FINAL REPORT

TO THE

FISHING INDUSTRY RESEARCH AND DEVELOPMENT COUNCIL

DEVELOPMENT OF CELLULAR FATTY ACID ANALYSIS FOR THE RAPID IDENTIFICATION OF BACTERIA FROM FISH

PROJECT 89/90

QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES

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PROJECT TITLE:	Development of cellular fatty acid analysis for the rapid identification of bacteria from fish
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SECTION 1

SUMMARY OF FINDINGS

A system has been set up for the identification of aquatic *Vibrionaceae* by means of their cellular fatty acid profiles. The hardware consists of a Hewlett Packard gas chromatograph (HP 5890A), an automated gas-liquid chromatography system equipped with a capillary column and an automatic sampler, and attached to a computer monitor and printer. A commercial software package, the MIDI Microbial Identification System (MIS), was purchased with funds provided by FIRDC to run the gas chromatograph. The software contained both a systems operation and a library generation programme for the establishment of a user created library based on the total cellular fatty acid profiles of reference bacteria. This was used to generate a cellular fatty acid library of species of *Vibrionaceae* named the "Vibrio" library.

In addition, departmental funds were used to purchase the "Aerobe" library, a cellular fatty acid library containing the profiles of a range of aerobic bacteria. This library initially contained ten species of *Vibrionaceae*, increasing to 38 species and subspecies with regular updates.

A collection of reference strains of *Vibrionaceae* was assembled and their identities checked using conventional morphological and biochemical tests. Following confirmation of their identities, the profiles of 69 strains of *Vibrionaceae* were entered in the data base to generate the "Vibrio" library. This library confirmed the identities of six of 48 field isolates, the "Aerobe" library failing to identify any isolates. At the same time, the "Aerobe" data base was searched with the profiles of 41 of the reference strains used to generate the "Vibrio" library, correctly identifying three of them.

Following revised instructions from the MIS manufacturers, a new "Vibrio" library was generated from the 69 confirmed *Vibrionaceae* with a minimum of 20 entries for each species using a standardised 40 mg packed wet cell mass for the preparation of each FAME. This required the preparation of multiple extracts of each isolate, a separate culture plate being harvested for each sample. The revised "Vibrio" library correctly identified 63 of 126 field *Vibrionaceae* tested, 76% of the correct identifications being in four species, namely, *Listonella anguillarum*, *Vibrio cholerae*, *V. alginolyticus* and *V. harveyi*. The "Aerobe" library correctly identified 12 of the 69 reference strains used to generate the "Vibrio" library and 15 of the 126 field isolates.

The poor performance of the purchased "Aerobe" library suggests that its data base is not sufficiently large to adequately identify all strains of a species. Although the "Vibrio" library contained only 69 strains in its data base, it clearly outperformed the "Aerobe" library in the identification of aquatic *Vibrionaceae*, possibly reflecting the aquatic origins of the majority of its strains. This highlights the need for as broad a data base as possible for each species using the maximum available number of strains from as diverse as possible a range of sources. Although further expansion of the data base is required to provide adequate identification of all aquatic *Vibrionaceae*, the "Vibrio" library has the potential to provide the cheap, rapid accurate identification required for disease control in aquaculture.

SECTION 2

BACKGROUND INFORMATION

With its warm temperatures and extensive coastline, there has been a rapid development of prawn and fish aquaculture in Queensland. This industry will be important in extending traditional fishing activities into new economic developments at local levels by providing new exports and opportunities for import substitution.

Disease is a major problem in the aquaculture industry, particularly in intensive aquaculture where fish are densely stocked and subject to stress (Austin and Allen-Austin, 1985). Bacterial diseases have a major negative influence on marine prawn hatchery production. Although some bacteria have been identified as strict fish pathogens, a vast array of bacteria are a normal part of the aquatic environment, but can become pathogens when fish are stressed by sub-optimal conditions. It is primarily these opportunistic bacteria proliferating under intensive culture environments that infect stressed or weakened hosts.

Rapid identification of the bacteria occurring both in the environment and on the fish, and the ability to interpret the importance of the isolate is essential in disease diagnosis. The identification and study of environmental bacteria is also important in post-harvest microbial spoilage. Yet, the taxonomy and ecology of the bacteria that can be isolated from fish and their environment has been fairly neglected, adversely affecting the identification of potential pathogens and an understanding of the epidemiology of fish disease (Lambert *et al* 1983; Austin and Allen-Austin, 1985; Ward *et al* 1986).

Traditionally, bacteria isolated from fish and their environment have been identified by the use of identification tables and keys constructed for the pathogenic bacteria associated with man and terrestrial animals, such as those prepared by Cowan (1974) and Lennette *et al* (1985). However, the bacteria that are isolated from fish can possess an array of biochemical, phenotypic and genotypic properties vastly different from those associated with such classic diseases of man and animals as whooping cough, tetanus, fowl cholera or blackleg.

An additional source of complexity is that the strict division between pathogenic and non-pathogenic bacteria, which is a feature of the bacteriology of man and terrestrial animals, does not necessarily apply with fish (Frerichs 1984). Hence, fish bacteriology often involves the classification and identification of a bewildering array of bacteria, many of which are environmental isolates which cause problems only in conjunction with stress factors such as overcrowding and poor water quality (Frerichs 1984). These environmental bacteria are often poorly studied, and do not feature in the traditional identification keys.

The deficiencies in classical bacteriological identification schemes have been widely recognised (Kämpfer *et al* 1987), and alternative techniques have been developed. These alternatives

include the development of more appropriate phenotypic tests, such as enzymatic hydrolysis of a range of chromogenic substrates or the utilisation of a spectrum of carbon substrates (Baumann *et al* 1984; Kämpfer *et al* 1987; Carnahan *et al* 1989) and techniques based on the development of monoclonal antibodies, (Hanna *et al* 1991; Chen *et al* 1992) or nucleic acid probes (Glenn Morris *et al* 1987).

However, each of these approaches has disadvantages. The use of an extensive array of substrate utilisation tests requires a major commitment in media preparation and equipment that may be practicable only in reference laboratories. The use of monoclonal antibodies and nucleic acid probes can provide rapid, specific identification. This very specificity can, however, be a problem. Thus, the only bacteria that can be identified by these techniques are those for which a unique specific antibody or probe are available (Welch 1991). Very few such specific reagents are commercially available and they are expensive and time consuming to produce "in house". Consequently, this technology is practicable for the identification of only a very limited range of bacteria. As well, with nucleic acid probes, the current labelling techniques involve the use of radioisotopes which pose safety problems, and generally have only a limited shelf life.

The availability of rapid and accurate identification of fish bacteria would make a major contribution to the Australian fishing industries, greatly enhancing both disease diagnosis and research. Accurate and rapid monitoring of the environmental bacterial population of intensive aquaculture operations such as prawn farming could provide a major contribution towards predicting, or even avoiding, the spectacular mass mortalities associated with such problems as bacterial necrosis. The benefits of such a rapid diagnostic tool are obvious where control of disease spread is critical.

The *Vibrionaceae* are one of the major bacterial families found in the aquatic environment. They comprise the genera *Aeromonas*, *Listonella*, *Photobacterium*, *Plesiomonas* and *Vibrio* and include 58 species and sub-species at this time. Identification using conventional morphological and biochemical tests requires between 80 and 100 tests which are both expensive and time consuming. Overall, the *Vibrionaceae* are easy to cultivate, but difficult to accurately identify. This makes them an ideal group of organisms for the development of a rapid identification scheme.

An ideal identification technique for bacteria would be one that is rapid, capable of handling large numbers of isolates, safe to use in routine laboratories and based on a single approach applicable to a broad range of organisms. Gas chromatography meets all of these requirements and is an ideal criterion for identification (Miller 1982). In addition, a useful chemotaxonomic marker should involve for its biosynthesis a relatively high number of genes to attain a certain stability. Lipopolysaccharides, in particular, fulfil these requirements. They are large and complex molecules of vital importance to the cell and require at least 20 biosynthetic enzymes (Jantzen 1984). As such, they provide a stable chemotaxonomic marker reflecting the basic genomic makeup of the cell. Because of the large number of fatty acids found in the cell wall and cell membranes of bacteria, and because the composition of cellular fatty acids is a very stable genetic trait that is highly conserved within a taxonomic group, fatty acid composition can be successfully used for identification of bacteria (Stager and Davis, 1992).

The usefulness of cellular fatty acid analysis for identification has been well established with coryneform bacteria (Athalye *et al.*, 1985), *Bacillus* spp. (Lawrence *et al* 1991), *Clostridium* spp. (Ghanem *et al* 1991), *Flavobacterium* spp. (Dees *et al.*, 1986), *Leptospiraceae* (Cacciapuoti *et al* 1991), coagulase-negative staphylococci (Kotilainen *et al* 1991) and *Alcaligenes*, *Pseudomonas* and other nonfermenting gram-negative bacteria (Dees and Moss, 1975; Veys *et al* 1989). In addition, fatty acid "fingerprints" have been used as a tool for rapid sorting of strains of environmental bacteria that did not conform to described species (Tearle and Richard, 1987). Moore *et al* (1987), in this laboratory, applied the technique of cellular fatty acid analysis to differentiate *Bordetella avium*, a newly recognised poultry pathogen, from closely related species which were difficult to separate using conventional identification techniques.

At the time this project commenced, only limited studies had been carried out on the differentiation of *Vibrionaceae* on the basis of their cellular fatty acid content. Oliver and Colwell (1973) examined the cellular fatty acid content of a range of marine isolates and found significant differences in the fatty acid patterns of the various genera which could be used for rapid differentiation. Lambert *et al* (1983) examined the cellular fatty acid content of 27 strains, comprising 16 species of *Vibrionaceae* and Urdaci *et al* (1990) similarly characterised 56 isolates, comprising 22 *Vibrio* species. In both of these studies, a decision tree was proposed for the separation of species, or groups of species, based on either the occurrence, or the relative proportions, of specific cellular fatty acids The strains examined in these studies, however, were isolated from pathological conditions in terrestrial animals and man. Prior to this study, no-one had yet attempted to establish cellular fatty acid profile identification schemes specifically based on aquatic bacteria. It remains to be seen whether their cellular fatty acid profiles have significant differences from their terrestrial counterparts. Oliver and Colwell (1973) reported differences in the saturated to unsaturated ratio of the cellular fatty acids of marine and estuarine isolates when compared with terrestrial isolates of the same species.

Recently, an automated gas-liquid chromatography system with a computer interface and software for the identification of bacteria on the basis of their total cellular fatty acid composition has been introduced. This allows for a more sensitive analysis of bacterial isolates with the potential for finer differentiation. To date, there has been no attempt to construct an identification system for *Vibrionaceae* based on the complete cellular fatty acid profiles of bacteria.

The aim of this project was to use the analysis of the total cellular fatty acid composition of bacteria for the development of an identification scheme for aquatic *Vibrionaceae*. Asthe work developed, *Vibrionaceae* could be cultivated, screened to the family level and processed through the gas chromatograph within three days of receipt of samples. This represents a considerable saving in time on the 7-14 days required to identify *Vibrionaceae* using the conventional characterisation techniques currently in use. Once established, the system has the potential to be easily transferred to other laboratories servicing the fishing and aquaculture industries.

SECTION 3

AIMS OF THE PROJECT

The proposed plan of operation of the project was summarised as:

Phase 1: Assemblage of a collection of reference fish bacteria
An extensive range of already characterised and identified bacteria was to be collected from laboratories around Australia and large reference collections overseas. Preliminary contact with some of the Australian laboratories had indicated that a substantial collection could be established.

Phase 2: Confirmation of the identities of the reference bacteria
A comprehensive array of the classical morphological and biochemical tests used for their original characterisation would be used to confirm the identities of reference strains.

Phase 3: Analysis of the cellular fatty acid profiles of the reference bacteria
A commercial gas chromatography system (the Hewlett Packard 5898A Microbial Identification System) purchased by the Department of Primary Industries just prior to commencement of this project was to be used. This commercial system allowed the use of a computer software package to establish an identification scheme based on the total cellular fatty acid profiles of the reference bacteria. This was to be backed up by a gas chromatograph-mass spectrometer (Finnigan 1020B) which could provide accurate identification of all the relevant fatty acids where needed.

Phase 4: Storage and identification of field isolates
Relevant field isolates of aquatic and terrestrial bacterial isolates from both the research and diagnostic activities performed by the department were to be collected and stored. These isolates would firstly be comprehensively characterised using conventional identification techniques and would include organisms that had been fully identified to species level as well as those which could not be identified.

Phase 5: Characterisation of field isolates by cellular fatty acid profiles
The bacteria collected in phase 4 were to be analysed to determine their cellular fatty acid profiles and the profiles examined using the cellular fatty acid profile identification scheme established in phase 3. The results of conventional identification would be compared with identification using cellular fatty acid profiles. The data base of the cellular fatty acid identification scheme established in phase 3 would be expanded with the addition of more identified isolates and the identification scheme would be extended if new taxa were revealed amongst those organisms unable to be identified by conventional techniques.

The various phases were to be carried out as follows:

Year 1

Collection of reference bacteria
Characterisation of reference bacteria
Installation of fatty acid library generation software onto gas chromatograph
Analysis and storage of fatty acid profiles of reference bacteria
Collection of field isolates

Year 2

Collection of reference bacteria
Characterisation of reference bacteria
Analysis and storage of fatty acid profiles of reference bacteria in data base
Collection of field isolates
Analysis of fatty acid profiles of field bacteria
Comparison of identification of field isolates by conventional means and using cellular fatty acid profiles

Year 3

Collection of reference bacteria
Characterisation of reference bacteria
Analysis and storage of fatty acid profiles of reference bacteria in data base
Collection of field isolates
Analysis of fatty acid profiles of field bacteria
Comparison of identification of field isolates by conventional means and using cellular fatty acid profiles
Addition of profiles of comprehensively identified field isolates to the cellular fatty acid data base
Expansion of the data base with the profiles of newly recognised taxa

SECTION 4

CONVENTIONAL CHARACTERISATION TESTS

4.1 INTRODUCTION

There have been several taxonomic studies of the *Vibrionaceae* in recent years resulting in an increase in the number of recognised species rising from five in 1974 to a current level of 48 (West *et al* 1983; Lee and Donovan 1985; Bryant *et al* 1986a). In this study, the identification of *Vibrionaceae* was based on the tests used by Bryant *et al* (1986a) for the numerical classification of *Vibrionaceae*. Bryant *et al* (1986b) subsequently constructed a computer matrix for the probabilistic identification of species of *Vibrionaceae* based on this data. Of the 111 tests used in their previous study, Bryant *et al* (1986b) selected 30 tests to form a basis for routine identification and a further 51 tests for the separation of 38 phenons of *Vibrionaceae* at a certainty level of 98% or greater. This program, the probabilistic identification of bacteria (PIB), was purchased and installed for the conventional identification of *Vibrionaceae* in this study. Seventy-three of the 81 tests in the identification matrix were set up, together with a further 16 auxiliary tests. These tests were used for the confirmation of the identities of reference strains and the comprehensive identification of field isolates using conventional morphological and biochemical tests.

4.2 MATERIALS AND METHODS

4.2.1 *Bacterial Strains*

The *Vibrionaceae* used in this study were from diagnostic and research activities at the Animal Research Institute as well as cultures obtained from other Australian and overseas laboratories. A total of 90 reference and previously characterised field isolates were obtained from other institutions for construction of the cellular fatty acid library. These included 59 strains from Dr. L. Sly, Microbiology Department, University of Queensland, 25 strains from Dr. J. Carson, Dept. Primary Industries, Mt. Pleasant Laboratories, Kings Meadows, Tasmania, three strains from Dr. Zhonglin Chai, Division of Biological Sciences, Deakin University, Geelong, Victoria and three strains from Dr. R. Whittington, Elizabeth Macarthur Agricultural Institute, Camden, New South Wales. In addition, other *Vibrionaceae* isolated from aquatic sources were obtained during the project from Dr. A. Thomas, Oonoonba Veterinary Laboratory, Townsville, Queensland, Dr. C.D. Anderson, Wallaceville Animal Research Centre, Mount Hutt, New Zealand and Dr. R. Callinan, Dept. of Agriculture and Fisheries, Wollongbar, New South Wales.

4.2.2 *Storage of bacterial cultures*

Vibrionaceae from diagnostic and research activities were immediately stored as low passage isolates both at -70°C and in liquid Nitrogen in Luria - Bertani medium (Maniatis *et al* 1982) modified with the addition of 20.0% glycerol and an additional 1.0% NaCl for marine isolates. Cultures obtained from other laboratories were subcultured on receipt, and similarly stored.

4.2.3 *Maintenance medium*

Vibrionaceae used in this study were subcultured from stocks held at -70°C frozen in Luria - Bertani medium. The frozen culture was scraped and a sample of the soft ice removed with a bacteriological loop and deposited onto an agar plate taking care not to allow the frozen culture to melt. Cultures were routinely subcultured and incubated at 25°C onto either Trypticase Soy Agar (TSA) (BBL® or TSA with added Electrolyte Supplement Solution (ESS) to satisfy the ionic growth requirements for marine bacteria (Furniss *et al* (1978). Similarly, broth cultures were grown in Trypticase Soy Broth (TSB) with ESS added for marine isolates.

4.2.4 *Bacterial Identification*

4.2.4.i *Screening tests*

Cultures were initially screened as described below to confirm they were *Vibrionaceae* before being subjected to comprehensive characterisation:

Cellular morphology: Motility and cellular morphology were determined from phase contrast microscopy of 4-18 hour broth cultures
Gram reaction: Overnight agar cultures were gram stained to ensure isolates were gram negative rods (straight or curved)
Oxidase: Overnight agar cultures were tested with Oxidase Reagent Droppers (Becton Dickinson®) containing tetramethyl-p-phenylene diamine dihydrochloride (1.0%) to determine the oxidase reaction
Oxidation/fermentation: Two steamed and cooled oxidation /fermentation tubes (casein hydrolysate 0.1%, yeast extract 0.01.%, $MgSO_4.7H_2O$ 0.3%, $CaCl_2.2H_2O$ 0.15%, NaCl 0.8%, KCl 1.0%, glucose 1.0%, phenol red 0.001%, agar 0.3%) were stab inoculated and one tube subsequently covered with sterile molten petrolatum. Tubes were examined after 1-2 days incubation to ensure fermentative growth and check for gas production.

4.2.4.ii *Comprehensive identification tests*

The following tests were carried out on the confirmed *Vibrionaceae*. All incubations were carried out at 25°C unless otherwise stated. Saline suspensions of overnight agar cultures were prepared as inocula. Broth tests were carried out in miniaturised sets of media prepared in 200 l volumes in microtitre trays and stored at -20°C prior to use Trays were inoculated with either 10 or 20 l volumes of the saline suspension then sealed with tape to prevent evaporation. Tests performed on solid media were inoculated using a plate replicator with up to eight cultures being spot inoculated onto each plate.

Salt tolerance: Isolates were tested for their ability to grow in a 1% tryptone water containing 0, 3, 6, 8, 10 and 12%(w/v) final concentration of NaCl as described by Lee et al (1979).

Indole production: Isolates were tested as described by Furniss *et al* (1978) using Kovacs reagent (Cowan 1974).

Swarming: Marine Agar (Furniss *et al* 1978) was prepared and spot inoculated with the isolates. Plates were examined for swarming after overnight incubation.

Antibiotic sensitivity: Resistance to O/129 (10 and 150 g), Ampicillin 10 g and Polymyxin 50 IU was determined using plates of blood agar base with added ESS. Discs for O/129 and Ampicillin testing were purchased (Oxoid ®). Polymyxin discs containing 50 IU were prepared, dried and stored at -5°C. Sensitivity test plates were prepared from Blood Agar Base (Oxoid ®) (with added ESS for marine isolates) and dried for 30 minutes at 37°C just prior to use. A saline suspension of an overnight culture was prepared and adjusted to a turbidity equal to Mc Farlane tube 0.5. This was spread over the surface of the plate. When the inoculum had dried on the plate the test discs were pressed onto the surface of the agar. The plates were examined for resistance to the antibiotic after overnight incubation.

Decarboxylation tests: Decarboxylation of arginine, lysine and ornithine were determined by modifying the method of Møller (1955) with the addition to the medium of 0.02% KH2PO4 as buffer and ESS (where required for marine isolates). Tests were read after 1-2 days incubation.

Nitrate reduction: Agar (0.1%) and ESS, (where required), were added to the medium of Lee *et al* (1979) providing a semi-solid agar deep. The cultures were inoculated and read as described by Lee *et al* (1979) after 3-5 days incubation.

Gluconate oxidation: The method of Cowan (1974) was used with ESS being added to the medium for marine organisms. Cultures were tested with Clinitest tablets (Ames & Co.®).

ONPG test: The method of Lee and Donovan (1985) was used.

Urea hydrolysis: The method of Cowan (1974) was used with the addition of ESS to the medium where required.

Voges-Proskauer test: The method of Lee *et al* (1979) was used with the addition of ESS to the medium where required.

Sugar fermentation: Fermentation of L-arabinose, arbutin, inositol, D-mannitol, mannose, trehalose, salicin, sorbitol, sucrose and glucose were tested in peptone water sugars containing bromcresol purple as an indicator (with added ESS for marine isolates) as described by Lee and Donovan (1985). A Durham tube was added to the glucose medium to detect gas production.

Luminescence: The medium of Baumann (1984) was used and plates were read as described by Lee *et al* (1979).
Tellurite inhibition: Cultures were spotted onto TSA plates (with added ESS for marine isolates) containing 0.0005% potassium tellurite and examined for growth after 1-2 days incubation.
Haemolysis: Plates were prepared with sheep blood (5%) in a blood agar base (Oxoid®) and spot inoculated with isolates. Haemolysis was read after 1 days incubation as described by Furniss *et al* (1978).
Extracellular enzymes: Production of alginase, chitinase, DNAse, lecithinase, amylase and lipase (Tween 20 and Tween 80) activity was tested as described by Furniss *et al* (1978) using blood agar base (supplemented with ESS where required) in place of marine agar.
Aesculin hydrolysis: The method of Lee and Donovan (1985) was used with the addition of ESS to the medium where required.
Casein hydrolysis: The method of Cowan (1974) was used with the addition of ESS to the medium where required.
Xanthine degradation: The method of Cowan (1974) was used with the addition of ESS to the medium where required.
Gelatin hydrolysis: The method of West and Colwell (1981) was used with the addition of ESS to the medium where required. Plates were spot inoculated and gelatinase activity read as a zone of opacity around the inoculum after 1-2 days incubation. This was confirmed by the occurrence of a clear zone around the inoculum after flooding the plate with 30% Trichloracetic acid (Cowan 1974).
Carbon substrate utilisation: A range of carbon substrates were added to the basal medium agar of Baumann (1984) at a final concentration of 0.2% (sugars) and 0.1% (other substrates). Substrates tested were L-arabinose, cellobiose, fructose, galactose, glucose, mannose, maltose, melibiose ,lactose, melizitose, sucrose, trehalose, D-xylose, ethanol, glycerol, inositol, 1-propanol, D-sorbitol, D-galacturonate, gluconate, glucuronate, amygdalin, arbutin, L-citrulline, L-hydroxyproline, L-leucine, D-glucosamine, N-acetylglucosamine, glutarate, DL-3-hydroxybutyrate, α-ketoglutarate, malonate, succinate, L-rhamnose, D-mannitol, γ-aminobutyrate, pyruvate and putrescein. Tests were interpreted as described by Lee *et al* (1979).
Temperature tolerance: Isolates were tested for growth at 4°C, 25°C, 30°C, 37°C and 42°C on TSA or TSA with added ESS.

4.3 RESULTS

4.3.1 *Confirmation of reference and identified field isolates from other institutions*

Using the PIB of Bryant *et al* (1986b), the results of 73 morphological and biochemical tests were analysed. This program identified *Vibrionaceae* at a confidence level of 98% or higher. Where necessary, the results of the additional 16 auxiliary tests were used to help with species differentiation.

Conventional morphological and biochemical testing confirmed the identities of 66 of the 90 reference and field cultures obtained from other institutions (Table1). The remaining 24 cultures comprised 11 identified as different species of *Vibrio* to the name under which they were supplied, eight as *Vibrionaceae* which could not be identified at the species level and five which were not *Vibrionaceae.*

4.3.2 *Conventional characterisation of field isolates*

Comprehensive morphological and biochemical characterisation was carried out on *Vibrionaceae* isolated from diagnostic and research activities at the Animal Research Institute as well as on isolates obtained from other institutions during the course of this project. Table 2 lists 129 species, subspecies or as yet unnamed phenotypes of *Vibrionaceae* comprehensively identified and stored for further cellular fatty acid analysis.

4.4 DISCUSSION

Our inability to confirm the identities of 24 of the 90 reference strains received from other institutions highlights the difficulty of identification of *Vibrionaceae* using conventional morphological and biochemical tests. Eleven of the 19 cultures confirmed as *Vibrionaceae* were identified as different species to the name under which they were supplied, while eight could not be identified at the species level. Variations could be due to a number of factors including differences in the manner in which tests are performed in different laboratories, different sets of characters selected or different clustering methods used for identification. In addition, it is known that the maintenance of a strain within a laboratory by repeated subculture can result in a decrease in its vigour and the loss of some characteristics (Bryant *et al* 1986a). This could result in the properties of a reference strain changing with time. In order to minimise the risks of cultures either changing or becoming contaminated during repeated subculturing, *Vibrionaceae* were stored immediately at low temperatures in Luria - Bertani medium containing glycerol (20.0%) following their isolation in our laboratory or receipt from other laboratories. This provided a frozen culture of soft ice crystals from which culture could readily be removed without thawing of the culture, allowing repeated sampling for tests without the need for subculturing of the isolate.

Table 1. *Vibrionaceae* reference strains and named field isolates from other institutions confirmed by conventional morphological and biochemical tests

Species name	Number tested	Culture identification code	Supplied by
Aeromonas caviae	1	F241	JC[1]
Aeromonas hydrophila	1	F229(ATCC† 7966)*	LS[2]
Aeromonas salmonicida	3	F250, F251, F252	JC
	3	F364, F365, F366	ZC[3]
Listonella anguillarum	4	F4(ATCC 19264)*, F116, F127, F137	LS
	5	F204, F205, F208, F209, F210	JC
Listonella pelagia	1	F200(ATCC 25916)*	JC
Photobacterium damsela	1	F191	JC
Photobacterium fischeri	1	F196	JC
Plesiomonas shigelloides	1	F230(ATCC 14029)*	LS
Vibrio alginolyticus	4	F122, F123(ATCC 17749)*, F124, F125	LS
Vibrio cholerae	5	F138, F141, F142, F143, F139(ATCC 14035)*	LS
Vibrio diazotrophicus	3	F133(ATCC 33446)*, F134, F136	LS
Vibrio fluvialis	3	F146, F147, F149	LS
Vibrio furnissii	4	F150, F151, F152, F153	LS
Vibrio gazogenes	1	F201(ATCC 29958)*	JC
Vibrio harveyi	4	F6, F156, F157(ATCC 14126)*, F158	LS
Vibrio hollisae	1	F231(ATCC 33565)	LS
Vibrio mediterranei	1	F195	JC
Vibrio metschnikovii	2	F144(ATCC•1), F145(ATCC 7708)*	LS
Vibrio mimicus	1	F155(ATCC 33653)*	LS
Vibrio natriegens	2	F162, F163	LS
Vibrio nereis	1	F164(ATCC 25917)*	LS
Vibrio ordalii	1	F202(ATCC 33509)*	JC
Vibrio parahaemolyticus	4	F166, F167, F168(ATCC 43996)	LS
		F246(ATCC 17802)*	LS
Vibrio splendidus I	1	F199(ATCC 33125)*	JC
Vibrio tubiashii	3	F169(ATCC 19109)*, F170, F171	LS
Vibrio vulnificus	4	F173, F174, F175(ATCC 33148), F176	LS
Total:	66		

†ATCC - American Type Culture Collection
* - Type Strain
[1] JC - obtained from Dr. J. Carson
[2] LS - obtained from Dr. L. Sly
[3] ZC - obtained from Dr. Z. Chai

Table 2. *Vibrionaceae* field isolates identified by conventional morphological and biochemical tests

Species name	Number identified
Aeromonas caviae	2
Aer. hydrophila	3
Aer. salmonicida subsp. *achromogenes*	1
Aer. salmonicida subsp. *salmonicida*	1
Sucrose negative Aeromonad	1
Listonella anguillarum	22
List. pelagia	1
Photobacterium damsela	18
Plesiomonas shigelloides	1
Vibrio alginolyticus	30
V. campbellii	1
V. cholerae	10
V. fluvialis	3
V. harveyi	9
V. mimicus	7
V. nereis	3
V. ordalii	4
V. parahaemolyticus	3
V. proteolyticus	1
V. splendidus I	1
V. tubiashii	5
V. vulnificus	2
Total:	129

SECTION 5

CELLULAR FATTY ACID ANALYSIS

5.1 INTRODUCTION TO THE PRINCIPLES

For gas chromatographic analysis of the cellular fatty acids of bacteria, the organisms are cultivated on a suitable agar medium and the growth harvested and washed. The cellular fatty acids are released by alkaline saponification and esterified. The fatty acid methyl esters (FAME's) thus formed is then loaded into the gas chromatograph. The sample is injected into a stream of heated carrier gas (the mobile phase), where the components are volatilised and swept through a fused silica capillary chromatographic column which contains the stationary phase. This is a thermostable differential solvent which selectively retards the components in the mixture according to their chemical composition. The separated components enter a detector connected to a printer/plotter and are consecutively registered as chromatographic peaks as they emerge. The area beneath the peaks indicates the relative proportions of the various components in the injected sample (Larsson and Odham, 1984). FAME's are identified by comparison of their retention time with known standards.

An automated gas chromatography system was recently developed with the capacity to reduce the component identity of the total cellular fatty acid composition of bacteria to a form suitable for archiving in a library of profiles. This allows for the storage of the cellular fatty acid profiles of representative species identified by conventional means, against which the profiles of an unknown can be searched. Software for the generation of such a library was developed by the MIDI corporation for commercial sale.

The aim of this component of the project was to use this automated system to archive the cellular fatty acid profiles of reference strains of *Vibrionaceae* confirmed by conventional means into a library, and to identify field isolates by comparison against this library. The results would then be compared with identification by conventional morphological and biochemical means.

In addition, a library containing the cellular fatty acid profiles of a wide range of aerobic bacteria was commercially available from MIDI. An additional aim of this project was to compare this library with the one generated in our laboratory.

5.2 MATERIALS AND METHODS

5.2.1 *Gas chromatograph and software*

For this study, a commercial identification system, the MIDI Microbial Identification System (MIS) was purchased with funds provided by FIRDC. The hardware of this system, purchased from QDPI funds, comprised a Hewlett Packard gas chromatograph (HP 5890A), an automated gas-liquid chromatography system equipped with a capillary column and an automatic sampler. This was attached to a computer monitor and printer. The software package supplied by MIDI contained both a systems operation and a library generation programme for the establishment of a user created library based on the total cellular fatty acid profiles of reference bacteria. The library so generated in this work was referred to as the "Vibrio" library (see Section 5.2.6). In addition, departmental funds were used to purchase the "Aerobe" library, a library of profiles of common aerobic bacteria containing 10 species of *Vibrionaceae*, namely, *Aeromonas caviae*, *Aer. hydrophila*, *Plesiomonas shigelloides*, *Vibrio alginolyticus*, *V. anguillarum*, *V. cholerae*, *V. fluvialis*, *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus*. With periodic updates from the manufacturers, this library has since expanded to include 38 species or subspecies of *Vibrionaceae* (Table 3).

5.2.2 *Preparation of bacterial cell suspensions*

Bacterial strains were subcultured from stocks held at -70°C in Luria-Bertani medium onto TSA (or TSA with ESS) and incubated for 18-24 hours. The overnight culture was then used to inoculate a Trypticase Soy Blood Agar (TSBA) plate, containing TSA with 5% added sheep blood. The culture was spread over the surface of the TSBA and incubated at 25°C overnight. The following day the growth was harvested, washed once in sterile saline (0.85%) and stored at -20C.

5.2.3 *Extraction of samples for gas chromatographic analysis*

Procedure A Initially, the technique of Lambert *et al* (1983) was used. The cell suspensions were thawed and subjected to alkaline saponification to release the cellular fatty acids as Sodium salts. They were mixed with 4.0 ml of 5.0% NaOH in 50.0% aqueous methanol, heated to 100C for five minutes, mixed again then heated to 100°C for another 30 minutes. This was followed by methylation to convert the fatty acids to the more volatile fatty acid methyl ester (FAME). Five millilitres of 15.0% HCl methanol was added, mixed and the tube heated to 100C for 15 minutes. The FAME thus formed was then extracted from the acidic aqueous phase, transferred to an organic phase and concentrated. One millilitre of saturated NaCl and 10.0 ml diethyl ether/ hexane (1:1) were added to the 1.0 ml saline cell suspension, mixed and the aqueous (bottom) layer removed and discarded. This extraction was repeated and the two organic layer samples pooled then concentrated with a stream of nitrogen gas. This was finally washed (to remove free fatty acids and residual reagents from the organic extract) and dried. Half a millilitre of 0.1M phosphate buffer was added, mixed, the organic layer, (top), removed

Table 3. *Vibrionaceae* species and subspecies currently in the "Aerobe" data base

Aeromonas caviae	*V. carchariae*
Aer. enteropelogenes	*V. cholerae* :01
Aer. hydrophila	*V. cholerae* non:01
Aer. ichthiosmia	*V. costicola*
Aer. salmonicida subsp. *masoucida*	*V. cincinnatiensis*
Aer. salmonicida subsp. *salmonicida*	*V. diazotrophicus*
Aer. schubertii	*V. fluvialis*
Aer. sobria	*V. furnissii*
Aer. trota	*V. gazogenes*
Aer. veronii	*V. harveyi*
Listonella anguillarum	*V. mediterranei*
List. pelagia	*V. metschnikovii*
Photobacterium angustum	*V. mimicus*
Photobact. damsela	*V. nereis*
Photobact. leiognathi	*V. ordalii*
Plesiomonas shigelloides	*V. parahaemolyticus*
Vibrio aestuarianus	*V. proteolyticus*
V. alginolyticus GC subgroup A,B	*V. tubiashii*
V. campbellii	*V. vulnificus*

and 1.0 ml of the ether/hexane mixture added to it. This was dried by passing through anhydrous Na_2SO_4 and collecting the extract into a gas chromatograph (GC) autosampler bottle. A cap was crimped onto the autosampler bottle and it was stored at -20°C for later GC analysis.

Procedure B The extraction technique was later modified to conform to the exact conditions used by MIDI in the extraction of samples for the construction of the "Aerobe" library. Alterations were as follows:
In the saponification procedure, 1.0 ml of a 15.0% solution of Na OH in 50.0% aqueous methanol was added to a washed packed cell suspension of 40mg (± 10mg). The final incubation step was performed at 100°C for 25 minutes.
The methylation procedure was carried out using 2.0 ml of 6.0N HCl in 50.0% aqueous methanol at a temperature of 80°C for 10 minutes. The FAME was then extracted with 1.25 ml of a mixture of hexane/methyl-tert butyl ether (1:1) and mixed for 10 minutes before discarding the aqueous (bottom) phase. Finally, the organic phase was washed for five minutes with 3.0 ml of a mild base solution of 1.2% Na OH and the organic (top) layer removed and stored as previously described.

5.2.4 *Gas chromatographic analysis of the FAME*

Gas chromatography was performed using a Hewlett Packard HP Ultra 2 polymeric silicone column containing 95% methyl silicone and 5% phenyl. The systems operation software determined the set points for the various heated zones of the GC, and of the oven temperature programme. The carrier gas was hydrogen. The profile produced for each culture was printed out with each component registered as a chromatographic peak with the retention time indicated for each peak. A standard provided by MIDI, comprising a mixture of 10-15 long chain FAME's was run through the GC after every six samples to check the calibration of the instrument. Initial calibration of the instrument (prior to the processing of sample batches) was ensured by repeated analysis, if necessary, of this standard. Column head pressure and oven calibration temperature were adjusted with reference to a table to ensure that the equivalent chain lengths (ECL's) of the polar hydroxy fatty acids in the mixture were centred on the expected ECL's listed in a peak naming table. This procedure allowed for some time-dependant drift in the absolute retention time of chromatographic peaks while still correctly naming the peaks. In addition, a reference culture of *Xanthomonas maltophilia* (ATCC 13637) with a known profile was run with every new batch of chemicals to ensure the standardisation of the procedure.

5.2.5 *Preparation of washed bacterial cells for fatty acid extraction*

5.2.5.i *Harvesting total growth on TSBA*

The protocol recommended by MIDI for the harvesting of cultures required scraping the growth from the third quadrant of the TSBA plate with a sterile 4 mm bacteriological loop, with a heaped loopful providing approximately 40 mg of live wet cells. As the majority of cultures used in this study produced insufficient growth to be harvested in this manner, the complete growth from the surface of a TSBA plate was harvested to obtain an adequate amount of live wet cells from the isolates.

5.2.5.ii *Harvesting standardised 40 mg (± 10 mg) from TSBA*

Following revised instructions from MIDI, a standardised mass of 40 mg (± 10 mg) packed wet cell suspension of each culture was harvested from TSBA plates for extraction. Cultures exhibiting poor growth on TSBA were inoculated onto multiple TSBA plates to provide sufficient mass for harvest.

5.2.6 *Construction of the "Vibrio" library: "Vibrio"-(a) library*

A cellular fatty acid library, the "Vibrio"-(a) library, was generated using 69 cultures of confirmed identity. These included the 66 confirmed reference and field strains from other institutions listed in Table 1 and three strains isolated from diagnostic submissions to this laboratory and comprehensively identified as previously described. These were one strain each of *V. harveyi*, *Aeromonas salmonicida* subsp. *salmonicida* and *Aer. salmonicida* subsp. *achromogenes*. Initially, a single preparation of each culture was harvested from the total growth from an overnight TSBA plate and the FAME extracted by the method of Lambert *et al* (1983) as described in extraction procedure A.

5.2.7 *Identification of "Vibrio"-(a) reference strains by the "Aerobe" library*

The "Aerobe" data base was searched with the fatty acid profiles of a range of 41 of the reference strains used to generate the "Vibrio"-(a) library to ascertain its capacity for identification of aquatic *Vibrionaceae*.

5.2.8 *Field isolate identification by the "Vibrio"-(a) and "Aerobe" libraries*

Forty-eight field isolates of *Vibrionaceae*, identified by conventional morphological and biochemical tests, were processed and analysed by gas chromatography with the technique of harvesting cells and FAME extraction used for generation of the "Vibrio"-(a) library. The profiles were compared against both the purchased "Aerobe" library and the constructed "Vibrio"-(a) library.

5.2.9 *Construction of the "Vibrio" library: "Vibrio"-(b) library*

A new cellular fatty acid library, the "Vibrio"-(b) library, was generated from the 69 confirmed reference strains according to the revised instructions received from MIDI. A minimum of 20 entries was prepared for each species of *Vibrionaceae* using a standardised 40mg (±10mg) of packed wet cell mass for the preparation of each FAME. This required the preparation of multiple extracts of each isolate, a separate TSBA plate being harvested for each entry. The extraction procedure B was used for preparation of the FAME.

5.2.10 *Identification of "Vibrio"-(b) reference strains by the "Aerobe" library*

The "Aerobe" data base was searched with the fatty acid profiles of the reference strains used to generate the "Vibrio" (b) library. Where multiple entries were prepared from a single strain, each profile produced was analysed and compared against the "Aerobe" library.

5.2.11 *Field isolate identification by the "Vibrio"-(b) and "Aerobe" libraries*

One hundred and twenty-six field isolates comprehensively characterised by conventional means were cultured and the FAME extracted by the method used for generation of the "Vibrio"-(b) library. The extracts were analysed and the profiles compared against both the purchased "Aerobe" library and the constructed "Vibrio"-(b) library.

5.3 RESULTS

5.3.1 *Generation of a similarity index for identification*

The MIS library search was carried out immediately following gas chromatographic analysis of the FAME and a similarity index (SI) produced for the most likely matches of the unknown to library entries. Where more than one possible match occurred, the suggested identities were listed in descending probability with the most probable genus being listed first. The SI produced was a numerical value that expressed how closely the composition of an unknown isolate compared with the fatty acid composition of the library matches. A SI of 0.500 or greater was considered to have a strong probability of being correct, while an index of 0.300 or greater indicated an organism which could be related to the MIS match. For this project, a SI of 0.300 or greater was taken to indicate possible identification.

5.3.2 *Identification of "Vibrio"-(a) reference strains by the "Aerobe" library*

The results of identification of the "Vibrio"-(a) reference strains by the "Aerobe" library are listed in Table 4. Of 41 strains tested, representing 18 species of *Vibrionaceae*, the "Aerobe" library correctly identified three strains, one each of *Plesiomonas shigelloides, Vibrio fluvialis* and *V. ordalii*. One strain each of *Aeromonas hydrophila* and *Listonella anguillarum* were identified but had equivalent SI's to other species of *Aeromonas* and *Vibrio* respectively. One strain of *V. cholerae* and two of *V. fluvialis* were correctly identified, but with similarity indices less than 0.300.

5.3.3 *Field isolate identification by the "Vibrio"-(a) and "Aerobe" libraries*

Table 5 gives the results of cellular fatty acid identification of 48 conventionally identified *Vibrionaceae* field isolates against the "Vibrio"-(a) and the "Aerobe" libraries. Six isolates, of *Vibrio cholerae* were correctly identified by the "Vibrio"-(a) library. One strain of *V. alginolyticus* was correctly identified, but had an equivalent SI with *V. mediterranei*. None of these field isolates were identified by the "Aerobe" library.

5.3.4 *Identification of "Vibrio"-(b) reference strains by the "Aerobe" library*

The results of identification by the "Aerobe" library of the 69 reference strains used for the construction of the "Vibrio"-(b) library are listed in Table 6. Where replicate cultures were examined for individual strains, the SI varied only slightly between replicates for most samples and identification was taken as the average of results for all replicates. Twelve strains were correctly identified, including four of *Aeromonas salmonicida*, subsp. *salmonicida*, three of *V. cholerae* and one each of *V. fluvialis*, *V. furnissii*, *V. gazogenes*, *V. mediterranei* and *V. ordalii*. One strain each of *Aer. hydrophila*, *Listonella anguillarum*, *V. cholerae*, *V. metschnikovii* and *V. mimicus* were correctly identified, but had equivalent SI's with other species of *Vibrionaceae*. Three strains of *Aer. salmonicida* subsp. *salmonicida*, two of *V. fluvialis* and two of *V. furnissii* were correctly identified, but had SI's less than 0.300.

5.3.3 *Field isolate identification by the "Vibrio"-(b) and "Aerobe" libraries*

Table 7 gives the results of cellular fatty acid identification of 126 conventionally identified *Vibrionaceae* field isolates against the "Vibrio"-(b) and the "Aerobe" libraries. Sixty-three isolates were correctly identified by the "Vibrio" -(b) library including one *Aeromonas caviae* two *Aer. hydrophila*, 18 *Listonella anguillarum*, seven *Photobacterium damsela*, 25 of *Vibrio alginolyticus*, four *V. cholerae*, and six *V. harveyi*. Another four cultures correctly identified had equivalent SI's for other species of *Vibrionaceae*. They included one *Aer. hydrophila*, two *V. alginolyticus* and one *V. harveyi*. Nine cultures were correctly identified, but had SI's less than 0.300. These included three of *List. anguillarum*, two of *V. alginolyticus*, two of *V. parahaemolyticus* and one each of *V. harveyi* and *V. splendidus* I. The "Aerobe" library correctly identified 14 cultures including three *List. anguillarum*, one *Plesiomonas shigelloides* eight *V. cholerae* and two *V. fluvialis*. The "Aerobe" library additionally identified one culture of *V. mimicus*, the profile having an equivalent SI with *V. cholerae*, and one of *V. campbellii* with a SI of less than 0.300.

5.4 DISCUSSION

One of the first problems we had to overcome was poor growth on TSBA of some marine *Vibrionaceae*. The addition of ESS to the TSBA plates was found to considerably improve the growth of salt-requiring isolates. However, changes were observed in the relative proportions of various fatty acids in the profiles produced. This confirmed the results of Oliver and Colwell (1973), who found that alterations in the salt concentration produced differences in the C16:0 and C16:1 fatty acid content in Vibrios. As our user generated "Vibrio" library was intended to be compared with, and if possible complement the commercial "Aerobe" library, it was considered necessary to strictly adhere to the media formulation used in the construction of the "Aerobe" library, and the addition of ESS to the TSBA plates was discontinued.

However, according to the reasoning of Ghanem *et al* (1991), our strict adherence to the protocols used by MIDI for the development of the "Aerobe" library may have been detrimental to the generation of a practical identification system. They used the MIDI system for the identification of species of *Clostridium* and recommended that library entries should include reasonable variation that will be seen among properly identified strains of a species. They felt that slight variations would occur even under "standardised" conditions. These would include different incubation times, (16 versus 18 or 24 hours), different batches of media, replicate cultures prepared on the same or on different days and cultures prepared and extracted by different personnel

The analysis of 69 confirmed reference strains by the "Aerobe" library showed a very poor level of identification. Seven of the 12 strains identified belonged to two species, *Aeromonas salmonicida* subsp. *salmonicida* and *Vibrio cholerae*. The "Aerobe" library identified four of the five reference strains used to generate the *V. cholerae* profiles in the "Vibrio" library, although one had an equivalent SI for *V. fluvialis*. However, the one strain which it failed to identify was the type strain. Comparison of the cellular fatty acid profile of this strain with that of other published data for the same strain showed that it lacked the 16:1 isomer of which Lambert *et al* (1983) reported 12%. This result was confirmed using a gas chromatograph/mass spectrometer to obtain better resolution of the profile. In our comprehensive characterisation studies using conventional biochemical tests, this strain, although identified as *V. cholerae* at a 98% confidence level, differed from published results in that it was negative in the Voges-Proskauer test and failed to utilise trehalose, fructose or galactose in carbon substrate utilisation tests. As a positive Voges-Proskauer reaction is one of the two major tests differentiating *V. cholerae* from *V. mimicus* in the original species description for *V. mimicus*, it would seem likely that the culture of the type strain of *V. cholerae* we received in our laboratory has either changed due to subculturing under laboratory conditions or is a different culture to the type strain.

The type strain of *Vibrio mimicus* could not be identified by the "Aerobe" library which was unable to differentiate between identifications of *V. cholerae* and *V. mimicus*. This appeared to be due to the failure of the chromatographic system to resolve a second 16:1 isomer from the major 16:1 isomer. The quantity of the second isomer in the type strain was at the level at which resolution just became possible using the MIDI system. This is significant, as Lambert *et al* (1983) used the relative amounts of the 16:1 isomers to differentiate between species of *Vibrio*. The "Aerobe" library subsequently failed to correctly identify five biochemically identical strains of *V. mimicus* isolated from two disease outbreaks in the Redclaw freshwater crayfish and another isolated from a freshwater fish skin lesion, all being identified by it as *V. cholerae*.

The only species for which the "Aerobe" library performed significantly better than the "Vibrio" library" was *Vibrio cholerae*. The "Aerobe" library identified eight field isolates including four terrestrial isolates, two from pigs and two from ducks . It was of interest to note that each of these four terrestrial isolates contained detectable levels of the 16:1 isomer. This isomer was detected in only two of five aquatic *V. cholerae* isolates examined. This suggests there could be differences in the cellular fatty acid profiles of aquatic isolates when compared with terrestrial strains. Oliver and Colwell (1973) observed differences between the cellular fatty acid profiles of marine and terrestrial Vibrios, the unsaturated to saturated C16 and C18 ratios being lower in

marine strains. They felt that as the optimal growth for *Vibrio cholerae* is 37C, the production of a greater proportion of unsaturated fatty acids could be the result of compensation for the low growth temperature of 25C.

The poor performance by the "Aerobe" library with Australian *Vibrionaceae*, identifying 14 of 126 isolates tested, appears to suggest that the data base is too narrow for the species represented. This could be the result of insufficient numbers of strains entered or of strains drawn from too limited a geographical range of sources. Details of the numbers and source of strains used for the generation of the "Aerobe" library were not available to us. If the "Aerobe" library contained the recommended minimum of 20 strains of each species in it s data base, it would appear that this number is not adequate to produce a sufficiently broad data base for identification of all strains of the species.

The results obtained in this study highlight the need for the generation of a large data base of fatty acid profiles in the library for accurate and reliable identification results. The initial "Vibrio"-(a) library which had only single entries of reference cultures, correctly identified only six of 48 field isolates representing 13 species of *Vibrionaceae*. Following reconstruction of the library with cultural and extraction techniques standardised to the protocol used by MIDI for the construction of the "Aerobe" library and with a minimum of 20 entries per species, the "Vibrio"-(b) library correctly identified 63 of 127 field isolates, representing eight of the 20 species tested. Closer examination of these results shows that 76% of the correct identifications occurred in four species, namely, *Listonella anguillarum*, *Vibrio cholerae*, *V. alginolyticus* and *V. harveyi*. The "Vibrio" library contained profiles from between four and nine strains for each of these species. This suggests that the range profiles in the data base of the current library is still far too narrow for most of the species tested.

The "Vibrio" library profiles of *Listonella anguillarum*, generated from three Australian and six imported strains, identified 20 of 21 Australian isolates and one of three which originated in Japan. By comparison, the "Aerobe" library identified one of the Australian isolates and two of the three from Japan. The "Aerobe" library found 17 of the 21 Australian isolates had profiles most closely matched to *V. cholerae*, but with SI's less than 0.300.

The five strains of *Vibrio cholerae* used to generate the profiles entered in the "Vibrio" library were all isolated from aquatic sources and comprised two serotype:01 (including the type strain) and three non:01 serovars. However, the profile of the type strain differed sufficiently from the profiles of the other four entries to warrant their separate entries in the library as a subgroup of *V. cholerae*, referred to as *V. cholerae* type A. Four terrestrial isolates, two from pigs and two from ducks, were included in a batch of 10 field isolates analysed by the GC. All had been previously identified by conventional morphological and biochemical tests as *V. cholerae,* and had been serotyped as non:01 The "Vibrio" library identified four aquatic isolates as *V. cholerae* type A but failed to identify any of the four terrestrial isolates. The "Aerobe" library identified all four terrestrial isolates as well as four of the six aquatic isolates as *V. cholerae* :01. As the "Vibrio" library was prepared from aquatic isolates, these results suggest that there are significant differences in the cellular fatty acid content between isolates from terrestrial and aquatic sources, and that the "Aerobe" library appears to contain predominantly terrestrial

strains. However, the serological differences between the serotype:01 and non:01 strains did not appear to be reflected in the fatty acid profiles, as neither library was able to differentiate between these strains.

The "Vibrio" library demonstrated very high levels of identification for both *Vibrio alginolyticus* and *V. harveyi* field isolates. *V. alginolyticus* profiles were generated from two imported and two Australian strains. Of 30 isolates tested, 25 were correctly identified, two more were identified but had an equivalent SI for *V. harveyi* and two more were identified with SI's of less than 0.300. In contrast, the "Aerobe" library failed to identify any of the 30 *V. alginolyticus* isolates tested. The "Vibrio" library, containing the fatty acid profiles of five strains of *V. harveyi*, identified six of eight of *V. harveyi* field isolates, with another isolate having an equivalent SI for *V. alginolyticus* and the remaining isolate with a SI of less than 0.300. The "Aerobe" library, however, failed to identify any of the eight *V. harveyi* field isolates.

Vibrio carchariae, a species associated with clinical disease in captive sharks, (Grimes *et al* 1984), has similar biochemical properties to *V. harveyi*, and our two reference strains were unable to be distinguished from *V. harveyi* with the range of conventional biochemical tests used in this study. As a result of this, the "Vibrio" library did not contain any library entries for *V. carchariae* in its data base, and identified these two isolates as *V. harveyi*. The "Aerobe" library contained *V. carchariae* in its data base but was unable to identify either of these two isolates.

Only one strain of *Photobacterium damsela*, isolated in Tasmania, was entered into the "Vibrio"-(b) data base, as other strains held at the time, although identified by conventional biochemical testing as *Photobacterium* spp., could not be confirmed in their identities at species level. The "Vibrio" library identified six of 16 field isolates of *Photobact. damsela* from a single outbreak of disease in eels in Queensland (Ketterer and Eaves 1992). These eels had been captured from the wild and held in high concentration in a holding tank prior to export. Isolates fell into one of two biotypes, identical biochemically, except for their ability to decarboxylate lysine. All of the lysine negative isolates, including the five from this outbreak and one isolated in Tasmania, were identified as *Photobact. damsela* by the "Vibrio" library while only one of 12 lysine positive isolates was identified by the "Vibrio" library. Although this would appear to correlate well with the classical description of *Photobact. damsela* as being lysine negative, this correlation fails, as the one strain of *Photobact. damsela* entered into the "Vibrio" library was lysine positive.

Early studies on the use of cellular fatty acid profiles for bacterial identification were based on the development of decision trees for separation of species or groups of species (Lambert *et al* 1983; Urdaci *et al* 1990). There, the presence or absence, or else the relative proportions of specific fatty acids, was taken as the criterion for separation. This procedure ignored differences in the levels of other fatty acid components in the sample which may have been significant. The development of a library based on the total cellular fatty acid profiles of bacteria produces a system which has a very high specificity but may have a low sensitivity at the species level. Within a species, cultures falling outside the range of profiles in the data base may be either mis-identified or not be matched to any species. This highlights the need for the expansion of the data base with profiles from the maximum obtainable number of organisms of each species from as wide a range of strains as possible. Ideally, each library entry should be broad enough to

include real variation within species, yet narrow enough to differentiate each species from all other species. However, this may not be possible for some species, as the profiles for some species may not be sufficiently distinct for clear separation from other groups.

There are, however, advantages to the high specificity of this identification system. Analysis of the total cellular fatty acid content of bacteria produces a profile characteristic of the organism that can be used epidemiologically as well as for identification to the species level. The profile provides a "fingerprint" that can be used to compare isolates of the same species to determine whether a strain causing disease is re-occurring, or whether a disease outbreak is due to a new strain of the same species. Similarly, it could be used to trace back the source of a disease outbreak.

Although it contained profiles from only 69 strains of *Vibrionaceae*, the constructed "Vibrio" library clearly outperformed the commercial "Aerobe" library for the identification of aquatic *Vibrionaceae*. This may reflect the fact that most of the reference strains used for its construction were from aquatic sources, many being Australian in origin. The "Vibrio" library as constructed produced a high level of identification in four species, *Listonella anguillarum*, *Vibrio cholerae*, *V. harveyi* and *V. alginolyticus*, but could not be relied on for identification of the majority of species of *Vibrionaceae*.

With further expansion of the data base, the system shows the potential to provide an accurate, rapid, cheap identification system. It has the added advantage of using a single standardised approach applicable to the identification of all bacteria. Once the data base is established, it could be transferred to any other laboratory which contained a suitable gas chromatograph The benefits of such a rapid diagnostic tool are obvious where control of spread of disease is critical, as in aquaculture operations.

Table 4. Identification by the "Aerobe" library of reference strains used for the generation of the "Vibrio"-(a) library

Species name	Total tested	Number identified[†] by "Aerobe" library
Aeromonas caviae	1	0
Aer. hydrophila	1	0 (1)[§]
Listonella anguillarum	7	0 (1)
Photobacterium damsela	1	0
Photobact. fischeri[*]	1	0
Plesiomonas shigelloides	1	1
Vibrio alginolyticus	4	0
V. cholerae	5	0 [1‡]
V. fluvialis	4	1 [2]
V. harveyi	1	0
V. mediterranei	1	0
V. metschnikovii	1	0
V. mimicus	3	0
V. nereis	1	0
V. ordalii	1	1
V. parahaemolyticus	3	0
V. tubiashii	1	0
V. vulnificus	4	0
Total:	41	3 (2) [3]

† - Identification based on a similarity index of 0.300 or greater

* - Species not in "Aerobe" data base

§ - Bracketed number indicates the number of strains with an equivalent similarity index for another species of *Vibrionaceae*

‡ - Superscript number indicates the number of strains correctly identified with a similarity index <0.300

Table 5. Identification of field isolates by the "Vibrio"-(a) and "Aerobe" cellular fatty acid libraries

Species name	Total tested	Number identified[†] by "Vibrio"-(a) library	"Aerobe" library
Aeromonas caviae	2	0	0
Aer. hydrophila	4	0	0
Photobacterium damsela	2	0	0
Plesiomonas shigelloides	1	0	0
Vibrio alginolyticus	22	0 (1)[§]	0
V. cholerae	6	6	0
V. harveyi	2	0	0
V. mimicus	1	0	0
V. nereis	2	0	0
V. parahaemolyticus	2	0	0
V. proteolyticus	1	0	0
V. tubiashii	1	$0^{1‡}$	0
V. vulnificus	2	0	0
Total:	48	$6 (1)^{1}$	0

[†] - Identification based on a similarity index of 0.300 or greater

[§] - Bracketed number indicates the number of strains with an equivalent similarity index for another species of *Vibrionaceae*

[‡] - superscript number indicates the number of strains correctly identified with a similarity index <0.300

Table 6. Identification by the "Aerobe" library of reference strains used for the generation of the "Vibrio"-(b) library

Species name	Total tested	Number identified[†] by "Aerobe" library
Aeromonas caviae	1	0
Aer. hydrophila	1	0 (1)
Aer. salmonicida subsp. *salmonicida*	7	4 [3‡]
Aer. salmonicida subsp. *achromogenes* [*]	1	0
Listonella anguillarum	9	0 (1)
List. pelagia	1	0
Photobacterium damsela	1	0
Photobact. fischeri [*]	1	0
Plesiomonas shigelloides	1	0
Vibrio alginolyticus	4	0
V. cholerae	5	3 (1)
V. diazotrophicus	3	0
V. fluvialis	3	1 [2]
V. furnissii	4	1 [2]
V. gazogenes	1	1
V. harveyi	5	0
V. hollisae [*]	1	0
V. mediterranei	1	1
V. metschnikovii	2	0 (1)
V. mimicus	1	0 (1)
V. natriegens [*]	2	0
V. nereis	1	0
V. ordalii	1	1
V. parahaemolyticus	4	0
V. splendidus I [*]	1	0
V. tubiashii	3	0
V. vulnificus	4	0
Total:	69	12 (5) [7]

[†] - Identification based on a similarity index of 0.300 or greater

[*] - Species not in "Aerobe" data base

[§] - Bracketed number indicates the number of strains with an equivalent similarity index for another species of *Vibrionaceae*

[‡] - Superscript number indicates the number of strains correctly identified with a similarity index <0.300

Table 7. Identification of field isolates by the "Vibrio"-(b) and "Aerobe" cellular fatty acid libraries

Species name	Total tested	Number identified† by "Vibrio"-(b) library	Number identified† by "Aerobe" library
Aeromonas. caviae	2	1	0
Aer. hydrophila	3	2 (1)§	0
Sucrose negative Aeromonad*	1	0	0
Listonella anguillarum	22	18 3‡	3
List. pelagia	1	0	0
Photobacterium damsela	18	7	0
Plesiomonas shigelloides	1	0	1
Vibrio alginolyticus	30	25 (2) 2	0
V. campbelli	1	0	0 1
V. cholerae	10	4	8 1
V. fluvialis	3	0	2
V. harveyi	8	6 (1) 1	0
V. mimicus	7	0	0 (1)
V. nereis	3	0	0
V. ordalii	4	0	0
V. parahaemolyticus	3	0 2	0
V. proteolyticus	1	0	0
V. splendidus I*	1	0 1	0
V. tubiashii	5	0	0
V. vulnificus	2	0	0
Total:	126	63 (4) 9	14 (1) 2

† - Identification based on a similarity index of 0.300 or greater

* - Species or phenotype not in "Aerobe" data base

§ - Bracketed number indicates the number of strains with an equivalent similarity index for another species of *Vibrionaceae*

‡ - Superscript number indicates the number of strains correctly identified with a similarity index <0.300

SECTION 6

CONCLUSIONS

The steady decline in wild fish stocks in the oceans will see an ever increasing role for aquaculture operations to replace this food resource. Disease will always present a major problem for the aquaculture industry, particularly where fish are densely stocked and subject to stress. The potential for rapid spread of disease and associated mass mortalities is much higher in the aquatic environment than in terrestrial situations and it is not acceptable to pour antibiotics into ponds to control disease in fish species being cultivated for human consumption.

Rapid identification of disease causing organisms will become increasingly important to the understanding and control of factors involved in disease outbreaks. The *Vibrionaceae* are one of the major bacterial families found in the aquatic environment and are often associated with disease outbreaks. Their identification using conventional morphological and biochemical tests is expensive and time consuming. Using cellular fatty acid analysis, *Vibrionaceae* could be cultivated, screened to the family level and processed through a gas chromatograph within three days of receipt of sample, saving seven to ten days on the time taken for conventional identification. The benefits of such a rapid diagnostic tool are obvious where control of disease spread is critical.

The results of this project demonstrated the inadequacies of the commercially available cellular fatty library for identification of aquatic *Vibrionaceae*. The specificity of this identification system highlights the need for a broad data base containing the maximum possible numbers of aquatic strains from as diverse sources as possible. The "Vibrio" library generated during the three years of this project, although limited in size, clearly outperformed the "Aerobe" library. It produced a high level of identification for a few species of *Vibrionaceae*, but needs further expansion of the data base for other species to provide an adequate identification system for all aquatic *Vibrionaceae*. This has the potential to provide the rapid identification of bacteria needed to service the aquaculture industry.

Without technical assistance, future progress will be slow in expanding the "Vibrio" cellular fatty acid data base. However, once established, this identification system can be readily transferred to any laboratory which has a suitable gas chromatograph with an autosampler attached to a computer monitor and printer. This set-up could be purchased for approximately $30,000, a relatively small price for a complete identification scheme. Although careful attention to detail is required in the processing of cultures and subsequent chemical extraction, the procedure is technically simple, and well within the capacity of most diagnostic laboratories.

This technique represents an important technological advance in the identification of bacteria. Once the library generation software has been installed, it can be used to generate a cellular fatty acid data base for any group of bacteria for which conventional identification has proved difficult.

SECTION 7

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SECTION 8

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