    32  2.2
AC060788 AC060788 Homo sapiens chromosome 8, clone CTD-2008O...    32  2.2
AE001025 AE001025 Archaeoglobus fulgidus DSM 4304 section 82...    32  2.2
AP001680 AP001680 Homo sapiens genomic DNA, chromosome 21q, ...    30  8.7
AP000942 AP000942 Homo sapiens genomic DNA, chromosome 11 cl...    30  8.7
AF205888 AF205888 Homo sapiens AXIN2 (AXIN2) mRNA, complete ...    30  8.7
AC113168 AC113168 Homo sapiens chromosome 3 clone RP11-268B2...    30  8.7
AC023825 AC023825 Homo sapiens chromosome 16 clone RP11-322D...    30  8.7
CNS09ZLG BX832686 Arabidopsis thaliana Full-length cDNA Comp...    30  8.7
```

Query= Vv-P3
gCTATTTCACCgCCgCTCAC

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AF376033 AF376033 Vibrio vulnificus isolate IF Vv11 cytolysi...    40  0.009
AF376032 AF376032 Vibrio vulnificus isolate IF Vv10 cytolysi...    40  0.009
AF376031 AF376031 Vibrio vulnificus isolate IF Vv24 cytolysi...    40  0.009
AF376030 AF376030 Vibrio vulnificus isolate IF Vv18 cytolysi...    40  0.009
AF376029 AF376029 Vibrio vulnificus isolate CNRVC 970121 cyt...    40  0.009
AF376028 AF376028 Vibrio vulnificus isolate CNRVC 970120 cyt...    40  0.009
AF376027 AF376027 Vibrio vulnificus strain CIP 75.4T cytolys...    40  0.009
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    40  0.009
AB124803 AB124803 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AF116529 AF116529 Caenorhabditis elegans synthetic multivulv...    32  2.2
AF000195 AF000195 Caenorhabditis elegans cosmid C32F10, comp...    32  2.2
AE014752 AE014752 Bifidobacterium longum NCC2705 section 139...    32  2.2
AE009878 AE009878 Pyrobaculum aerophilum strain IM2 section ...    32  2.2
SME591785 AL591785 Sinorhizobium meliloti 1021 complete chro...    30  8.7
AE016989 AE016989 Shigella flexneri 2a str. 2457T section 12...    30  8.7
```

Query= Vv-P4
CCgCAgAgCCgTAAACCgAA

```
VIBVVHAB M34670 V.vulnificus cytolysin (vvhA) and vvhB gene ...    40  0.009
AY046900 AY046900 Vibrio vulnificus cytolysin (vvhA) gene, p...    40  0.009
AP005348 AP005348 Vibrio vulnificus YJ016 DNA, chromosome II...    40  0.009
AE016809 AE016809 Vibrio vulnificus CMCP6 chromosome II sect...    40  0.009
AB124802 AB124802 Vibrio vulnificus vvhB, vvhA genes for hyp...    40  0.009
AL606824 AL606824 Mouse DNA sequence from clone RP23-9G13 on...    32  2.2
AY426744 AY426744 Phytophthora sojae putative phosphatidylin...    32  2.2
AF449619 AF449619 Phytophthora sojae isolate P6497 exonuclea...    32  2.2
AE003616 AE003616 Drosophila melanogaster chromosome 2L, sec...    32  2.2
AE003431 AE003431 Drosophila melanogaster chromosome X, sect...    32  2.2
AC023747 AC023747 Drosophila melanogaster X BAC RP98-38L1 (R...    32  2.2
AC023742 AC023742 Drosophila melanogaster X BAC RP98-43C24 (...    32  2.2
AC012675 AC012675 Drosophila melanogaster, chromosome 2L, re...    32  2.2
AE003431 AE003431 Drosophila melanogaster chromosome X, sect...    32  2.2
AE003616 AE003616 Drosophila melanogaster chromosome 2L, sec...    32  2.2
AE003639 AE003639 Drosophila melanogaster chromosome 2L, sec...    30  8.7
```

Oakey *et al*. (2003) – *Vibrio harveyi* 16S rRNA gene

Primer VH-1
AACgAgTTATCTgAACCTTC

```
VSP508300 AJ508300 Vibrio sp. SR2 partial 16S rRNA gene. 9/2003    40  0.009
VD16SRNA X99762 V.diabolus 16S ribosomal RNA. 6/2003               40  0.009
AY456924 AY456924 Vibrio parahaemolyticus strain EcGS020801 ...    40  0.009
AY344374 AY344374 Unidentified bacterium clone K2-S-4 16S ri...    40  0.009
AY321532 AY321532 Marine bacterium 13706 16S ribosomal RNA g...    40  0.009
AY264928 AY264928 Vibrio harveyi strain OVL 0-53535-C 16S ri...    40  0.009
AY038544 AY038544 Uncultured gamma proteobacterium clone CD4...    40  0.009
AF388389 AF388389 Vibrio parahaemolyticus clone Vp 27 16S ri...    40  0.009
AF388388 AF388388 Vibrio parahaemolyticus clone F44 16S ribo...    40  0.009
AF388387 AF388387 Vibrio parahaemolyticus clone Vp16 16S rib...    40  0.009
AF388386 AF388386 Vibrio parahaemolyticus clone Vp23 16S rib...    40  0.009
AF343953 AF343953 Marine bacterium NBF32 16S ribosomal RNA g...    40  0.009
AF319770 AF319770 Vibrio sp. Ex97 16S ribosomal RNA gene, pa...    40  0.009
AF319769 AF319769 Vibrio sp. Ex25 16S ribosomal RNA gene, pa...    40  0.009
AB038030 AB038030 Vibrio splendidus biovar II gene for 16S r...    40  0.009
AB031630 AB031630 Uncultured gamma proteobacterium TIHP368-0...    40  0.009
AB031614 AB031614 Uncultured gamma proteobacterium TIHP302-2...    40  0.009
AB031591 AB031591 Uncultured gamma proteobacterium TIHP302-0...    40  0.009
AY038544 AY038544 Uncultured gamma proteobacterium clone CD4...    40  0.009
AY580861 AY580861 Uncultured gamma proteobacterium clone PI_...    40  0.009
```

Primer VH-2
gCAgCTATTAACTACACTACC

```
VS16SRRNZ Z22999 Vibrio sp. (A065) gene for 16S ribosomal RN...    42  0.007
VS16SRRNY Z22998 Vibrio sp. (A063) gene for 16S ribosomal RN...    42  0.007
VS16SRRNW Z22996 Vibrio sp. (A060) gene for 16S ribosomal RN...    42  0.007
VS16SRRND Z22973 Vibrio sp. (A068) gene for 16S ribosomal RN...    42  0.007
VF16SRRNX Z22990 V.fluvialis (ATCC 33812) gene for 16S ribos...    42  0.007
AY456924 AY456924 Vibrio parahaemolyticus strain EcGS020801 ...    42  0.007
AY374391 AY374391 Uncultured Vibrio sp. clone 2-129 16S ribo...    42  0.007
AY345423 AY345423 Bacterium K23 16S ribosomal RNA gene, part...    42  0.007
AY264934 AY264934 Vibrio harveyi strain ACMM 645 16S ribosom...    42  0.007
AY264930 AY264930 Vibrio harveyi strain UQM 2724 16S ribosom...    42  0.007
AY264926 AY264926 Vibrio harveyi strain OVL 99-52331-A 16S r...    42  0.007
AY264925 AY264925 Vibrio harveyi strain OVL 0-53535-F 16S ri...    42  0.007
AY245195 AY245195 Vibrio vulnificus isolate 7 16S ribosomal ...    42  0.007
AY245189 AY245189 Vibrio vulnificus isolate 9 16S ribosomal ...    42  0.007
AY245188 AY245188 Vibrio parahaemolyticus isolate L21 16S ri...    42  0.007
AY245178 AY245178 Vibrio parahaemolyticus isolate 13 16S rib...    42  0.007
AY241409 AY241409 Vibrio sp. UST010723-015 16S ribosomal RNA...    42  0.007
AF540018 AF540018 Vibrio parahaemolyticus strain RLUH-1 16S ...    42  0.007
AF537958 AF537958 Vibrio alginolyticus strain VM102 16S ribo...    42  0.007
AF465376 AF465376 Vibrio sp. UST991130-022 16S ribosomal RNA...    42  0.007
```

Osorio *et al*. (1999) - *Photobacterium damselae* 16S rRNA gene, hemi-nested

Primer Car1
gCTTgAAgAgATTCgAgT

```
VO16SR275 X71834 V.ordalii (ssp. MSC 275) gene for 16S ribos...    36  0.14
VD16SRRNA X74700 V.damsela (ATCC 33539T) gene for 16S riboso...    36  0.14
PP16SRRW X78106 P.damselea (wild isolate) 16S rRNA gene. 2/1995    36  0.14
PHR16SRD2 D25308 Photobacterium histaminum 16S rDNA gene. 2/...    36  0.14
PDA18496 Y18496 Photobacterium damselae 16S rRNA gene. 7/1999      36  0.14
AY227004 AY227004 Arctic sea ice bacterium ARK10167 isolate ...    36  0.14
AY198105 AY198105 Uncultured bacterium clone ARKMP10 16S rib...    36  0.14
```

```
AY147861 AY147861 Photobacterium damselae ssp. damselae st...    36  0.14
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    36  0.14
AF468350 AF468350 Arctic sea ice bacterium ARK10299 16S ribo...    36  0.14
AF468348 AF468348 Arctic sea ice bacterium ARK10172 16S ribo...    36  0.14
AF468347 AF468347 Arctic sea ice bacterium ARK10170 16S ribo...    36  0.14
AF468344 AF468344 Arctic sea ice bacterium ARK10150 16S ribo...    36  0.14
AF468241 AF468241 Uncultured bacterium clone ARKDMS-28 16S r...    36  0.14
AF441962 AF441962 Uncultured gamma proteobacterium clone CD1...    36  0.14
```

Primer Car2
CACCTCgCggTCTTgCTg

```
UBA307936 AJ307936 uncultured bacterium mRNA for 16S ribosom...    36  0.14
CDI582264 AJ582264 Uncultured candidate division OP8 bacteri...    36  0.14
CDI582245 AJ582245 Uncultured candidate division OP8 bacteri...    36  0.14
BSPZ77636 Z77636 Bacterium 2-400 C2.37 partial 16S rRNA gene...    36  0.14
AY508339 AY508339 Uncultured bacterium clone HH41_12 16S rib...    36  0.14
AY147861 AY147861 Photobacterium damselae ssp. damselae st...    36  0.14
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    36  0.14
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    36  0.14
AF523927 AF523927 Uncultured bacterium clone RCP1-64 16S rib...    36  0.14
AF050586 AF050586 Uncultured eubacterium WCHB1-82 16S riboso...    36  0.14
AF050585 AF050585 Uncultured eubacterium WCHB1-71 16S riboso...    36  0.14
AB116468 AB116468 Uncultured gamma proteobacterium gene for ...    36  0.14
AF050585 AF050585 Uncultured eubacterium WCHB1-71 16S riboso...    36  0.14
AF050586 AF050586 Uncultured eubacterium WCHB1-82 16S riboso...    36  0.14
AY571810 AY571810 Uncultured Rubrobacter sp. clone 347E 16S ...    36  0.14
AE015936 AE015936 Clostridium tetani E88, section 1 of 10 of...    32  2.2
```

Primer Nest-car1
ggTCTTgCTgCCCTCTg

```
AC005578 AC005578 Homo sapiens chromosome 19, cosmid F20887,...    34  0.56
UPL241008 AJ241008 uncultured Planctomycetes Sva0500 16S rRN...    34  0.56
UBA488085 AJ488085 Uncultured bacterium partial 16S rRNA gen...    34  0.56
PDA18496 Y18496 Photobacterium damselae 16S rRNA gene. 7/1999      34  0.56
AY508339 AY508339 Uncultured bacterium clone HH41_12 16S rib...    34  0.56
AY508338 AY508338 Uncultured bacterium clone HH41_11 16S rib...    34  0.56
AY508290 AY508290 Uncultured bacterium clone HH41_4 16S ribo...    34  0.56
AY147861 AY147861 Photobacterium damselae ssp. damselae st...    34  0.56
AY147860 AY147860 Photobacterium damselae ssp. piscicida s...    34  0.56
AY147859 AY147859 Photobacterium damselae ssp. piscicida s...    34  0.56
AY147858 AY147858 Photobacterium damselae ssp. piscicida s...    34  0.56
AY147857 AY147857 Photobacterium damselae ssp. piscicida s...    34  0.56
AY147856 AY147856 Photobacterium damselae ssp. piscicida s...    34  0.56
AJ583205 AJ583205 uncultured bacterium partial 16S rRNA gene...    34  0.56
AF110276 AF110276 Beggiatoa sp. MS-81-1c 16S ribosomal RNA g...    34  0.56
AB026844 AB026844 Photobacterium damselae ssp. piscicida g...    34  0.56
AC122795 AC122795 Mus musculus BAC clone RP24-226D9 from chr...    32  2.2
HSJ828K20 AL049737 Human DNA sequence from clone RP5-828K20 ...    32  2.2
AC010883 AC010883 Homo sapiens BAC clone RP11-339H12 from 2,...    30  8.7
```

Osorio *et al.* (2000) - *Photobacterium damsela*e ssp. *damsela*e and *piscicida*, *ureC* and 16S genes, multiplex format

Primer Ure-3
CTTgAATATCCATCTCATCTgC

```
LDU40071 U40071 Listonella damsela urease large subunit (ure...    44  0.002
AY363684 AY363684 Yersinia kristensenii urease gene locus, a...    40  0.036
AY363683 AY363683 Yersinia intermedia urease gene locus, and...    40  0.036
AC122866 AC122866 Mus musculus BAC clone RP23-145J18 from 8,...    36  0.56
AC068544 AC068544 Homo sapiens BAC clone RP11-369J9 from 2, ...    36  0.56
U32736 U32736 Haemophilus influenzae Rd section 51 of 163 of...    36  0.56
AC124814 AC124814 Mus musculus chromosome 1, clone RP24-119P...    36  0.56
BX322656 BX322656 Mouse DNA sequence from clone RP23-146D13 ...    34  2.2
AC122203 AC122203 Mus musculus BAC clone RP23-69J7 from 5, c...    34  2.2
AC108773 AC108773 Mus musculus chromosome 5, clone RP23-118L...    34  2.2
HS354N19 AL022722 Human DNA sequence from clone RP3-354N19 o...    34  2.2
AC116010 AC116010 Homo sapiens chromosome 11, clone RP11-139...    34  2.2
AC078982 AC078982 Homo sapiens 3 BAC RP11-486O23 (Roswell Pa...    34  2.2
AC055878 AC055878 Homo sapiens chromosome 11, clone RP11-34N...    34  2.2
AC025160 AC025160 Homo sapiens 12 BAC RP11-526P6 (Roswell Pa...    34  2.2
AY363681 AY363681 Yersinia bercovieri urease gene locus, and...    34  2.2
AB119283 AB119283 Homo sapiens FSTL1 gene for follistatin-re...    34  2.2
AC069160 AC069160 Arabidopsis thaliana chromosome 1 BAC T9I1...    32  8.8
```

Primer Ure-5_
TCCggAATAggTAAAgCggg

LDU40071	U40071 Listonella damsela urease large subunit (ure...	40	0.009
AL513343	AL513343 Human DNA sequence from clone RP11-90O23 o...	34	0.55
AC133207	AC133207 Mus musculus BAC clone RP23-116L23 from ch...	30	8.7
AC136431	AC136431 Homo sapiens chromosome 16 clone RP11-148M...	30	8.7
AC108073	AC108073 Homo sapiens BAC clone RP11-713C19 from 4,...	30	8.7
AC104166	AC104166 Homo sapiens chromosome 3 clone RP11-199N2...	30	8.7
AC099674	AC099674 Homo sapiens chromosome 1 clone RP11-143H1...	30	8.7
AC092326	AC092326 Homo sapiens chromosome 16 clone RP11-14N9...	30	8.7
AC079790	AC079790 Homo sapiens BAC clone RP11-484E6 from 2, ...	30	8.7
AC020716	AC020716 Homo sapiens BAC clone RP11-449G13 from 16...	30	8.7
AC003689	AC003689 Homo sapiens Chromosome 11q12.2 PAC clone ...	30	8.7
SYCSLLLH	D64006 Synechocystis sp. PCC 6803 DNA, complete gen...	30	8.7
AE005457	AE005457 Escherichia coli O157:H7 EDL933 genome, co...	30	8.7
L78837S2	L78838 Homo sapiens T cell surface glycoprotein CD6...	30	8.7

A4.8 Summary of the Numbers of Potential Secondary Structures Determined for each Primer Pair Using NetPrimer®.

Primer Name and Sequence (5′ to 3′)	Hairpin	Dimer	Cross Dimer	Palindrome	Repeat & Run
Arias *et al.* (1995) – *Vibrio vulnificus* 23S rRNA gene					
118V					
ACCCAAAggggTAAgCC	2	1	2	0	3
1037R					
CATCgCTTTAAggAACAgC	1	2	2	0	1
Dvu9V					
CCACTggCATAAgCCAg	1	1	0	0	0
Dvu45R					
CTACCCAATgTTCATAgAA	0	2	0	0	1
Brasher *et al.* (1998) – *Vibrio vulnificus cth* cytolysin/haemolysin gene					
L-CTH					
TTCCAACTTCAAACCgAACTATgAC	1	1	1	0	1
R-CTH					
gCTACTTTCTAgCATTTTCTCTgC	2	3	1	0	2
Coleman *et al.* (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene					
Vv-Primer 1					
CgCCgCTCACTggggCAgTggCTg	3	3	3	0	1
Vv- Primer 2					
gCgggTggTTCggTTAACggCTgg	0	1	3	1	1
Coleman and Oliver (1996) - *Vibrio vulnificus cth* cytolysin/haemolysin gene					
VvPCR1					
CgCCgCTCACTggggCAgTggCTg	3	3	5	0	1
VvPCR2					
CCAgCCgTTAACCgAACCACCCgC	0	1	5	0	1
Conejero & Hedreyda (2003) - *Vibrio harveyi tox*R gene					
Vh-toxR-F					
TTCTgAAgCAgCACTCAC	1	1	2	0	0
Vh-toxR-R					
TCgACTggTgAAgACTCA	1	2	2	0	0
Dalla Valle *et al.* (2002) – *Photobacterium damsela*e ssp. *piscicida*, unique RAPD					
PdP1					
CCCCTAATAggCCCCATTCTT	1	3	1	0	2
PdP2					
CCgTCCCCTTgATggTggCACgA	1	2	1	0	1
PdP5					
TTgCgATgACgACAgCTATg	0	1	1	0	0
PdP6					
TCTggCgACCAACAAACgTA	0	1	1	0	1
González ***et al.* (2003) - *Vibrio anguillarum rpoN* gene**					
rpoN-ang5'					
gTTCATAgCATCAATgAggAg	1	1	3	0	0
rpoN-ang3'					
gAgCAgACAATATgTTggATg	0	1	3	0	0
Hirono *et al.* (1996) - *Vibrio anguillarum vah*1 haemolysin gene					
VaH1-P1					
ACCgATgCCATCgCTCAAgA	1	1	2	0	0
VaH1-P2					
ggATATTgACCgAAgAgTCA	1	2	2	0	0

Primer Name and Sequence (5′ to 3′)	Hairpin	Dimer	Cross Dimer	Palindrome	Repeat & Run
Iwamoto *et al.* (1995) - *Vibrio 'trachuri'* (*Vibrio harveyi*) unique *Pst*I fragment					
Pst I-1a					
TgCgCTgACgTgTCTgAATT	0	3	6	0	0
Pst I-1b					
AAgCAgCgATgACAAgCAgT	0	0	6	0	0
Kim and Jeong (2001) – *Vibrio vulnificus* 16S rRNA gene, tri-primer					
TriVib 1					
gTggTAgTgTTAATAgCACT	1	3	2	0	0
TriVib 3R					
gCTCACTTTCgCAAgTTggCC	1	3	2	0	1
Kvitt et al. (2002) - *Photobacterium damsela*e ssp. *piscicida* 16S rRNA gene					
Pdp-P1f					
TAgTgTAgTTAACACCTgCAC	1	4	1	1	0
Pdp-P2r					
ACACTCgAATCTCTTCAAgT	0	3	1	0	0
Pdp-P3f					
AAgCTTgAAgAgATTCgAgTg	0	2	3	1	0
Pdp-P4r					
CCACCTCgCggTCTTgCTgC	0	1	3	0	0
Lee et al. (1998) - *Vibrio vulnificus vvh* cytolysin/haemolysin gene					
Vv-P1					
gACTATCgCATCAACAACCg	0	0	3	0	0
Vv-P2					
AggTAgCgAgTATTACTgCC	0	1	3	0	0
Vv-P3					
gCTATTTCACCgCCgCTCAC	0	0	2	0	1
Vv-P4					
CCgCAgAgCCgTAAACCgAA	0	0	2	0	1
Oakey *et al.* (2003) – *Vibrio harveyi* 16S rRNA gene					
VH-1					
AACgAgTTATCTgAACCTTC	0	1	2	0	0
VH-2					
gCAgCTATTAACTACACTACC	0	2	2	0	0
Osorio *et al.* (1999) - *Photobacterium damsela*e 16S rRNA gene, hemi-nested					
Car1					
gCTTgAAgAgATTCgAgT	0	1	2	0	0
Car2					
CACCTCgCggTCTTgCTg	0	1	2	0	0
Nest-car1					
ggTCTTgCTgCCCTCTg	0	0	2	0	1
Osorio *et al.* (2000) - *Ph. damsela*e ssp. *damsela*e & *piscicida*, *ureC* & 16S rRNA gene, multiplex format					
Ure-5'					
TCCggAATAggTAAAgCggg	1	2	0	1	1
Ure-3'					0
CTTgAATATCCATCTCATCTgC	0	1	0	0	

A4.9 *Vibrio* Identification Workshop - Laboratory Schedule

VIBRIO IDENTIFICATION WORKSHOP

Laboratory Schedule

Tuesday 15/6/04

1. Identification of Vibrios using MicroSys V36

Purpose: Set up of MicroSys V36 for identification of an unknown *Vibrio* species.

Materials provided: Freeze dried V36 panels
V36 set up instructions
Volumes of sterile 2% NaCl
Swabs
Pipettors and sterile tips
Sterile paraffin oil
Sealing tape
Mueller-Hinton agar
Antibiotic discs
24 hour cultures on Johnson's Marine Agar (JMA) labelled: 165, 172, 186, 279, 396, 412, 448, 683

Task: Take any two cultures, perform a Gram stain, oxidase test and set up for identification as given in the V36 instructions. Record the reactions on the work sheets provided.

Note: (i) Some of the strains provided are zoonotic while others are exotic to Australia. Exercise due care!
(ii) None of the strains provided are strict halophilies and do not require the use of 3% NaCl in the inoculum.
(iii) None of the strains are nutritionally fastidious and do not require supplementation.
(iv) All the strains are to be incubated at 25°C

2. Vibrios in diagnostic cases

Purpose: Identification *and* Interpretation of V*ibrio* species from a diagnostic case

Materials provided: V36 set up instructions
Volumes of sterile 2% NaCl
Swabs

Pipettors and sterile tips
Sterile paraffin oil
Sealing tape
Mueller-Hinton agar
Antibiotic discs

Primary plates of diagnostic cases on JMA and TCBS, labelled Case 1, Case 2, Cased 3 and Case 4
Purity plates on JMA, 24 hr old, of target organisms
Case notes as lab accessions

Task: Select a case. Examine primary plates and determine a course of action; discuss if necessary. Purity plates, 24 hr old of target organisms are available for identification using MicroSys V36. Set up identifications as appropriate. Record results on the work sheets provided.

Note: (i) None of the strains provided are strict halophiles and do not require the use of 3% NaCl in the inoculum.
(ii) None of the strains are nutritionally fastidious and do not require supplementation.
(iii) All the strains are to be incubated at 25°C

3. *Vibrio* phenotyping

Purpose: Examine range of phenotyping tests used for *Vibrio* characterisation

Materials provided: 96 well phenotyping panel, pre-inoculated and incubated with test strains
Detection reagents
Pipettors
Tips
Microfuge tubes
Test interpretation guide
Record sheets

Task: Using the interpretation guide, record the reactions for the range of tests in the phenotyping panel. Record findings on the record sheet provided. Keep record sheets for later interpretation.

4. Culture characteristics - Diversity in the Vibrios

Purpose: Examine a range of *Vibrio* species to determine cultural variation

Materials provided: 48 hour cultures of the following species:

M. viscosa	winter ulcer disease in salmonids
V. alginolyticus	swarming
V. agarivorans	agarolysis
V. anguillarum	classical vibriosis in salmonids
V. gazogenes	red pigmentation
V. lentus	vibriosis in octopus
V. nigripulchritudo	black pigmentation
V. ordalii	vibriosis in salmonids
V. salmonicida	Hitra's disease in salmonids

Task: Examine culture plates for colony appearance. For *M. viscosa* use a loop to determine colony stickiness - hence the species name *viscosa*.

Conference Room
Round up at end of day

Wednesday 16/6/04

5. PCR for *Vibrio* Identification

Purpose: Identification and differentiation of *Photobacterium damselae* by PCR.

Materials: PCR grade water
Magnesium sulphate
Invitrogen Platinum Taq polymerase and buffer
Primers Car1, Car2, Ure-3' and Ure5'
DNA controls of *Ph. damselae* ssp. *damselae* and *Ph. damselae* ssp. *piscicida*
Culture plates of *Ph. damselae* ssp. *damselae* and *Ph. damselae* ssp. *piscicida*
Cultures stored on FTA cards
Negative control as pooled DNA from 20 species of *Vibrio*
Electrophoresis buffer, gels, 100bp DNA ladder, ethidium bromide, Polaroid camera and film.

Task: Run PCR assays using control DNA for both subspecies of *Ph. damselae*.
Titrate control DNAs to determine threshold of detection.
Determine the effect of aged cultures on assay performance
Run PCR assays using DNA stored on FTA cards

6. Test Interpretation (24 hour)

6.1 Unknown identifications

Record results of V36 panel at 24 hours. Do not remove the sealing tape. Do not read any tests that require addition of reagents. Final reading of tests will be undertaken the next day.

6.2 Cases

Record results of V36 panel at 24 hours. Do not remove the sealing tape. Do not read any tests that require addition of reagents. Final reading of tests will be undertaken the next day.

6.3 Antibiotic sensitivities

Measure zone diameters and record; interpret as sensitive or resistant.

7. Factors Affecting Phenotype

Purpose: Various factors can influence expression of phenotype and, depending on severity, may have a profound and misleading affect on identification outcome. Provided are identification panels for several species which have been manipulated to demonstrate this affect.

Materials: Paired microtitre trays inoculated and incubated to show the affects listed in the table.

Task: Examine the paired phenotyping panels and note the differences in profile.

Species	Factor	Condition
Ph damselae ssp. *damselae*	Nutritional fastidiousness	Addition of Casamino acids enables full expression of sole carbon utilisation capability
V. anguillarum	Culture vigour	Recently revived culture has reduced expression of phenotype compared to subcultured population
V. halioticoli	NaCl concentration	Sub-optimal NaCl concentration will reduce expression of phenotype: compare the use of 2% and 3% NaCl as inocula
V. harveyi	Pre-inoculum medium	Pre-inoculum media affects test outcome: compare results for blood agar, ZoBell's marine agar and Johnson's marine agar
V. logei	Incubation temperature	Incubation at 25°C reduces expression phenotype compared to incubation at 15°C

8. PCR analysis

Look at the negative controls; if no bands are evident then examine the test lanes on the gel. With reference to the 100bp markers estimate band size for the *Ph. damselae* ssp. *damselae* and *Ph. damselae* ssp. *piscicida*.

Ph. damselae ssp. *damselae*: 2 bands of 267 and 448 bp, 16S rRNA gene and *ure*C.
Ph. damselae ssp. *piscicida* 1 band of 267 bp, 16S rRNA gene.

Questions

- Did the titration of purified DNA give you an idea of the threshold of detection (sensitivity)?
- Were you able to clearly differentiate between the 2 sub-species?
- Were the FTA (dried) controls useful?
- Did the age of the cultures have much affect on the PCR results?
- Did anyone titrate the purified DNA to get an idea of threshold of detection?

Thursday 17/6/04

9. Case and Culture Interpretation

9.1 Record colour change tests on the lid of the microtitre tray.
9.2 Carefully remove the sealing tape and discard carefully - the underside is contaminated.
9.3 Add reagents to wells A10, A11 and A12 and record reactions. For the gelatin test use an unused empty well on the tray for the reagent addition.
9.4 Using an inverted magnifying mirror, record the results of the sole carbon utilisation tests.
9.5 Enter all the data on to the record sheet.
9.6 Obtain an identification using PIBWin. Using the statistics provided, consider the quality of the identification and your acceptance of the suggested identification.
9.7 For the case study, determine the significance of the finding.

Conference Room

Round up and finish

APPENDIX 5: DRAFT ANZSDP FOR THE IDENTIFICATION OF *VIBRIONACEAE*

Identification of *Vibrionaceae* from Australian aquatic animals using phenotypic and PCR procedures

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SUMMARY

The Vibrionaceae *is a large and complex group of marine bacteria that are important to the health of aquatic animals. A range of pathogenicity is seen across the species but a consistent feature is opportunistic basis of infection. Nearly all phases of production are affected from larval rearing through to competent adult animals. Disease outbreaks may occur in disparate animal groups from marine mammals, through to fin fish, crustacea, molluscs and zooxanthellae of coral. Some strains however can act as probionts and have proved effective as a means of controlling disease caused by other species of Vibrionaceae.*

Identification*: Routine, high-volume identification is realistically achieved by phenotyping using standardised tests. To accommodate the large number of taxa and the phenotypic diversity that exists intra-species, identification is only practicable using computer-assisted probabilistic methods. The use of molecular tools for identification remains limited but PCR for several species is useful as a means of rapid screening or confirmatory identification.*

Status of Australia and New Zealand*: The range of Vibrionaceae associated with aquatic animals in Australia is relatively small despite the diversity of habitats, geographic range and climatic variation. Major pathogens encountered are* Photobacterium damselae *ssp.* damselae*,* Vibrio anguillarum *and* Vibrio harveyi*. More unusual species isolated are* Photobacterium damselae *ssp.* piscicida, Vibrio scophthalmi *and* Vibrio penaeicida.

Introduction

The *Vibrionaceae* is an important and ubiquitous group of bacteria in marine and estuarine environments[57]. Characteristically they have a close association with aquatic animals either as symbionts, normal flora or as pathogens. The ability of the group to occupy a wide diversity of habitats means that the *Vibrionaceae* have direct and indirect affects on aquaculture either as pathogens that are the cause of major disease, low grade infections that erode

productivity or beneficial effects as probionts like some strains of *Vibrio alginolyticus*[4] or *V. mediterranei*[24]. To varying degrees, *Vibrionaceae* have an intimate relationship with all farmed marine species of aquatic animals from live feed inputs such as *Artemia* and rotifers, crustaceans, bivalved and single shelled molluscs through to fin fish. The myriad of types, the range of species and their diverse role in aquaculture make the *Vibrionaceae* an important but challenging group of bacteria.

Taxonomy

The *Vibrionaceae* comprise five genera: *Enterovibrio, Grimontia, Moritella, Photobacterium* and *Vibrio.* The genus *Listonella* has also been proposed and although a validly published name, there is now little evidence to justify its status[3], and use of the name is declining. The taxonomy of the family is based largely on phylogenetic analysis of 16S rDNA, supported by fluorescent amplified fragment length polymorphism data[53,54]. FAFLP has proved particularly useful as a means of species delineation because of the high level of 16S rDNA sequence similarity that exists between many species of the *Vibrionaceae*. Currently some 89 species across the five genera are recognised; most of the species occur in the genus *Vibrio*. Phenotypes, when based on numerical taxonomy, are generally useful and predictive[14,15] but where kit systems have been used to define species, invariably descriptions are poor and insufficient for the purpose of identification. There is marked genomic plasticity within the *Vibrionaceae* which is seen as pronounced phenotypic diversity between and within species[52,58]. As a consequence, finding traits that differentiate species continues to be a problem, particularly those delineated by molecular taxonomy.

Disease in Aquatic Animals

Central to disease involving *Vibrionaceae* is the opportunistic basis of infection. The pathogen may have an intimate association with the host as normal flora but only exhibits its pathogenic capacity once host defences are breached. This may occur either from stress events that lead to immunosuppression, physical damage to the integument, or the emergence of aggressive biovars within a population. Severity of disease may range from fulminating septicaemias, typical of disease outbreaks, through to chronic infections that affect just a few individuals.

Species of *Vibrionaceae* have an obligate requirement for NaCl[19] that ranges from as little as 5-15mM for *V. cholerae* and *V. metschnikovii* to 300-400 mM for *V. splendidus*[7,9]. A critical factor for potential pathogens is the coincidence of physiologies between host and pathogen. For osmoregulators such as fin fish, pathogens must have an optimum NaCl requirement close to physiological levels of vertebrates while for osmoconformers such as molluscs and crustacea, pathogens have optimum NaCl concentrations close to that of seawater. Extracellular pathogenicity factors such as siderophores, haemolysins, cytotoxins and proteases have been described in several species of *Vibrionaceae* and appear to be important in expression of disease[1,29,63].

Host Range

Development of an exhaustive list of Australian host species serves little purpose. More importantly, cognisance of the range of host types is central to obtaining an understanding of the pathogenic versatility of the *Vibrionaceae*. In nearly every group of marine species can examples be found of *Vibrionaceae* acting as pathogens. Major groups affected are: marine mammals, teleost fish, crustacea, molluscs, both univalve and bivalve, and plants represented by the zooxanthellae of coral[10].

Species Enzootic in Australia

Listing *Vibrionaceae* known to occur in Australia provides a guide to species that have been found associated with aquatic animals, Table 1. Not all the species listed were found as pathogens.

Table 1. *Vibrionaceae* enzootic in Australia

Photobacterium damselae ssp. *damselae*
Photobacterium damselae ssp. *piscicida*
Photobacterium iliopiscarium

Vibrio alginolyticus
Vibrio anguillarum
Vibrio chagasii
Vibrio cholerae non-O1
Vibrio cyclitrophicus
Vibrio diazotrophicus
Vibrio fischeri
Vibrio furnissii
Vibrio fluvialis
Vibrio halioticoli
Vibrio harveyi
Vibrio ichthyoenteri
Vibrio lentus
Vibrio mediterranei
Vibrio mimicus
Vibrio mytili
Vibrio natriegens
Vibrio navarrensis
Vibrio nereis
Vibrio parahaemolyticus
Vibrio pelagius
Vibrio penaeicida
Vibrio proteolyticus
Vibrio scophthalmi
Vibrio splendidus biovar I
Vibrio tasmaniensis
Vibrio tubiashii
Vibrio vulnificus biovar I

Species Exotic to Australia

Several pathogens, as listed in Table 2, have not been detected in association with aquatic animals and are considered exotic.

Table 2. *Vibrionaceae* exotic to Australia

Species	Primary host
Moritella viscosa	salmonids
Vibrio ordalii	salmonids
Vibrio pectenicida	scallops
Vibrio salmonicida	salmonids
Vibrio tapetis	clams
Vibrio vulnificus biovar II	eels
Vibrio wodanis	salmonids

Zoonotic Agents

Some species of *Vibrionaceae* are the cause of zoonoses[17], Table 3. Infection invariably is the result of physical trauma arising from puncture wounds or the result of ingesting uncooked seafood. Life threatening conditions have been reported occasionally in the immunocompromised.

Table 3. Zoonotic *Vibrionaceae*

Photobacterium damselae ssp. damselae
Vibrio alginolyticus
Vibrio cholerae non-O1
Vibrio cincinnatiensis
Vibrio fluvialis
Vibrio furnissii
Vibrio harveyi
Vibrio mimicus
Vibrio parahaemolyticus
Vibrio vulnificus

Characteristics of the *Vibrionaceae*

Sodium chloride is pivotal for the growth of *Vibrionaceae*. Many species have an obligate requirement for sodium ions while growth of nearly all species is stimulated by NaCl, even those which have a low requirement for NaCl such as *V. cholerae*[45]. Species of the *Vibrionaceae* do not in general have fastidious growth requirements and can be readily grown on peptone based media as long as NaCl requirements are met. More reliable growth is obtained with blood enriched media for *Moritella viscosa, V. salmonicida* and *V. wodanis*. In defined media some *Vibrionaceae* require supplementation with vitamins[8]; most strains however, even in complex media, respond well to the addition of low levels of yeast extract[47]. As the natural habitat of the *Vibrionaceae* is the marine environment, better growth is obtained at a slightly alkaline pH in the range of 7.5-7.8. Many species and strains will form distinctive curved rods but this characteristic is not diagnostic of the *Vibrionaceae*. In tissue smears rods can appear preternaturally large or pleomorphic but on culture will assume more typical form and proportions.

Uniformly the *Vibrionaceae* are facultative anaerobes that ferment glucose. All species are oxidase positive with the exception of *V. metschnikovii* which is oxidase negative. Species are sensitive to the vibriostat 0/129, a pteridine derivative related to trimethoprim[34]. Some species however, such as *V. lentus*, may appear resistant if inappropriate test media are used or if strains have acquired resistance to trimethoprim from the *drfA1* gene in plasmid class I integrons[56]. Most species of *Vibrionaceae* will grow at 25°C except for *M. marina, M. viscosa, Ph. iliopiscarium, Ph. phosphoreum, V. salmonicida* and *V. wodanis* which grow at 15°C. Zoonotic species will all grow at 35-37°C.

Isolation Strategies

For osmoregulators, cultures from internal sites should be made on media enriched

with blood such as Blood Agar Base No. 2 (Oxoid) with 7% defibrinated sheep's blood as a non-selective medium, and thiosulphate citrate bile sucrose (TCBS; Oxoid[37]) agar as a selective indicator medium for *Vibrionaceae*. TCBS should only be used in conjunction with a non-selective medium as not all species will grow on TCBS. For external sites on osmoregulators or any site of an osmoconformer, samples should be plated on either ZoBell's 2216E[40] or Johnson's marine agar[25] (see Appendix 1) as non-selective media and TCBS as the selective medium. For salmonids, particularly during periods of low water temperatures, samples from external sites should be plated on blood agar supplemented with 1.5% NaCl[31]. Plates should be incubated for 2-3 days at 25°C or at 15°C for up to 7 days for psychrophiles.

Preservation

Cultures of *Vibrionaceae* can be held frozen at -80°C or in liquid nitrogen. A cryopreservative (see Appendix 1) based on peptone and glycerol is suitable[59]. Long term storage based on freeze-drying is effective but the menstruum must be based on *meso*-inositol[43] to regulate membrane phase transition temperature effects and protein stabilisation[28] during freeze-drying and rehydration. Some species, notably *M. marina* and *V. salmonicida* have proved refractory to freeze-drying and are best preserved frozen. Generally, cultures recover well from preservation. ZMA or JMA are suitable recovery media but for more fastidious species particularly *M. marina, M. viscosa* and *V. salmonicida* blood agar supplemented with 2% NaCl should be used. A prudent strategy for recovery from freeze-drying is to use *Vibrio* Recovery Medium (Appendix 1) which contains sodium pyruvate[12,35] which has been found useful in repair of damaged cells.

Identification Strategies

Phenotyping: Typically the *Vibrionaceae* exhibit a wide diversity of phenotype not only between species but importantly within species. This heterogeneity in phenotype means that identification using a small number of tests either with keys or tables is unreliable. A more dependable strategy is to use a simultaneous polythetic approach[48] combined with computer-assisted probabilistic identification[61].

An identification matrix, VibEx7 (Appendix 5), has been developed for species of *Vibrionaceae* associated with a diversity of aquatic animals in Australia from both temperate and tropical regions[15]. The matrix can identify 61 species and biovars and a further 25 as yet un-named protospecies of *Vibrionaceae* from aquatic animals. The panel of tests consists of 39 biochemical and 5 antibiotic sensitivity tests; details of the tests and formulations are given in Appendix 2. The biochemical tests are in conventional tube or plate format. Alternatively, the panel of tests is available commercially in miniaturised format as MicroSys® V36 (DPIW, Launceston, Tasmania).

Probabilistic identification is undertaken using an implementation of Bayes Theorem[61]. An identification is reached if the Willcox probability value $P \geq 0.99$ and the modal likelihood score ≥ 0.001. The Willcox probability is a measure of the most likely identification, while the modal likelihood is a measure of the goodness-of-fit of the unknown to the nominated species[20]. Probabilistic identification is undertaken using PIBWin[13], an intuitive software package, available freely from the University of Southampton, UK at: http://www.som.soton.ac.uk/staff/tnb/pib.htm

Molecular: Identification of *Vibrionaceae* using molecular methods is controversial. Amplified fragment length polymorphism (AFLP) and multilocus sequence typing[51] have proved important in establishing the taxonomic structure of the *Vibrionaceae* but these procedures are not as yet suitable for routine identification purposes. PCR amplification of sequence motifs characteristic of species is a practical means of molecular identification but the scope of application is limited. The most widely described and conserved construct is the 16S rRNA gene but for the *Vibrionaceae*[44] sequence divergence is only 9% which

greatly limits the possibilities of identifying motifs that are species unique. Sequences other than 16S rRNA have been identified, typically virulence factors, but the constancy of these targets across strains of a species is unknown and their suitability as constructs for identification purposes is questionable.

Recommended PCR primer sets relevant for *Vibrionaceae* of aquatic animals in Australia are for *Ph. damselae* and *V. harveyi*. Based on the 16S rRNA gene both primer sets are suitable for species identification. The PCR for *Ph. damselae* is in multiplex format[41] with one primer pair specific for *Ph. damselae sensu lato* and a second primer pair for the urease gene *ure*C that is specific for *Ph. damselae* ssp. *damselae*. Evidence of *Ph. damselae* ssp. *piscicida* is inferred by the absence of a *ure*C amplicon. The primer pair for *V. harveyi*[39] is compromised to some extent by known cross reaction with some, but not all, strains of *V. alginolyticus*. For the primers to be truly discriminating, positive PCR reactions must be verified using at least one of the phenotypic tests listed in Table 4.

Table 4. Differential phenotypic tests for *Vibrio harveyi* and *Vibrio alginolyticus* for confirming a positive PCR reaction for 16S rRNA *V. harveyi* primers.

Species	PNPG	Aesculin[2]	Putrescine[3]
V. alginolyticus	16%	10%	100%
V. harveyi bv I	95%	95%	2%
V. harveyi bv II	100%	100%	0%

[1]PNPG: 2-nitrophenyl β-D-galactopyranoside; [2]Aesculin hydrolysis; [3]Utilisation of putrescine

Identification by PCR should be limited to pure cultures and used as a means of confirming the identity of strains with atypical phenotypes. Performance of the primers has not been validated for the purpose of direct detection in tissues or environmental samples.

A range of primer sets for other constructs in the *Vibrionaceae* have been described, of which some have been critically evaluated. Of these primers for the *cth* cytolysin/haemolysin gene of *V. vulnificus*[11] and the *vah*1 haemolysin gene of *V. anguillarum*[23] appear robust. Use of these primers for identification in the absence of other species defining characteristics is not recommended since the frequency at which the targets occur intra-species is not known. The primers may have value however for screening purposes, particularly for strains where the target is known to occur or for establishing the presence of virulence factors.

Quality Control

Identification outcome is dependent on several factors of which the most important is the use of standardised tests. Substitution with tests of different format should not be made unless extensive testing has been undertaken to verify test equivalence. It is important to recognise and identify sources of error which, if not well controlled, can result in unreliable identification outcomes[48]. Intrinsic error is associated with some tests and some species that can result in variable test outcomes. A second form of error arises from procedural deficiencies, particularly interpretation of weak positive tests. Regular use of quality control organisms (Table 5) is recommended together with trend analysis to identify drift in performance. With practice, intra-laboratory test error of 2% is achievable[15,27,49].

Table 5. *Vibrio* Quality Control Strains

Vibrio anguillarum ATCC 19264^T
Vibrio fluvialis NCTC 11327^T
Vibrio mediterranei CIP 103203^T
Vibrio parahaemolyticus ATCC 17802^T
Vibrio tubiashii NCIMB 1340^T

Limitations

The identification matrix VibEx7 reflects the diversity of *Vibrionaceae* associated with aquatic animals in Australia. It does not however include all the validly published species of *Vibrionaceae* as listed in Table 6 and these species will not be identified using the matrix.

Data for new species as they occur can be readily added to the VibEx7 matrix. The ability to differentiate the new species from those already in the matrix can be assessed using the IDSC tool in the PIBWin software.

Table 6. Species not in the VibEx7 matrix

Species	Host
Enterovibrio coralii	Plant
Enterovibrio norvegicus	Animal
Moritella abyssi	Environment
Moritella japonica	Environment
Moritella profunda	Environment
Moritella yayanosii	Environment
Photobacterium aplysiae	Animal
Photobacterium frigidiphilum	Environment
Photobacterium ganghwense	Environment
Photobacterium halotolerans	Environment
Photobacterium indicum	Environment
Photobacterium lipolyticum	Environment
Photobacterium profundum	Environment
Photobacterium rosenbergii	Plant
Vibrio aerogenes	Environment
Vibrio brasiliensis	Animal
Vibrio coralliilyticus	Plant
Vibrio crassostreae	Animal
Vibrio diabolicus	Environment
Vibrio ezurae	Animal
Vibrio fortis	Animal
Vibrio gallicus	Animal
Vibrio gigantis	Animal
Vibrio hepatarius	Animal
Vibrio hispanicus	Animal
Vibrio kanaloae	Animal
Vibrio neonatus	Animal
Vibrio neptunius	Animal
Vibrio pacinii	Environment
Vibrio pomeroyi	Animal
Vibrio ponticus	Animal
Vibrio rotiferianus	Animal
Vibrio ruber	Environment
Vibrio superstes	Animal
Vibrio xuii	Animal

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APPENDICES

Appendix 1
Maintenance Media

Johnson's Marine Agar[25]

Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous (II) sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g

Agar	12.0 g
Aged seawater	900 ml
Distilled water	100 ml
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

Sheep Blood Agar+2% NaCl

Blood agar base no.2 (Oxoid CM0271)	40.0 g
NaCl	15.0 g
Distilled water	1000 ml
pH	7.4±0.2

Autoclave at 121°C for 15 minutes and cool to 50°C; aseptically add 70 ml of sterile defibrinated sheep's blood, mix gently and pour as plates.

Vibrio Recovery Medium[15]

Peptone (Oxoid LP0037)	5.0 g
Yeast extract	1.0 g
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.2 g
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	0.3 g
Sodium pyruvate	1.0 g
Bacteriological charcoal	2.0 g
Aged seawater	900 ml
Distilled water	100 ml
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

ZoBell's Marine Agar 2216E[40]

Peptone (Oxoid LP0037)	5.0 g
Yeast extract (Oxoid LP0021)	1.0 g
Ferric (III) phosphate ($FePO_4 \cdot 4H_2O$)	0.1 g
Aged seawater	900 ml
Distilled water	100 ml
Agar	12.0 g
pH	7.5-7.6

Autoclave at 121°C for 15 minutes

Cryopreservative (freezing)[59]

Proteose peptone no.3 (Difco)	1.0 g
Glycerol	8 ml
Distilled water	92 ml

Aliquot as 5 ml volumes and autoclave at 121°C for 15 minutes.

Cryopreservative (freeze-drying)[18]

Nutrient Broth No.2 (Oxoid CM067B)	16 ml
meso-inositol	2.4 g

Filter sterilise and add aseptically to 32 mL of sterile inactivated horse serum. Mix and divide into 3 mL aliquots. Store at -20°C.

Appendix 2
Culture Media for Identification

Note: Media formulations are based on the cited source references but have been modified in some instances for use with the *Vibrionaceae*. In most cases the modification relates to the addition of NaCl and supplementation with yeast extract.

The tests as formulated should not be substituted with variants. The media as described are standardised for the identification of *Vibrionaceae* using the identification matrix VibEx7.

Arginine Dihydrolase[55,60]

Peptone (Oxoid LP0037)	0.1 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	1.5 g
K_2HPO_4	0.03 g
Phenol red (1% *aq*)	0.1 ml
L-arginine hydrochloride	1.0 g
Agar	0.3 g
Distilled water	100 mL
pH	6.8

Distribute as 4ml volumes in 12x90mm culture tubes. Autoclave at 121°C for 15 minutes.

Fermentation Test Medium[2]

Peptone (Oxoid LP0037)	10.0 g
Yeast extract (Oxoid LP0021)	1.0 g
Lab Lemco (Oxoid LP0029)	3.0 g
NaCl	15.0 g
Bromocresol purple 0.5%[1]	8 ml
Distilled water	900 ml
pH	7.2

[1]0.5 g bromocresol purple in 100 ml 50:50 v/v ethanol/distilled water

Dissolve the ingredients, check and adjust pH and distribute as 90 ml volumes in screw cap bottles. Sterilise by autoclaving at 121°C for 15 minutes.

Prepare filter sterilised stocks of the following sugars as 10% stocks in distilled water: arbutin, mannitol, salicin, sucrose and β-gentiobiose.

To 90 ml of sterile base add 10 ml of 10% sugar stock to give a final concentration of 1%. Aliquot medium as 3 ml volumes in 12 x 90mm diameter sterile tubes.

Tolerance to NaCl[60]

7% NaCl

Tryptone (Oxoid L0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	7.0 g
Distilled water	100 mL
pH	7.2

10% NaCl

Tryptone (Oxoid L0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	10.0 g
Distilled water	100 mL
pH	7.2

Autoclave at 121°C for 15min as 100ml volumes in sealed bottles to prevent evaporation. Dispense as 3ml volumes in 12x90mm sterile tubes.

Amylase[60]

Nutrient broth No. 2 (Oxoid CM0067)	2.5 g
NaCl	1.0 g
Soluble starch	0.1 g
Agar	1.5 g

Distilled water	100 ml
pH	7.5

Dissolve all the ingredients except for the agar. Warm to assist solution of the starch if required. Add the agar. Autoclave at 121°C for 10 minutes. Pour as plates.

Acetoin (Voges-Proskauer) Test[18]

Tryptone(Oxoid LP0042)	0.7 g
Soya peptone (Oxoid LP0044)	0.5 g
Yeast extract (Oxoid LP0021)	0.1 g
Glucose	1.0 g
NaCl	1.5 g
Agar	0.3 g
Distilled water	100 ml
pH	7.0

Dissolve all the ingredients including the agar. Aliquot as 3 ml volumes in 12x90mm tubes. Autoclave at 115°C for 10 min. Cool as butts.

Gelatin[60]

Nutrient broth No. 2 (Oxoid CM0067)	2.5 g
NaCl	1.0 g
Gelatin	0.5 g
Agar	1.5 g
Distilled water	100 ml
pH	7.5

Dissolve all the ingredients except for the agar. Warm to assist solution of the gelatin. Add the agar. Autoclave at 115°C for 20 min. Pour as plates.

Indole[21,60]

Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	1.5 g
L-tryptophan	0.04 g
Distilled water	100 ml
pH	7.5

Dissolve ingredients. Aliquot as 3 ml volumes in 12x90mm tubes. Autoclave at 121°C for 15min.

Chromogen Tests[30]

Nutrient Base

Tryptone (Oxoid LP0042)	4.0 g
Yeast extract (Oxoid LP0021)	0.4 g
NaCl	6.0 g
Distilled water	300 ml
pH	7.5

Dissolve ingredients and aliquot as four volumes of 75 ml. Autoclave at 121°C for 15 min.

Phosphate buffer: 0.01M, pH 7.5

Stock 0.1M buffer

$NaH_2PO_4 \cdot 2H_2O$	0.245 g
$Na_2HPO_4 \cdot 12H_2O$	3.022 g
Distilled water, made up to	100 ml
pH	7.5

Prepare a stock of 0.01M buffer, pH 8.0

0.1M phosphate buffer	10 ml
Distilled water	90 ml

Phosphate buffer: 0.01M, pH 8

Prepare a x10 stock of 0.1M buffer

$NaH_2PO_4 \cdot 2H_2O$	0.083 g
$Na_2HPO_4 \cdot 12H_2O$	3.395 g
Distilled water, made up to	100 ml
pH	8.0

Prepare a stock of 0.01M buffer, pH 8

0.1M phosphate buffer	10 ml
Distilled water	90 ml

Chromogen stocks

IXP medium

3-indoxyl phosphate	0.08 g
0.01M phosphate buffer, pH 8	25 ml

Filter sterilise. Aseptically add to 75 ml of nutrient base. Aseptically aliquot as 2 ml volumes in 12x90mm sterile tubes.

PNPG medium

4-nitrophenyl α-D-galactopyranoside	0.06 g
0.01M phosphate buffer, pH 7.5	25 ml

Filter sterilise. Aseptically add to 75 ml of nutrient base. Aseptically aliquot as 2 ml volumes in 12x90mm sterile tubes.

LGN medium

L-Glutamic acid 5-(4-nitroanilide)	0.06 g
0.01M phosphate buffer, pH 7.5	25 ml

Filter sterilise. Aseptically add to 75 ml of nutrient base. Aseptically aliquot as 2 ml volumes in 12x90mm sterile tubes.

NPS medium

4-nitrophenyl sulphate	0.04 g
0.01M phosphate buffer, pH 7.5	25 ml

Filter sterilise. Aseptically add to 75 ml of nutrient base. Aseptically aliquot as 2 ml volumes in 12x90mm sterile tubes.

Protect media from light.

Aesculin Hydrolysis[16]

Tryptone (Oxoid LP0042)	1.0 g
Yeast extract (Oxoid LP0021)	0.1 g
NaCl	1.5 g
Aesculin	0.1 g
Ferric citrate	0.05 g
Distilled water	100 mL
pH	7.5

Dissolve ingredients and aliquot as 3 ml volumes in 12x90mm tubes. Autoclave at 115°C for 10min. Protect medium from light.

Carbon Source Utilisation Tests[9]

Inorganic nitrogenous base

Buffer base

TRIS (basic)	6.1 g
Distilled water	500 ml
pH	7.5

Adjust pH with concentrated HCl

Salts solution

NH_4Cl	1.0 g
$K_2HPO_4 \cdot 3H_2O$	0.075 g
$FeSO_4 \cdot 7H_2O$	0.028 g
NaCl	11.7 g
$MgSO_4 \cdot 7H_2O$	12.3 g
KCl	0.75 g
Yeast extract (Oxoid LP0021)	0.015 g
$CaCl_2 \cdot H_2O$	1.45 g
Distilled water	400 ml

Weight out all the salts and combine except for the calcium chloride. Add the distilled water; once the salts are fully dissolved add the calcium chloride. Combine the buffer base and salts solution.

Divide the medium into 10 x 90 ml volumes. To each volume add 1.2 g of *purified* agar (Oxoid LP0028) and autoclave at 121°C for 15 min; cool to 55°C.

Carbon sources

Prepare 2% w/v or v/v concentrations in distilled water, of the carbon substrates listed; filter sterilise.

α-ketoglutarate	L-histidine
Acetate	DL-3-hydroxybutyrate
D-alanine	trans-4-hydroxy-L-proline
Citrate	DL-lactate
L-citrulline	D-lactose
D-galactose	Propionate
D-gluconate	Putrescine
D-glucosamine	Succinate
D-glucose	Sucrose
D-glucuronate	Water (control)
Glycerol	

Complete medium

To a 90 ml volume of cooled molten inorganic nitrogenous base, add 10 ml of carbon source. Mix well and pour as plates.

Supplement for nutritionally fastidious strains

To the nitrogenous base add 0.15 g Casamino Acids (Difco, 0230-15).

Decarboxylase Test[36,46,60]

Difco Decarboxylase broth base (Møller)	300 ml
Yeast extract (Oxoid LP0021)	0.3 g
NaCl	4.5 g
pH	6.5

Divide the base into three volumes of 100 ml each. To one volume add 1.0 g of L-lysine and to the second volume 1.0 g of L-ornithine; the third volume is a control. Check and adjust the pH to 6.5 if required. Autoclave at 121°C for 10 min. Check the pH of the media and, if required, adjust aseptically with 1N NaOH or HCl. Aseptically aliquot the media as 3 ml volumes into 12x90mm sterile tubes.

Urease[33]

Broth base

Peptone (Oxoid LP0037)	0.1 g
Glucose	0.1 g
Sodium chloride	1.5 g
Na_2HPO_4 (anhydrous)	0.12 g
KH_2PO_4 (anhydrous)	0.08 g
Phenol red (0.01%)	4 ml
Distilled water	95 ml
pH	6.8

Dissolve the ingredients and autoclave as a single volume at 115°C for 20 min.

Urea stock (40% w/v)

Urea	8 g
Distilled water	20 ml

Dissolve the urea and filter sterilise.

Complete medium

To the cool sterile base add 5 ml of sterile urea stock; mix. Aliquot aseptically as 2 ml volumes in 12x90mm sterile tubes.

Shelf Life of Media

Agar plates have a shelf life of 4 weeks and liquid media have a shelf life of 8 weeks when stored at 2-8°C.

Saturated Ammonium Sulphate (Gelatin test)[42]

Ammonium sulphate	10 g
Distilled water	10 mL

Store at room temperature.

Coblentz Reagents (Acetoin test)[32]

Reagent 1

α-naphthol	0.5 g
Ethanol	10 mL

Store refrigerated in a dark bottle.

Reagent 2

Creatine	0.03 g
KOH	4.0 g
Distilled water	10 mL

Store refrigerated.

Iodine (Starch test)[22]

Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

Dissolve the potassium iodide in 20 ml of water and then add the iodine. Once dissolved, make up to 300 ml with water.

Store at room temperature in a dark bottle.

Kovács' Reagent (Indole test)[5]

p-dimethylaminobenzaldehyde	2 g
pentan-1-ol (*n* amyl alcohol)	30 ml
Concentrated HCl	10 ml

Dissolve the aldehyde in the alcohol by gently warming at 50-55°C. Cool and slowly add the acid. Protect from light and store at 4°C.

Note: *iso* amyl alcohol is not the same as *n* amyl alcohol. The *iso*- form of pentanol cannot be used for Kovács' reagent.

Appendix 3
Identification by Phenotype

Principle

Identification of *Vibrionaceae* is made using the entire panel of tests listed in Table 7 using the standardised test formulations given in Appendix 2. An identification is made by matching the phenotype of an unknown against the probability data matrix VibEx7 and determining the most likely identification using the computer software package PIBWin.

Table 7. Panel of tests for the identification of *Vibrionaceae*

Arginine dihydrolase
Lysine decarboxylase
Ornithine decarboxylase
Acid (fermentation)
Arbutin
Mannitol
Salicin
Sucrose
Gentiobiose
Growth
7% NaCl
10% NaCl
Acetoin
Indole
Alkaline phosphatase, pH 8.0
Oxidase
Hydrolysis
2-nitrophenyl β-D-galactopyranoside
L-glutamic acid 5-(4-nitroanilide)
4-nitrophenyl sulfate
Aesculin
Agar
Gelatin
Starch
Sole carbon utilisation:
α-ketoglutarate
Acetate
D-alanine
Citrate
L-citrulline
D-galactose
D-gluconate
D-glucosamine
D-glucose
D-glucuronate
Glycerol
L-histidine
DL-3-hydroxybutyrate
trans-4-hydroxy-L-proline
DL-lactate
D-lactose
Propionate
Putrescine
Succinate
Sucrose
Resistance
0/129 10µg
0/129 150µg
Ampicillin 10µg
Carbenicillin 100µg
Novobiocin 5µg

Sample Requirements

Pure cultures less than 48 hours old should be used for the inoculum. Cultures recovered from preservation by freezing or drying must be subcultured at least twice before commencing identification.

Test Procedures

Perform an oxidase test by a preferred method. Observe agarolytic activity as pitting of colonies on maintenance agar such as JMA or ZMA.

Inocula: Prepare two inocula in 3ml volumes of 2% saline: one to a density equal to McFarland 0.5, the other to McFarland 2.

Inoculation of media: Inoculate the decarboxylase test media with 100µL of McFarland 2 density cell suspension. Inoculate the remaining liquid media with 100µL of McFarland 0.5 density cell suspension. For the acetoin and arginine dihydrolase tests, inoculate the semi-solid media with a straight wire.

Sole carbon source media are spot inoculated with 2µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 30.

Gelatin and starch plates are spot inoculated with 2µL of McFarland 0.5 suspension or with a multipoint inoculator. Maximum number of inocula on a plate should not exceed 6 well separated inoculum points.

Tests for arginine dihydrolase and decarboxylases are overlayed with 20-25mm of sterile liquid paraffin.

Sensitivity tests are undertaken on Mueller-Hinton agar supplemented with 2% w/v

NaCl. The medium is inoculated with the McFarland 0.5 suspension.

Incubation: Tests are incubated at 25°C for 48 hours. Observe tests daily and record changes. Sensitivity tests are incubated for 24 hours and the diameter of the zone of inhibition measured. Psychrophilic species are tested at 15°C for 8 days; sensitivity tests for 3-4 days. Known psychrophiles are listed in Table 7.

Table 8. *Vibrionaceae* requiring incubation at 15°C

Moritella marina
Moritella viscosa
Photobacterium iliopiscarium
Photobacterium phosphoreum
Vibrio logei
Vibrio wodanis
Vibrio salmonicida

Test Interpretation

Arginine dihydrolase: A positive reaction is a pink/red colour; yellow to orange is negative, Figure 1.

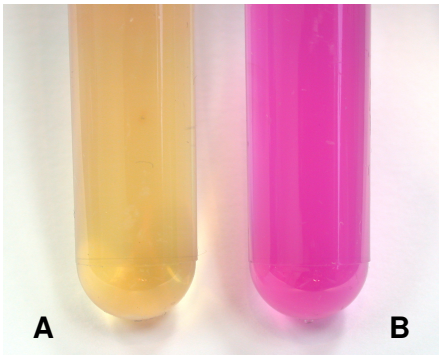

Figure 1. Arginine dihydrolase. **A**: negative; **B**: positive

Decarboxylase tests: The negative control should be yellow for the test to be valid; a positive test for ornithine or lysine decarboxylase is purple, Figure 2.

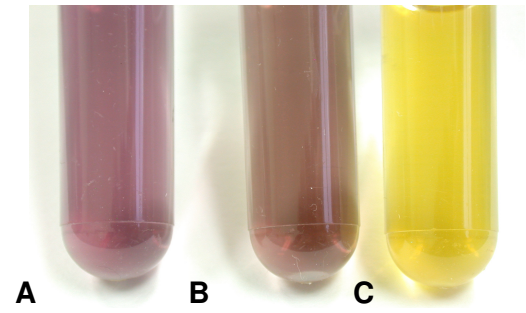

Figure 2. Decarboxylase test. **A**: positive ornithine; **B**: positive lysine; **C**: negative control

Acid from carbohydrates: A dirty yellow to bright yellow colour is positive; pale purple to deep purple is negative, Figure 3. Protein deamination may occur with prolonged incubation and may cause positive tests to appear negative due to alkaline pH shifts; ignore reversions.

Figure 3. Fermentation test. **A**: positive; **B**: negative

NaCl tolerance tests: Any signs of growth is a positive reaction.

Acetoin: Overlay semi-solid medium with 200µL of α-naphthol followed by 100µL of KOH/creatine. Leave at room temperature for up to 30 minutes. Pale pink to red layer is positive; yellow to tan is negative, Figure 4.

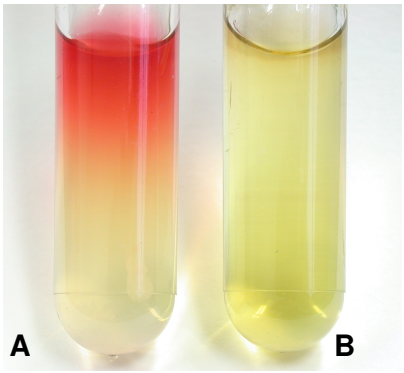

Figure 4. Acetoin (Voges-Proskauer) test. **A**: positive; **B**: negative

Indole: In a microtitre tray well add 100µL of Kovács' indole reagent to an equal volume of culture. Mix the contents of the well by careful aspiration with a pipette. If a pink/red colour is visible record as positive, Figure 5. If in doubt, remove contents with a glass Pasteur pipette and allow the phases to separate in the pipette body; record the reaction based on the top phase only.

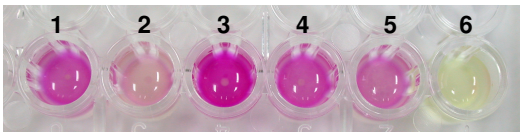

Figure 5. Indole test. Wells 1-5 is a range of positive reactions; well 6 is negative.

Gelatin hydrolysis: Flood plate with saturated ammonium sulphate. Any zone of clearing around inocula is positive.

Starch hydrolysis: Flood plate with Gram's iodine. Zones of yellow to light tan colour around inocula is positive; black to dark blue is negative.

Urease: A pink/red colour is positive; yellow is negative, Figure 6.

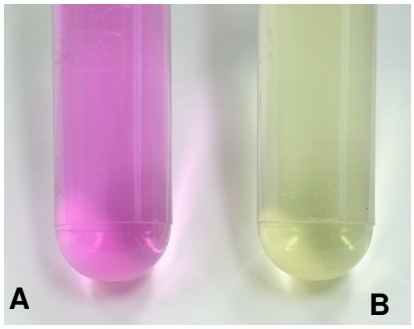

Figure 6. Urease test. **A**: positive; **B**: negative

Chromogens:
Indoxyl alkaline phosphatase: Any blue/black colour is positive; no colour is negative, see Figure 7.

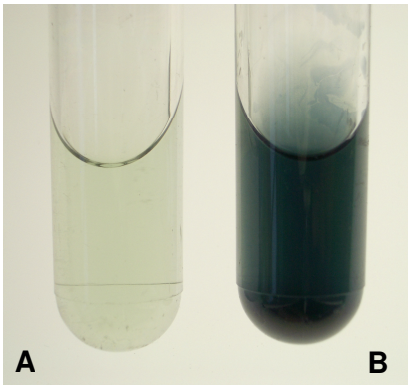

Figure 7. Indoxyl phosphate for alkaline phosphatase test, pH 8.0. **A**: negative; **B**: positive.

Nitrophenol & nitroaniline chromogens: Any yellow colour is positive; no colour is negative, see Figure 8.

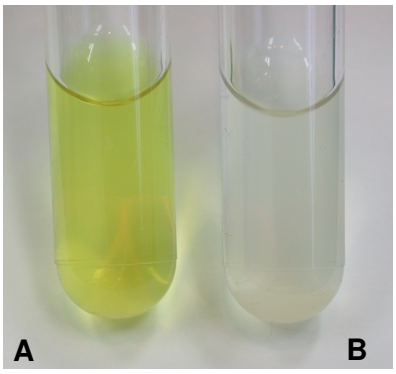

Figure 8. Nitrophenol and nitroanilide chromogen test. Substrate: 2-nitrophenyl β-D-galactopyranoside (PNPG). **A**: positive; **B**: negative.

Aesculin: A brown to black colour is positive for hydrolysis; a very light tan to colourless is negative; see Figure 9.

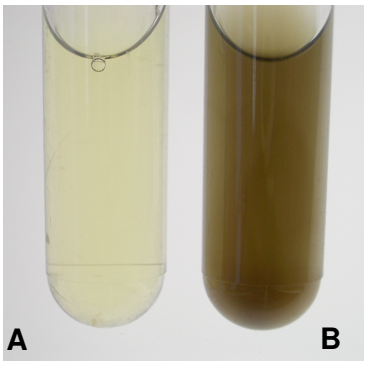

Figure 9. Aesculin test. **A**: negative; **B**: positive

Sole carbon source tests: Examine the negative control plate; some strains and some species may show very slight growth even when using purified agar, Figure 10. With reference to the control plate examine growth on the remaining test plates. Growth

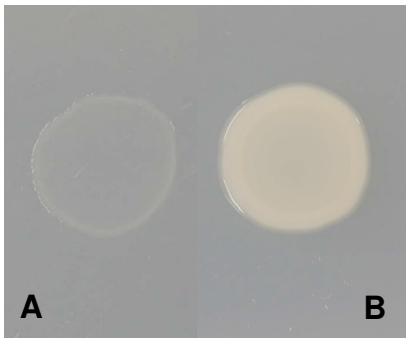

Figure 10. Sole carbon source test. **A**: negative control; **B**: glucose. Test organism *Vibrio fluvialis* NCTC 11327^{T}. Differentiation between positive and negative tests.

in excess of the negative control is read as positive. If there is no growth with glucose the strain may be nutritionally fastidious.

Retest with sole carbon source media containing 0.015g/L Casamino acids.

Antibiotic sensitivity test: The diameter of the zone of inhibition is interpreted using the data in Table 9; disc size is 6mm.

Table 9. Zone size interpretation for diagnostic antibiotics

Test	Resistant	Sensitive
0/129 10µg	≤15mm	≥16mm
0/129 150µg	No zone	Any zone
Ampicillin 10µg	≤13mm	≥14mm
Novobiocin 5µg	≤16mm	≥17mm
Carbenicillin 100µg	≤22mm	≥23mm

Probabilistic identification

An identification is obtained using the software PIBWin and the probability matrix VibEx7. An identification is accepted if the Willcox probability score *P* is ≥0.99 and the modal likelihood score (MLS) is ≥0.001[15].

If *P* is ≥0.99 but the MLS is <0.001, the unknown may represent an outlier of the nominated species. Differences between expected and observed reactions should be inspected to determine if the suggested identification can be accepted. If test variance for unexpected results is between 30-70% then the identification can be accepted but should be reported as a poor fit that represents a species outlier. If observed and expected reactions for two or more tests are in complete disagreement then the suggested identification is erroneous and the unknown should be reported as unidentifiable.

Each species in the data matrix is proceeded by the number of strains used to establish the phenotypic range. For taxa based on only a few strains an acceptable match will occur only if the unknown is very similar to the species description. Since the estimate of diversity is narrow, it is likely that strains of the species may appear as outliers.

In some instances, an unknown may appear to span two near related taxa such as biovars of a species. If the first and second most likely identifications are for related taxa then the probability values can be summed[6], and if P_{sum} is ≥0.99, the unknown can be assigned to the level of taxon complex or group.

Care must be exercised when accepting a computer assisted identification. Ultimately professional judgement and micro-biological sense must be used for interpretation.

Appendix 4
Identification by PCR

Introduction

The primer sets have been evaluated and optimised for the purpose of culture identification. It should be recognised that the protocols represent optimal conditions but minor refinements may be required to account for variation in the characteristics of different thermal cyclers.

Extraction of DNA

Reference DNA: Extract and purify DNA from control strains using a QIAamp DNA mini-kit (cat. no. 51304, QIAGEN). Purified control DNA is used at a concentration of 50pg μl^{-1}.

Sample DNA: To 100µl of PCR grade 18MΩ water in a 1.5 ml microfuge tube, suspend sufficient cells to a density equivalent to McFarland 1. Hold the tube at 100°C in a dry-heat block for 10 minutes and then cool rapidly in a cool block for 5 minutes. Pellet the cells at 10,000 rcf for 5 minutes and collect the supernatant containing liberated DNA. The extracted DNA is suitable for amplification without purification.

PCR reaction volume

All PCR reactions are as 25µl volumes in 200µl thin walled tubes.

Standard PCR reagents

Standard reagents for the PCR primer sets are listed in Table 10.

Table 10. Standard PCR reagents

PCR grade water, 18MΩ
50mM magnesium chloride
10x Invitrogen Platinum® Taq buffer
16mM dNTP stock (4mM each dNTP)
Invitrogen Platinum® Taq DNA polymerase
Primers (20µM stock)

Electrophoresis of amplicon

Amplicon is visualised by electrophoresis using 2% agarose gel containing 0.5µg ml^{-1} ethidium bromide and 1x TBE buffer,

Table 11. Use a 100bp ladder as a comparative index of amplicon size. Gels should be run at 7 volts cm^{-1} constant voltage.

Table 11. 10x Tris-Boric-EDTA buffer

Tris (base)	108.0 g
Boric acid	55.0 g
EDTA	8.3 g
pH	8.0

Photobacterium damselae[41]

Targets: 16S rRNA gene for species identification and *ure*C gene for subspecies identification.

Primers:

Primer	Sequence 5' → 3'
Car1	gcttgaagagattcgagt
Car2	cacctcgcggtcttgctg
Ure-5'	tccggaataggtaaagcggg
Ure-3'	cttgaatatccatctcatctgc

DNA controls:
Ph. damselae ssp. *damselae* NCIMB 2184^T
Ph. damselae ssp. *piscicida* NCIMB 2058^T
Ph. iliopiscarium ATCC 51760^T

Master mix:

Component	Volume
Water	11.15 µl
50mM $MgCl_2$	1 µl
10x reaction buffer	2.5 µl
16mM dNTPs	1.25 µl
20µM Car1	2 µl
20µM Car2	2 µl
20µM Ure-5'	2 µl
20µM Ure-3'	2 µl
5U $µl^{-1}$ Taq polymerase	0.1 µl
Template DNA	1 µl
Volume	25 µl

Cycle parameters:

Cycle parameters	
95°C	4min.
30 cycles	
95°C	1min.
60°C	1min.
72°C	40sec.
72°C	5min. final extension

Interpretation:
Ph. damselae ssp. *damselae*. Species specific amplicon at 267bp for 16S rDNA with an additional amplicon at 448bp for the *ure*C gene that is diagnostic for the subspecies *damselae*.

Ph. damselae ssp. piscicida. A single amplicon at 267bp for 16S rDNA. Absence of an amplicon for *ure*C is diagnostic for the subspecies *piscicida*.

Limitations:
A single band of 267bp is diagnostic of *Ph. damselae sensu lato*. An identification of *Ph. damselae* ssp. *piscicida* is inferred if only this band is present but some caution needs to be used where an identification is reached on the basis of absence. Corroborating phenotypic evidence and complete 16S rRNA sequence should be obtained if the identification represents a new finding for a region or host not previously associated with *Ph. damselae* ssp. *piscicida*.

Vibrio harveyi[39]

Target: 16S rRNA gene

Primers:

Primer	Sequence 5' → 3'
VH-1	AACgAgTTATCTgAACCTTC
VH-2	gCAgCTATTAACTACACTACC

DNA controls:
V. harveyi ATCC 14126^T
V. alginolyticus ATCC 17749^T

Master mix:

Component	Volume
Water	18.15 µl
50mM $MgCl_2$	1.5 µl
10x reaction buffer	2.5 µl
16mM dNTPs	1.25 µl
20µM VH-1	0.25 µl
20µM VH-2	0.25 µl
5U $µl^{-1}$ Taq polymerase	0.1 µl
Template DNA	1 µl
Volume	25 µl

Cycle parameters:

Cycle parameters	
95°C	2min.
40 cycles	
94°C	1min.
65°C	1min.
72°C	2min.
72°C	5min. final extension

Interpretation:
A single band of 413bp is characteristic of *V. harveyi*.

Limitations:
On the basis of testing undertaken, the primers are specific for strains of both *V. harveyi* biovar I and biovar II associated with blister disease in abalone. Some strains

of *V. alginolyticus* are known to cross react because of sequence similarity with the primer regions. A positive finding must be corroborated by phenotype using the tests given in Table 4.

Vibrio vulnificus[11]

Target: *cth* cytolysin/haemolysin gene.

Primers:

Primer	Sequence 5' → 3'
L-CTH	ttccaacttcaaaccgaactatgac
R-CTH	gctactttctagcattttctctgc

DNA controls:
V. vulnificus ATCC 27562^T
V. parahaemolyticus ATCC 17802^T

Master mix:

Component	Volume
Water	16.375 µl
50mM $MgCl_2$	1.25 µl
10x reaction buffer	2.5 µl
16mM dNTPs	1.25 µl
20µM L-CTH	1.25 µl
20µM R-CTH	1.25 µl
5U μl^{-1} Taq polymerase	0.125 µl
Template DNA	1 µl
Volume	25 µl

Cycle parameters:

Cycle parameters	
95°C	3min.
30 cycles	
94°C	1min.
57°C	1min.
72°C	3min.
72°C	5min. final extension

Interpretation:
A single band of 205bp is characteristic of the cytolysin/haemolysin gene in *V. vulnificus*.

Limitations:
An identification of *V. vulnificus* is inferred by the presence of the *cth* gene which appears to be specific for *V. vulnificus*. It is noteworthy that the forward primer L-CTH is unique to *V. vulnificus* while R-CTH is homologous with the thermolabile haemolysin gene of *V. parahaemolyticus*[50]. Based on limited testing the primers appear specific for *V. vulnificus*, however the frequency with which the gene occurs within the species is not known and may not be sufficiently reliable for the purpose of identification in the absence of *a priori* information.

Vibrio anguillarum[23]

Target: *vah*1 haemolysin gene.

Primers:

Primer	Sequence 5' → 3'
VaH1-P1	accgatgccatcgctcaaga
VaH1-P2	ggatattgaccgaagagtca

DNA controls:
V. anguillarum ATCC 19264^T
V. parahaemolyticus ATCC 17802^T

Master mix:

Component	Volume
Water	14.4 µl
50mM $MgCl_2$	0.75 µl
10x reaction buffer	2.5 µl
16mM dNTPs	1.25 µl
20µM VaH1-P1	2.5 µl
20µM VaH1-P2	2.5 µl
5U μl^{-1} Taq polymerase	0.1 µl
Template DNA	1 µl
Volume	25 µl

Cycle parameters:

Cycle parameters	
94°C	4min.
30 cycles	
94°C	30sec.
55°C	30sec.
72°C	60sec.
72°C	5min. final extension

Interpretation:
A single band of 490bp is characteristic of the haemolysin gene of *V. anguillarum*.

Limitations:
The *vah*1 gene appears to be specific to *V. anguillarum* despite having a common ancestry with haemolysins from other species of *Vibrionaceae*. From limited testing however it appears that not all strains of *V. anguillarum* possess the *vah*1 gene and its use as a primary means of identification is limited. Primers for the *vah*1 gene should be limited to determining the presence of the haemolysin gene in strains or as a supporting test.

Appendix 5: VibEx7 probability matrix for the identification of *Vibrionaceae*

Data as % strains positive		*A. sobria HG7*	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leiognathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
Arginine dihydrolase		86	1	1	57	1	99	99	99	99	75	99	99	1	3	96	99
Acid:	Arbutin	14	1	1	1	1	1	1	1	1	1	1	1	14	3	1	67
	Mannitol	99	1	1	1	99	1	1	1	33	1	1	99	99	99	98	50
	Salicin	14	1	1	1	1	1	1	1	1	1	1	1	57	20	1	75
	Sucrose	57	1	1	1	1	1	1	1	33	50	1	99	1	99	98	75
	Gentiobiose	14	1	1	1	99	1	1	1	1	1	1	1	99	1	1	75
Growth:	7% NaCl	1	99	99	1	99	86	99	1	99	50	99	67	29	99	93	25
	10% NaCl	1	25	99	1	1	1	1	1	1	1	1	1	1	99	3	1
Amylase		99	1	1	99	1	27	14	20	99	1	1	99	43	99	96	99
Voges Proskauer (Acetoin)		86	1	1	1	1	99	99	99	99	99	99	1	14	99	94	1
Gelatinase		71	1	1	99	1	24	33	20	1	25	1	67	1	99	77	50
Indole		86	75	1	1	1	3	1	1	1	1	1	99	1	99	93	99
IXP alkaline phosphatase		86	50	1	13	99	99	85	20	33	25	99	99	43	99	88	50
PNPG α-D-galactosidase		29	1	99	1	1	3	1	20	1	1	99	99	99	16	53	99
LGN γ-glutamyl transpeptidase		71	1	99	99	1	48	14	20	1	75	1	1	29	96	15	50
NPS sulphatase		14	1	99	1	99	1	1	1	1	25	1	1	14	40	1	1
Aesculin hydrolysis		71	1	1	20	50	31	1	1	1	25	1	33	99	10	63	99
Utilisation:	α-ketoglutarate	14	50	99	1	99	3	1	20	1	1	99	67	1	99	36	25
	Acetate	29	99	99	88	99	93	1	60	99	50	1	67	43	96	51	99
	Alanine	1	99	99	25	1	10	1	99	1	1	1	99	14	99	76	99
	Citrate	43	99	1	1	99	1	1	1	99	1	1	99	1	96	95	99
	Citrulline	1	1	1	38	1	1	1	1	1	1	1	1	1	20	16	99
	Galactose	57	99	1	1	99	93	28	60	99	25	99	99	71	13	71	99
	Gluconate	99	75	99	1	1	3	14	20	99	99	1	99	14	99	96	75
	Glucosamine	99	25	1	50	99	99	42	60	99	99	99	99	86	99	95	99
	Glucuronate	1	1	1	1	1	1	1	1	33	1	1	1	1	1	1	1
	Glycerol	99	75	99	99	99	99	42	60	99	75	99	99	1	99	95	99
	Histidine	29	50	1	88	1	1	1	1	1	1	1	1	1	99	93	50
	DL-3-hydroxybutyrate	1	1	1	1	1	1	1	1	1	25	1	1	1	1	1	99
	Hydroxyproline	1	1	1	1	99	1	1	1	1	1	1	1	1	99	1	75
	DL-lactate	14	99	99	99	1	79	1	1	1	99	99	99	29	99	91	99
	Lactose	1	1	1	1	1	1	1	20	1	1	99	1	1	1	1	1
	Propionate	14	99	1	1	1	1	1	1	1	1	1	67	1	99	6	99
	Putrescine	14	1	1	1	1	1	1	1	99	1	1	1	1	99	1	50
	Succinate	86	75	99	99	99	89	42	40	99	75	99	99	29	99	93	75
	Sucrose	57	1	1	1	1	1	1	1	33	50	1	99	1	99	96	75
Oxidase		99	99	99	99	99	99	99	99	99	99	1	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	99	1	1	1
Resistance:	0/129 10µg	99	1	99	38	1	1	14	1	1	1	1	1	1	70	3	1
	0/129 150µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Data as % strains positive		*A. sobria HG7*	*G. hollisae*	*M. marina*	*M. viscosa*	*Ph. angustum*	*Ph. damselae* ssp. *damselae* biovar I	*Ph. damselae* ssp. *damselae* biovar II	*Ph. damselae* ssp. *piscicida*	*Ph. iliopiscarium*	*Ph. leiognathi*	*Ph. phosphoreum*	*V. aestuarianus*	*V. agarivorans*	*V. alginolyticus*	*V. anguillarum*	*V. calviensis*
Test	*No. strains*	7	4	1	8	1	29	7	5	3	4	1	3	7	30	60	4
	Ampicillin 10µg	57	1	1	1	99	65	14	20	1	75	1	1	1	99	91	1
	Novobiocin 5µg	86	25	1	1	50	27	14	20	33	50	1	67	14	90	11	1
	Carbenicillin 100µg	17	1	1	1	99	89	42	20	33	75	1	33	1	99	90	1
Lysine decarboxylase		99	1	1	38	99	62	57	1	99	1	1	1	1	99	1	25
Ornithine decarboxylase		29	1	1	1	1	1	1	1	1	1	1	1	1	66	1	1
Urease		14	1	1	1	99	99	99	1	1	25	1	1	1	1	1	1

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	No. strains	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
Arginine dihydrolase		1	95	1	1	99	99	1	1	99	99	1	1	15	1	1	1
Acid:	Arbutin	1	1	1	66	1	99	1	1	99	1	99	1	29	1	1	1
	Mannitol	50	99	99	99	85	99	40	87	99	99	33	99	94	83	60	99
	Salicin	75	5	1	66	1	99	40	40	99	1	99	1	79	4	1	1
	Sucrose	1	43	99	66	99	99	20	1	99	99	99	67	65	1	66	99
	Gentiobiose	50	1	1	33	14	1	60	90	11	1	1	1	95	9	1	1
Growth:	7% NaCl	99	95	58	99	99	99	1	70	99	99	99	67	99	99	33	67
	10% NaCl	1	10	1	66	1	50	1	1	56	90	66	33	5	4	16	1
Amylase		75	99	75	66	99	25	40	55	99	70	99	1	99	99	1	1
Voges Proskauer (Acetoin)		1	5	58	33	1	1	1	1	11	1	99	1	2	75	1	1
Gelatinase		99	99	67	33	99	25	20	1	44	30	99	33	97	83	16	1
Indole		99	95	99	1	85	99	20	1	99	99	1	33	97	99	1	1
IXP alkaline phosphatase		99	99	92	99	85	99	80	99	99	99	50	33	99	99	99	99
PNPG α-D-galactosidase		1	76	75	99	99	99	1	20	33	44	99	99	95	99	1	1
LGN γ-glutamyl transpeptidase		99	86	99	1	71	99	1	50	99	99	1	1	94	87	1	1
NPS sulphatase		1	99	8	1	71	25	60	99	11	1	1	1	60	61	16	33
Aesculin hydrolysis		99	99	22	99	99	99	80	99	88	10	99	33	95	99	66	1
Utilisation:	α-ketoglutarate	99	99	99	1	99	1	1	1	99	99	1	1	97	1	1	1
	Acetate	25	99	99	33	99	99	80	1	99	90	99	33	89	99	83	99
	Alanine	50	99	67	1	99	99	20	1	99	99	33	1	99	99	33	1
	Citrate	99	95	92	99	99	99	1	60	99	99	66	1	95	99	16	1
	Citrulline	25	62	1	1	85	1	1	1	56	10	1	1	6	1	1	1

Data as % strains positive		*V. campbellii*	*V. chagasii*	*V. cholerae*	*V. cincinnatiensis*	*V. cyclitrophicus*	*V. diazotrophicus*	*V. fischeri* biovar I	*V. fischeri* biovar II	*V. fluvialis*	*V. furnissii*	*V. gazogenes*	*V. halioticoli*	*V. harveyi* biovar I	*V. harveyi* biovar II	*V. ichthyoenteri* biovar I	*V. ichthyoenteri* biovar II
Test	No. strains	4	21	12	3	7	4	5	10	9	10	3	3	62	23	6	3
	Galactose	1	99	83	66	85	99	40	99	99	99	1	99	73	74	1	1
	Gluconate	1	67	99	66	99	99	1	20	99	99	33	1	99	99	99	1
	Glucosamine	1	99	99	99	99	75	20	99	99	99	33	99	84	96	99	99
	Glucuronate	1	14	25	1	1	25	1	1	78	10	99	1	61	13	99	1
	Glycerol	99	99	99	99	99	1	40	90	99	99	99	1	85	83	1	1
	Histidine	1	10	67	1	71	99	1	1	89	99	1	1	8	1	1	1
	DL-3-hydroxybutyrate	1	1	1	1	14	1	40	1	78	80	1	1	1	1	1	1
	Hydroxyproline	1	10	1	1	71	1	1	1	11	1	1	1	50	1	1	1
	DL-lactate	75	99	99	66	99	99	1	1	99	99	66	99	99	99	66	99
	Lactose	1	1	1	1	28	50	1	1	1	1	1	1	1	1	1	1
	Propionate	1	95	58	1	99	75	1	1	99	99	66	1	94	78	16	1
	Putrescine	1	5	1	1	1	99	1	1	33	90	1	1	2	1	1	1
	Succinate	99	99	75	99	99	75	40	90	99	90	99	1	97	99	1	99
	Sucrose	1	43	99	66	99	99	60	1	89	99	99	67	65	4	66	99
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	33	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	25	19	8	66	28	25	1	1	44	60	99	1	82	91	16	67
	0/129 150µg	1	1	1	1	1	1	1	1	1	10	1	1	1	1	1	1
	Ampicillin 10µg	99	99	25	1	1	1	60	80	33	80	1	33	97	1	33	1
	Novobiocin 5µg	99	19	17	50	71	75	1	10	99	99	99	33	94	74	66	1
	Carbenicillin 100µg	99	99	33	1	1	1	99	80	11	80	1	33	95	1	33	1
Lysine decarboxylase		50	5	99	1	28	1	20	55	1	1	1	1	98	99	1	1
Ornithine decarboxylase		1	1	99	1	28	1	20	33	11	1	1	1	99	1	1	1
Urease		1	10	1	1	1	1	99	99	1	1	1	1	63	1	1	1

Data as % strains positive		*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchrituudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test	*No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Arginine dihydrolase		99	1	84	99	1	99	1	1	99	1	1	99	6	99	1	1
Acid:	Arbutin	1	1	1	1	1	25	83	75	1	1	1	1	1	1	1	1
	Mannitol	75	99	99	99	86	99	99	99	67	1	1	99	99	1	99	99
	Salicin	1	1	80	1	1	99	92	50	1	99	1	17	18	1	1	1
	Sucrose	13	1	95	99	1	99	99	99	99	1	99	99	11	1	99	80
	Gentiobiose	1	1	21	1	1	99	99	25	1	50	1	1	1	1	1	40
Growth:	7% NaCl	38	1	74	80	86	99	99	99	99	1	1	67	99	1	80	80
	10% NaCl	1	1	5	40	14	99	75	1	67	1	1	1	89	1	1	1
Amylase		38	1	84	99	1	99	92	99	67	67	50	99	99	99	20	80

Data as % strains positive	*V. lentus*	*V. logei*	*V. mediterranei*	*V. metschnikovii*	*V. mimicus*	*V. mytili*	*V. natriegens*	*V. navarrensis*	*V. nereis*	*V. nigripulchritudo*	*V. ordalii*	*V. orientalis*	*V. parahaemolyticus*	*V. pectenicida*	*V. pelagius* biovar I	*V. pelagius* biovar II
Test *No. strains*	9	2	19	5	7	5	12	4	6	3	6	6	18	2	10	5
Voges Proskauer (Acetoin)	1	1	1	99	1	1	1	1	17	1	1	1	1	1	1	1
Gelatinase	63	1	32	60	71	1	42	75	17	99	99	67	99	1	20	80
Indole	63	1	95	60	99	1	27	99	83	67	1	99	99	1	1	99
IXP alkaline phosphatase	75	99	89	40	86	99	75	99	67	67	40	83	99	99	99	99
PNPG α-D-galactosidase	50	1	99	99	43	75	75	1	1	99	1	67	17	1	99	99
LGN γ-glutamyl transpeptidase	38	99	99	1	99	60	67	50	99	67	99	83	99	1	80	99
NPS sulphatase	25	99	79	1	33	1	58	1	1	1	1	1	56	1	70	60
Aesculin hydrolysis	71	99	95	99	1	99	99	75	1	50	1	20	11	1	99	99
Utilisation: α-ketoglutarate	50	99	37	1	99	20	67	99	99	99	17	1	89	1	20	20
Acetate	88	50	99	60	99	99	92	75	99	67	1	67	99	1	90	80
Alanine	75	1	99	60	99	99	99	75	99	99	67	99	99	99	99	99
Citrate	13	99	99	40	99	99	92	99	99	99	99	99	94	1	80	99
Citrulline	1	1	11	1	1	20	83	1	83	1	1	1	17	1	60	99
Galactose	25	99	99	40	86	80	83	1	33	99	1	67	89	1	99	99
Gluconate	25	99	5	99	99	99	92	99	99	33	1	99	99	1	99	99
Glucosamine	13	99	99	20	99	99	99	99	99	99	50	83	99	50	70	99
Glucuronate	1	1	58	1	86	1	33	25	1	33	1	1	17	1	1	1
Glycerol	38	99	99	99	99	99	92	99	99	99	33	83	99	99	99	99
Histidine	1	1	89	1	99	99	99	99	99	99	33	17	99	1	80	1
DL-3-hydroxybutyrate	1	1	16	1	1	1	83	1	99	99	1	50	6	1	20	1
Hydroxyproline	1	1	1	1	1	1	25	1	1	1	1	83	83	1	1	1
DL-lactate	63	1	99	40	99	99	99	99	99	99	17	99	99	99	99	99
Lactose	1	1	99	40	1	20	8	1	1	99	1	1	1	1	10	20
Propionate	13	1	99	1	50	99	92	99	99	67	1	67	89	1	60	80
Putrescine	13	1	99	1	1	1	83	1	83	1	1	83	89	1	99	99
Succinate	50	99	95	60	99	80	99	99	83	99	1	99	89	50	99	99
Sucrose	1	1	99	99	1	99	99	99	99	1	83	99	11	1	99	80
Oxidase	99	99	99	1	99	80	99	99	99	99	99	99	99	99	99	99
Agarolysis	1	1	5	1	1	1	8	1	1	1	1	1	1	1	1	1
Resistance: 0/129 10µg	99	1	1	1	1	99	99	25	33	1	1	1	61	1	60	1
0/129 150µg	1	1	1	1	1	1	1	1	1	1	1	1	6	1	1	1
Ampicillin 10µg	13	99	1	40	14	1	8	50	1	1	50	1	94	50	10	1
Novobiocin 5µg	1	1	11	40	57	60	99	75	80	1	1	17	94	1	70	80
Carbenicillin 100µg	25	99	42	40	43	1	8	75	1	1	17	1	94	99	20	1
Lysine decarboxylase	1	99	22	60	99	1	1	1	1	1	1	1	99	1	1	1
Ornithine decarboxylase	13	99	1	1	99	1	1	1	1	1	1	1	99	1	1	1
Urease	1	1	1	1	1	1	1	1	1	1	1	1	17	1	1	1

Data as % strains positive		*V. penaeicida*	*V. proteolyticus*	*V. rumoiensis*	*V. salmonicida*	*V. scophthalmi*	*V. splendidus* biovar I	*V. splendidus* biovar II	*V. tapetis*	*V. tasmaniensis*	*V. tubiashii*	*V. vulnificus* biovar I	*V. vulnificus* biovar II	*V. wodanis*	Phenon 6	Phenon 8	Phenon 10
Test	*No. strains*	4	12	3	1	11	37	3	1	8	7	7	5	1	7	7	6
Arginine dihydrolase		1	99	99	1	9	99	99	1	99	99	1	1	1	99	99	17
Acid:	Arbutin	1	1	1	1	1	1	1	1	1	1	71	99	1	99	1	1
	Mannitol	1	99	33	99	10	97	99	1	71	99	71	1	1	99	99	99
	Salicin	1	1	1	1	1	8	1	1	25	99	57	99	1	99	1	33
	Sucrose	1	1	99	1	99	8	1	1	25	99	1	1	99	86	99	83
	Gentiobiose	1	1	1	1	1	1	1	1	1	99	28	40	1	40	1	1
Growth:	7% NaCl	1	99	67	99	36	71	33	1	75	57	85	20	1	99	99	99
	10% NaCl	1	99	33	1	1	11	1	1	1	1	1	1	1	99	99	83
Amylase		33	99	67	1	1	84	99	99	13	99	99	99	99	99	71	83
Voges Proskauer (Acetoin)		1	99	1	1	1	1	1	1	1	1	1	1	1	14	1	67
Gelatinase		1	99	67	1	1	89	99	1	13	99	99	80	1	14	99	99
Indole		1	75	1	1	1	95	66	1	63	99	99	1	99	99	86	83
IXP alkaline phosphatase		50	92	99	1	82	92	99	1	99	86	85	99	1	99	99	99
PNPG α-D-galactosidase		99	1	67	1	9	92	1	1	1	99	99	80	1	14	29	50
LGN γ-glutamyl transpeptidase		1	75	99	1	18	50	99	1	13	99	1	1	1	99	86	67
NPS sulphatase		1	1	1	1	1	79	1	1	99	1	1	1	1	86	86	50
Aesculin hydrolysis		1	1	33	1	99	97	99	1	99	99	42	25	1	99	1	83
Utilisation:	α-ketoglutarate	99	99	67	1	9	99	99	99	38	1	99	80	1	99	99	99
	Acetate	99	99	67	99	45	76	99	1	99	86	85	99	99	99	99	99
	Alanine	99	99	99	1	1	97	99	1	99	99	99	99	1	99	99	99
	Citrate	99	99	99	99	91	97	99	99	99	99	99	99	99	99	99	99
	Citrulline	1	25	1	1	1	24	1	1	1	99	1	1	1	71	14	17
	Galactose	99	1	99	99	36	92	1	1	1	99	99	99	1	1	43	67
	Gluconate	99	99	67	99	82	84	1	1	99	99	99	99	1	99	99	99
	Glucosamine	99	99	99	1	99	89	66	99	99	99	99	80	1	99	86	99
	Glucuronate	25	1	1	1	64	21	1	1	1	86	85	80	1	1	1	17
	Glycerol	99	99	99	99	1	99	99	99	99	99	85	99	99	99	99	99
	Histidine	99	99	33	1	1	3	1	1	1	99	28	1	1	86	99	83
	DL-3-hydroxybutyrate	25	1	1	99	18	1	1	1	1	99	1	1	1	99	1	1
	Hydroxyproline	1	99	1	1	1	1	1	1	1	86	1	1	1	1	1	99
	DL-lactate	99	99	67	1	99	97	66	99	99	99	99	99	1	99	99	99
	Lactose	99	1	33	1	1	21	1	1	1	99	1	1	1	1	1	1
	Propionate	75	99	33	1	18	39	33	1	13	86	99	80	1	99	86	99
	Putrescine	1	99	1	99	1	5	1	1	1	29	1	1	1	99	14	17
	Succinate	99	99	99	99	91	99	99	99	99	86	99	99	99	99	86	99
	Sucrose	1	8	99	99	99	3	33	1	25	99	1	1	99	86	99	83
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Resistance:	0/129 10µg	25	67	1	1	9	29	99	1	63	14	1	1	1	1	57	99
	0/129 150µg	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10µg	1	58	1	99	1	3	99	1	1	1	1	1	1	1	57	99
	Novobiocin 5µg	1	99	1	1	27	3	33	1	1	57	57	20	1	99	57	99
	Carbenicillin 100µg	1	1	33	99	1	18	99	1	1	1	1	1	1	1	71	99
Lysine decarboxylase		1	8	33	1	1	1	1	1	1	1	99	60	1	29	1	83
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	99	1	1	29	1	99
Urease		1	1	33	1	1	1	1	1	1	1	1	1	1	14	1	1

Data as % strains positive		Phenon 15	Phenon 19	Phenon 20	Phenon 21	Phenon 24	Phenon 25	Phenon 26	Phenon 27	Phenon 29	Phenon 36	Phenon 41	Phenon 42	Phenon 43	Phenon 45	Phenon 46	Phenon 52
Test	*No. strains*	8	4	6	8	4	14	16	17	17	6	6	4	11	4	6	3
Arginine dihydrolase		99	99	83	88	99	99	99	99	99	50	99	99	99	99	99	33
Acid:	Arbutin	99	1	83	99	1	21	1	1	1	1	1	1	9	1	1	1
	Mannitol	99	99	67	99	99	77	99	99	88	50	1	50	91	25	99	1
	Salicin	1	1	99	99	1	57	1	1	99	1	1	1	27	1	1	1
	Sucrose	99	99	99	99	99	99	99	99	12	1	99	99	82	25	1	1
	Gentiobiose	86	1	83	99	25	57	1	1	1	1	1	1	9	1	1	1
Growth:	7% NaCl	63	25	83	99	99	43	99	88	88	50	83	75	91	25	99	99
	10% NaCl	1	1	1	1	1	1	31	1	1	17	1	1	55	1	1	67
Amylase		63	99	99	99	99	99	93	94	99	67	99	99	73	50	99	67
Voges Proskauer (Acetoin)		25	1	1	1	1	1	1	1	1	1	17	99	9	1	1	67
Gelatinase		13	1	99	99	75	99	99	99	94	1	67	50	55	25	99	99
Indole		99	99	99	88	1	93	99	99	99	50	33	1	91	25	99	1
IXP alkaline phosphatase		99	25	83	99	99	99	99	99	99	1	83	99	99	99	99	67
PNPG α-D-galactosidase		99	99	17	99	99	99	99	88	99	99	99	1	1	1	1	33
LGN γ-glutamyl transpeptidase		50	99	50	99	1	14	99	82	94	67	99	99	55	25	17	1
NPS sulphatase		88	1	17	88	99	93	99	99	99	1	1	1	55	50	50	1
Aesculin hydrolysis		99	99	67	99	1	99	99	99	99	40	50	1	99	1	99	99
Utilisation:	α-ketoglutarate	38	1	99	99	25	99	99	69	99	99	1	1	9	25	99	1
	Acetate	38	75	99	88	99	57	99	88	99	99	99	50	73	99	99	67
	Alanine	13	99	83	99	50	99	99	94	99	83	99	99	99	99	1	33
	Citrate	88	99	83	99	50	99	99	99	94	99	67	75	91	99	1	33
	Citrulline	1	25	40	88	1	1	99	1	99	50	67	25	55	1	1	1
	Galactose	99	99	83	99	99	99	99	88	99	99	99	1	9	50	99	1
	Gluconate	50	1	83	99	99	79	99	94	99	33	83	25	99	75	99	33
	Glucosamine	99	99	99	99	99	99	99	94	99	99	1	99	99	99	99	67
	Glucuronate	13	1	1	1	99	1	6	53	99	17	1	1	1	1	1	1
	Glycerol	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	67
	Histidine	1	1	33	13	1	1	18	12	1	67	17	1	9	1	1	99
	DL-3-hydroxybutyrate	1	75	67	1	1	1	1	6	6	33	1	1	1	1	1	33
	Hydroxyproline	1	1	99	1	1	1	1	1	1	1	83	1	18	1	1	33
	DL-lactate	99	99	99	99	25	99	99	94	99	99	99	99	99	75	99	33
	Lactose	1	25	1	1	1	1	1	6	6	1	1	1	1	1	1	1
	Propionate	1	99	67	99	1	7	68	88	94	83	99	99	91	75	1	67
	Putrescine	99	1	17	1	1	1	6	1	1	33	1	1	9	1	1	1
	Succinate	99	99	83	99	99	93	99	99	99	99	99	99	91	99	99	1
	Sucrose	99	99	99	99	99	99	99	99	18	17	99	99	82	25	1	99
Oxidase		99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
Agarolysis		13	1	1	1	1	1	1	1	6	1	1	1	9	1	1	1
Resistance:	0/129 10µg	1	1	1	1	1	14	1	12	1	1	1	1	1	1	33	67
	0/129 150µg	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Ampicillin 10µg	1	1	1	1	1	1	1	1	1	1	99	75	9	25	1	33
	Novobiocin 5µg	1	50	17	1	1	1	18	1	1	1	67	99	18	1	1	99
	Carbenicillin 100µg	1	1	1	13	1	21	6	1	65	1	99	99	9	25	1	1
Lysine decarboxylase		25	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1
Ornithine decarboxylase		1	1	1	1	1	1	1	1	1	1	1	1	9	1	1	1
Urease		1	1	50	1	1	1	6	1	1	1	1	1	1	1	1	1

Data as % strains positive		Phenon 53	Phenon 57	Phenon 58	Phenon 59	Phenon 69	Phenon 83
Test	*No. strains*	9	4	4	14	5	6
Arginine dihydrolase		11	99	25	1	80	99
Acid:	Arbutin	1	1	1	1	1	1
	Mannitol	99	99	99	1	99	1
	Salicin	13	1	1	7	20	1
	Sucrose	99	1	1	14	99	1
	Gentiobiose	1	25	1	93	1	1
Growth:	7% NaCl	89	75	25	21	20	1
	10% NaCl	11	1	1	1	1	1
Amylase		1	99	1	1	80	99
Voges Proskauer (Acetoin)		1	1	1	1	99	1
Gelatinase		11	75	1	21	1	99
Indole		99	75	1	7	99	1
IXP alkaline phosphatase		99	99	99	93	1	33
PNPG α-D-galactosidase		1	99	99	99	80	99
LGN γ-glutamyl transpeptidase		33	1	1	1	1	66
NPS sulphatase		44	99	50	99	20	16
Aesculin hydrolysis		56	50	1	93	1	1
Utilisation:	α-ketoglutarate	1	25	1	1	80	33
	Acetate	99	50	50	7	99	99
	Alanine	1	99	1	1	80	1
	Citrate	89	75	99	99	99	50
	Citrulline	1	1	1	1	1	1
	Galactose	1	99	99	99	60	83
	Gluconate	1	75	25	7	99	1
	Glucosamine	99	99	99	99	20	99
	Glucuronate	44	75	1	1	1	1
	Glycerol	44	99	99	99	99	99
	Histidine	1	1	1	1	60	50
	DL-3-hydroxybutyrate	89	1	1	1	60	1
	Hydroxyproline	11	1	1	1	1	1
	DL-lactate	99	75	1	1	60	99
	Lactose	1	75	99	99	20	1
	Propionate	99	1	1	1	40	1
	Putrescine	1	1	1	1	99	1
	Succinate	78	99	99	93	99	99
	Sucrose	99	1	1	7	80	1
Oxidase		99	99	99	99	99	99
Agarolysis		1	1	1	1	1	1
Resistance:	0/129 10µg	89	1	1	1	40	1
	0/129 150µg	1	1	1	1	1	1
	Ampicillin 10µg	11	75	50	14	1	1
	Novobiocin 5µg	89	1	1	1	50	1
	Carbenicillin 100µg	11	99	99	71	1	1
Lysine decarboxylase		1	99	99	93	1	1
Ornithine decarboxylase		1	1	1	1	1	1
Urease		1	75	99	99	80	1

APPENDIX 6: PUBLICATIONS AND PRESENTATIONS

Oral presentations at conferences

Carson, J., Wilson, T., Gudkovs, N. & Bryant, T. (2002) Vibrios of Aquatic Animals: Towards a National Standard Diagnostic Technology. Conference of the FRDC Atlantic Salmon Aquaculture Subprogram, 8 July, 2002, Hobart, Tasmania

Carson, J., Wilson, T., Gudkovs, N., Higgins, M. & Bryant, T. (2003) Vibrios of Aquatic Animals: Towards a National Standard Diagnostic Technology. Conference of the FRDC Aquatic Animal Health Subprogram, 8th-10th October 2003, Geelong, Victoria

Carson, J., Wilson, T., Gudkovs, N., Higgins, M. & Bryant, T. (2005) The identification of *Vibrionaceae* from aquatic animals: a practical method for diagnostic laboratories. European Association of Fish Pathologists, 12th International Conference on 'Diseases of Fish and Shellfish', 11th-16th September 2005, Copenhagen, Denmark.

Refereed papers

Bryant, T.N. (2004) PIBWin - software for probabilistic identification. *Journal of Applied Microbiology* 97: 1326-1327

Vidgen, M., Carson, J., Higgins, M. and Owens L. (2006) Changes to the phenotypic profile of *Vibrio harveyi* when infected with the bacteriophage *Vibrio harveyi* myovirus-like (VHML). *Journal of Applied Microbiology* 100: 481-487

Battaglene, S.C., Morehead, D.T., Cobcroft, J.M., Nichols P.D., Brown M.R., Carson, J. (2006) Combined effects of feeding enriched rotifers and antibiotic addition on performance of striped trumpeter (*Latris lineata*) larvae. *Aquaculture* 251: 456-471

APPENDIX 7: ABBREVIATIONS

ANZSDP	Australian New Zealand Standard Diagnostic Procedure
ANGIS	Australian National Genomic Information Service
ATCC	American Type Culture Collection
BASIC	Beginners All-purpose Symbolic Instruction Code
BLASTn	Basic Local Alignment Search Tool, nucleotide
bp	base pair
bv	biovar
CECT	Colección Española de Cultivos Tipo
CIP	Collection de l'Institut Pasteur
csu	carbon substrate utilisation
CSV	comma separated value
E	Expect value
dNTP	deoxynucleotide triphosphate
DPIW	Department of Primary Industries & Water
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
FAFLP	Flurorescent Amplified Fragment Length Polymorphism
FASTA	Fast all
GBEST	Software utility for selecting tests of greatest differential capability
HMO	Hypothetical median organism
IDSC	Software utility that calculates a Willcox probability value for an HMO (q.v.) for a taxon in probability matrix
IXP	Indoxyl phosphate
JMA	Johnson's marine agar
LGN	L-glutamic acid 5-(4-nitroanilide)
LPN	L-proline 4-nitroanilide
LMG	Laboratorium voor Microbvlogie, University of Ghent, Belgium
MLS	Modal likelihood score
MLST	Multilocus sequence typing
NCBI	National Center for Biotechnology
NCIMB	National Collection of Industrial & Marine Bacteria
NCTC	National Collection of Type Cultures
NIBHT	National Institute of Bioscience and Human Technology, Tsukuba, Japan
NPS	4-nitrophenyl sulfate
nt	nucleotide
ONPG	2-nitrophenyl β-D-galactopyranoside
OTU	operational taxonomic unit
OVERMAT	Software to determine the extent of overlap between taxa in a probability matrix
P	Willcox probability value
p	average test error
p_i	individual test error
PCR	polymerase chain reaction
PIBWin	Probabilistic identification of bacteria [for] Windows; software program
PNPG	2-nitrophenyl β-D-galactopyranoside
pNPP	4-nitrophenyl phosphate
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RTF	rich text format
S	Bit score, S
S_i	Gyllenberg separation index
S_{max}	Theoretical maximum separation value for an individual test
S_t	Gyllenberg separation value for an individual test
S_J	Jaccard's similarity coefficient
S_{SM}	Simple matching coefficient
SCFA	Standing Committee for Fisheries and Aquaculture
SBA2	Sheep's blood agar with 2% NaCl
TCBS	Thiosulphate citrate bile sucrose [agar]
TCFB	Tasmanian Collection of Fish Bacteria
T_m	Melting temperature of DNA
TTC	2,3,5 triphenyltetrazolium chloride
U	units of enzyme activity

UPGMA	unweighted pair-group method with arithmetic average
V36	panel of phenotypic tests for the identification of *Vibrionaceae*
VibEx7	probability matrix for the identification of *Vibrionaceae*
VP	Voges Proskauer test for the production of acetoin
VRM	*Vibrio* recovery medium
ZMA	ZoBell's marine agar 2216E
16S rRNA	16S unit of bacterial ribosomal RNA
[xxxx]	GenBank sequence accession number