Aquatic Animal Health Subprogram: Rapid strain identification of the bacterial fish pathogen *Streptococcus iniae* and development of an effective polyvalent vaccine for Australian barramundi

Andrew C Barnes
Centre for Marine Studies and School of Biological Sciences, The University of Queensland

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Australian Government
Fisheries Research and Development Corporation

Project 2007/226

Copyright

Disclaimer
The authors do not warrant that the information in this document is free from errors or omissions. The authors do not accept any form of liability, be it contractual, or otherwise, for the contents of this document or for any consequences arising from its use or any reliance placed upon it. The information, opinions and advice contained in this document may not relate, or be relevant, to a readers particular circumstances. Opinions expressed by the authors are the individual opinions expressed by those persons and are not necessarily those of the publisher, research provider or the FRDC.

The Fisheries Research and Development Corporation plans, invests in and manages fisheries research and development throughout Australia. It is a statutory authority within the portfolio of the federal Minister for Agriculture, Fisheries and Forestry, jointly funded by the Australian Government and the fishing industry.

Title: Aquatic Animal Health Subprogram: Rapid strain identification of the bacterial fish pathogen *Streptococcus iniae* and development of an effective polyvalent vaccine for Australian barramundi.
Author: Andrew C Barnes

First Published February 2010 by FRDC and The University of Queensland

25 Geils Court
Deakin
ACT 2600

School of Biological Sciences
Brisbane
QLD 4072
Australia

ISBN: 978-0-646-53019-2

Table of Contents

		Page
1.	Non-Technical Summary	1
2.	Acknowledgments	3
3.	Background	4
4.	Methods	6
5.	Results	12
6.	Discussion	19
7.	Benefits and Adoption	25
8.	Further Developments	26
9.	Planned Outcomes	28
10.	Conclusions	30
11.	References	31
12.	Appendix 1 – Intellectual Property	33
13.	Appendix 2 - Staff	34
14.	Appendix 3 – Raw data	35
15.	Appendix 4 – Molecular Serotyping of *S. iniae* – A Laboratory Manual v1.1	49

1. Non-Technical Summary

2006/227	**Aquatic Animal Health Subprogram: Rapid strain identification of the bacterial fish pathogen *Streptococcus iniae* and development of an effective polyvalent vaccine for Australian barramundi**

PRINCIPAL INVESTIGATOR **Prof. Andrew C. Barnes**
ADDRESS **Centre for Marine Studies and School of Biological Sciences**
The University of Queensland
Brisbane QLD4072
a.barnes@uq.edu.au

OBJECTIVES

1 Characterise the genetic and molecular basis by which biotypes of *S. iniae* vary in relation to capsular and surface protein antigen presentation and strain variation

2 Develop and implement a rapid antigen typing scheme for *S. iniae* and transfer rapid identification technology to regional laboratories

3 Develop a polyvalent vaccine against all known Australian strains of *S. iniae*

4 Verify the effectiveness of the vaccine in experimental challenge studies initially using the intraperitoneal injection route of immunisation in comparison with immersion.

NON-TECHNICAL SUMMARY

OUTCOMES ACHIEVED TO DATE

The genetic and molecular basis for serotype variation in *S. iniae* has been determined and serotype has found to be controlled by a limited number of genes within the large capsular operon. This information has enabled development of a reliable typing system that has been used to determine the variability of *S. iniae* isolates from farms in Australia. This knowledge has enabled several outcomes. Firstly, we have been able to direct formulation of current vaccines to ensure that vaccines for particular farms contain the necessary isolates making them more effective. Secondly, we have written a manual to enable accurate typing of future isolates from outbreaks of disease in Australia such that veterinary laboratories may advise vaccine companies when new strains arise. Finally, by determining the scope of variability amongst strains in Australia, we can recommend a vaccine formulation that should protect against all current isolates in Australia. This will permit registration of a universal (or generic) vaccine for use in Australia.

Need

Fish can be routinely vaccinated against diseases caused by bacteria in the same way that other animals and humans are vaccinated. Vaccination exploits the natural immune system of the animal to remember and respond to a particular antigen if it encounters it again. *Streptococcus iniae* is a bacterium that causes disease in farmed fish similar to meningitis and blood poisoning in humans. Treating this infection with antibiotics is difficult because antibiotics have to be fed to the fish and when the fish are sick, they don't eat the medicated feed. There is also the problem of bacteria becoming resistant to antibiotic treatment, and this resistance can be transmitted to other bacteria that may be harmful to humans. Furthermore,

specific food safety requirements such as maximum residue levels (MRLs) must be met before farmers are able to sell food animals, so a 'withdrawal period' may be required. This causes problems for the farmer, particularly if the fish are close to market size.

Vaccinating against *S. iniae* is possible in fish but it is not always successful because *S. iniae* can change its surface antigen (coat or capsular polysaccharide), meaning that vaccinated fish may not recognise and respond to the new strains and still become diseased. This is similar to flu in humans, where different strains arise each year and new vaccines have to be manufactured and distributed. With flu, it is very easy to type the virus based on two proteins on the surface, H (the haemagglutinin) and N (the neuraminidase), thus two genes can be identified to determine the virus type or types (eg H5N1) that are circulating through a population and therefore ensure that these types are in the 'flu shot' vaccine. Typing the surface of *S. iniae* is much more difficult as it has a carbohydrate coat which is indirectly controlled by a large suite of genes (more than 20).

The objectives of the present project were severalfold: Firstly, to see how many of these genes vary in *S. iniae*. Then to determine how many variants of each gene there are from farmed fish in Australia. With this information we can a) make sure that all of the correct variants from a particular farm are included in the vaccines for that farm; and b) potentially design a vaccine containing all relevant variants in Australia that can be produced as a single vaccine to be used on all farms.

Results and Discussion

The variability of the structure of the surface capsule (sugar coat) of *S. iniae* can be attributed to changes in 5 genes. Three of these genes are involved in determining the *amount* of capsule. One gene determines the *type* of sugars (the ratio of glucose to galactose) in the capsule, and one gene determines the *length* of the sugar chains. All three of these features can potentially change the way that the surface is 'seen' by the fish immune system, although a vaccination trial indicated that the gene that controls polymer chain length may be less important in determining how the fish immune system responds to the capsule. Even though variability was limited to few genes, the degree of variability in the genes that regulate the *amount* of capsule was unexpectedly high.

A reproducible and accurate typing system was developed and a manual drafted and disseminated to veterinary laboratories. This will enable accurate detection of any new isolates should they arise in the future

Objective 3 was partially met: Based on the information generated from the typing it is possible to recommend a range of isolates that should offer broad protection against Australian *S. iniae* isolates and therefore formulate a polyvalent generic vaccine.

We were unable to meet objective 4, as the challenge model for *S. iniae* was insufficiently robust for vaccine testing and we were unable to resolve this during the present project lifespan or budget.

Future Direction

Based on results in this report it is possible to recommend formulation of a generic vaccine for Australian barramundi. Future work should include further typing of isolates, but it should focus predominantly on testing generic vaccine formulations in fish to ensure cross protection and on generating a dossier to permit registration of the vaccine.

KEYWORDS: Barramundi, aquaculture, disease, *Streptococcus iniae*, vaccines, diagnosis.

2. Acknowledgements

Many people have contributed to this project to varying degrees, and it could not have been completed without help from those acknowledged below.

- The Australian Barramundi Farmers Association for its strong and continued support for the project and interest throughout.
- Justice Baiano was employed full time on this project and performed the PCR and sequencing of the strains, and analysed and managed the data.
- John Humphrey (formerly of Berrimah Veterinary Laboratories), Glenn Schipp (Darwin Aquaculture Centre) and Shannon McBride (Formerly of GFB Fisheries, Bowen, QLD) co-writing the original FRDC Aquatic Animal Subprogram proposal and pushing it forward in its various revisions and incarnations.
- Ian Anderson and his team at DEEDI QLD Oonoonba Veterinary Laboratory, especially Annette Thomas and Judy Forbes-Faulkner, for their patient repeated supply of strains.
- Matt Landos, Future Fisheries Veterinary Services, Lennox Head, for his excellent liaison work with the industry and with Allied Biotechnology.
- Mark White, Allied Biotechnology, Sydney, for supplying strains, making vaccines and generally keeping us in the loop. Without the link between Matt and Mark, the project would have lost some (perhaps most) of its industry relevance and direction.
- Richard Whittington, University of Sydney, for interesting and productive discussions on direction.
- Suresh Benedict, Berrimah Veterinary Laboratories, for comments on milestone reports, supply of many strains and being very helpful and cooperative.
- Lynn Shewmaker, Centers for Disease Control, Atlanta for supply of strains and helpful advice.
- Roger Chong, DEEDI Queensland Veterinary Laboratory, Yeerongpilly, Nikki Buller, Department of Fisheries, Western Australia, Christian Michel, Jouy-en Josas, France, Olivier Decamp, INVE Technologies BV, Belgium for supply of some strains into our collection
- Candy Chan and Shan (Summer) Liang, UQ undergraduate students for helping out with vaccination, sampling and ELISA.

3. Background

The present project was developed in response to the increasing occurrence and distribution of the septicaemic bacterial disease streptococcosis in the Australian barramundi industry and its serious adverse impacts on productivity and the economics of farming barramundi.

Streptococcosis can be controlled through the use of antibiotics, however, this is not a satisfactory solution for the industry due to (1) recurrence of disease on withdrawal of the antibiotic, (2) cost of medication, (3) withholding periods prior to market and (4) the preference to move to non-antibiotic means of disease prevention and control. Although vaccination offers a safe, effective and economic control strategy to prevent streptococcosis in barramundi, major impediments currently exist in adopting vaccination for the sustainable management of the disease in the Australian barramundi industry as recent vaccination failures through reliance on autogenous vaccines have demonstrated.

Vaccination against streptococcosis is problematic as the causative organism, *Streptococcus iniae*, expresses a highly variable capsule (polysaccharide coat), and, unless a particular capsular serotype is incorporated into a vaccine, it will not protect the fish from disease. The project was developed following a joint approach from the Darwin Aquaculture Centre (DAC) and Good Fortune Bay (GFB) Fisheries to The University of Queensland (UQ), identifying that there was an urgent need for vaccine technology to prevent streptococcosis in farmed barramundi and that uncertainties existed regarding serotype identification for vaccine manufacture and the efficacy of vaccination. Prior to the project, UQ had conducted serotyping of *S. iniae* on behalf of veterinary diagnostic laboratories and contracted vaccine manufacturers on an *ad-hoc* basis in order to inform them on which strains to include in autogenous vaccine preparations. With other large farmed barramundi producers considering vaccination, the limitations of the present arrangements and methodologies were apparent, highlighting the need for a concerted collaborative effort to develop rapid methods for serotyping/genotyping of *S. iniae* isolates for vaccine manufacture. The techniques developed had to be directly relevant to serotype so that veterinary diagnostic laboratories could rapidly inform vaccine companies of strains to include in vaccines.

The barramundi farming industry and the vaccine producers are the key beneficiaries of the present project. The outcomes from the present project guide formulation of vaccine products, and results indicate that a multivalent preparation containing sufficient strains to be effective at most farm sites is possible, subject to the Australian Pesticides and Veterinary Medicines Authority (APVMA) approval. This outcome would provide a significantly more affordable product for Australian farmers as use of the same vaccine on more sites will allow larger scale, and thus more cost effective, vaccine production.

Consultation

The Australian Barramundi Farmers Association (ABFA) recognised that streptococcosis is a major limiting factor in the commercial production of farmed barramundi in both marine and freshwater systems. The ABFA were fully supportive of the proposed research. As well, individual barramundi farmers impacted by streptococcosis supported the research. State and Territory Fisheries and Aquaculture management in the NT, and Qld, recognised the problem and fully supported the proposal. Vaccine companies (Allied Diagnostics and Intervet (now part of Merial/Merck/Schering-Plough Animal Health)) are interested in the commercialisation of a vaccine and private veterinary consultants vested with responsibility for disease control and prevention to the industry also expressed full support for the project.

Need

Streptococcus iniae is the major cause of streptococcosis, the most important bacterial disease affecting Australian farmed barramundi. Streptococcosis is a major limiting factor in production of barramundi in freshwater and marine systems. Economic loss occurs through high mortalities, loss of marketable product, lost feed costs and high costs of treatment, control, prevention and cleanup. Eradication of the disease from the farm environment is not possible. Vaccination offers an inexpensive, reliable and safe method of preventing the disease; however, current strategies rely on autogenous vaccines, i.e., vaccines prepared from specific strains of the organisms causing disease on a particular farm. Provision for preventative vaccination of stock in Australia is currently hampered by the lack of a commercially licensed generic Streptococcus vaccine. Further, the use of autogenous vaccines may not protect the fish from disease caused by newly emergent strains and the reproduction of vaccines in response to each separate occurrence is time consuming, and relies on the disease outbreak occurring first. At least seven strains of *S. iniae* are recognised in Australia, with further strains likely to emerge. Current vaccines are usually strain specific and therefore cross protection against all isolates in all locations in Australia may not be afforded. It has been demonstrated on at least one occasion in Darwin that a vaccine developed against one strain was ineffective against another. A need exists to: understand the underlying molecular mechanisms whereby new strains of *S. iniae* evolve; develop technologies that will provide rapid typing of existing or new strains of *S. iniae* and; provide a strategy for rapid incorporation of new strains into a polyvalent vaccine for national industry use.

Outcomes

1. Provide a cost-effective and reliable vaccine against streptococcosis in barramundi.
2. Development of an understanding of the molecular epidemiology in the occurrence and spread of disease will provide a rational basis for implementation of disease control and translocation strategies.
3. A commercially available vaccine for use on farms and in nurseries to produce fish immunised against streptococcosis will provide major economic benefits to industry.
4. Technology transfer to regional veterinary laboratories of a rapid reliable molecular means of determining serotype that is not dependent on raising fish antibodies will facilitate prompt changes to vaccines as required.

Objectives

1 Characterise the genetic and molecular basis by which biotypes of *S. iniae* vary in relation to capsular and surface protein antigen presentation and strain variation

2 Develop and implement a rapid antigen typing scheme for *S. iniae* and transfer rapid identification technology to regional laboratories

3 Develop a polyvalent vaccine against all known Australian strains of *S. iniae*

4 Verify the effectiveness of the vaccine in experimental challenge studies initially using the intraperitoneal injection route of immunisation in comparison with immersion.

4. Methods

4.1. Determining which genes within the *S. iniae* capsular operon are variable

S. *iniae* isolates from a diverse range of geographic origins and hosts were selected for an initial screen of capsular operon diversity (Table 1). Strains were included that had been isolated from documented cases of vaccine failures in Australia and Israel (Bachrach *et al*., 2001), and the Type strain from an Amazon freshwater dolphin, isolated from a display aquarium in San Francisco in 1970 was also included as the main reference strain (Pier & Madin, 1976).

Table 1. Strains used in preliminary screen study.

Isolate	Source	Geographic Origin
QMA0072	*Lates calcarifer*, seawater	Townsville, Queensland
QMA0076	*Lates calcarifer*, freshwater	Townsville, Queensland
QMA0083	*Lates calcarifer*, freshwater	Fremantle, Western Australia
QMA0140^T	*Inia geoffrensis,* freshwater	USA
QMA0155	*Lates calcarifer*	New South Wales
QMA0165	*Lates calcarifer*, seawater	Bowen, Queensland
QMA0177	*Lates calcarifer*, seawater	Northern Territory
QMA0191	*Lates calcarifer*, seawater	Northern Territory
QMA0188 (KFP173)	*Oncorhynchus mykiss*	Israel
QMA0186 (KFP404)	*Oncorhynchus mykiss*	Israel

T – Type strain.

DNA was extracted by enzymatic lysis appropriate for *Streptococcus* species as previously described (Gardiner *et al*., 1998).

Primers for PCR and sequencing were designed using the *S. iniae* capsule operon sequence available on GenBank (sequence accession number AY904444). The primers used are listed in Table 2.

Long distance PCR was carried out to amplify the capsule operon in two overlapping segments of 7kb and 14kb. Elongase enzyme (Invitrogen, USA) was used in accordance of the manufacturer's recommendations.

Capsular operon genes were amplified using a proofreading DNA polymerase (PrimeStar HS DNA polymerase, Takara, Japan) in 25 μL reactions composed of: 5 μL of 5 x PCR buffer, 0.5 μL of 4 x 2.5 mM dNTP's, 100 ng of each primer, 0.15 μL of PrimeStar DNA HS DNA polymerase, 100-200 ng of extracted bacterial DNA, to volume with sterile Milli-Q water. Reactions were carried out at temperatures appropriate to each primer pair (Table 3). A "hot start" technique was employed to reduce the likelihood of non-specific amplification products prior to cycling as follows: 2 min at 94 °C for one cycle, followed by 35 cycles of denaturation for 15 s at 94 °C, 30 s at an appropriate annealing temperature, and extension at 72 °C of 1 min for every expected kb of amplicon. Reaction mixtures were held at 4 °C until analysed by agarose gel electrophoresis.

Where a single amplicon was produced, 0.6 μL of PCR product was added to 0.3 μL of a 1:2 mixture of Exonuclease I and Shrimp Alkaline Phosphatase and 3.1 μL of sterile Milli-Q water and incubated at 37 °C for 30 min followed by heating at 85 °C for 15 min before DNA sequencing using BigDye Terminator v3.1 chemistry by the Australian Genome Research Facility, Brisbane. Where more than one amplicon was observed, the remainder of the PCR

reaction was separated in a 1% agarose gel, run at 60 V for 1 h, excised from the gel with a sterile scalpel blade and then extracted from the gel slice using a kit (MEGA-Spin Gel Extraction Kit, Intron Biotechnology, Korea). The purified product was then sequenced.

Table 2. Oligonucleotide Primer sequences used in this study.

Primer	Sequence 5'-3'
CPS Y F	TTATATTTCCTTTTTTTGTGTCAATTTGA
CPS Y R	ATGAGAATACAACAATTACATTACA
CPS A F	TGATTGGAGTTAAAAAGTAATG
CPS A R	ATCGCATAGGATGGCAATTCA
CPS B F	CAGTAATGGGAGGAAAGTAAATG
CPS C R	TTGTGACATCCTTAACCTC
CPS D R	TCACTTTCTGGAATGTTTTTTAC
CPS E F	ATGAAAAGAAGTCAAAAAAGAGTAATC
CPS E R	TTACTCCTGTTTAGCGTCATTTA
CPS F F	ATGTATCCTTATATTAAACGAC
CPS F R	TCACTTCTCCTTATTTTGCTCTG
CPS G F	ATGAAAAAAGTACTTATTACAGGTGC
CPS G R	CTATATTTTTTCATTGTTTCTTTCC
CPS H F	ATGAAAAAATATAGTAGAAGTGTG
CPS I R	AATCTCCTATTTTTTCCCACAT
CPS J F	ATGAAAATACTTGTGACAGGTG
CPS K F	ATGATAACTGTTTGTATGGCAAC
CPS K R	TTACCATTTTTTATTTTTCCTTTTTGTTT
CPS L F	ATGAAAGTAGCGTTTTATTTAGAT
CPS L F RC	ATCTAAATAAAACGCTACTTTCAT
CPS L R	GTGTTCATCTGGTATTTTTTATTAA
CPS L R RC	TTAATAAAAAATACCAGATGAACAC
ORF276 F	ATGAGGGTATCTATAAAAAGTATAA
ORF193 F	ATGTTGCTGTTACTGACATTAACATT
ORF193 F RC	AATGTTAATGTCAGTAACAGCAACAT
ORF193 i R	AAACTAGAACCTGAAAGAAAAATACC
ORF151 R	TCATAGATACTCCTTTTCCTGTT
CPS M F RC	AATTCAATTTTATACTGTTTTTTTTCAT
CPS M R	ATATAAGAACTAAAAAATTCAAGTCAAATTAA
CPS N F	ATGAAAAAAATAGCAGTTGCTGG
CPS N R	TTAATCCCGGCCGAAAAGGT
ORF183 R	TTAGACTTCCTGAGCTGCGAA

Table 3. Groupings used to amplify capsule operon genes.

Block	Genes in Block	Primers used for PCR	Annealing Temperature	Expected Product Size
1	*cpsY-cpsA*	CPS Y F, CPS A R		2544 bp
2	*cpsB-cpsD*	CPS B F, CPS D R		2159 bp
3	*cpsE*	CPS E F, CPS E R		1802 bp
4	*cpsF-cpsG*	CPS F F, CPS G R		1468 bp
5	*cpsH-cpsI*	CPS H F, CPS I R		1605 bp
6	*cpsJ-cpsK*	CPS J F, CPSK R		1576 bp
7	orf276-orf151	ORF276 F, CPSLF RC		2314 bp
8	*cpsL-cpsM*	CPS L F, CPS M R		3997 bp
9	*cpsN*-orf183	CPS N F, ORF100 R		1895 bp

4.2. Development of the most efficient molecular serotyping method for *S. iniae*

Based on the results of the initial screen, a large number of Australian isolates were analysed (Table 4), concentrating only on those genes that were determined to be variable in order to establish the degree of variability amongst these genes and thus devise the optimum typing strategy for future molecular serotyping of *S. iniae* isolates from Australian barramundi

farms. DNA was extracted as described above, and *cpsY, cpsB, cpsC. cpsD, cpsE, cpsG* and *cpsH* were amplified using the primer sets indicated in Table 5, using Takara PrimeScript proof reading *taq* polymerase and 25 μL reactions as described above. Annealing temperatures were adjusted according to the primer set used (Table 5). Based on the results obtained from this widespread screen, a manual has been prepared, tested and revised to enable accurate molecular serotyping of Australian isolates of *S. iniae* and is included in the Appendix.

Table 4. *S. iniae* strains used in broad range molecular serotyping.

Isolate	Source	Geographic Origin	Source
142	*Lates calcarifer*,	Northern Territory (SW)	Berrimah Veterinary Laboratory
150	*Lates calcarifer*,	Northern Territory (SW)	Berrimah Veterinary Laboratory
153	*Lates calcarifer*,	Northern Territory (SW)	Berrimah Veterinary Laboratory
158	*Lates calcarifer,*	South Australia site 1?	Allied Biotechnology
159	*Lates calcarifer*	South Australia site 2?	Allied Biotechnology
160	*Lates calcarifer*,	South Australia site 3	Allied Biotechnology
164	*Lates calcarifer*,	Queensland site 1 (FW)	TAAHL, Oonoonba
170	*Lates calcarifer*,	Queensland site 2 (SW)	TAAHL, Oonoonba
173	*Lates calcarifer*	South Australia site?	AVM
189	Unknown	Réunion Island	Christian Michel, INRA, France
190	Unknown	Thailand	Christian Michel, INRA, France
216	*Lates calcarifer*	Queensland site Q1?	Allied Biotechnology
218	*Lates calcarifer*	Queensland site Q2?	Allied Biotechnology
220	*Lates calcarifer*	New South Wales	Allied Biotechnology
233	*Lates calcarifer*	New South Wales	Allied Biotechnology
243	*Lates calcarifer*	South Australia	Allied Biotechnology
244	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
245	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
246	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
247	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
248	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
249	*Lates calcarifer*	South Australia site 3	Allied Biotechnology
250	*Lates calcarifer*	New South Wales	Allied Biotechnology
251	*Lates calcarifer*	New South Wales	Allied Biotechnology
252	*Lates calcarifer*	New South Wales	Allied Biotechnology
253	*Lates calcarifer*	New South Wales	Allied Biotechnology
254	*Lates calcarifer*	New South Wales	Allied Biotechnology

Table 5. Annealing temperatures and expected PCR product sizes for targeted genes.

Forward primer	Reverse primer	Gene/s targetted	Annealing temperature (ºC)	Expected product size (kb)
HKi F	CPSA R	*cpsY*	65	3.0
CPSB F	CPSD R	*cpsB-D*	55	2.2
CPSE F	CPSE R	*cpsE*	58	1.8
CPSF F	CPSH R	*cpsF-H*	64	2.5

4.3. Development of a *Streptococcus iniae* challenge model in barramundi.

Experimental animals and husbandry

Juvenile barramundi, mean weight 35 grams, were obtained from Ecofish Pty Ltd, Caloundra, Queensland. Fish were stocked at 25 fish per tank in 160 litre food-grade circular plastic tanks

containing 150 L brackish water (5 ppt) prepared from marine salts (Ocean Nature, Aquasonic, NSW) and reverse osmosis filtered fresh water. Each tank was connected to an independent biofilter and pump (Eheim 2217) and thus each tank represented an individual experimental unit (Figure 1, below). Water was recirculated at 1000 L per hour and biofilters were established for 6 weeks prior to stocking with fish. Fish were acclimated for 14 days prior to commencement of the challenge and fed twice daily at 6am and 6 pm with a commercial pelleted diet (Ridley Aqua Feeds, Narangba, Qld). Daily water changes (30-50%) were made at least 2 hours after the morning feed. A 12 hour daylight cycle was maintained for the duration of the challenge. Water temperature was recorded daily and maintained at 24 ± 1°C via the aquarium room air conditioner. Ammonia, nitrite and nitrate were assayed daily using commercial kits (Aquasonic, NSW) and pH was recorded by taking a 5 mL water sample and analysed using a laboratory pH meter.

Challenge inoculum

Streptococcus iniae strain QMA00165, isolated from a marine barramundi farm in Queensland in 2006 was selected for this study. The isolate was cultured from stock (-80°C, 20% glycerol in vegetable peptone broth) on Columbia agar base containing 5% defibrinated sheep blood overnight at 37°C. Identity was verified by PCR of the lactate oxidase gene as previously described. For preparation of the challenge inoculum, a single colony was selected from the confirmed overnight agar culture and suspended in 1 mL sterile Phosphate Buffered Saline (PBS). Aliquots (100 μL) were then spread onto sheep blood agar plates and incubated overnight at 37°C. From these plates, culture was removed with a cotton swab and suspended in sterile PBS. Cells were washed once in PBS and resuspended to an optical density of 1.00 at 600nm, previously estimated to be equivalent to approximately 5 x 10^8 cfu mL^{-1}. Serial tenfold dilutions of this suspension were prepared in PBS and 100μL aliquots of 10^4, 10^5 and 10^6-fold dilutions spread onto tryptone soya agar for subsequent viable counting to accurately determine the challenge dose employed.

Injection challenge model

Three independent replicate challenge models were performed. Fish were removed from their tanks using a hand net and anaesthetised in MS222 diluted 1 part in 10,000 in clean aquarium water. In each model, 3 doses (undiluted, 10 fold and 100 fold dilutions) of the above inoculum were employed, such that 18 fish were injected intraperitoneally with 100μL for each dose and distributed into 3 tanks. A fourth tank contained 18 unchallenged control fish subject to anaesthesia and handling. In all, 10 tanks were employed, 3 for each challenge dose and 1 control, each tank containing 18 animals. Fish were observed 4 times daily, every 6 hours and mortalities were recorded. Any fish showing acute signs of disease (swimming upside down) were euthanased by overdose of anaesthetic. All mortalities were sampled and plated onto Tryptic Soy Agar (TSA). Overnight growth on TSA plates was subsequently tested for direct-lysis PCR of the lox gene to confirm cause of death. The trial was terminated when no mortalities had been recorded for 4 consecutive days.

Analysis of results

Mortality data were analysed by Kaplan-Meyer survival analyses using Prism version 4 for Macintosh (GraphPad Ltd, California).

4.4. Antibody response following vaccination with *S. iniae* CPS variants

In the absence of a suitable challenge model, and to ensure that some information was derived on the effects of vaccines containing different capsular genotypes, a vaccine trial was conducted using 8 experimental vaccines prepared from different capsular genotypes.

However instead of challenging the fish, sera were collected at a time to correspond with peak secondary antibody response following a booster vaccination, and this response was measured by ELISA.

Experimental animals
Barramundi (*Lates calcarifer*) of various sizes were obtained from a commercial fish farm and were kept in brackish water (5 ppt) in a recirculating system. Fish were maintained in aerated recirculating water at 30 ± 1 °C and fed twice daily with a commercial extruded diet (Ridley Aqua Feeds, Narangba).

Bacterial strains and culture
S. iniae strains 155, 158, 159, 177, 191, 216, 218, 236, recovered from outbreaks of Streptococcosis in farmed barramundi in Australia, with known capsular operon sequences were used in this study (Table 6). Bacteria were maintained as stocks in 20% glycerol at minus 80 °C. For routine culture, one loopful of frozen stock was taken and grown on blood agar overnight at 28°C. Colonies were picked from the agar plate to make a even cell suspension using 500 μL Veggietone broth (Oxoid), and vortexed to mix well. Aliquots (200 μL) of the suspension was transferred into a 50 mL Falcon tube containing 25 mL Veggietone broth and incubated at 28°C overnight with gentle agitation (130 rpm).

Table 6. *S. iniae* strains and capsular genotype used in vaccine trial

Strain	Site	*cpsY*	*cpsC*	*cpsD*	*cpsE*	*cpsG*	*cpsH*
155	NSW1	1	1	3	3	2	1
158	SA1	1	1	3	6*	2	1
159	SA2	1	1	3	3	2	1
177	NT1	2	2	3	3	3	1
191	NT1	2	1	3	3	2	1
216	QLD1	1	1	3	8	-	-
218	QLD2	1	1	3	3a	2	2
236	NSW1	1	1	1	5a*	-	-

**indicates mutation that results in early termination of the gene*

Preparation of vaccine
Eight cultures, prepared as described above, were chilled immediately on ice and then inactivated with formalin (40% formaldehyde in water) to a final concentration of 0.5% v/v formalin (equivalent to 0.2% formaldehyde) for 24 hours at 4°C. Aliquots (200 μL) were spread onto blood agar plates and incubated for 48 hours to confirm inactivation. Inactivated preparations were then washed in sterile PBS and resuspended in 1 mL sterile PBS. The resulting suspension was then emulsified in an equal volume of Freund's incomplete adjuvant by shaking at 4000 rpm for 10 sec in MagnaLyser (Roche Diagnostics). Vaccine emulsions were stored at 4 °C until required.

Vaccination of fish
Fish, acclimated and maintained as described above, were anaesthetised with MS 222 (0.005-0.01%) and vaccinated by intraperitoneal injection of 100 μL of oil adjuvanted vaccine. Fish were allowed to recover in clean aquarium water before being returned to their respective tanks. After 900 degree days, fish were administered a booster vaccination using the same vaccines and protocol as the primary vaccination.

Collection of antisera
Two hundred degree days post vaccination, the fish were euthanased by lethal overdose of anaesthetic (Aqui-S®) and exsanguinated by caudal venipuncuture. Blood samples were allowed to clot at 4°C overnight and were centrifuged at 6000 x g for 5 min to separate the serum from the red blood cells. The serum was collected and aliquoted into 50 μL volumes, and stored at - 20°C for subsequent analysis.

Enzyme linked Immunosorbent assay
A whole-cell ELISA was used to detect *S. iniae*-specific antibody in sera from vaccinated fish essentially as described previously (Delamare-Debouttevillle *et al*., 2006). Briefly, vaccines prepared as above were washed in tris-buffered saline and then resuspended to an optical density of 1.00 in carbonate-bicarbonate buffer pH 9.0. A suspension of QMA0177 was then used to coat high binding capacity ELISA plates using neat, 10-fold and 100-fold dilutions for optimization. After blocking in 2% normal goat serum, Positive control and negative control primary antiserum were used at 10, 100 and 1000 x to optimize primary antibody concentration. Secondary antibody was a commercial mouse anti barramundi IgM monoclonal antibody (Aquamab F-02, Aquatic Diagnostics) diluted to 4 mg/ mL in tris buffered saline containing Tween 20 (TBST). Tertiary antibody was a commercial goat vs mouse polyclonal antibody alkaline phosphatase conjugate diluted 1:30,000 in TBST. Colour was developed for 30 min. using *p*-nitrophenyl phosphate and plates read at 595 nm with a BMG Fluostar Optima microplate reader.

Following optimisation, 4 plates each were coated with each of the washed vaccine preparations at 10-fold dilution. Each primary antibody was also diluted 10 x for the assay, based on the results obtained by optimization with positive control serum. Secondary and tertiary antibodies were used as described above, following the manufacturers' recommendations. Antisera from 4 or 5 fish per vaccine were analysed individually and the results expressed as a mean OD ± standard error.

5. Results

5.1. Capsular sequence variation in *Streptococcus iniae*

Initially ten strains of diverse origin within Australia and overseas were selected and their entire capsular operon sequenced (Table 7). Sequences obtained from the capsular genes were complete, except where the amplification primer was used for sequencing leaving *ca*. 35 bp of unresolved data at the ends of each block including primer sites. Genes that were amplified and analysed from each of the isolates can be found in Table 7. In all cases, the sequevar indicated is defined as differing in relation to the type strain QMA0140 (ATCC29178) and other previous designations for other mutations. The total number of sequevars obtained for each gene in this initial preliminary screen of the entire operon is shown in the last column.

Table 7. Sequence types for each gene within the complete capsular operon of *S. iniae* isolates

Gene	Strain number and origin									
	72	76	83	140	155	165	177	191	188	186
	QLD	QLD	WA	USA	NSW	QLD	NT	NT	ISR	ISR
cpsY	1	1	1	1	1	1	2	2	1	1
cpsA	1	1	1	1	1	1	1	1	1	1
cpsB	1	1	1	1	1	1	1	1	1	1
cpsC	1	1	1	1	1	1	2	1	1	1
cpsD	3	3	3	1	3	3	3	3	3A	2
cpsE	3	4	3	1	3	3A	3	3	2	2
cpsF	1	1	1	1	1	1	1	1	1	1
cpsG	1	1	2	1	2	1	3	2	X	X
cpsH	1	1	1	1	1	2	1	1	1	1
cpsI	1	1	1	1	1	1	1	1	1	1
cpsJ	1	1	1	1	1	1	1	1	1	1
cpsK	1	1	1	1	1	1	1	1	1	1
orf276	1	1	1	1	1	1	1	1	1	1
orf193	1	1	1	1	1	1	1	1	1	1
orf151	1	1	1	1	1	1	1	1	1	1
cpsL	1	1	1	1	1	1	1	1	1	1
cpsM	1	1	1	1	1	1	1	1		
cpsN	1	1	1	1	1	1	X	1	1	1
orf183	1	1	1	1	1	1	X	1	1	1

Mutations given discreet numbers represent coding mutations compared to the type strain. Letters indicate a silent mutation.

Variations in gene sequences were found to be limited to the first 9 kb of the operon with a high degree of variability in particular in the *cpsDE* region (Table 7). More than one sequevar was found in the genes *cpsC*, *cpsY*, *cpsD*, *cpsE*, *cpsG* and *cpsH* (Tbale 7). Initial absence of an amplicon for the *cpsGH* block for the Israeli isolates 188 (KFP173) and 186 (KFP404) was resolved to indicate that *cpsG* is absent in these strains, whilst *cpsH* is present. The lack of a product in the *cpsN*-orf183 block for isolate 177 was re-tested using primer pair cps N F and cps N R and still yielded no product, confirming its absence.

In the control strain (QMA0140), a previously undetected transposase was found in orf276. This may be another site for genetic recombination, however the significance of this observation requires further investigation.

Based on the initial screen, almost all of the diversity in the capsular operon could be accounted for in 6 genes. Thus, the diversity of the *cpsY, cpsC, cpsD, cpsE, cpsG*, and *cpsH* genes was further investigated in a broad range of isolates from Australia and some additional representatives from overseas (Table 8). In addition, the *cpsB* and *cpsF* genes were also examined for diversity. Sequence analysis of genes from key isolates revealed that there was still significant genetic diversity found in these strains, in particular in the *cpsE* gene, which is responsible for transport of capsular components out of the cell, for which and additional 4 variants were found (Table 8, Table 9). In comparison, no new sequevars were found for the other genes. Thus the full extent of the diversity of these other genes, apart from *cpsE,* from our Australian isolate collection is likely to have been exhausted.

A range of strains used in vaccines were provided by the vaccine manufacturer Allied Biotechnology Pty Ltd and serotypic variability was determined by targeted sequencing of the variable *cps* genes using the methods outlined in the previous report. Twelve strains, supplied were analysed using the *cps* sequence typing methods as defined in the Manual.

Consistent with previous results, a type 2 *cpsY* gene was found only in NT isolates. Strain 189 from Réunion Island had a deleted *cpsG* gene which was previously found to be absent in the two Israeli strains. Strains 233 and 236 were isolated from bone samples from a farm in NSW. They have two features: a type 1 *cpsD* gene and a type 5a *cpsE* gene neither of which had been found previously in Australia. The differences between a type 5 *cpsE* and a type 5a *cpsE* are subtle – a one nucleotide change after a premature termination codon. The lack of selective pressure to retain the sequence after the stop codon is likely to result in changes after this point without affecting the already altered protein.

Strains 243-248 are vaccine strains used to control outbreaks at Farm A in SA. The vaccine was effective until strain 249 arose. Strain 249 contains a type 1 *cpsD* gene. The change from *cpsE* type 3 to *cpsE* type 5 gene incorporates an early termination codon and presumably results in a truncated protein.

Similarly, strains 250-252 are vaccine strains that had been used successfully at Farm B in NSW until strains 253 and 254 arose. Although the data for *cpsD* in strain 253 is absent, both strains contain a new *cpsE* type 5a which results in a truncated protein. Strain 254 also contains the *cpsD* type 1.

Data for the strains 233, 236, 249, 253, and 254 *cpsG* and *cpsH* genes was vigorously pursued without success. Neither the *cpsFGH* region nor the individual *cpsG* and *cpsF* genes could be amplified by PCR using the primers defined in the manual. The deletion of a number of *cps* genes in *S. iniae* is not without precedent, so an attempt was also made to amplify the *cpsE-cpsM* region assuming that a similar deletion event to that of ATCC29177 had occurred in between these genes in the new Australian strains. This test also was negative. Some additional tests for other *cps* genes occurring late in the capsular operon are currently ongoing.

Strikingly, the *cps* profiles for isolates 244 to 248 from Farm A and 250 to 252 from Farm B are identical. These isolates also share their profile with isolates 83 (from WA), 155 (NSW), 159 (SA), and 173 (SA). The co-ascension of the type 1 *cpsD* and *cpsE* types 5/5a in recent isolates from the two farms is intriguing and suggests a common source is likely for these isolates.

From the above results, the incorporation of these new strains in a multivalent vaccine is warranted as there has been an increase in the number of sequevars for the *cpsD* gene.

Table 8. *S. iniae cps* gene diversity. Of the 23 genes in the operon only genes exhibiting diversity are shown.

Strain	*cpsY*	*cpsC*	*cpsD*	*cpsE*	*cpsG*	*cpsH*	State (site)	Date
Type Strain								
140	1	1	1	1	1	1	USA	1970
Australian Isolates								
72	1	1	3	3A	1	1	QLD TSW	1995
76	1	1	3	4	1	1	QLD TFW	1998
78	1	1	3	3	1	1	QLD CFW	2001
165	1	1	3	3A	1	2	QLD B	2006
170	1	1	3	3A	1	2	QLD B	2006
164	1	1	3	3A	1	2	QLD K	2006
216	1	1	3	8			QLD BW	2007
218	1	1	3	3A	1	2	QLD B	2007
155	1	1	3	3	2	1	NSW	8/12/2005
220	1	1	3	3	2	2	NSW	2006
233	1	1	1	5A*	X	X	NSW	11/2008
236	1	1	1	5A*	X	X	NSW	03/2009
250	1	1	3	3	2	1	NSW	1/11/2007
251	1	1	3	3	2	1	NSW	20/06/2008
252	1	1	3	3	2	1	NSW	20/06/2008
253	1	1	1	5A*	X	X	NSW	16/01/2009
254	1	1	1	5A*	X	X	NSW	16/01/2009
142	2	1	3	3	1	1	NT	07/2005
150	2	1	3	3	1	1	NT	08/2005
153	2	1	3	3	1	1	NT	09/2005
177	2	2	3	3	3	1	NT	06/2006
191 (v)	2	1	3	3	2	1	NT	04/2005
158	1	1	3	6	2	1	SA(1)	14/02/2006
159	1	1	3	3	2	1	SA(2)	14/02/2006
160	1	1	3	3	1	2	SA(R)	12/1999
173	1	1	3	3	2	1	SA(?)	2006(r)
243	1	1	3	3	2	1	SA(R)	05/2006
244	1	1	3	3	2	1	SA(R)	10/2008
245	1	1	3	3	2	1	SA(R)	10/2008
246	1	1	3	3	2	1	SA(R)	03/2009
247	1	1	3	3	2	1	SA(R)	03/2009
248	1	1	3	3	2	1	SA(R)	03/2009
249	1	1	1	5*	X	X	SA(R)	05/2009
83	1	1	3	3	2	1	WA	2004
International Isolates								
186	1	1	2	2	X	1	Israel	
188	1	1	3A	2	X	1	Israel	
189	1	1	4	9	X	1	Réunion Is.	
190	1	1	1	5*	1		Thailand	

(v) Used in autogenous vaccine July 2005

(r) denotes UQ received date NOT isolation date

Mutations given discreet numbers represent coding mutations compared to the type strain. Letters indicate a silent mutation. *Mutation results in an early stop codon terminating expression of the protein. X indicates a complete gene deletion. Empty cells indicate that sequence data has not been obtained using the standard methodology in the manual. This may mean substantial alteration of the genes, or multiple gene deletions and remains to be confirmed.

Table 9. *cpsE* sequevar mutations

cpsE sequevar	Mutation/s (amino acid changes)
E_5	Single nt deletion plus 4 nt change plus G to E, V to F
E_{5A}	Single nt deletion plus 4 nt change plus G to E, V to F and silent C to A after early termination
E_6	Single nt deletion plus 4 nt change
E_7	A to V plus a silent mutation
E_8	K to T

Analysis of the new sequevars of *cpsE* showed that two strains had a one nucleotide deletion which would result in a truncated protein roughly three-fifths the normal size. This would make significant changes to the function of the protein. The other new sequevars had a combination of mutations resulting in amino acid substitutions and/or silent mutations that were different to those previously determined. Table 9 contains information on the changes to create these new *cpsE* sequevars.

5.2. Development of a challenge model for S. iniae in barramundi

Adverse event

During the acclimation period, 5 days prior to commencement of the challenge model, the entire stock of fish (25) in tank 5 were lost between 2 am and 6 am. Ammonia levels in the tank were high (5 ppm), but no higher than in the other tanks. The pH however was high (8.1) compared to 6.5 in all other tanks. At this pH ammonia becomes highly toxic to fish and thus probably explains the sudden death of all the fish. Subsequent physiological analysis of the fish confirmed ammonia toxicity as the most likely cause of death. The cause of the sudden rise in pH remains cryptic as all the remaining (15) tanks in the system were functioning normally, it was decided to proceed with the challenge models, but with a reduced number of fish (18 per tank).

Challenge inoculum

Viable count indicated that the undiluted challenge inoculum contained $8.6 \pm 0.5 \times 10^8$ cfu/mL. Thus fish given the high dose received 8.6×10^7 cells per fish, those given the medium dose received 8.6×10^6 and the low dose fish received 8.6×10^5 viable cells.

Mortalities

Mortalities in the high and medium dose tanks commenced 36 hours post challenge and continued for 4 days. No mortalities occurred in the control fish. Kaplan-Meyer survival curves showing percentage survival for each of the three replicate challenge models are given in Figure 1. In two of the three independent models, a significant challenge resulted with a clear dose-dependent effect and a significant difference between each dose (P=0.0018) Moreover the replication between these two models in terms of percentage mortality and times to death at each dose was very high (Figure 1). In the third model, no mortality was detected in high, medium or control groups. Cause of death was confirmed by isolation of *S. iniae* and identification by PCR.

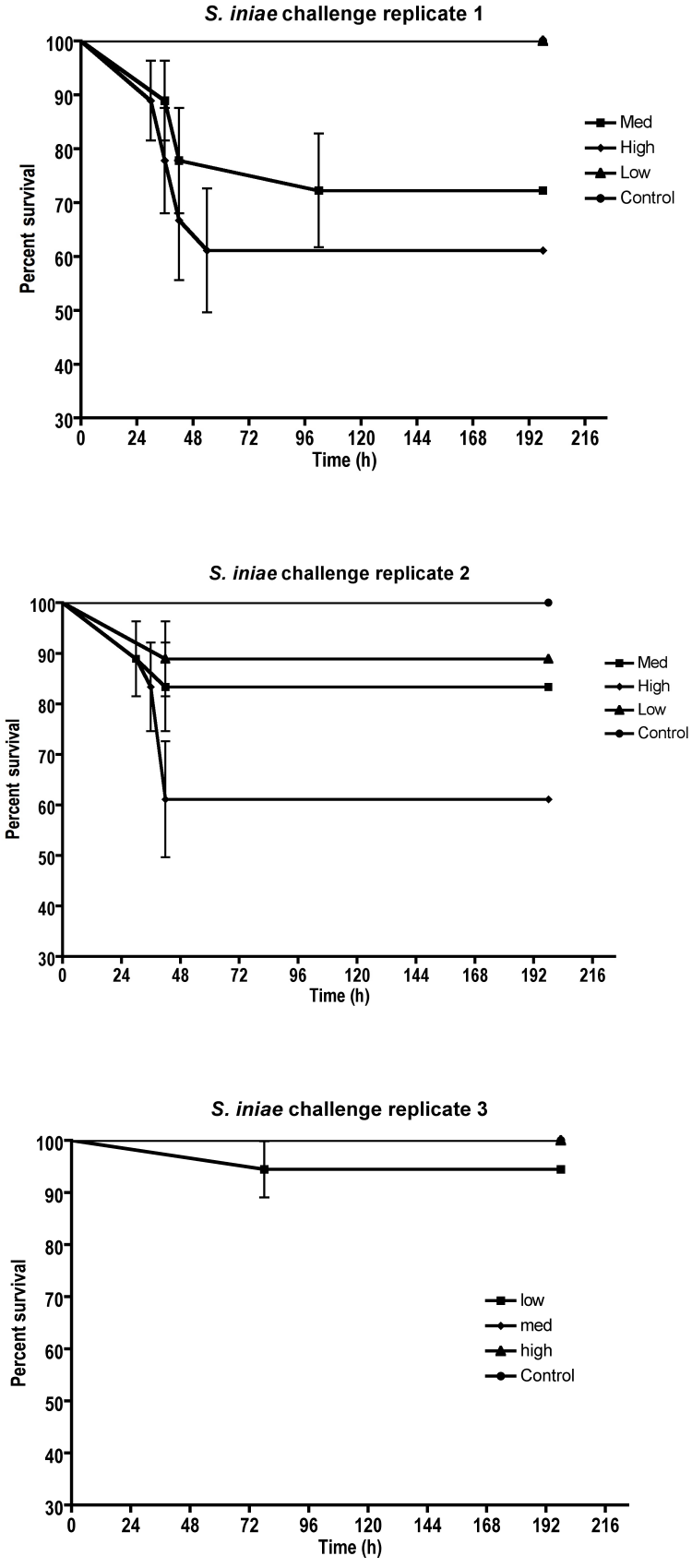

Figure 1. Kaplan-Meyer survival curves for 3 replicate *S. iniae* challenge models

5.3. Antibody response following vaccination with *S. iniae* CPS variants

In the absence of a suitable challenge model, and to ensure that some information was derived on the effects of vaccines containing different capsular genotypes, a vaccine trial was conducted using 8 experimental vaccines prepared from different capsular genotypes. However instead of challenging the fish, sera were collected at a time to correspond with peak secondary antibody response following a booster vaccination, and this response was measured by ELISA.

The results of the ELISA are presented in Fig. 2. Negative control sera gave very low optical densities by ELISA against all of the different coating strains of *S. iniae* (Fig. 2). As with all experiments involving barramundi, there was high variability in the response amongst animals vaccinated with the same vaccine leading to large standard errors. In general, *S. iniae* strains in which the *cpsE* gene had a mutation leading to early termination of the gene product (158, 236) failed to elicit a high antibody response in fish suggesting that this mutation reduces the immunogenicity of the strain (Table 1, Fig. 2). Furthermore, strain 216 also failed to elicit a high antibody response. Whilst the *cpsE* gene in this strain is functional, we were unable to amplify *cpsG* or *cpsH*. Whilst there was insufficient time to investigate further, it seems likely that a large deletion of multiple genes, similar to that found in 236 has occurred. It appears therefore, that in order to elicit a strong antibody response in fish, both a functional *cpsE* and functioning *cpsG* and H genes are required. It may be that these genes are critical for capsular expression. There was some evidence of cross reactivity amongst some of the strains largely in line with similar capsular genotype, though this was not always the case.

Three of the strains included in the trial had very similar capsular genotypes, (indeed two were identical (155, 159) and one differed in only its *cpsH* gene (218). Cross reactivity between 155 and 159 was high, serving to validate the central hypothesis. Strain 218 differed from 155 and 159 only in its *cpsH* gene, and cross reactivity was again high, suggesting that the amino acid changes in *cpsH* between type 1 and type 2 are probably not serologically relevant. Therefore, whilst extremely useful for epidemiological tracing of strains (monitoring of spread between facilities), *cpsH* may not be a good indicator of varying serotype.

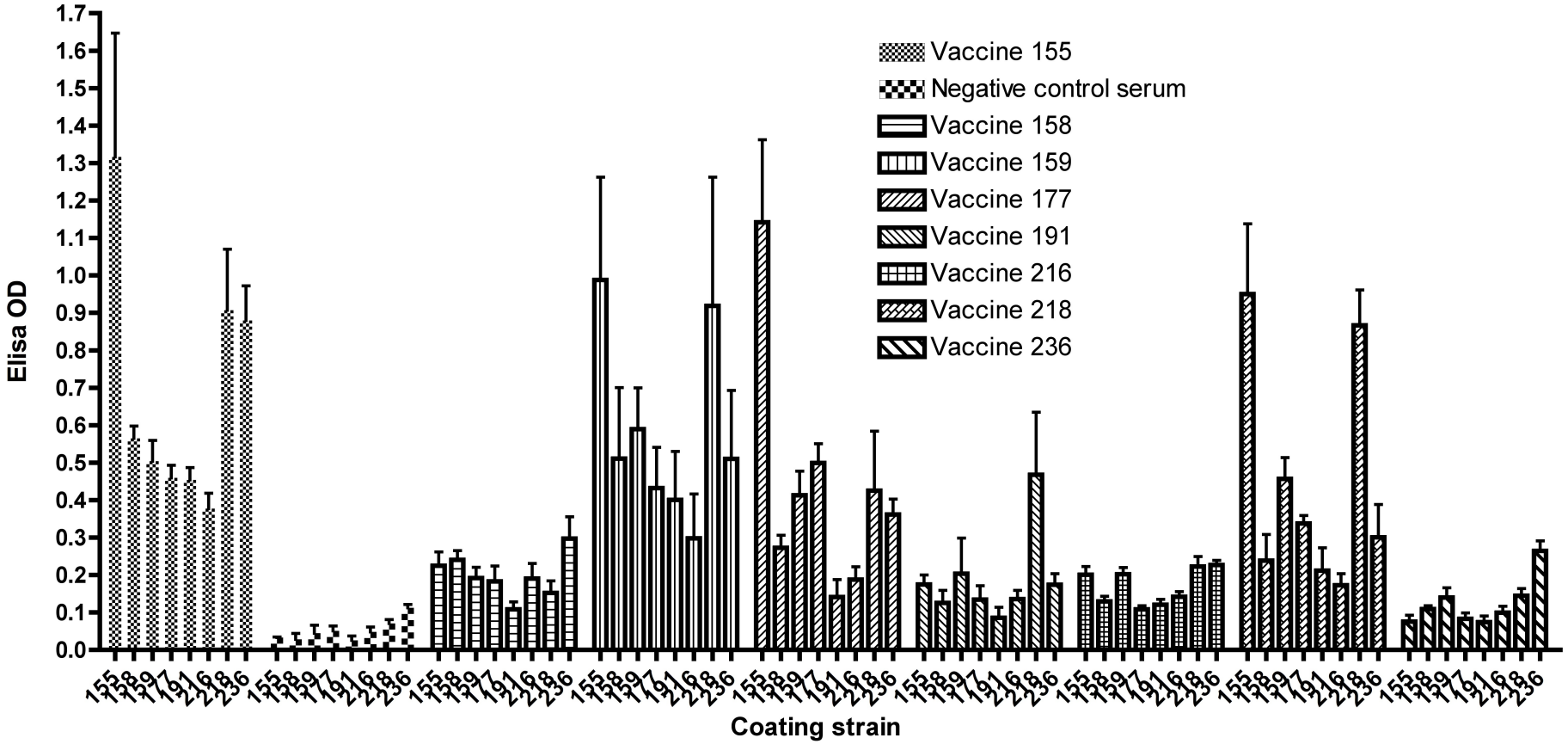

Figure 2. Secondary antibody response of barramundi (n=4 or n=5) following booster vaccination with different strains of *S. iniae.* Antibodies were tested for cross-reactivity against each of the different strains used in the individual vaccines. ELISA OD mean ± standard error.

6. Discussion

6.1 Discussion format

The scope of the discussion for this project is quite diverse and includes some quite complex molecular biochemistry involving gene and enzyme function. To make the discussion more readable by non-biochemists, it has been divided into sections to separate the biochemistry from the more functional aspects of what is happening on the farm following vaccination of fish. The first section provides a general overview, which is then followed by a number of case studies. In this way it is hoped that that non-biochemists can gain maximum benefit from reading the case studies and recommendations without the meaning being clouded by jargon. For the biochemists, the theory behind what may be happening at the cell surface is included in its own section.

6.2 *Streptococcus iniae* is very variable

Streptococcus species have a well deserved reputation for high variability, even within an individual species, dating back to Lancefield's seminal work in the 1920s (Lancefield, 1928a; b; c). Indeed it was Lancefield that determined that the variability stemmed not only from proteins, but from non-protein substances, probably carbohydrates in type-specific extracts from *S. haemolyticus* (now *S. pyogenes*). *S. iniae* is no exception amongst the streptococci, with indications of molecular variability first reported in 1997 (Weinstein *et al*., 1997). Antigenic variability was first associated with vaccine failures in *S. iniae* in trout in Israel during the 1990s (Bachrach *et al*., 2001). Subsequently, presence of capsular polysaccharide and attribution of antigenic variability to polysaccharide, rather than protein antigens, was reported (Barnes *et al*., 2003b). There is also widespread genetic variability amongst *S. iniae* strains in Australia, although at the onset of this project this had only been determined at the whole genome level by Pulsed Field Gel Electrophoresis (PFGE) (Nawawi *et al*., 2008), in line with earlier research in US and Canadian clinical isolates (Facklam *et al*., 2005) and the link between molecular variability and serotypic variability had not been made.

As serotype is evidently based largely on the capsule in *S. iniae* (the M-like proteins that are highly variable in other Streptococci are highly conserved and homogeneous in *S. iniae* at both the antigenic (Barnes *et al*., 2003a) and genetic level (Baiano *et al*., 2008)), analysis of the genes encoding capsular biosynthesis provides a reproducible way of typing strain in a manner that should be relevant to the serotype. However, capsular biosynthesis and the links between genes and capsule type are complex. Unlike protein structures where changes in genes may lead directly to changes in the expressed protein, and thus direct correlation to structure and antigenicity, the capsule is polysaccharide. Thus the genes encoding its biosynthesis do so indirectly – they encode a suite of proteins that have functions in regulation, biosynthesis and transport of the final complex polysaccharide structures on the surface. In spite of this complexity, the technique, known as molecular serotyping has been applied very effectively in the group B Streptococci (GBS)(Kong *et al*., 2008).

The background for the current project was therefore clear: *S. iniae* infections in vaccinated fish were reported from the field in Australia. Previous infection of vaccinated fish in Israel was attributed to serotypic shift. Australian *S. iniae* isolates were variable at the genetic level based on whole genome analysis by PFGE, but it was hard to correlate PFGE variability with serotypic variability, as many variations seen in PFGE are likely to be in non-coding DNA, or genes that code for elements not associated with the surface. Previous research on Streptococci in general, and *S. iniae* in particular, indicated that capsular polysaccharide was

likely to be the major variable surface antigen. Therefore the present project set out to determine the degree of variability in the machinery associated with the manufacture, transport and anchoring of the capsular polysaccharide onto the surface of *S. iniae* with the aim of directing vaccine formulation to provide protection against a broad range of Australian isolates.

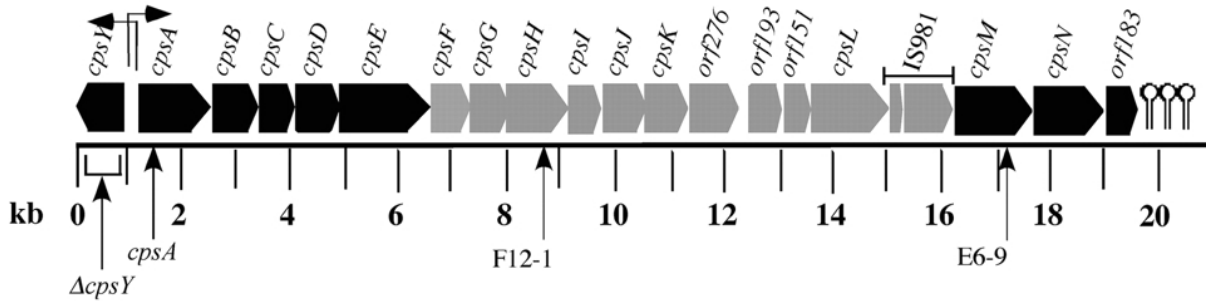

Figure 3. The 21 kb capsular operon of *S. iniae* type strain showing known genes and gene orientation. (Miller & Neely, 2005)

Capsular biosynthesis in *S. iniae* is under the control of a 21kb operon (cassette of genes), the cps operon, containing around 20 genes (Fig. 3). The operon has been fully sequenced (Miller & Neely, 2005) and characterised to some degree using site directed mutagenesis (Lowe *et al*., 2007). To have the best possible chance of detecting potential variation between isolates, an initial screen of 10 isolates from very diverse origins was performed, in which the entire capsular operon was sequenced very accurately using a special enzyme (proof-reading *taq* polymerase) that checks for mistakes in the process of amplifying the genes. This enables very accurate detection of small (single nucleotide) changes in individual genes throughout the operon. The probability of obtaining results that permitted clear interpretation was further enhanced by ensuring that the isolates from the serotypic vaccine failure in Israel and a vaccine failure in Australia were included amongst the first strains chosen for sequencing.

Sequencing of the operon resulted in a number of surprising findings. Firstly, the number of silent (non-coding) mutations across the 21kb operon was incredibly low, far below the average polymorphisms across normal bacterial genomes. When mutations did occur, they were almost always coding, resulting in amino acid changes in the expressed proteins. Secondly, mutations were restricted to a limited set of genes within the operon. That is, the mutations were not evenly distributed across all of the 21 kb, but were confined to a limited set of 6 genes, *cpsY, cpsC, cpsD, cpsE, cpsG* and *cpsH* (Table 7). The functions of these genes will be discussed later. The limitation of the variability to a few genes permitted more rapid screening of isolates from specific cases and from diverse locations across Australia and was also used to direct formulation of polyvalent vaccines as described in the case studies below. In all, 39 isolates were characterised, including the complete operon sequences for the 10 strains listed in Table 7. Widespread screening revealed that *cpsE* had the largest diversity (that is the largest number of variant sequence types (ST)). This did not, however, mean that cpsE was the most frequently modified gene in the operon within specific sites or regions. Indeed, switching between ST1 and ST2 within the *cpsG* and *cpsH* genes occurred more frequently (Table 8) in Australian isolates. *cpsD* was also variable, with 4 different coding sequence types found amongst the isolates sequenced. However, diversity of *cpsD* was lower in Australia, with only 2 sequence types found. Indeed ST3 was universal across all states in

Australia, with a sequence type ST1, identical to the type strain from dolphins, only arising under high selective pressure in two cases in NSW and SA (Table 8, see case histories below). The *cpsY* and *cpsC* genes also had low variability, with only 2 coding sequence types each. For *cpsY*, all isolates examined carried ST1 with the exception of the isolates from NT, which, without exception, carried the ST2 *cpsY* gene. It is intriguing that a new sequence type should only be found in NT, especially when movements of fingerlings interstate are common. This suggests that *S. iniae* infections arose locally in the fish after they were shipped and the bacterium was not co-transported with the fingerlings from the Darwin hatchery. *cpsY* ST2 would currently appear to be a robust diagnostic indicator for NT origin of the strain.

cpsC was also relatively highly conserved, with only 2 sequence types detected across the 39 isolates examined. Indeed ST1 was almost ubiquitous globally, with ST2 only arising in one case under selective pressure from vaccination (Table 8, see case studies)

6.3. Case studies

6.3.1. Northern Territory 2005-2006

In April 2005, streptococcosis emerged as a cause of high mortalities in farmed barramundi in Port Hurd, NT. The disease was initially controlled, but not eliminated, through the use of antibiotics. In July 2005, vaccination of fingerlings before transport to the farm was initiated using an autogenous vaccine prepared from the isolate obtained from the index case and subsequently designated NT1 (Strain 191 in the UQ strain collection (Table 8)).

Further cases of clinical streptococcosis were recorded in the fish at Port Hurd in July, August and September, as well as isolated from brain and kidney in sub-clinically infected fish. All these cases were in non-vaccinated fish and are represented by strain numbers 142, 150 and 153 (Table 8).

Following the use of the vaccine in fingerlings, monitoring and surveillance of the fish at the farm failed to demonstrate clinical streptococcosis in vaccinated fish and it was concluded that the disease was successfully controlled by the use of the vaccine.

In January 2006, an outbreak of clinical streptococcosis occurred in multiple cohorts of fish across the farm, including fish vaccinated with the autogenous NT1 strain (191, Table 8). Subsequent examination of the isolates from this disease event showed a different strain designated NT2 to be the cause (represented by strain 177, table 8).

Analysis of the capsular genotypes of the strains indicated a consistent molecular serotype during 2005, that was used to produce the autogenous in the vaccine and explains the efficacy of the vaccine. Strains of identical molecular serotype were only isolated from unvaccinated cohorts during 2005 (Table 8). When disease arose across the site in early 2006, more than 6 months after vaccination, mortality resulted from strains with multiple coding changes in the capsular operon, including in the regulatory gene *cpsC* and in the *cpsG* which controls the glucose/galactose ratio in the capsule (Table 8). Serologically, strain 191 (in the vaccine) did not elicit particularly high homologous antibody response, as detected in whole cell ELISA (Fig. x), and this already low response had low cross reactivity with strain 177, explaining the re-emergence of disease on this farm.

6.3.2. New South Wales 2006-2008

Recirculation systems present an interesting problem when disease arises amongst a cohort of fish. Theoretically, best practice would be to close down the system, disinfect it completely,

dismantle and clean pipework, pumps and filtration, then restart with clean stock. In practice this is not possible as multiple size-classes are generally kept within the same system to allow continual supply of table-sized fish for customers. Shutting down, cleaning, then restarting, with time for re-establishing biofiltration would cause significant financial stress to most small and medium sized businesses and destroy their market unless they had multiple separate systems. Thus, outbreaks of *S. iniae* may be controlled with careful antibiotic treatment, and vaccinated stock re-introduced into the system. However, this means that the reservoir of infection is never really eradicated from the system, providing ample opportunity for re-infection, should immunity become compromised.

Fish at the farm were vaccinated with an autogenous vaccine based on strain 155. An outbreak in 2006 resulted from a strain with a shift in *cpsH* from ST1 to ST2 (strains 220, 250, 251 252). Autogenous vaccines incorporating these strains were successfully used to vaccinate new stock in January (strain 250) and July 2008 (strains 251 and 252).

In November 2008, a veterinarian investigated a bone deformity issue in the barramundi in the recirculation system where the fish were getting spinal fractures at around 25g. In some vaccinated fish, these spinal fractures were being colonised by *S. iniae* thus perpetuating the osteomyelitis although not all fractures were infected. The brain and head kidney of nine fish were sampled for bacteria , but no *S. iniae* was recovered, nor was *S. iniae* seen in the kidney during histology. *S. iniae* was only seen in, and recovered from, the bone lesions. It was apparent that the vaccinated fish were able to wall off the infection into the bone area only.

The isolates that caused this unusual pathology were found to have a frameshift mutation in the *cpsE* gene that resulted in an early stop codon. That is to say, the protein resulting from the gene would be severely truncated and may not function. Moreover, all of the capsular operon genes from *cpsF-cpsM* were deleted in these isolates.

It is likely that such a serious mutation in the capsular operon results in complete cessation of the capsular biosynthesis machinery and therefore creates a capsule deficient mutant. This may explain the unusual pathology and very low mortality rate in the fish as a capsule deficient strain, whilst not recognised by the vaccine-induced antibodies, would be highly susceptible to phagocytic attack and may only be able to seek refuge in the bone of already compromised fish.

6.3.3. South Australia 1999-2009

In another recirculating aquaculture facility producing barramundi, an isolate from an outbreak in December 1999 was initially used in an autogenous vaccine against *S. iniae* (Strain 160). A later outbreak in autumn of 2006 resulted from a new strain (Strain 243) and again in the spring of the same year (strains 244, 255) and autumn of 2009 (Strains 24 6-248) all exhibiting a shift in both the *cpsG* (ST2-ST1) and *cpsH* (ST1-ST2) genes (Table 8). A new autogenous vaccine incorporating both strain types was used to vaccinate new stock. In May 2009 low level mortality was detected in vaccinated stock exhibiting similar symptoms to the case in NSW. The new strain was typed and found to carry the frameshift mutation in *cpsE* (*cpsE* ST5) and the deletion of *cpsF* through *cpsM* similar to that found in the NSW case. The development of these very similar cases suggests the possibility of a common origin for the isolate. However, a silent mutation after the early termination signal in *cpsE* was found in the NSW isolates (hence *cpsE* ST5A) hinting that the SA case and NSW case may have arisen independently. Interestingly, the unusual truncation of cpsE has only previously been found

outside Australia in a strain from Thailand. Whether this type has been introduced or whether it is a natural adaptation to host specific immunity that occurs independently is unknown.

6.4. What do the highly variable genes in the capsular operon do?

The functions of the genes of the capsular operon of *S. iniae* have, in most cases, not been determined directly. However some work employing site-directed mutagenesis has be conducted in *S. iniae* (Lowe *et al*., 2007), and a substantial work has been conducted in other *Streptococcus* species (Chaffin *et al*., 2000; Chaffin *et al*., 2005; Cieslewicz *et al*., 2005; Guidolin *et al*., 1994; Morona *et al*., 2002). By comparing the genes in the *S. iniae* capsular operon with those that have been characterised and analysed functionally in other streptococci it is possible to identify putative function and to speculate on the effects of changes amongst these genes. In order to remain focused, only genes with mutations in the *S. iniae* operon will be considered here in detail.

6.4.1. *cpsY*

The product of *cpsY* is homologous to transcriptional regulatory proteins CpsY in *S. pneumoniae* and *S. agalactiae*, members of the LysR family of transcriptional regulators, and was postulated to be part of the promoter region of the *cps* operon in *S. iniae* (Lowe *et al*., 2007). However, knockout mutation of the *cpsY* gene in *S. agalactiae* had no effect on capsular expression, though the knockout strain grew poorly in methionine deficient media compared to the wild type (Shelver *et al*., 2003). Mutational analysis in *S. iniae* revealed no change in growth in vitro in knockout mutants, but in virulence assays, the *cpsY* deletion mutant showed significantly reduced dissemination to brain and spleen and reduced virulence in a mortality-over-time assay (Lowe *et al*., 2007). Interestingly, presence of the wild type strain was able to complement the deficiency, and more recently a two-component system has been shown to regulate capsular biosynthesis (Bolotin *et al*., 2007), perhaps indicating a role for soluble secreted factors and cell-cell communication. The role of *cpsY*, is therefore unclear, but evidence indicates that it is involved in regulation or signal transduction rather than capsule structure, transport or anchoring and may therefore play little role in the antigenic nature of the cell surface.

6.4.2. *cpsC*

The product of *cpsC* in *S. iniae* is homologous to *cpsC* in *S. pneumoniae* (Lowe *et al*., 2007), and *epsC* in *S. thermophilus* (Minic *et al*., 2007). It is essential for the phosphorylation of CpsD (Minic *et al*., 2007).

6.4.3 *cpsD*

CpsD is a self-phosphorylating tyrosine kinase and works in conjunction with the products of *cpsB* (phosphatase) and *cpsC* to directly control the activity of CpsE (Minic *et al*., 2007). Interestingly, its complementary phosphatase, *cpsB* is very highly conserved in *S. iniae* with no mutations found in any of the isolates screened.

6.4.4 *cpsE*

In *S. iniae, cpsE* encodes a UDP-glucose dependent glycosyl transferase that transfers the sugar molecules from the activated donor molecules (in this case the nucleoside UDP) to specific acceptor molecules during the assembly of the mature polysaccharide. This is the priming phosphogalactosyltransferase initiating synthesis and export of the capsular polymer. The products of *cpsBCD* work together to regulate *cpsE*. Mutations in this gene cluster are the most common mutations amongst the *S. iniae* isolates recovered in Australia and it is likely that they change the amount of polysaccharide capsule. The amount of capsular

polysaccharide produced can have profound effects on the tissue distribution of the organism during disease (Lowe *et al*., 2007)

6.4.5 *cpsG*

CpsG is a UDP-glucose 4 epimerase that converts UDP-glucose to UDP-galactose and is highly specific in this role (ie. It only recognizes one substrate and can only create one product). Mutations in this gene that alter enzyme efficiency may change the rate of conversion and thus change the ratio of glucose:galactose in the final capsular polymer with resulting effects on surface epitopes.

6.4.6 *cpsH*

cpsH encodes a putative polysaccharide repeating unit polymerase. Mutations in this gene that effect enzyme efficiency are likely to affect polysaccharide polymer chain length for a given energy/nutrient source and are therefore potentially capable of affecting surface epitopes.

Coding mutations found in the *S. iniae* capsular operon are restricted predominantly to the 6 genes above and, with the exception of *cpsY*, seem to be positively selected by vaccination. *cpsY* does not appear to be directly involved in capsular structure from previous research but does impact on the tissue distribution. In contrast all of the other genes control the final molecular structure of the exopolysaccharide capsule on the surface of *S. iniae*: *cpsCDE* are directly involved in capsule ***quantity***, *cpsG* determines the ***type*** *of sugar* monomer subunit, and *cpsH* determines the ***length*** of the polymers

7. Benefits and Adoption

7.1 Accurate strain typing

A procedure has been developed and exploited to accurately type *S. iniae* isolates from barramundi farms in Australia in a functionally relevant yet highly reproducible manner. A manual containing complete operating procedures for typing of new strains as they arise at farm sites has been prepared and is included in the Appendix. Advantages of 'molecular serotyping' are several:

- Results may be compared completely accurately over the internet without the requirement for transfer of reference strains or reagents between laboratories.
- All reagents required may be ordered 'off the shelf', and are less susceptible to problems of batch variation and associated revalidation than polyvalent antisera.
- The method is universal. That is, it is capable of detecting and documenting any possible new variants as they arise at Australian farms without recourse to new reagents or methods.
- The procedure has been successfully tested in the laboratory by undergraduate students who are inexperienced in the technique. Most were able to reproduce results accurately, with some practice, suggesting transferability to other labs.

The major disadvantages with the protocol are the financial and time costs associated with it. Sequencing is expensive and reagents required to ensure accuracy are also expensive. Moreover, it takes several days (minimum of 6) of full time work from receipt of strains to delivery of results, although multiple strains can be analysed simultaneously.

7.1.1 Beneficiaries of strain typing

The major beneficiaries of the strain typing performed during this project were the farmers using vaccines produced by Allied Biotechnology Pty Ltd based on strains typed using this system. Accurate typing enables fewer cultures to be included in autogenous vaccines, as inclusion of multiple, identical isolates is avoided. Moreover it ensures that all variability previously encountered on a particular site is accounted for in the vaccine. Vaccine companies also benefit. Whilst Allied Biotechnology has been the only company to take up the typing offer during this project, and the feedback of results to the company was a little slow in some cases as difficulties in the typing system were overcome, the effect of multiple strain inclusion was illustrated convincingly in the SA and NSW case studies above. Even with as few as two types included in the vaccine (SA), *S. iniae* is effectively prevented from causing mortality, and this was under farming conditions highly suitable to S. iniae propagation and maintenance with continuous reservoirs of infection within the system. The probability of success therefore for a multivalent generic vaccine would seem high.

8. Further Development

Consultation with stakeholders (vaccine company, veterinarians and farmers) indicates that there are several options for future development that may suit the farmers and be viable for the vaccine producers, bearing in mind market size, projected growth and the outcomes from the current project.

Currently, UQ can type strains accurately based on sequencing as outlined in the Manual written in 2008 and attached in the Appendix. Using this method strains from farms, vaccines and from vaccinated fish have been typed. This has shown that there is an unexpectedly high rate of change in the strains. Following a vaccine trial, some of the changes seen may not be relevant to vaccine performance, while others are critically important. Based on this information a recommendation can be made for the formulation for a polyvalent vaccine based on between 5 and 7 strains that should protect against all strains currently in Australia.

The market for Streptococcus vaccine in Australia is 200-300 L per annum at the very most. Even with projected growth of the industry, it would be very hard to recover the cost of licensing a generic vaccine (licensing can cost between $300-400K) during the lifespan of the product. Current autogenous vaccines are reasonably priced and they allow flexibility. Rapid typing of strains is important as it can reduce the number of strains used in any autogenous preparation by identifying which isolates from a particular farm are the same. This reduces cost. It also ensures optimal protection. This is a critical issue where a large number of isolations have been made from a particular farm, and without knowledge of the strain types, the temptation would be to include them all in the vaccine. A recent example where this would have caused serious problems arose during the typing of 7 isolates from a single farm. Of these isolates, 6 were identical and one was significantly different. Including all isolates in the autogenous vaccine would have diluted the single novel strain 6 fold, raising the risk that the vaccine would be ineffective against this strain. By only including the 2 types in the vaccine, antigenic concentrations could be maintained.

The current typing system, though accurate and able to identify any novel strains that may arise, is probably too expensive outside of the current FRDC funding support to be done on a cost-recovery basis without seriously impacting on the cost of the vaccine. This leaves two options:

1) The industry continues to use autogenous vaccines, the formulation being guided by a typing scheme, probably run by the state vet labs on a fee for service basis. This may require subsidising the current methodology.

2) Government funding is sought to offset the cost of registration of a generic vaccine. This could be achieved by subsidising a current vaccine producer to develop the registration dossier. The advantage to this is that current producers will be familiar with the requirements for registration, will have Good Manufacturing Practice (GMP) and Australian Pesticides and Veterinary Medicines Association accredited production facilities and controlled and reproducible fermentation systems. The disadvantage is that it grants a monopoly to a single producer, and little return would be seen by the FRDC and by the farmers. A means of passing on the benefit of the registration cost-offset to farmers would need to be found.

There are pros and cons to both scenarios:

The advantage of the autogenous vaccines is their flexibility - For example, if a farmer wants Flexibacter in the vaccine as well, it can be added specifically for him or her as an autogenous preparation, specified by a vet. That could not be done with a generic, licensed vaccine.

The disadvantage of the autogenous vaccines is that farmer has to have a problem before a solution can be custom-prepared; it isn't available off the shelf directly when needed in order to prevent the problem arising in the first place. There is also some cost saving in the production of a generic vaccine as it doesn't cost proportionately more to produce 200 L than 5 L, thus the actual cost per litre is lower, though storage costs and recovery of registration costs would need to be accounted for in the final vaccine price. The major drawback, of course, is that you need to treat existing problems with antibiotics and can only vaccinate when you introduce new stock as the vaccine becomes available. Thus, autogenous vaccines can never really completely remove antibiotic use from the industry, in the way that a generic vaccine can.

Whichever direction is taken by the industry, the typing system is absolutely critical: If the autogenous route continues, the correct strains must be in the vaccine for it to be effective, and unnecessary inclusion of identical strains should be avoided to prevent dilution of critical antigens. If a generic vaccine is chosen, typing isolates as they emerge in the field, particularly if they arise in vaccinated fish, is a critical component of stewardship of the product. The system developed during this project is suitable for this within the limitations of time and cost indicated above. Other technologies are available that lend themselves to accurate typing. High resolution melt-curve analysis (HRM), a real-time PCR system based method for detection of genetic changes in specific PCR products is ideal for the types of changes we see in the *S. iniae* capsular operon. It is very fast and inexpensive (aside from the initial equipment and software costs). However, it is only really cost effective for examining large numbers of strains at a time and as they arise only sporadically in the field it may end up costing more than the current accurate and reliable method.

9. Planned Outcomes

9.1. Characterise the genetic and molecular basis by which biotypes of *S. iniae* vary in relation to capsular and surface protein antigen presentation and strain variation.

This objective has been achieved with clear indication of which genes amongst the capsular operon are responsible for variability amongst Australian *S. iniae* isolates. One question that remains to be answered is whether vaccination is driving evolution of a particular strain on a site, or whether multiple strains co-exist at a particular farm, with the most virulent dominating initially, until vaccination selects for an alternate strain from the resident pool. This question could only be answered with much more intensive sampling and sequence type analysis.

9.2. Develop and implement a rapid antigen typing scheme for *S. iniae* and transfer rapid identification technology to regional laboratories.

This objective has been partially achieved. A 'rapid' and very accurate typing system has been developed and optimised. A manual has been written to enable adoption of the methodologies by third parties. However, this has yet to be implemented in any of the state veterinary laboratories. It is thought that this simply comes down to time and money in laboratories that are already operating at full capacity. Reducing the cost of the process in terms of labour and expenditure on consumables, particularly sequencing, without compromising the reliability and effectiveness of the system seems difficult at present.

9.3. Develop a polyvalent vaccine against all known Australian strains of *S. iniae*.

A vaccine has not been 'developed'. However, a polyvalent generic vaccine can be designed using the information generated in this project through the sequence typing, vaccine case histories, and response to vaccination in the *in vivo* trial.

9.4. Verify the effectiveness of the vaccine in experimental challenge studies initially using the intraperitoneal injection route of immunisation in comparison with immersion.

An injection challenge model was developed *in vivo*. Mortalities were not high, in spite of using a recent virulent isolate. Although mortalities were reproducible in two replicate challenges, the third replicate did not reproduce the previously observed mortality pattern. Coupled with the low challenge, this made statistical validation of potential vaccine efficiency impossible. Research was substantially delayed by difficulty in getting permission from the animal ethics committee at UQ to develop any form of challenge model. As a result of both the delay in gaining permission, and the restrictive nature of the conditions applied by the AEC, optimisation of the models was impossible beyond a single small-scale dose titration study by injection, within the timescale of the current project. Further trials are proposed for 2010, subject to ethical approval, CI time and availability of sufficient laboratory funds.

10. Conclusions

Disease caused by *Streptococcus iniae* continues to be an expensive problem on Australian barramundi farms. As a result of this project we know why vaccination with autogenous bacterins sometimes does not work. Generally disease in vaccinated fish is caused by strains that are different from those in the autogenous preparation. The autogenous preparations seem to be very effective against the strains that they contain.

Antigenic variability in *S. iniae* is a result of changes in the capsular polysaccharide on the surface of the cell. Control of biosynthesis of this capsule at the genetic level is complex, but the variability seen in *S. iniae* strains in response to vaccination is seen at the genetic level in the capsular operon in a limited number of the 21 genes.

Of the six genes that vary in Australian isolates, *cpsY* does not seem to be in response to vaccination, with the single variant type occurring only in the Northern Territory and seems to be diagnostic for NT origin. Thus the major antigenic variation results from changes in *cpsC, cpsD, cpsE*, *cpsG* and *cpsH*. *cpsCDE* affect immunogenicity by changing the amount of capsule on the surface. *cpsG* changes the actual sugar composition in terms of the glucose/galactose ratio, and *cpsH* changes the polymer length. It appears that changes in *cpsH* may not greatly affect the antigenic nature of the capsule although further antibody work is required to confirm this.

Variability in *cpsE* is highest (ie the largest number of different types). However, switches in *cpsG* (or complete deletion) are common in strains occurring in outbreaks following to vaccination and have resulted reinfection of vaccinated stock. A polyvalent generic vaccine should certainly contain all three *cpsG* sequence types.

The typing system based on sequencing is robust, accurate and reproducible. A manual has been produced that contains detailed operating procedures to enable transfer of the method to other laboratories. It is relatively rapid (results in 6-7 days), but expensive due to quite intensive labor costs, inexplicably high sequencing costs in Australia, and the practical issue of strains tending to be received for typing in low numbers.

Further work is required in challenge model development. A robust and reproducible challenge model is critical for future vaccine development, and for corroboration of results generated during the present study. Results from Atlantic salmon research presented at this years European Asssociation of Fish Pathologists' conference in Prague highlighted the importance of the challenge model and particularly the route of challenge for accurately evaluating vaccine efficacy. Subject to development of an appropriate challenge model, information from the present project can be used to formulate a generic vaccine for use in Australian barramundi. Moreover, with the typing system in place, stewardship of the vaccine in the field is greatly facilitated, providing early warning of any potential evolution of novel strains and accurately guiding their incorporation into revision of the vaccine. This will provide a long term solution for the Australian barramundi farmers that can evolve with the industry as it grows.

11. References

Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D. L. & Eldar, A. (2001). Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a streptococcus vaccine. *Appl Environ Microbiol* **67**, 3756-3758.

Baiano, J. C., Tumbol, R. A., Umapathy, A. & Barnes, A. C. (2008). Identification and molecular characterisation of a fibrinogen binding protein from *Streptococcus iniae*. *BMC Microbiol* **8**, 67.

Barnes, A. C., Horne, M. T. & Ellis, A. E. (2003a). *Streptococcus iniae* expresses a cell surface non-immune trout immunoglobulin-binding factor when grown in normal trout serum. *Fish Shellfish Immunol* **15**, 425-431.

Barnes, A. C., Young, F. M., Horne, M. T. & Ellis, A. E. (2003b). *Streptococcus iniae*: serological differences, presence of capsule and resistance to immune serum killing. *Dis Aquat Org* **53**, 241-247.

Bolotin, S., Fuller, J. D., Bast, D. J., Beveridge, T. J. & de Azavedo, J. C. (2007). Capsule expression regulated by a two-component signal transduction system in *Streptococcus iniae*. *FEMS Immunol Med Microbiol*.

Chaffin, D. O., Beres, S. B., Yim, H. H. & Rubens, C. E. (2000). The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. *J Bacteriol* **182**, 4466-4477.

Chaffin, D. O., Mentele, L. M. & Rubens, C. E. (2005). Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. *J Bacteriol* **187**, 4615-4626.

Cieslewicz, M. J., Chaffin, D., Glusman, G., Kasper, D., Madan, A., Rodrigues, S., Fahey, J., Wessels, M. R. & Rubens, C. E. (2005). Structural and Genetic Diversity of Group B Streptococcus Capsular Polysaccharides. *Infect Immun* **73**, 3096-3103.

Delamare-Deboutteville, J., Wood, D. & Barnes, A. C. (2006). Response and function of cutaneous mucosal and serum antibodies in barramundi (Lates calcarifer) acclimated in seawater and freshwater. *Fish Shellfish Immunol* **21**, 92-101.

Facklam, R., Elliott, J., Shewmaker, L. & Reingold, A. (2005). Identification and characterization of sporadic isolates of *Streptococcus iniae* isolated from humans. *J Clin Microbiol* **43**, 933-937.

Gardiner, D. L., Goodfellow, A. M., Martin, D. R. & Sriprakash, K. S. (1998). Group A streptococcal Vir types are M-protein gene (emm) sequence type specific. *J Clin Microbiol* **36**, 902-907.

Guidolin, A., Morona, J. K., Morona, R., Hansman, D. & Paton, J. C. (1994). Nucleotide sequence analysis of genes essential for capsular polysaccharide biosynthesis in Streptococcus pneumoniae type 19F. *Infect Immun* **62**, 5384-5396.

Kong, F., Lambertsen, L. M., Slotved, H. C., Ko, D., Wang, H. & Gilbert, G. L. (2008). Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. *J Clin Microbiol* **46**, 2745-2750.

Lancefield, R. C. (1928a). The antigenic complex of streptococcus haemolyticus. I. Demonstration of a type- specific substance in extracts of streptococcus haemolyticus. *Journal of Experimental Medicine* **47**, 91-103.

Lancefield, R. C. (1928b). The antigenic complex of streptococcus haemolyticus. II. Chemical and immunological properties of the protein fractions. *Journal of Experimental Medicine* **47**, 469-480.

Lancefield, R. C. (1928c). The antigenic complex of streptococcus haemolyticus. III. Chemical and immunological properties of the species-specific substance. *Journal of Experimental Medicine* **47**, 481-491.

Lowe, B. A., Miller, J. D. & Neely, M. N. (2007). Analysis of the Polysaccharide Capsule of the Systemic Pathogen *Streptococcus iniae* and Its Implications in Virulence. *Infect Immun* **75**, 1255-1264.

Miller, J. D. & Neely, M. N. (2005). Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. *Infect Immun* **73**, 921-934.

Minic, Z., Marie, C., Delorme, C., Faurie, J. M., Mercier, G., Ehrlich, D. & Renault, P. (2007). Control of EpsE, the phosphoglycosyltransferase initiating exopolysaccharide synthesis in Streptococcus thermophilus, by EpsD tyrosine kinase. *J Bacteriol* **189**, 1351-1357.

Morona, J. K., Morona, R., Miller, D. C. & Paton, J. C. (2002). Streptococcus pneumoniae Capsule Biosynthesis Protein CpsB Is a Novel Manganese-Dependent Phosphotyrosine-Protein Phosphatase. *J Bacteriol* **184**, 577-583.

Nawawi, R. A., Baiano, J. C. F. & Barnes, A. C. (2008). Genetic variability amongst *Streptococcus iniae* isolates from Australia. *J Fish Dis* **31**, 305-309.

Pier, G. B. & Madin, S. H. (1976). *Streptococcus iniae* sp nov, a beta-hemolytic Streptococcus isolated from an amazon freshwater dolphin, Inia geoffrensis. . *Int J Syst Bacteriol* **26**, 545-553.

Shelver, D., Rajagopal, L., Harris, T. O. & Rubens, C. E. (2003). MtaR, a regulator of methionine transport, is critical for survival of group B streptococcus in vivo. *J Bacteriol* **185**, 6592-6599.

Weinstein, M. R., Litt, M., Kertesz, D. A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B. M., Borczyk, A. & Low, D. E. (1997). Invasive infections due to a fish pathogen, *Streptococcus iniae*. *N Engl J Med* **337**, 589-594.

Appendix 1

Intellectual Property

Data contained herein can be used to design a generic polyvalent vaccine for *Streptococcus iniae* in Australia, and potentially overseas, with some adaptation. There is prior IP in respect of polyvalent *S. iniae* vaccines owned by USDA, but this is restricted to specific named strains and there is no prior basis for using molecular serotyping to guide the formulation of the vaccine. It is proposed that IP is developed and protected based on the following preliminary claims:

1. A vaccine preparation that contains multiple strains of *Streptococcus iniae* inactivated in one of a number of ways known to those familiar in the art.
2. Preferably including strains of different types.
3. Preferably strains of which the type has been defined by sequences of the genes within the capsular operon.

The typing system may have some merit in terms of a combined diagnostic and typing 'kit', but it is hard to determine what, amongst the methods, would constitute new IP that was not already obvious to those familiar in the art. It is the sequences themselves that constitute new IP.

Appendix 2

Staff:

In-kind commitment

Dr Andrew Barnes designed the study, wrote milestone 1, co-wrote milestone reports 2, 3 and 4, authored the final report, liaised with industry including presentations to ABFA. In addition Dr Barnes obtained all permits, ran the challenge model experiment and the vaccination trial including all animal husbandry and analysed the data from these components.

Employed under contract to UQ using FRDC funding.

Justice C. F. Baiano was employed full time on this project at level A6-A7 over the operating duration of the project. Justice was employed for his technical ability in amplification and sequencing of potentially difficult bacterial target genes. Dr Baiano's salaries, on-costs and termination accounted for most of the expenditure on the current project. Dr Baiano generated and analysed all of the sequence data included in this report, and co-wrote milestone reports 2, 3, 4.

Short-term hourly casual employment

Roslina Ahmad Nawawi received all strains and placed them to stock in the UQ laboratory collection. She prepared all genomic DNA from *S. iniae* isolates analysed in this project.

Unpaid student assistants

Candy Chang and Shan Liang provided technical help with the ELISA. Candy Chang also helped with vaccination of the fish in the antibody response study and shared animal husbandry duties with Dr Barnes.

Appendix 3

Raw sequence data in FASTA format.
Sequence data for all sequence types of cps genes sequenced in the current study.

>CPSY1
ATGAGAATACAACAATTACATTACATTATCAAAATCGTTGAATGTGGCTCAATGAATGAAGC
TGCAAAGCAACTTTATATTACACAACCCAGTTTATCAAATGCTGTTAAGGACCTTGAACAAG
AAATGGGAATAACCATTTTTATTCGAAATCCTAAGGGCATCACTCTGACCAAAGATGGTGTT
GAATTTCTTTCTTACGCTAGACAAATCATTGAACAAACCTCTTTGTTGGAGGAACGTTATAA
AAATCATGACAGCAACCGCCAGCATTTTAGTGTTTCGTCTCAACACTATGCCTTTGTGGTTA
ATGCCTTTGTGTCCCTTTTAAAAGAGACAGACATGACCAAATATGAACTTTTTCTTCGAGAA
ACAAGAACTTGGGAAATCATTGATGATGTCAAAAACTTCCGCTCAGAAATTGGTGTTCTCTT
CATCAATGACTACAACCGAGATGTTTTAACTAAGCTTTTTGATGAAAATCAATTACAAGCAA
ACAAACTCTTCCAAACAAGACCCCATATTTTCGTTAGTAACAAACACCCCTTAGCAGACCGC
TCTAGTTTAGATGTTGAGGACTTGCAAGCTTATCCTTATTTAAGCTATGACCAAGGCATTCA
TAACTCCTTTTATTTCTCAGAAGAGATGAAAGCACAGATGCCCCATACCAAATCCATTGTCG
TTAGTGACCGTGCGACACTTTTTAACCTCATGATTGGTTTAGACGGCTACACCGTAGCTAGT
GGGGTTTTAAATAGTAAATTAAATGGTGATCAAATTGTTGCCATCCCACTAAATGTTCCAGA
TATTATCGATGTTATCTATATTAAACATGAGAAGGCTAACCTCTCCAAAATAGGTGAAAAAT
TTATAGATTACCTCTTAAAAGAGGTCAAATTGACACAAAAAAAGGAAATATAA
>CPSY2
ATGAGAATACAACAATTACATTACATTATCAAAATCGTTGAATGTGGCTCAATGAATGAAGC
TGCAAAGCAACTTTATATTACACAACCCAGTTTATCAAATGCTGTTAAGGACCTTGAACAAG
AAATGGGAATAACCATTTTTATTCGAAATCCTAAGGGCATCACTCTGACCAAAGATGGTGTT
GAATTTCTTTCTTACGCTAGACAAATCATTGAACAAACCTCTTTGTTGGAGGAACGTTATAA
AAATCATGACAGCAACCGCCAGCATTTTAGTGTTTCGTCTCAACACTATGCCTTTGTGGTTA
ATGCCTTTGTGTCCCTTTTAAAAGAGACAGACATGACCAAATATGAACTTTTTCTTCGAGAA
ACAAGAACTTGGGGAATCATTGATGATGTCAAAAACTTCCGCTCAGAAATTGGTGTTCTCTT
CATCAATGACTACAACCGAGATGTTTTAACTAAGCTTTTTGATGAAAATCAATTACAAGCAA
ACAAACTCTTCCAAACAAGACCCCATATTTTCGTTAGTAACAAACACCCCTTAGCAGACCGC
TCTAGTTTAGATGTTGAGGACTTGCAAGCTTATCCTTATTTAAGCTATGACCAAGGCATTCA
TAACTCCTTTTATTTCTCAGAAGAGATGAAAGCACAGATGCCCCATACCAAATCCATTGTCG
TTAGTGACCGTGCGACACTTTTTAACCTCATGATTGGTTTAGACGGCTACACCGTAGCTAGT
GGGGTTTTAAATAGTAAATTAAATGGTGATCAAATTGTTGCCATCCCACTAAATGTTCCAGA
TATTATCGATGTTATCTATATTAAACATGAGAAGGCTAACCTCTCCAAAATAGGTGAAAAAT
TTATAGATTACCTCTTAAAAGAGGTCAAATTGACACAAAAAAAGGAAATATAA
>cpsA1
AACGAAAGAGTTCTTCGAAAGAGAAACATAATCTAAGCTTAATAAATATCCTATTGTTAATA
ATTTATACAGGTCTTTCAATAATTATTAGCTTTTGGATGTACCTCTATAACTTCCTTGCTTT
TAGGCAGCTAAACCTTGTTTTAAGCATTGGCTTAGTCCTTGTTTTTTTTTATGTGTCTTTTTC
TCATTATCAAGAAAAAGCTTAAGGGATTGACCAGTCTTATTTTGGTTATTAGCACTATTTTG
TTAGCTATTATGCTATTTACCTTTAAATCAACAATTGACTTTACAGCTGAAATCAATAAAAC
AGCCTCTTTCTCAGAGATTGAAATGTCTGTTATTGTTCCCAAAGAGAGTGCAATCAATTCTA
TATCAGAATTAGAGACAGTTCAAGCACCTCTAAAAAATGACTCAGAGAATATCGACTCACTC
ATAAAGCACATTAAAGCGGCTAAAAAGAAAGAGTTAAAACTTGAAGAAGTTGCTTCTTATCC
AGAAGCCTATCAAAAAATGCTAAGCAATCAGTCTCAAGCAATGGTTATGAACAGTGCTTATA
TGTCATTATTAGGGCAAGAAGATTTACAGTTTAGTGATAAGGTTAAAACCATCTATAGTTAT
AAAATTAAAAAAGACATTAAGGCTAAAAAGTCACATGTCACTAAGGCAGGCGTTTATAACAT

CTATATCAGCGGTATTGATACTTATGGTCCGATTTCAACCGTTTCTAGATCAGATGTTAATA
TTGTCATGACAGTTAATATGAACACTCATAAAGTGCTTTTAACAACAACACCAAGAGATTCT
TATGTGAAGATTCCAAATGGTGGTGGTGATCAATTTGATAAATTGACCCACGCAGGAATTTA
TGGTGTAGAAACGTCAATGGCAACCTTAGCAAATCTCTATGGTATTGAAATGACTGATTATG
CTAGAATCAATTTCACGTCCTTTTTGAACTTAATTGATATGCTAGGTGGCATTGAAGTTGTT
AATGACAAAGCCTTCTCAGCAGGTGGTTTTGATTATCCTGTTGGAAAGATTTCACTTAATTC
ACAAGAAGCCTTGATGTTTGTGCGTGAGCGAAAATCATTAGAAGGCGGAGATGGTGATCGTG
GTAAAAATCAAGAAAAAGTTGTTAGTGCCATCATTCGTAAACTAACAATGATTAAATCGCCA
GCCCAATTCTCAGAAATCGTGACAGGATTGCAAACTTCTATTCAAACCAATCTAACATTAGA
TCAGCTCATGACAATTGCAAATGCCCAATTGGAAAGCAACCAGTCTTATGACATTACCTCAC
AAGA
>cpsBCD1_140
CGTTTTCGATGTGGATGATGGTCCCTTAACAATTGATGACAGTTTAGCATTAATTGGAGAAA
GTTACCGACAAGGTGTTCGAACCATTGTTTCAACATCTCACAGACGAAAAGGCATGTTTGAA
ACACCTGAGGATGATATTTACAATAAATTTCTTCACCTTAAAAGAGAGGCTGAAAAAGAATA
TGAGGGATTAACCATTCTATATGGAGGAGAACTCTATTACACCGAAGATATCCTTGATAAAT
TGGCTCAGAATCTTATTCCTAGAATGAATGACACACGCTTTGCCCTCATTGAATTTTCCATG
AACACGCCATGGCGCGATATTCATACGGCACTTTCTCAAGTTATTATGCTTGGTATTACACC
AATCATTGCTCATATAGAACGTTACGATGCTTTGGCCTTAAATGCTAAACGGGTTCAGGAAC
TCATTAATATGGGATGTTACACTCAAGTCAATAGTTCTCATGTGTTGAAAGCCAAGTTATTT
GGAGATAGTTTAAAAGTCTTTAAAAAACGTGCTAAGTTTTTCTTAGATGAAAATTTAGTGCA
CTGTATTGCAAGTGACATGCACAATTTAAAAAAACGCCCACCCTTTATGCAAGAAGCTTATC
AACATGTGACGAAACATTATGGTAGAAAACGCGCTAGAGAACTTTTTATCACAAACCCTCAA
ACCCTGATTGAAAATGATTATTTATAGGAGAAAAGATGAACACAAGCGAAAACACATCAATT
GAAATTGATATTCTTAGTCTATTAAAGAGAATTTGGCAGAAAAAAGTAGTGATTCTATTTGT
GACGCTACTAGCTGGTTTTTTAGCCTTGGTGGCAAGTATGTTTCTCATTAAACCATCTTACA
CCTCAACAACAAGGTTATATGTTATCAATCGTCAACAGTCAGATAACCTTACGGCGACCGAT
TTGCAGGCTGGTGGTTACTTGGTTAATGACTACAAGGAAATCATTACCTCGCGTGATGTGAT
GCATGATGTTATTGCTAAGGAAAATGTTAGCATGTCACCAGAAGAGTTGAGCCAAATGATTA
CAGTAACTGTACCGGCAGATACCCGTGTTATCTCAATTTCTGTAAATAACCATGAACCACAA
AAAGCTAAAGATTTAGCGAATGCCGTTCGTGAAGTAGCCTCAGAAAAAATCAAAGACGTGAC
GAAAGTTCAAGATGTCACTGCTTTAGAAAAAGCTCAACTTCCAACCAAACCGTCATCGCCAA
ATAGTAAACGCAATGCAGTGATGGGACTTCTAGTTGGTGCTGTTTTAAGTATATTTGCAGTT
ATCCTAAAAGAAGTTTTAGATGACCGTGTTAAAAGTCCAGAAGATGTTGAAGATGTACTTGG
TATGACATTACTAGGTATGGTTCCAAACACAAATAAAATGTAAGAGGTTAAGGATGTCACAA
TTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTATAATTCCAT
TCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTTCTGTTCAAC
CTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAAGCAGGTTTT
AAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTTTAAATCTGA
TGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAAGTGTTATTT
CAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCCACCAAATCCAACA
ACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTTATTTGACTA
TATTATCATTGATACCCCACCAATAGGCTTGGTGATTGATTCTGCCATCATTGCGCAAAAAG
CAGACGCCACTATCTTGGTAACTGAAGCAGGTTCTATTAAACGTCGTTTTGTGCAAAAGGCA
AAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAAAGTAGATCA
ACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTATGGCAAA
>cpsC1
ATGAACACAAGCGAAAACACATCAATTGAAATTGATATTCTTAATCTATTAAAGAAAATTTG
GCAGAAAAAAGTAGTGATTCTATTTGTGACGCTACTAGCTGGTTTTTTAGCCTTGGTGGCAA
GTATGTTTCTCATTAAACCATCTTACACCTCAACAACAAGGTTATATGTTATCAATCGTCAA
CAGTCAGATAACCTTACGGCGACCGATTTGCAGGCTGGTGGTTACTTGGTTAATGACTACAA
GGAAATCATTACCTCGCGTGATGTGATGCATGATGTTATTGTTAAGGAAAATGTTAGCATGT

CACCAGAAGAGTTGAGCCAAATGATTACAGTAACTGTACCGGCAGATACCCGTGTTATCTCA
ATTTCTGTAAATAACCATGAACCACAAAAAGCTAAAGATTTAGCGAATGCCGTTCGTGAAGT
AGCCTCAGAAAAAATCAAAGACGTGACGAAAGTTCAAGATGTCACTGCTTTAGAAAAAGCTC
AACTTCCAACCAAACCGTCATCGCCAAATAGTAAACGCAATGCAGTGATGGGACTTCTAGTT
GGTGCTGTTTTAAGTATATTTGCAGTTATCCTAAAAGAAGTTTTAGATGACCGTGTTAAAAG
TCCAGAAGATGTTGAAGATGTACTTGGTATGACATTACTAGGTATGGTTCCAAACACAAATA
AAATGTAA
>cpsC2
ATGAACACAAGCGAAAACACATCAATTGAAATTGATATTCTTAGTCTATTAAAGAGAATTTG
GCAGAAAAAAGTAGTGATTCTATTTGTGACGCTACTAGCTGGTTTTTTAGCCTTGGTGGCAA
GTATGTTTCTCATTAAACCATCTTACACCTCAACAACAAGGTTATATGTTATCAATCGTCAA
CAGTCAGATAACCTTACGGCGACCGATTTGCAGGCTGGTGGTTACTTGGTTAATGACTACAA
GGAAATCATTACCTCGCGTGATGTGATGCATGATGTTATTGCTAAGGAAAATGTTAGCATGT
CACCAGAAGAGTTGAGCCAAATGATTACAGTAACTGTACCGGCAGATACCCGTGTTATCTCA
ATTTCTGTAAATAACCATGAACCACAAAAAGCTAAAGATTTAGCGAATGCCGTTCGTGAAGT
AGCCTCAGAAAAAATCAAAGACGTGACGAAAGTTCAAGATGTCACTGCTTTAGAAAAAGCTC
AACTTCCAACCAAACCGTCATCGCCAAATAGTAAACGCAATGCAGTGATGGGACTTCTAGTT
GGTGCTGTTTTAAGTATATTTGCAGTTATCCTAAAAGAAGTTTTAGATGACCGTGTTAAAAG
TCCAGAAGATGTTGAAGATGTACTTGGTATACTAGGTATGGTTCCAAACACAAATAAAATGT
AA
>cpsD1
ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTA
TAATTCCATTCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTT
CTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAA
GCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTT
TAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAA
GTGTTATTTCAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCCACCA
AATCCAACAACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTT
ATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGGTGATTGATTCTGCCATCATTG
CGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCAGGTTCTATTAAACGTCGTTTTGTG
CAAAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAA
AGTAGATCAACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTATGGCAAA
>cpsD2
ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTA
TAATTCCATTCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTT
CTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAA
GCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTT
TAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAA
GTGTTATTTCAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCCACCA
AATCCAACAACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTT
ATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGTTGATTGATTCTGCCATCATTG
CGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCCGGTTCTATTAAACGTCGTTTTGTG
CAAAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAA
AGTAGATCAACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTATGCAAAACC
>cpsD3
ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTA
TAATTCCATTCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTT
CTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAA
GCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTT
TAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAA
GTGTTATTTCAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCTACCA
AATCCAACAACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTT

ATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGTTGATTGATTCTGCCATCATTG
CGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCAGGTTCTATTAAACGTCGTTTTGTG
CAAAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAA
AGTAGATCAACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTATGGCAAAACCA
AAAAATCATCCAAAAGTAAAAAA
>cpsD3a
ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTA
TAATTCCATTCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTT
CTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAA
GCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTT
TAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAA
GTGTTATTTCAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCTACCA
AATCCAACAACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTT
ATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGTTGATTGATTCTGCCATCATTG
CGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCCGGTTCTATTAAACGTCGTTTTGTG
CAAAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAA
AGTAGATCAACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTAT
>cpsD4
ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTA
TAATTCCATTCGCACTAATATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTT
CTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGATTAACTTAGCCATTTCCTTTGCAAAA
GCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCAGGTGCCTT
TAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAA
GTGTTATTTCAAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCCACCA
AATCCAACAACCTTACTTCAAAATAGTAATTTTAATTTTATGATTGACACAGTCAAAGAGTT
ATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGGTGATTGATTCTGCCATCATTG
CGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCAGGTTCTATTAAACGTCGTTTTGTG
CAAAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAA
AGTAGATCAACAACTTGGTTCTTATGGCGCTTACGGTTCCTACGGAGATTATGGCAAAACCA
AAA
>cpsE1
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGAAACTTTTATTACTCTTTTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT

GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGATTAGACCAAT
>cpsE2
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTTTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATTATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGACTAGACCAAT
>cpsE3
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT

AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGATTAGACCAAT
>cpsE3a
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGACTAGACCAAT
>cpsE4
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT

GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCTTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGACTAGACCAAT
>cpsE5
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGAAACTTTTATTACTCTTTTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATTGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTATAGTCAGCAGCACATAAGCATGT
GCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAATG
TTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGCT
GTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCTT
AAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGTG
GAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGAC
TTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAGG
TTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTCG
ACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGAA
TCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCTC
AGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTCC
TTTCCATTGTAAATGGATTAGACCAAT
>cpsE5a
TGTTAGATGTGTTAATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATAT
AGTAATAACTTAACTGACAAGGAAACTTTTATTACTCTTTTTGTAACACTTTTTATTTACAC
CCTTCTGGGCATTCGATTTAATGTCTTTTCTATTATTAATTGGTTTACAGACTTAAAAACAA

TCTCCTTAGTAGTCTTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTT
TTCTTAGACAGTTTTAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGT
ATCAGCCCCTAGAATAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAG
GAAAAAAAGAAAAGAAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATC
CAAACTGTTTTGAATAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAA
TAAATTAGGCACTTACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGAT
TGGTTGCTGAATATGAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGG
CGTGAGGCCATCTTAGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCAT
TGAAAATATTGTCATGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACC
TATTAAGCAGGAATGAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAA
ACGGTCTTGGTTACAGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCA
CTTTAAGCCTAAACAAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATA
GAGAACTGAATGCTAAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAG
GACCGTGACTTAATGTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTATAGTCAGCA
GCACATAAGCATGTGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTT
CGGAACAAAAAATGTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGT
CAACTGACAAGGCTGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATG
ATTGTAACTGGCTTAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGT
TCTAGGGAGTCGTGGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAG
TAACCGTCACTGACTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTT
GTTATCCAAGCAGGTTTTAAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCC
TGTAAAAATTCTCGACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGA
TTGCTATTGTTGAATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACC
AGCGAACGTGTCTCAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACC
TTTGGACCAAGTCCTTTCCATTGTAAATGGATTAGACCAATTATCGGATAAAGAACTAA
>cpsE6
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTATAGTCAGCAGCACATAAGCATGT
GCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAATG
TTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGCT
GTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCTT
AAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGTG
GAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGAC
TTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAGG
TTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTCG
ACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGAA
TCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCTC

AGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTCC
TTTCCATTGTAAATGGATTAGACCAAT
>cpsE7
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACAGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATAAGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT
TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGATTAGACCAAT
>cpsE8
ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAAC
TGACAAGGGAACTTTTATTACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTC
GATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATCTCCTTAGTAGTC
TTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTAGACAGTTT
TAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAA
TAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAG
AAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCAAACTGTTTTGAA
TAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATAAATTAGGCACTT
ACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATAT
GAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTT
AGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCA
TGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTATTAAGCAGGAAT
GAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTTGGTTAC
AGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAAC
AAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCT
AAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGACCGTGACTTAAT
GTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAGCACATACGCATG
TGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAAT
GTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGC
TGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCT

TAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTTCGGTAATGTTCTAGGGAGTCGT
GGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCACTGA
CTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAG
GTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTC
GACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGATTGCTATTGTTGA
ATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCAGCGAACGTGTCT
CAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTC
CTTTCCATTGTAAATGGATTAGACCAAT
>cpsE9
GTTAGATGTGTTATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAG
TAATAACTTAACTGACAAGGGAACTTTTATTACTCTTTTTGTAACACTTTTTATTTACACCC
TTCTGGGCATTCGATTTAATGTCTTTTCTATTATTAATCGGTTTACAGACTTAAAAACAATC
TCCTTAGTAGTCTTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTT
CTTAGACAGTTTTAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTAT
CAGCCCCTAGAATAACTTGGCGATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGA
AAAAAAGAAAAGAAAAGGATTTTGGTAGTCGGTGCAGGTGAAGGGGGTTCCACCTTTATCCA
AACTGTTTTGAATAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATCAATA
AATTAGGCACTTACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTG
GTTGCTGAATATGAAGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCG
TGAGGCCATCTTAGACATTTGTCGTCAGGCTAATGTTCCAGTAAACAATATGCCAAGCATTG
AAAATATTGTCATGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGACATCGCTGACCTA
TTAAGCAGGAATGAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAAC
GGTCTTGGTTACAGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACT
TTAAGCCTAAACAAATCTTATTATTAGGACATGGGGAGAACTCTATTTATTTAATTAATAGA
GAACTGAATGCTAAATATTCTGATAACATCATAATTACTCCGATTATTGCTGATATCCAGGA
CCGTGACTTAATGTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGCAG
CACATAAGCATGTGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTC
GGAACAAAAAATGTTGCAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTC
AACTGACAAGGCTGTTAATCCTCCAAATATCATGGGGGCAACAAAGCGTTTTGCTGAAATGA
TTGTAACTGGCTTAAATGAAGCTGGAAAAACGCAATTTGTAGCAGTCCGCTTCGGTAATGTT
CTAGGGAGTCGTGGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGT
AACCGTCACTGACTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTG
TTATCCAAGCAGGTTTTCAAGCTAGTGGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCT
GTAAAAATTCTCGACTTAGCTAAAAAAGTGATTAAGTTGAGTGGGCACACTGAAGATGAGAT
TGCTATTGTTGAATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCA
GCGAACGTGTCTCAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCT
TTGGACCAAGTCCTTTCCATTGTAAATGGATTAGACCAATTATCGGATAAAGAACTAA
>cpsG1
ATGAAAAAAGTACTTATTACAGGTGCAAATTCTTATATAGGAACTTCCCTTGAAAAATGGCT
ACAACAATCAGAGGAACAATATCATGTTGATACTTTAGACATGATTGATCCAAACTGGAAAA
CGTTTGACTTTTCACCTTATGACAGCATTTTTCATGTGGCAGCTATTGTTCATAAAAATGAA
AAGCAAATGAACTCAGACCTCTATGAAAAGGTTAATACCAAACTTCCTATAGAGTTGGCTAC
CATTGCTAAACATTCTGGTCTAAGACAGTTTATATTTTTAAGTAGTATGAGTGTCTATGGTA
ATGATACAGAAGAAATAACACGTGAAACTAGGGAAAATCCATCAAGTTATTATGGAAAAAGT
AAACTAGCAGCAGAGATAGGTTTAAAAGATTTACAATCAGATAGTTTTAAAGTACTTATTCT
ACGCCCGCCAATGGTTTATGGACCACAGGCAACAGGTAATTACAGTAGATTATCAAAGTTAT
CAAAGTTTACGCCAATTTTTCCCAAAGTTGCTAATAAACGAAGCATGATTTACCTTGATAAT
CTTTTAGAATTTGTTCGCTTGTCAATTGAAACAGAATTGAGTGGAATCCATTTTCCACAAAA
TAAAGATTATGTGACAACAAGTCAATTAGTAAACGTCATTCGTCAAGTTAATGGAAAAAGCA
CACTGTTAACTTCTCTCTTTAACCCAATCATTAAATCTTTAAAAGGTTTTAGTCAAATCAAT
AAGCTCTTTGGGAATTTAGTTTATAGTAAAGAAATGTCACAAGAAGCTTTTGACTATAATGT
TACAGGATTTGAAGAGTCAATACGAATATCGGAAAGAAACAATGAAAAAATATAG

>cpsG2
ATGAAAAAAGTACTTATTACAGGTGCAAATTCTTATATAGGAACTTCCCTTGAAAAATGGCT
ACAACAATCAGAGGAACAATATCATGTTGATACTTTAGACATGATTGATCCAAACTGGAAAA
CGTTTGACTTTTCACCTTATGACAGCATTTTTCATGTGGCAGCTATTGTTCATAAAAATGAA
AAGCAAATGAACTCAGACCTCTATGAAAAGGTTAATACCAAACTTCCTATAGAGTTGGCTAC
CATTGCTAAACATTCTGGTCTAAGACAGTTTATATTTTTAAGTAGTATGAGTGTCTATGGTA
ATGATACAGAAGAAATAACACGTGAAACTAGGGAAAATCCATCAAGTTATTATGGAAAAAGT
AAACTAGCAGCAGAGATAGGTTTAAAAGATTTACAATCAGATAGTTTTAAAGTACTTATTCT
ACGCCCGCCAATGGTTTATGGACCACAGGCAACAGGTAATTACAGTAGATTATCAAAGTTTA
CGCCAATTTTTCCCAAAGTTGCTAATAAACGAAGCATGATTTACCTTGATAATCTTTTAGAA
TTTGTTCGCTTGTCAATTGAAACAGAATTGAGTGGAATCCATTTTCCACAAAATAAAGATTA
TGTGACAACAAGTCAATTAGTAAACGTCATTCGTCAAGTTAATGGAAAAAGCACACTGTTAA
CTTCTCTCTTTAACCCAATCATTAAATCTTTAAAAGGTTTTAGTCAAATCAATAAGCTCTTT
GGGAATTTAGTTTATAGTAAAGAAATGTCACAAGAAGCTTTTGACTATAATGTTACAGGATT
TGAAGAGTCAATACGAATATCGGAAAGAAACAATGAAAAAATATAG
>cpsG3
ATGAAAAAAGTACTTATTACAGGTGCAAATTCTTATATAGGAACTTCCCTTGAAAAATGGCT
ACAACAATCAGAGGAACAATATCATGTTGATACTTTAGACATGATTGATCCAAACTGGAAAA
CGTTTGACTTTTCACCTTATGACAGCATTTTTCATGTGGCAGCTATTGTTCATAAAAATGAA
AAGCAAATGAACTCAGACCTCTATGAAAAGGTTAATACCAAACTTCCTATAGAGTTGGCTAC
CATTGCTAAACATTCTGGTCTAAGACAGTTTATATTTTTAAGTAGTATGAGTGTCTATGGTA
ATGATACAGAAGAAATAACACGTGAAACTAGGGAAAATCCATCAAGTTATTATGGAAAAAGT
AAACTAGCAGCAGAGATAGGTTTAAAAGATTTACAATCAGATAGTTTTAAAGTACTTATTCT
ACGCCCGCCAATGGTTTATGGACCACAGGCAACAGGTAATTACAGTAGATTATCAAAGTTAT
CAAAGTTATCAAAGTTTACGCCAATTTTTCCCAAAGTTGGTAATAAACGAAGCATGATTTAC
CTTGATAATCTTTTAGAATTTGTTCGCTTGTCAATTGAAACAGAATTGAGTGGAATCCATTT
TCCACAAAATAAAGATTATGTGACAACAAGTCAATTAGTAAACGTCATTCGTCAAGTTAATG
GAAAAAGCACACTGTTAACTTCTCTCTTTAACCCAATCATTAAATCTTTAAAAGGTTTTAGT
CAAATCAATAAGCTCTTTGGGAATTTAGTTTATAGTAAAGAAATGTCACAAGAAGCTTTTGA
CTATAATGTTACAGGATTTGAAGAGTCAATACGAATATCGGAAAGAAACAATGAAAAAATAT
AG
>cpsH1
ATGAAAAAATATAGTAGAAGTGTGTTACAAGGGAAAAAAATTCTTTTTTTCTCTCCCTCTTT
CTTTAATTATGAAAATGTAATAAAAGATAAAATGGTCGAACTAGGAGCAGACGTTTATTTCT
TTGATGAACGTCCATTTTCATCAGTTTATAGAAAAGCATTACTAAAACTTAATCCTAATGTA
TTTTCAAAAAGTACCGAAAAATATTTTGATTTGATTTTTAATAATGTTTCAGATATTTGTTT
TGATTATGTCTTTTTTTTTGAAGTGTGAAACTCCAACATTAAAAGTATTAAGGAAATATAGAG
CATATTTTAAGAATGCGAAATTTTGCTTATATATGTGGGATTCGATATCAAATGTAAAAAAT
ATTGAAAAAAAAATTAATTTATTTTGATATTATATCTTCATTTGATAAAAAAGACAGTGAAGA
GCGAGGATTTAACTTTAGACCGTTATTTTATAGTGATGAGTATGCGAAACCATATAAGAAAC
AATTTTATAAATATGATATTTGTTCATTTGGCACAATTCATTCAGATAGATATAGTATCTTG
ACAAAATTTGTTAATTATTCAAAAAAAAATAATCTTAAATTTTATTTTTTTAATTTTCTTCA
AGGGAAGTTTATGTTTTATTTTTACAAAATTGTAAAAAAAGATTTTTTAAAAGCAAACATTT
CAGAATTTAGTTTTGTAAAGAAAAATAGTCAAGAAATTATTAAAACAATTCTAGATTCAAAA
GTTGTTTTAGATATTCAACATCCAAATCAAACTGGCTTAACGATGCGAACAATTGAAATGAT
TGGATTGAATAAAAAAATAATTACGACTAATAATTCAATCGTAAACTATGATTTTTACAATA
AAAACAATATTTTAATTATTGATCGACATAATATTGAAATTGATAGAGAATTCTTTGAAACA
GAATATTCTGCATTAAATCAAGATGTTTATAAAAAATATAGTTTAGAATTTTGGTTATACGA
TACTTTAGGGATTAAACAGGATGATGAATAA
>cpsH2
ATGAAAAAATATAGTAGAAGTGTGTTACAAGGGAAAAAAATTCTTTTTTTCTCTCCCTCTTT
CTTTAATTATGAAAATGTAATAAAAGATAAAATGGTCGAACTAGGAGCAGACGTTTATTTCT

TTGATGAACGTCCATTTTCATCAGTTTATAGAAAAGCATTACTAAAACTTAATCCTAATGTA
TTTTCAAAAAGTACCGAAAAATATTTTGATTTGATTTTTAATAATGTTTCAGATATTTGTTT
TGATTATGTCTTTTTTTTTGAAGTGTGAAACTCCAACATTAAAAGTATTAAGGAAATATAGAG
CATATTTTAAGAATGCGAAATTTTGCTTATATATGTGGGATTCGATATCAAATGTAAAAAAT
ATTGAAAAAAAATTAATTTATTTTGATATTATATCTTCATTTGATAAAAAAGACAGTGAAGA
GCGAGGATTTAACTTTAGACCGTTATTTTATAGTGATGAGTATGCGAAACCATATAAGAAAC
AATTTTATAAATATGATATTTGTTCATTTGGCACAATTCATTCAGATAGATATAGTATCTTG
ACAAAATTTGTTAATTATTCAAAAAAAAATAATCTTAAATTTTATTTTTTTAATTTTCTTCA
AGGGAAGTTTATGTTTTATTTTTACAAAATTGTAAAAAAAGATTTTTTTAAAGCAAACATTT
CAGAATTTAGTTTTGTAAAGAAAAATAGTCAAGAAATTATTAAAACAATTCTAGATTCAAAA
GTTGTTTTAGATATTCAACATCCAAATCAAACTGGCTTAACGATGCGAACAATTGAAATGAT
TGGATTGAATAAAAAAATAATTACGACTAATAATTCAATCGTAAACTATGATTTTTACAATA
AAAACAATATTTTAATTATTGATCGACATAATATTGAAATTGATAGAGAATTCTTTGAAACA
GAATATTCTGCATTAAATCAAGATGTTTATAAAAAATATAGTTTAGAATTTTGGTTATACGA
TACTTTAGGGATTAAACAGGATGATGAATAA
>cpsJ
AACATTTATTAAATAGTGGACATCAAATTATTGCAACAGATTACAGATCGGAACTTATTGAC
AGCAGAGCAATAATAATTGAAGCAGATTTATTTGAAGTGAATGATCCATTCGAATATTTTGA
TAAACCAGATATTCTTTTGCATTTGGCTTGGAGGGATGGTTTTATTCATAATTCAGAATCTC
ATTTAAAAGACCTTCATAAACATTACGATTTTATTTCCAAAATGGTGAACTCTGGAATTAAA
CATGTAGCAGTTTTTGGAACAATGCATGAGATTGGTTTTTATGAGGGATCAATTAATGAAAG
CACTCCAGCAAATCCTCAAAGTTTGTATGGAATTAGTAAAAATGCGTTGAGACTTTCTATTC
AGGAGCTTTGTAAAGAGAACAATATTATTTTTCAATGGTTAAGAGGATTTTATATAGTAGGT
AATACACCAGATGGTAATTCAATTTTTTCTAAAATAATACAAGCAGCATCTAGAGGAGAAAA
AAAATTTCCATTTACTTCTGGTTTAAATCAATTCGACTTTATTAATTATGATGATTTTTGCG
ATAGTGTAACTAGAACAGTTTCTCAAAATAAAATAAATGGTATTATTAATATTTGTAATGGT
TTTCCAGAAAGTTTAGCTAATCGAGTAGAAAAATTCATTATAGAAAATAAGTTAGATATTGA
ATTAGAATATGGAGTGTTTCCAGATAGACCATATGATTCAAAAGCAGTATGGGGGAATTCAG
AAAAGTTGAAAAGCATAAAAAGAGGTTAGATAAATTGA
>cpsK
ATGGGGGAATTCAGAAAAGTTGAAAAGCATAAAAAGAGGTTAGATAAATTGACAATGATAAC
TGTTTGTATGGCAACTTTTAATGGTGAAAAATATATTGAAGAACAGATTATTAGTATTCTTT
CTCAATTAAATGATTATGATGAATTAATTATTTCAGATGACGGTTCAACAGATAGTACTGTA
GAAATAATAAAAAAGATAGATGATAAAAGAATTAATTTAGTATTTAATAAGAATGATAGAGG
TTACTCTTCAAATTTTTATAATGCAATGAGTATGGCAACTGGAGATTATATTTTTTTATCAG
ATCAAGATGATATATGGTTAGAAGACAAAGTCACTATCACATTGAAATATTTAAAAAGTTTT
GATTTTGTTGTATCAGATGCATTTGTAGTTGATTCAAACTTAAATCTATTAGAGGAATCACG
TTTTAAAACCTTCAATATTTCGAATGGCTTTTTACCTAATTTCATCAGAACTAGATACATTG
GATGTTGTATGGCATTTAATAGGAAAGTATTAGATGCACTATATCCATTCCCAGAAAAAAAA
TATAATATTCCACACGACTTGTGGATTACGCTAATATCTGAAAAGTATTATAAAACCTCTTT
AATTGATAATCAACTTATTAAATATAGGAGACATGATAATAATGTTTCCAATGGTGGACTAA
CTGTAAAAAACAACAAGAGGTCATTTTTTCAAATAATAAAAAGTAGAATAATT
>cpsL
TTGATTATTCTGACCCCGAAGCAGCTAATCCAGGAATAGGTGGCACACAGTTTTTAACTTAT
TCATTGAGTTATCATTTAGGTCAAACTGGTGAGATTGATGTGTCTTTATTTGTCAATGAAAT
GGCAAATTTTCCTGAAGAAATGAAGATTGAAATTGTAAGATCACTTGAAGAGTGTATTATAA
GAGTTAACGAAGATAATTTCGATTATTTAGTAATAAGAGGACCATTTATTCAAAAAAAAATA
ATGAATCAAATAGCTGAAAGCGGTGTTAAAATTATTACTTGGTCACATAATTTAGAAACATT
TCAGTTTTTTTCAAGGTTATTAAGTCCATCGATTGTAAAAAATATTTGTGTATCAAACTCTC
AAAGGCTATTATTAAAAGACTCAAAACTATTTTCAAAAAGTGTAACAATTTTAAATGGTATT
AACTTTAAAGATTACAGTCAATTGCCAATTAACAATGTTAAAAAATATGATGTTTGCTATAT
TGGAAATTTATATCCTAAAAGTGGCTATGAAGATGTATTAAAAGCATGGCCGAAAATAAAAA

AAACTATACCAAATGCAAATTTATGTATTATTGGCGGTAATAAACTATACAATAATAGGTTA
AATTCATCCTATAGTTCAAAGTCAATTAAAAAACTAAAAAAATTAGAAGAGAAAATTAATTT
AGAATTTAAAGACTCAATTGAATATGTCGGAGTGTTAGGTGGAAGAAAAAAACTAAGCAAAA
TGTCTGAGTCTACAATTGGAATTGCTAATATAACTCCGACTGGAGAAACTTTTGGATTAGCA
GCAATTGAGTTTCAAGCCTTGGGAGTACCTGTAGTATCAATCAATAAAGTTGGATTAAAAGA
GACAGTTAAGTCAAATGAAACTGGAATTTTAGTTAATAAAAAGAAAGATTTGGCTGATGCAA
TTATAAGTCTATTAATTGATAAGGATAAGACTAATCA
>cpsM
TGAAAGCGGTGTTAAAATTATTACTTGGTCACATAATTTAGAAACATTTCAGTTTTTTTCAA
GGTTATTAAGTCCATCGATTGTAAAAAATATTTGTGTATCAAACTCTCAAAGGCTATTATTA
AAAGACTCAAAACTATTTTCAAAAAGTGTAACAATTTTAAATGGTATTAACTTTAAAGATTA
CAGTCAATTGCCAATTAACAATGTTAAAAAATATGATGTTTGCTATATTGGAAATTTATATC
CTAAAAGTGGCTATGAAGATGTATTAAAAGCATGGCCGAAAATAAAAAAAACTATACCAAAT
GCAAATTTATGTATTATTGGCGGTAATAAACTATACAATAATAGGTTAAATTCATCCTATAG
TTCAAAGTCAATTAAAAAACTAAAAAAATTAGAAGAGAAAATTAATTTAGAATTTAAAGACT
CAATTGAATATGTCGGAGTGTTAGGTGGAAGAAAAAAACTAAGCAAAATGTCTGAGTCTACA
ATTGGAATTGCTAATATAACTCCGACTGGAGAAACTTTTGGATTAGCAGCAATTGAGTTTCA
AGCCTTGGGAGTACCTGTAGTATCAATCAATAAAGTTGGATTAAAAGAGACAGTTAAGTCAA
ATGAAACTGGAATTTTAGTTAATAAAAAGAAAGATTTGGCTGATGCAATTATAAGTCTATTA
ATTGATAAGGATAAGACTAATCAATTATCTATTAATGCAAAAGATTTTGTTAGAAGAAATTT
TGATATAAAATTTATTGTTAAAGATTGGATAGTTTTGTTTAATAGTTTACCTAACACTTCTT
TAAAAACTTGTCAAAGCAGTTTTTCAGATGACGATTTCTTGGAAAAATTAACTCAGAAGAAT
TTTAAGATTAAACAAAATAGATTATTAAGTTGGTTACCACCAATACAATTTTACAAATATGT
TCGCTATATATTTGTTAGGATTTTAGAAAAATCAAATATTATTTAA
>cpsNO
GGGCTTTCGATGGCCGTGCTTTTGGCACAACATCATCAGGTTATAGCGGTAGATATTGTTCC
TGAAAAGGTAGATTTGATTAATGCTAAGAAATCACCTATTCAAGATAAGGAAATTGAAGAGT
TTTTAGCCAGTAAAGAACTTCATTTAAAGGCAACATTGGATGCTGAGTCGGCTTATCAAGAT
GCTGATTTTGTTATTGTTGCAGCACCTACAAATTATGATAGCAAGCGTGATTTTTTTGACAC
GAAGGCTGTTGAAGCTGTTATTGAAGCAGTTCTTGCTGTTAACACTCAGGCAATCATTATCA
TTAAATCAACGATTCCAGTAGGATACACAGATAGTATTCGTCAAAAGTTCAATACAACTAAC
ATTATTTTTAGTCCAGAATTTCTAAGGGAATCACGGGCATTATACGATAATCTCTACCCAAG
TCGAATAATTGTTGGAACTGATTTATCTAATCAAAGATTAACACAAAAAGCGAATGAATTTG
CTCGCTTGTTACAAGAAGGCGCTATTAAAGAACCGATTGAAACCTTGATTATGGGCTTTACC
GAGGCTGAAGCGGTCAAACTCTTCTCAAATACCTATTTAGCTTTACGGGTGTCTTATTTCAA
TGAACTTGATACTTACGCTGAGACAAAAGGACTTGATACGAAAGCTATTATTGACGGTGTTG
GTCTTGATCCGAGAATTGGTGACCACTATAATAACCCATCTTTTGGTTATGGGGGGTATTGT
TTACCGAAAGATACCAAACAATTGTTGGCCAATTATAGAGATGTGCCTCAAAACATGATGAC
AGCTATTGTTGAGAGTAACCGCACTCGTAAAGACTACATTGCTGAGAAAGTCTTGGAATTAG
CAGGCGCTTATGAAGGCTCAAGTGAATTTGATCCCAATATGGAAAAAGAAGTGGTTATTGGG
GTTTACCGCTTAACAATGAAGAGCAATAGCGATAATTTTAGACAATCATCTATTCAAGGCAT
TATGAAACGGATTAAACTAAGGTGCAAAAGTTATTATTTTTGAACCAAGTTTAGATGACGTT
CAACTTTCTTTGGTAGTCAGGTGGTAAATGACCTTGAAACGTTCAAAAAAACATCTAATCAA
TCATTGCAAATCGTTCGATTCAGCACTTGATGATGTTCAAGAAAAGGTCTACACTCGCGACC
TTTTCGGCCGGGATTAATCCGAGTGTCTTGGTTAATTCTTATAGCATTTCATTGCCCTTTTA
GCTCTCGTATGTTATATTAATTATATGGATTTCTCTGTTTGAGTGTTGTGAAGGATGGTGTC
GTATGAGTGGAACTGAACAAAATAGTCATAAAACGTTAATTCAAAAATTATTGGTCAGTATT
CATTATTTGACCCTCTTCAAAGATGAGTTGAATTTGGTCGAAAGAACTCCATCTATCTTAGG
AAGTGAGTTCCCAAAGCATTTGGTGCAATCAGAACTTGGTGATATTGTAGCCATTATCAACA
CCTTAGATAAGCAACAGCGCTTAATTGAATCAACCTTCTTGTATGAAGAAAGTGCCTTCAAA
TTAATGAATAAAACCTTGAACATTGTTTCTAACTGGGTAAAAGGTCTGGAAGAGTTGATTAA
CCTTTGCCAGTCCAAGGAAGTTTTTCAAGCCATTTTAGGAGACAAAAGGGTTCGAGTCTTTG

GGGTTTTGATTGATGTGTTTTCATCACTGAAAGTTATCATCATGTCCCTCAAAGAAGAGCCT
GTGCCTCATGTTCTCTATGAGCATATCAAAATGGTCAACCTTGAAGAAGAAGCTTTTAGAAA
GCATTACCAGAGCCCAAAAA
>ORFs
TTATTCTCATA--GCTAGCTTGAGCGTAGTCAGCTTGATAAATCATTTCAAATT--------
---TACCTTTACGAA------TT--TG----TTGTCTGA--CA----AGTCCTCGTTTA-
AC----CT----CATTATG---------AATGGT-TTGAG---------G------CG----
-------CTTTACCTAGCAAGTTAGCG---------------AT-G-GCTCTATTG----
GACTTT----CCTTCTGACTT--CCATT--TCTCGATGA--AGTAACGCT--CA--
GCTATT----G----TCAAATGTTTTCCTTTTGGTGTATAA-TGTTC---
ATGCATCTCAAAGTGTCCTAT-CTTGT-GTTTG----TGG-------TGAAC-----TA---
CAAGTATATCTCTTTGAGATGTTTTTTCA---TACCCTCAG-
ATGGCTAACTTCATTTTAGAACTTTCTTTAAAGGTTTAAAAGCAATTAATGAAGAATATAAT
CAAAAAGTCATTGAAATTAATTTTATAGGAAAAAGTTCAACATTAGATGATTTAAAGAAGCT
TTCAGAAAAGCTAGAGTTGAAGAATGTGTACTTCCATGGAACGAAAACAACTGAGGAATTAA
ATGATTTATACGATAAATTTGACTTAGGCTTAGGTTGTCTAGCATTGCATAGAAGAGATGCA
GATATTGATACGACAATAAAAGTAATTGAATATTATTGTAGAGGAGTTGCAGTTCTGACTAG
TGGAGATAGTCCATTAGGAGAAATTTTCTCTAATACAACAATAAAAATTGCTTCCAATGAAG
ATATTATTGACCTAAATTATGTTTTATCAGAATTCGAAAAAATTACTCAGTCAGATATGCAA
CAAATTTCTCAGTTGAGTATAAATAAATTTTCGTGGAATAAAATTATATTTGATTTGAACTG
ATAAATATTATAGGAATAAATTAAGGAAATAAAATTGAAAAAAATAAAAATAAATTTTACTT
TTGAAGATATTTGTGCATGGTCAATAGTATTAAGTTGTATATTACTATATTATTAAAMCGCT
AYTTTCAGCTATGGAATGTTGCTGTTACTGACATTAACATTAATAAATATTTTTATACATAA
GAAATTATACATTGATATAAAACTTATCATTTTATGGCTTGGTATTTTTTTCCAACAAATGA
TTTCACTTATATTATTTAAATTGGATTTTGCTATTTTTATCAAAAATCTTTTTTCAATATTA
TTAATTATTATAATAACTTCAATAAGTCATGCAATTGTAGATAAAGATAGTTTTTTTTATAA
ATATCGAAATTTTGCTATTTTTTGTACTCTGATTATTATTGCTCAATCATTTGTTGTATATG
TTTTGAAAAAACCAATTAATGCAATTTTACTTTTACCTCAATCAGATAGCTCCAATTGGGTT
GAACAAAGTTTAAGACCGATGTCTCTTTTTTACCGAGCCTCAAACTTATTGTAGTTTTATTTT
TCCAGCTTTTTTACTATGTTATAAGAAAAAAGATTATTTTGGAACCTTTACTATTTTTCTAG
GTATTTTTCTTTCAGGTTCTAGTTTAGGCATTATGATGATTGTTTTTTTAGGACTAATTACT
TTTTTTAAATCTTCAATTTCTTTTTTTAAAAAATGTCTAATATTTTTCGGTGGAATATTTTC
AACGGTTGTTATTTTTTTACTACCAATTTTTAGTTTTATCAAATTAAAGATTTTAAGTCTTT
TTATGGAATTTTCTCTAACAAATTTTCAATTCAAGAGTACATTTTCATACACTAATTATTTG
AGGTTGATAAAAGGTTGGGTTACTTTCTATGAGCTACCACTTAAAGAAAAGATAGTTGGTTT
AGGTTTAAATAATCTTACAAATTATATGGAAATTACAGGAACTCGCTTTTCATGGAGTAAAC
AGTGGGCTGTTAATCATATTCAAAATGCGTATTTTAATTCTTCTTCAGGAATTTTTATTGAA
TGTGGTTTAGTAATCGGAATATTATATTACTATATATTGATAAAAAAAATAAAAATTGAAAA
TTCAATAAGTAAAAATATTTTAATTTTTATTATTTTTCAAGGTTTTGCAACGCAAATTTTTT
TCAATTCGATATTTATTTTTTGGATTCTAATGTAT-AC----

Appendix 4

Molecular serotyping manual for *Streptococcus iniae*

Capsular genotyping or molecular serotyping of *Streptococcus iniae*

A Laboratory Manual

Version 1.1

Justice C. F. Baiano and Andrew C. Barnes
With support from FRDC Project 2007-226

Version 1.0 September 2008
Version 1.1 November 2009

CONTENTS

Contents 51

Foreword 53

Background and principle 54

References 55

Outline Protocol 56

Equipment 56

Adjustable micropipettes 56
Microcentrifuge 56
Thermal Cycler (PCR machine) 56
Heating block or water bath 56
Horizontal electrophoresis and power supply 57
Gel documentation 57
Bioinformatics software 57
Personal protective equipment 57

Standard operating procedures 58

SOP0015. Reliable extraction of genomic DNA from Gram positive bacteria 58

Stock solutions 58
Lysozyme stock (100mg/mL) 58
Proteinase K stock (20 mg/mL) 58
Sodium dodecyl sulphate stock (SDS, 10%) 58
Procedure 58

SOP0008. Agarose gel electrophoresis for analysis of DNA fragments or amplicons up to 20kb in size 60

Tris Acetate EDTA buffer (TAE): 60
Preparation of 0.5M EDTA (pH 8.0) stock (500 mL) 60
Preparation of TAE (50 x stock): 60
Preparation of a 1% agarose gel 60
Electrophoretic separation of DNA fragments or amplicons 61

SOP0016. Confirmation of Presumptive Streptococcal isolates as *Streptococcus iniae* by polymerase chain reaction 63

PCR components: 63
Preparation of the reaction mixture 63
Reaction parameters: 63

SOP0030. Amplification of capsular genes for molecular serotyping *Streptococcus iniae* 65

PCR components: 65
PCR amplification reaction mix: 65
Reaction parameters: 65

SOP0025. PCR product cleanup protocol using ExoSAP 67

Enzyme mix: 67
Reaction protocol: 67

SOP0026. Preparation of reaction mixtures for sequencing of cleaned PCR products 68

APPENDIX 1. 69
Table A. Sequences of primers used in PCR. 69
APPENDIX 2 70
ALIGNMENT OF SEQUEVAR TYPES 70
cpsY sequevar alignment 70
cpsC sequevar alignment 70
cpsD sequevar alignment 72
cpsE sequevar alignment 73
cpsG sequevar alignment 77
cpsH sequevar alignment 77
Appendix 3 79
Suppliers 79
Takara polymerases and InTron Biotechnology products 79
Oligonucleotide Primers and Fine chemicals 79
Eppendorf equipment 79
Hoeffer, Amersham and GE Healthcare equipment and reagents 79

FOREWORD

This document is provided to enable rapid surface typing of *Streptococcus iniae* that is accurate, reproducible between laboratories and cost effective. In its current version, the manual represents a working document that will be revised and improved for both clarity and detail over the coming months. It is acknowledged that, having developed and successfully applied the methodology, the Authors are extremely familiar with the procedures and various tips and tricks required to ensure that the methods work successfully. Thus it is extremely important that the current document is revised in terms of content and format with help from interested parties that are not familiar with the techniques and protocols used. This may include members of the FRDC Aquatic Animal Health Subprogram, participating veterinary laboratories and other laboratories, possibly involving ring-testing over the remaining period of this project. We look forward to input from all sources to create a useable manual.

BACKGROUND AND PRINCIPLE

The streptococci represent a diverse group of Gram positive bacteria with low Mol% G+C. Many of the streptococci are pathogens of humans and animals and have consequently become the focus of intensive research. One of the defining characteristics of streptococcal pathogens is their high serotypic diversity. The major antigenic components of streptococcal pathogens were defined as a few surface proteins, including M and M-like proteins (reference), but the dominant protective antigens comprised the polysaccharides of the extracellular capsule – a key feature of virulent streptococcal pathogens. The primary method currently used to define serotype of these organisms is serotypic capillary precipitation devised by Lancefield in 1934 (Lancefield, 1934) and is based on capsular antigens, the major surface expressed opsonising antigens of the streptococci (Heard & Mawn, 1993; Paoletti et al., 1999). In spite of the long history of Lancefield typing, many streptococci are non-typeable by this method (Ramaswamy et al., 2006; Slotvedt et al., 2002; Slotvedt et al., 2007). This may result from mutation in the capsular genes (Ramswamy et al., 2006; Bentley et al., 2006), reversible nonencapsular phase variation (Cieslewicxz et al., 2001) or an uncharacterised capsule (Slotvedt et al., 2002), which represents the case for *Streptococcus iniae*.

Recently molecular serotyping based on sequencing of the capsular genes has been developing rapidly in human pathogenic streptococci (Bentley et al., 2006; Ramswamy et al., 2006). This method has now replaced traditional antibody based typing for some species (Luan et al., 2005) and has a number of advantages:

- Firstly, it allows typing of previously non-typeable isolates;
- Secondly, it removes the requirement for a continuous supply of highly homogeneous and thoroughly characterised antibodies;
- Perhaps most importantly the technique is highly precise and reproducible between laboratories, with results from separate laboratories in different states or even countries directly and immediately comparable over the internet without need for exchange of strains and sera.

The capsular operon of *Streptococcus iniae* is between 18 and 23 kB in length and contains up to xx genes that regulate, transport and synthesise the capsule (Miller & Neely, 2001). The capsular operon from diverse antigenically different *S. iniae* isolates from all continents where *S. iniae* occurs has been sequenced the in the first instance to determine which genes

are variable and which are conserved. The research then focused on Australian isolates to establish the variability in these key variable genes thus enabling rapid capsular genotyping within Australia in order to better direct formulation of autogenous multivalent vaccines.

The present manual contains an outline protocol for molecular serotyping of the ubiquitous fish pathogen *Streptococcus iniae*. Following the outline protocol there is a complete set of Standard Operating Procedures (SOPs) for each of the methods used in the protocol. The appendix contains sample data sequences of the key variant genes in the *S. iniae* capsular operon and further information to facilitate establishment of the methodology within any PCR-equipped veterinary or research laboratory.

References

Bentley et al., (2006) PLoS Genetics 2: e31.
Cieslewicxz et al., (2001) J. Biol. Chem. 276:139-146.
Heard & Mawn 1993) J. Clin Pathol. 46: 145-148.
Lancefield (1934) J. Exp. Med. 59: 441-458.
Luan et al., (2005). J. Clin. Microbiol. 43: 3727-3733.
Miller & Neely (2005) Infect. Immun. 73: 921-934.
Paoletti et al., (1999) J. Clin. Microbiol. 37: 3759-3760.
Ramswamy et al., (2006) J. Med Microbiol. 55:775-783.
Slotvedt et al., (2002) J. Clin Microbiol. 40: 1882-1883.
Slotvedt et al., (2007) J. Clin Microbiol. 45: 2929-2936.

OUTLINE PROTOCOL

1. Confirm identity of presumptive *Streptococcus iniae* by *Lox* PCR (SOP0016)
2. Purify genomic DNA from confirmed isolates of *S. iniae* (SOP0015)
3. Amplify by polymerase chain reaction capsular genes *cpsY, cpsB-D, cpsE, cpsF-H* (SOP0030)
4. Analyse amplicons by agarose gel electrophoresis (SOP0008)
5. Clean PCR products for sequencing (SOP0025)
6. Prepare reactions for Sanger sequencing (SOP0026) and send for sequencing by a commercial laboratory.
7. Assemble sequences using appropriate software and compare to reference sequences (Appendix)

EQUIPMENT

Adjustable micropipettes

Accurate, regularly calibrated micropipettes are essential. Ideally, a number of pipettes covering the following ranges should be available: 0.1-2 μL, 0.5-10μL, 0.5-20μL, 10-100 μL, 20-200 μL, 100-1000 μL. Pipettes should only be used within their accurate range and should be inspected monthly and serviced/calibrated at least once annually.

Equipment used in this research: Eppendorf Research Variable Pipettes.

Microcentrifuge

A microcentrifuge with a rotor capable of carrying 1.5 mL, 750 μL microcentrifuge tubes and 200 μL PCR tubes. Rotor should operate at up to 10,000 x g.

Equipment used in this research: Eppendorf Minispin Plus equipped with F45-12-11 rotor.

Thermal Cycler (PCR machine)

A programmable thermal cycler with a heated lid and preferably with gradient capability so that reaction parameters can be fine-tuned.

Equipment used in this research; Eppendorf Master Cycler Gradient EP-S

Heating block or water bath

Temperature control for some reactions is required. Many of these reactions may be carried out in a thermal cycler. For larger reaction volumes, or if the cycler is in use, a heating block or water bath may be used.

Equipment used in this research: Eppendorf Thermomixer with blocks for 1.5mL and 750 μL microcentrifuge tubes.

Horizontal electrophoresis and power supply

For separation of PCR products a horizontal electrophoresis system is required. A mini-system will be perfectly adequate, and two combs can be used if large numbers of samples need to be analysed.

Equipment used in this research: Hoeffer HE33 mini submarine system with EPS301 power supply (all from GE Healthcare Biosciences).

Gel documentation

Ethidium bromide-stained agarose gels should be analysed by ultraviolet transillumination. At its simplest, this may simply comprise an ultraviolet light source in a box with a glass or UV transparent lid. The image may be captured on a digital camera. Care must be taken to avoid skin exposure to UV. However for safety, enclosed gel documentation cabinets with built in video or stills capture are preferred.

Equipment used in this research: UVP BioDoc-It, UVP, Cambridge UK. ImageQuant400, GE Healthcare Biosciences.

Bioinformatics software

Software is required that allows sequence chromatogram analysis, assembly and alignment. Biomanager is widely used online in Australia, and Mega can be downloaded for Windows based PCs free of charge. We prefer Genecodes Sequencher (Supplied by Genesearch in Australia) for ease of use, reliability and support.

Equipment used in this research: Sequencher version 4.8 for Macintosh OS X.

Personal protective equipment

Laboratory coat. Latex gloves are suitable for protection of most of the purification, amplification and sequencing work. Nitrile gloves MUST be worn for any work that involves ethidium bromide.

STANDARD OPERATING PROCEDURES

SOP0015. RELIABLE EXTRACTION OF GENOMIC DNA FROM GRAM POSITIVE BACTERIA

Stock solutions

SUCET buffer (lysis buffer)(0.75 M Sucrose; 40mM EDTA; 40mM Tris base; pH8.0)

Weigh:

Sucrose (Sigma, S7903)	25.67 g
Tris Base (Sigma T6791)	0.485 g
EDTA (Sigma E5134)	1.49 g

Bring to 80 mL using reagent grade (Milli Q or equivalent) water. Bring to pH 8.0 using 5M HCl. Complete to 100 mL with reagent grade water in volumetric glassware. Autoclave (10 min 121°C) and store at 4°C. Use within 1 month.

Lysozyme stock (100mg/mL)

Weigh:

Lysozyme (Sigma L7651)	0.5 g

Dissolve in 5 mL sterile water. Place 100μl aliquots into in clean microcentrifuge tubes. Store at -20°C until required.

Proteinase K stock (20 mg/mL)

Weigh:

Proteinase K (Quantum Scientific 700-031)	100 mg

Dissolve in 5mL sterile reagent grade water. Aliquot into 100μl in clean microcentrifuge tunes. Store at -20°C until required.

Sodium dodecyl sulphate stock (SDS, 10%)

Weigh:

SDS (Sigma L3771)	5g

Make up to 50 mL with reagent grade water. Store at room temperature.

Procedure

1. From pure culture on appropriate solid medium remove sufficient growth to fill a 10μl disposable inoculation loop.
2. Suspend in 500μl SUCET buffer and disaggregate thoroughly
3. Add 20μl lysozyme stock and 0.5-1.5μl RNAse A (Invitrogen).
4. Incubate at 37°C for 1 hour in a thermoblock, incubator or clean water bath, preferably with agitation.
5. Add 50μl 10% SDS and 20μl proteinase K stock.
6. Incubate at 37°C for 1 hour.

7. Perform 3 freeze/thaw cycles at -20°C (or on dry ice) and 50°C for 5 min each.
8. Spin in a microcentrifuge at high speed for 2 min.
9. Transfer supernatant to a PCR cleanup column (Intron PCRquick spin, Cat No. 17201, supplied by Scientifix) and recover DNA according to manufacturer's protocol.
10. Determine concentration of DNA by spectrophotometry.
11. Store in aliquots at -20°C

SOP0008. AGAROSE GEL ELECTROPHORESIS FOR ANALYSIS OF DNA FRAGMENTS OR AMPLICONS UP TO 20KB IN SIZE

Tris Acetate EDTA buffer (TAE):

TAE electrophoresis buffers are used at a working concentration of 0.04M Tris, 0.001M EDTA at pH 7.5-7.8. For simplicity a 50 X stock solution can be prepared from Tris base, glacial acetic acid and a 0.5M EDTA, (pH 8.0) stock solution.

Preparation of 0.5M EDTA (pH 8.0) stock (500 mL)

- In a 500mL glass bottle, weigh: Ethylene diaminetetracetic acid disodium salt (Sigma E5134) 93.05 g
- Add 400 mL reagent grade water (Milli-Q or equivalent).
- Using a calibrated pH probe, measure the pH under continuous stirring.
- Adjust the pH to 8.0 with 1M NaOH dropwise using a disposable pasteur pipette, continuing to stir and monitor the pH. EDTA is only soluble at pH greater than 7.0, but will continue to acidify the solution as it dissolves, thus continuous stirring and monitoring of the pH is required, with adjustments to the pH made as necessary using 1M NaOH until the EDTA is completely dissolved.
- Bring to volume (500 mL) using volumetric glassware.

Preparation of TAE (50 x stock):

- In a 1 L bottle, weigh:
- Tris base (Sigma T6791) 242 g
- Add 500 mL reagent grade water (Milli-Q or equivalent)
- Add glacial acetic acid 57.1 mL
- Add 0.5 M EDTA (pH 8.0) 100 mL
- Bring to volume (1 Litre) with reagent grade water using volumetric glassware.
- Incubate mixtures at 37 °C for 40 minutes, then at 85 °C for 15 minutes.

Preparation of a 1% agarose gel

- Prepare 50 mL gel mixture for each mini-gel required. Volumes should be adjusted for larger gels and this must be determined for each electrophoresis system used.*
- Into a 250 mL bottle or flask weigh: DNA grade agarose (Bioline BIO-41025, or equivalent) 1 g.
- Add 100 mL 1 x TAE (prepared by diluting 50 X stock in reagent grade water).
- Dissolve by bringing to the boil in a microwave oven, or on the free-steam (no pressure) cycle in a laboratory autoclave.

- Allow to cool to approx 55 °C.
- Add 1 μL ethidium bromide (CAUTION. Teratogen. Wear nitrile gloves, and prepare in a defined safety area in accordance with laboratory OH&S guidelines).
- Pour 50 mL* gel mixture into electrophoresis gel casting assembly.
- *Insert the appropriate comb/combs in advance and ensure that the gel mixture is sufficiently deep to provide adequate sample wells, but the level does not rise above the top of the teeth in the combs as this will create a ridge in the gel and effect the migration of the samples.
- Allow the gel to set for a minimum of 1 hour at room temperature. This can be accelerated by transferring to a refrigerator or cold room at 4 °C. However, caution is advised as this may contaminate the fridge/cold room with ethidium bromide if any spillage occurs.

Electrophoretic separation of DNA fragments or amplicons

Once hardened, the gel should be transferred to the buffer tank of the electrophoresis system and the comb(s) removed.

Add 1 x TAE buffer to the tank to cover the gel to a depth of approximately 2mm. Buffer level should never exceed 4-5 mm above the surface of the gel or migration of the samples will be compromised.

- Take 5 μL PCR product and transfer to a clean PCR tube
- Add 1 μL of gel loading buffer (0.3% bromophenol blue, 50% glycerol in reagent grade water; or commercial equivalent)
- Load 5 μL into each well using an adjustable pipette being careful not to introduce bubbles into the wells, which may cause transfer of sample between wells. Change the pipette tip between samples.
- Assemble the electrophoresis system connecting the power supply.
- Separate the nucleic acids by electrophoresis at 80-100 V for 15-30 min. This should be determined empirically for each gel system.
- Disconnect the power.
- Examine the separated nucleic acids in situ by transferring the gel to a UV transilluminator or gel documentation system.

After recording the results gels should be discarded in a manner appropriate for hazardous waste containing ethidium bromide. Under EMS protocols, gels should be broken up and incinerated. Electrophoresis buffers will contain traces of ethidium bromide from the gels and

should be detoxified prior to suitable disposal. The TAE electrophoresis buffer may be left in the gel tank, but should be changed after 5 gels have been run in the tank.

SOP0016. CONFIRMATION OF PRESUMPTIVE STREPTOCOCCAL ISOLATES AS *STREPTOCOCCUS INIAE* BY POLYMERASE CHAIN REACTION

Identification of *S. iniae* by PCR is carried out by testing for the lactate oxidase gene (*lctO*) based on the methods of Mata *et al*. (2003). The primers are specific for the *S. iniae* lactate oxidase gene and do not cross react with other streptococcal species. All components should be frozen at -20°C in suitable aliquots sterile tubes.

PCR components:

- 10-100ng extracted DNA (SOP0015)
- PCR kit including $MgCl_2$, reaction buffer and *Taq* DNA polymerase (Bioline)
- 10 mM dNTP solution – 2.5 mM each of dATP, dCTP, dGTP, dTTP (Biotech International)
- Sterile Milli-Q grade water eg. Gibco Ultra Pure Molecular Biology Grade water (Invitrogen)
- LOX-1 and LOX-2 primers – see Appendix 1.

Preparation of the reaction mixture

PCR amplifications should be performed in 25 μL volumes containing the following per reaction:

- 0.75 μL 50 mM $MgCl_2$
- 2.5 μL 10X reaction buffer
- 0.25 μL 10 mM dNTPs
- 0.25 μL LOX-1 primer (200 ng/μL concentration)
- 0.25 μL LOX-2 primer (200 ng/μL concentration)
- 0.25 U Bioline *Taq* DNA polymerase
- 10-100ng extracted DNA
- Water to 25 μL

Reaction parameters:

Denaturation:

- 94 ºC for 2 min

Followed by 35 cycles of:

- 94 ºC for 30s
- 60 ºC for 1 min
- 72 ºC for 1 min

Then a final extension of:

- 72 ºC for 10 min
- 4 ºC hold

After cycling, load 5 μL of reaction mixture into a 50 mL 1X TAE 1% agarose gel containing 1 μL 10 mg/mL ethidium bromide. Run at 80V for 40 min and check under UV light (SOP0008). A band should be observed at either 869 bp or 920 bp. If no band is observed, the isolate is not *S. iniae* or you should troubleshoot.

SOP0030. AMPLIFICATION OF CAPSULAR GENES FOR MOLECULAR SEROTYPING *STREPTOCOCCUS INIAE*

Molecular serotyping is based on the capsule operon (*cps* gene series) in *S. iniae*. It is intended to give a very detailed analysis of an isolate's genetic makeup in regions that are known to be key antigens and have been shown to be variable. The hotstart protocol and proofreading DNA polymerase detailed below are critical to the success of this typing method. The specified DNA polymerase **is not to be substituted** with *Taq* DNA polymerase.

PCR components:

10-100ng extracted DNA

PCR kit (Takara PrimeStar DNA polymerase)

Sterile Milli-Q grade water eg. Gibco Ultra Pure Molecular Biology Grade water (Invitrogen)

Various primers for amplification of specific regions (Appendix 1)

PCR amplification reaction mix:

PCR amplifications should be performed in 25 μL volumes containing the following per reaction:

5 μL 5X reaction buffer

0.25 μL 10 mM dNTPs

0.25 μL Forward primer (200 ng/μL concentration)

0.25 μL Reverse primer (200 ng/μL concentration)

0.2 μL Takara PrimeStar DNA polymerase

10-100ng extracted DNA

Water to 25 μL

Prepare all reaction components except DNA and water as a master mix for use in the hotstart protocol below.

Reaction parameters:

Denaturation:

94 ºC for 1 min, hold and add mastermix, then 1 more minute at 94 ºC.

Followed by 35 cycles of:

94 ºC for 15s

X ºC for 30s

72 ºC for 1 min/kb of expected product size

Then a final extension of:

72 ºC for 10 min

4 ºC hold

Annealing temperature is dependent on the primer pair being used. Refer to Table 1 on the next page to determine both the annealing temperature and the extension time required for successful amplification of the *cps* gene/s of interest.

Table 1. Annealing temperatures and expected PCR product sizes for targeted genes.

Forward primer	Reverse primer	Gene/s targetted	Annealing temperature (ºC)	Expected product size (kb)
HKi F	CPSA R	*cpsY*	65	3.0
CPSB F	CPSD R	*cpsB-D*	55	2.2
CPSE F	CPSE R	*cpsE*	58	1.8
CPSF F	CPSH R	*cpsF-H*	64	2.5

After cycling, load 5 μL of reaction mixture into a 50 mL 1X TAE 1% agarose gel containing 1 μL 10 mg/mL ethidium bromide. Run at 80 V for 40 min and check under UV light (SOP0008).

SOP0025. PCR PRODUCT CLEANUP PROTOCOL USING EXOSAP

This protocol relies on the actions of Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (Exo) to remove any unincorporated primer from the PCR before it is sent for sequence analysis. Alternatively, PCR products can be cleaned using a kit.

Enzyme mix:

Mix the enzymes together in the ratio of 1 Exo : 2 SAP and store ozen at -20 °C. Do not add water to the mix as it will impair the activity of the enzymes after thawing.

Reaction protocol:

Add the following per reaction:

- 0.4 μL ExoSAP
- 0.7 μL PCR product
- 3.3 μL sterile water

Incubate mixtures at 37 ºC for 40 minutes, then at 85 ºC for 15 minutes.

SOP0026. PREPARATION OF REACTION MIXTURES FOR SEQUENCING OF CLEANED PCR PRODUCTS

Add 4 μL of purified PCR product mix, 3 μL of water, and 1 μL (25 ng/μL) appropriate primer. **Note: only one primer is required for each sequencing reaction. One reaction will need to be performed for each primer to obtain complete contigs.**

Primers used for sequencing will depend on which gene/s has/have been targeted. Table 2 shows the primers needed to obtain gene sequences. Typically, sequences obtained are 800-1000nt in length.

Table 2. Sequencing primers required for each PCR product.

PCR product	Primers required
HK-*cpsA*	HKi F, CPSY F, CPSY R
cpsB-D	CPSB F, CPSBi F, CPSC R, CPSD F, CPSD R
cpsE	CPSE F, CPSE R
cpsF-H	CPS F F, CPSG F, CPSH F, CPSH R

APPENDIX 1.

Table A. Sequences of primers used in PCR.

PRIMER	SEQUENCE (5'-3')
LOX-1	AAGGGGAAATCGCAAGTGCC
LOX-2	ATATCTGATTGGGCCGTCTAA
HKi F	CAGGAAGCAGATATTCCTTTGTT
CPSY F	TTATATTTCCTTTTTTTGTGTCAATTTGA
CPSY R	ATGAGAATACAACAATTACATTACA
CPSA R	ATCGCATAGGATGGCAATTCA
CPSB F	CAGTAATGGGAGGAAAGTAAATG
CPSBi F	CGTGCTAAGTTTTTCTTAGA
CPSC R	TTGTGACATCCTTAACCTC
CPSD F	ATGTCACAATTAAATTTAGTAAGAAGTAAACG
CPSD R	TCACTTTCTGGAATGTTTTTTAC
CPSE F	ATGAAAAGAAGTCAAAAAAGAGTAATC
CPSE R	TTACTCCTGTTTAGCGTCATTTA
CPSF F	ATGTATCCTTATATTAAACGACTATTAGCA
CPSG F	ATGAAAAAAGTACTTATTACAGGTGC
CPSH F	ATGAAAAAATATAGTAGAAGTGTG
CPSH R	TTATTCATCATCCTGTTTAATCCCTAAAGTA

APPENDIX 2
ALIGNMENT OF SEQUEVAR TYPES
cpsY *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGAGAATACAACAATTACATTACATTATCAAAATCGTTGAATGTGGCTCAATGAATGAAGCTGCAAAGCAACTTTATAT
cpsY1       ................................................................................
cpsY2       ................................................................................

            81        91        101       111       121       131       141       151
consensus   TACACAACCCAGTTTATCAAATGCTGTTAAGGACCTTGAACAAGAAATGGGAATAACCATTTTTATTCGAAATCCTAAGG
cpsY1       ................................................................................
cpsY2       ................................................................................

            161       171       181       191       201       211       221       231
consensus   GCATCACTCTGACCAAAGATGGTGTTGAATTTCTTTCTTACGCTAGACAAATCATTGAACAAACCTCTTTGTTGGAGGAA
cpsY1       ................................................................................
cpsY2       ................................................................................

            241       251       261       271       281       291       301       311
consensus   CGTTATAAAAATCATGACAGCAACCGCCAGCATTTTAGTGTTTCGTCTCAACACTATGCCTTTGTGGTTAATGCCTTTGT
cpsY1       ................................................................................
cpsY2       ................................................................................

            321       331       341       351       361       371       381       391
consensus   GTCCCTTTTAAAAGAGACAGACATGACCAAATATGAACTTTTTCTTCGAGAAACAAGAACTTGGGRAATCATTGATGATG
cpsY1       ...............................................................A................
cpsY2       ...............................................................G................

            401       411       421       431       441       451       461       471
consensus   TCAAAAACTTCCGCTCAGAAATTGGTGTTCTCTTCATCAATGACTACAACCGAGATGTTTTAACTAAGCTTTTTGATGAA
cpsY1       ................................................................................
cpsY2       ................................................................................

            481       491       501       511       521       531       541       551
consensus   AATCAATTACAAGCAAACAAACTCTTCCAAACAAGACCCCATATTTTCGTTAGTAACAAACACCCCTTAGCAGACCGCTC
cpsY1       ................................................................................
cpsY2       ................................................................................

            561       571       581       591       601       611       621       631
consensus   TAGTTTAGATGTTGAGGACTTGCAAGCTTATCCTTATTTAAGCTATGACCAAGGCATTCATAACTCCTTTTATTTCTCAG
cpsY1       ................................................................................
cpsY2       ................................................................................

            641       651       661       671       681       691       701       711
consensus   AAGAGATGAAAGCACAGATGCCCCATACCAAATCCATTGTCGTTAGTGACCGTGCGACACTTTTTAACCTCATGATTGGT
cpsY1       ................................................................................
cpsY2       ................................................................................

            721       731       741       751       761       771       781       791
consensus   TTAGACGGCTACACCGTAGCTAGTGGGGTTTTAAATAGTAAATTAAATGGTGATCAAATTGTTGCCATCCCACTAAATGT
cpsY1       ................................................................................
cpsY2       ................................................................................

            801       811       821       831       841       851       861       871
consensus   TCCAGATATTATCGATGTTATCTATATTAAACATGAGAAGGCTAACCTCTCCAAAATAGGTGAAAAATTTATAGATTACC
cpsY1       ................................................................................
cpsY2       ................................................................................

            881       891       901       911
consensus   TCTTAAAAGAGGTCAAATTGACACAAAAAAAGGAAATATAA
cpsY1       ........................................
cpsY2       ........................................
```

cpsC *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGAACACAAGCGAAAACACATCAATTGAAATTGATATTCTTARTCTATTAAAGARAATTTGGCAGAAAAAAGTAGTGAT
cpsC1       ..........................................A...........A.......................
cpsC2       ..........................................G...........G.......................

            81        91        101       111       121       131       141       151
consensus   TCTATTTGTGACGCTACTAGCTGGTTTTTTAGCCTTGGTGGCAAGTATGTTTCTCATTAAACCATCTTACACCTCAACAA
cpsC1       ................................................................................
cpsC2       ................................................................................
```

```
            161       171       181       191       201       211       221       231
consensus   CAAGGTTATATGTTATCAATCGTCAACAGTCAGATAACCTTACGGCGACCGATTTGCAGGCTGGTGGTTACTTGGTTAAT
cpsC1       ................................................................................
cpsC2       ................................................................................

            241       251       261       271       281       291       301       311
consensus   GACTACAAGGAAATCATTACCTCGCGTGATGTGATGCATGATGTTATTGYTAAGGAAAATGTTAGCATGTCACCAGAAGA
cpsC1       ...............................................T................................
cpsC2       ...............................................C................................

            321       331       341       351       361       371       381       391
consensus   GTTGAGCCAAATGATTACAGTAACTGTACCGGCAGATACCCGTGTTATCTCAATTTCTGTAAATAACCATGAACCACAAA
cpsC1       ................................................................................
cpsC2       ................................................................................

            401       411       421       431       441       451       461       471
consensus   AAGCTAAAGATTTAGCGAATGCCGTTCGTGAAGTAGCCTCAGAAAAAATCAAAGACGTGACGAAAGTTCAAGATGTCACT
cpsC1       ................................................................................
cpsC2       ................................................................................

            481       491       501       511       521       531       541       551
consensus   GCTTTAGAAAAAGCTCAACTTCCAACCAAACCGTCATCGCCAAATAGTAAACGCAATGCAGTGATGGGACTTCTAGTTGG
cpsC1       ................................................................................
cpsC2       ................................................................................

            561       571       581       591       601       611       621       631
consensus   TGCTGTTTTAAGTATATTTGCAGTTATCCTAAAAGAAGTTTTAGATGACCGTGTTAAAAGTCCAGAAGATGTTGAAGATG
cpsC1       ................................................................................
cpsC2       ................................................................................

            641       651       661       671       681
consensus   TACTTGGTATGACATTACTAGGTATGGTTCCAAACACAAATAAAATGTAA
cpsC1       ..........GACATT..................................
cpsC2       ..........------..................................
```

cpsD *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGTCACAATTAAATTTAGTAAGAAGTAAACGTGAGCATTATCAGCATGCAGAAGAATACTATAATTCCATTCGCACTAA
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................

            81        91        101       111       121       131       141       151
consensus   TATTCAGTTTAGTGGTCGTGATTACAAGGCAATTGTCTTAACTTCTGTTCAACCTGGTGAAGGAAAGTCAACCACATCGA
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................

            161       171       181       191       201       211       221       231
consensus   TTAACTTAGCCATTTCCTTTGCAAAAGCAGGTTTTAAAACCCTCCTCATTGATGCAGATGTTCGTAATTCAGTGATGTCA
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................

            241       251       261       271       281       291       301       311
consensus   GGTGCCTTTAAATCTGATGACAGGTATGAGGGTCTTTCAAGTTATTTATCAGGAAACGCTGAATTATCAAGTGTTATTTC
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................

            321       331       341       351       361       371       381       391
consensus   AAGAACGGATGTTCCAAATCTCATGTTGATCCCATCAGGTCAAGTGCYACCAAATCCAACAACCTTACTTCAAAATAGTA
cpsD2       ...............................................C...............................
cpsD3a      ...............................................T...............................
cpsD3       ...............................................T...............................
cpsD1       ...............................................C...............................

            401       411       421       431       441       451       461       471
consensus   ATTTTAATTTTATGATTGACACAGTCAAAGAGTTATTTGACTATATTATCATTGATACCCCACCAATAGGCTTGTTGATT
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................G.....

            481       491       501       511       521       531       541       551
consensus   GATTCTGCCATCATTGCGCAAAAAGCAGACGCCACTATCTTGGTAACTGAAGCMGGTTCTATTAAACGTCGTTTTGTGCA
cpsD2       ...................................................C...........................
cpsD3a      ...................................................C...........................
cpsD3       ...................................................A...........................
cpsD1       ...................................................A...........................

            561       571       581       591       601       611       621       631
consensus   AAAGGCAAAAGAACAAATGGAACAAAGTGGTGCACAGTTCTTAGGGGTTATTCTTAATAAAGTAGATCAACAACTTGGTT
cpsD2       ...............................................................................
cpsD3a      ...............................................................................
cpsD3       ...............................................................................
cpsD1       ...............................................................................

            641       651       661       671
consensus   CTTATGGCGCTTACGGTTCCTACGGAGATTAT
cpsD2       ............................
cpsD3a      ............................
cpsD3       ............................
cpsD1       ............................
```

cpsE *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGATTAGTTTCTCCCATCTTTCAGCCTATGCTTTTTTAATGGCATATAGTAATAACTTAACTGACAAGGGAACTTTTAT
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       ................................................................................
cpsE1       ............................................................................A.....
cpsE5       ............................................................................A.....
cpsE6       ................................................................................

            81        91        101       111       121       131       141       151
consensus   TACTCTTGTTGTAACACTTTTTATTTACACCCTTCTGGGCATTCGATTTAATGTCTTTTCTATTATTAATCGGTTTACAG
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       .......T........................................................................
cpsE1       .......T........................................................................
cpsE5       .......T........................................................................T.....
cpsE6       ................................................................................

            161       171       181       191       201       211       221       231
consensus   ACTTAAAAACAATCTCCTTAGTAGTCTTTAACCTCTTCCTAGCCTTTTTAGTTGGTTACTTCACTGACTTGTTTTTCTTA
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       ................................................................................
cpsE1       ................................................................................
cpsE5       ................................................................................
cpsE6       ................................................................................

            241       251       261       271       281       291       301       311
consensus   GACAGTTTTAGTCGTCGTTTTATCTTTTTGGCTTATATCTTTAGTTCTTTCTTGGTATCAGCCCCTAGAATAACTTGGCG
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       ................................................................................
cpsE1       ................................................................................
cpsE5       ................................................................................
cpsE6       ................................................................................

            321       331       341       351       361       371       381       391
consensus   ATTATGGCATGAATTCAATTTAAGCAAATACGAAAAAGGAAAAAAAGAAAAGAAAAGGATTTTGGTAGTCGGTGCAGGTG
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       ................................................................................
cpsE1       ................................................................................
cpsE5       ................................................................................
cpsE6       ................................................................................

            401       411       421       431       441       451       461       471
consensus   AAGGGGGTTCCACCTTTATCCAAACTGTTTTGAATAAGGGAAAAGATATTGAAATTGTTGGTATTGTTGATTCAGATATC
cpsE7       ................................................................................
cpsE8       ................................................................................
cpsE3       ................................................................................
cpsE3a      ................................................................................
cpsE4       ................................................................................
cpsE2       ................................................................................
cpsE1       ................................................................................
cpsE5       ................................................................................
cpsE6       ................................................................................

            481       491       501       511       521       531       541       551
consensus   AATAAATTAGGCACTTACCTCCATGGCATTAAAGTTATGGGGAACAAGTATTCTATCCCAAGATTGGTTGCTGAATATGA
cpsE7       ................................................................................
```

```
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            561       571       581       591       601       611       621       631
consensus   AGTTAATCAAGTAACTATTGCCATTCCAAGTCTTGACGGTAAAGGGCGTGAGGCCATCTTAGACATTTGTCGTCAGGCTA
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            641       651       661       671       681       691       701       711
consensus   ATGTTCCAGTAAACAATATGCCAAGCATTGAAAATATTGTCATGGGAAATGTTTCCCTCAATACCTTTGAAGAAATTGAC
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            721       731       741       751       761       771       781       791
consensus   ATCGCTGACCTATTAAGCAGGAATGAAGTTTTTCTAGATCAAAGTGCTTTGCAACCTTTTTTTAGTGAAAAAACGGTCTT
cpsE7       ..........................................................................A.....
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            801       811       821       831       841       851       861       871
consensus   GGTTACAGGTGCAGGAGGCTCTATTGGATCTGAGATATGTCGTCAAGTAGCCCACTTTAAGCCTAAACAAATCTTATTAT
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            881       891       901       911       921       931       941       951
consensus   TAGGACATGGGGAGAACTCTATTTATTTAATTAATAGAGAACTGAATGCTAAATATTCTGATAACATCATAATTACTCCG
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................T...............
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................


            961       971       981       991       1001      1011      1021      1031
consensus   ATTATTGCTGATATCCAGGACCGTGACTTAATGTTTAAGATTATGGCTGACTATAAACCAGATGTGGTTTACCATGCAGC
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
```

```
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................T-.GT....
cpsE6       ..........................................................................T-.GT....

            1041      1051      1061      1071      1081      1091      1101      1111
consensus   AGCACATAAGCATGTGCCATTAATGGAATATAATCCAAGAGAAGCTGTCAAAAATAATATTTTCGGAACAAAAAATGTTG
cpsE7       ..........................................................................
cpsE8       ........C.................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1121      1131      1141      1151      1161      1171      1181      1191
consensus   CAGAAGCAGCAAAAGCAGCTGGCATTCCAAAATTTGTGATGGTGTCAACTGACAAGGCTGTTAATCCTCCAAATATCATG
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1201      1211      1221      1231      1241      1251      1261      1271
consensus   GGGGCAACAAAGCGTTTTGCTGAAATGATTGTAACTGGCTTAAATGAAGCTGGAAAAACGCAATTTGCAGCAGTCCGCTT
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1281      1291      1301      1311      1321      1331      1341      1351
consensus   CGGTAATGTTCTAGGGAGTCGTGGAAGTGTTGTCCCTTTGTTTAAAGAACAGATTAAAAAGGGTGGTCCAGTAACCGTCA
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1361      1371      1381      1391      1401      1411      1421      1431
consensus   CTGACTTTAGAATGACCCGTTACTTTATGACAATTCCAGAAGCAAGTCGACTTGTTATCCAAGCAGGTTTTCAAGCTAGT
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ................T.........................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................


            1441      1451      1461      1471      1481      1491      1501      1511
consensus   GGTGGTGAGATTTTTGTACTTGATATGGGTGAGCCTGTAAAAATTCTCGACTTAGCTAAAAAAGTGATTAAGTTGAGTGG
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
```

```
cpsE6       ..........................................................................

            1521      1531      1541      1551      1561      1571      1581      1591
consensus   GCACACTGAAGATGAGATTGCTATTGTTGAATCTGGTATTAGACCAGGAGAAAAACTTTACGAGGAACTCTTATCAACCA
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1601      1611      1621      1631      1641      1651      1661      1671
consensus   GCGAACGTGTCTCAGAACAAGTTCATGACAAAATCTTTGTTGGCAGAGTAAGCTCAAAACCTTTGGACCAAGTCCTTTCC
cpsE7       ..........................................................................
cpsE8       ..........................................................................
cpsE3       ..........................................................................
cpsE3a      ..........................................................................
cpsE4       ..........................................................................
cpsE2       ..........................................................................
cpsE1       ..........................................................................
cpsE5       ..........................................................................
cpsE6       ..........................................................................

            1681      1691      1701
consensus   ATTGTAAATGGATTAGACCAAT
cpsE7       .....................
cpsE8       .....................
cpsE3       .....................
cpsE3a      ............C........
cpsE4       ............C........
cpsE2       ............C........
cpsE1       .....................
cpsE5       .....................
cpsE6       .....................
```

cpsG *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGAAAAAAGTACTTATTACAGGTGCAAATTCTTATATAGGAACTTCCCTTGAAAAATGGCTACAACAATCAGAGGAACA
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            81        91        101       111       121       131       141       151
consensus   ATATCATGTTGATACTTTAGACATGATTGATCCAAACTGGAAAACGTTTGACTTTTCACCTTATGACAGCATTTTTCATG
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            161       171       181       191       201       211       221       231
consensus   TGGCAGCTATTGTTCATAAAAATGAAAAGCAAATGAACTCAGACCTCTATGAAAAGGTTAATACCAAACTTCCTATAGAG
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            241       251       261       271       281       291       301       311
consensus   TTGGCTACCATTGCTAAACATTCTGGTCTAAGACAGTTTATATTTTTAAGTAGTATGAGTGTCTATGGTAATGATACAGA
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            321       331       341       351       361       371       381       391
consensus   AGAAATAACACGTGAAACTAGGGAAAATCCATCAAGTTATTATGGAAAAAGTAAACTAGCAGCAGAGATAGGTTTAAAAG
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            401       411       421       431       441       451       461       471
consensus   ATTTACAATCAGATAGTTTTAAAGTACTTATTCTACGCCCGCCAATGGTTTATGGACCACAGGCAACAGGTAATTACAGT
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            481       491       501       511       521       531       541       551
consensus   AGATTATCAAAGTTAT---------CAAAGTTTACGCCAATTTTTCCCAAAGTTGCTAATAAACGAAGCATGATTTACCT
cpsG1       ................................................................................
cpsG2       .......---------........................................................................
cpsG3       ................CAAAGTTAT.............................G.......................

            561       571       581       591       601       611       621       631
consensus   TGATAATCTTTTAGAATTTGTTCGCTTGTCAATTGAAACAGAATTGAGTGGAATCCATTTTCCACAAAATAAAGATTATG
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            641       651       661       671       681       691       701       711
consensus   TGACAACAAGTCAATTAGTAAACGTCATTCGTCAAGTTAATGGAAAAAGCACACTGTTAACTTCTCTCTTTAACCCAATC
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            721       731       741       751       761       771       781       791
consensus   ATTAAATCTTTAAAAGGTTTTAGTCAAATCAATAAGCTCTTTGGGAATTTAGTTTATAGTAAAGAAATGTCACAAGAAGC
cpsG1       ................................................................................
cpsG2       ................................................................................
cpsG3       ................................................................................

            801       811       821       831       841       851       861
consensus   TTTTGACTATAATGTTACAGGATTTGAAGAGTCAATACGAATATCGGAAAGAAACAATGAAAAAATATAG
cpsG1       ...........................................................................
cpsG2       ...........................................................................
cpsG3       ...........................................................................
```

cpsH *sequevar alignment*

```
            1         11        21        31        41        51        61        71
consensus   ATGAAAAAATATAGTAGAAGTGTGTTACAAGGGAAAAAAATTCTTTTTTTCTCTCCCTCTTTCTTTAATTATGAAAATGT
cpsH1       ................................................................................
cpsH2       ................................................................................

            81        91        101       111       121       131       141       151
```

```
consensus    AATAAAAGATAAAATGGTCGAACTAGGAGCAGACGTTTATTTCTTTGATGAACGTCCATTTTCATCAGTTTATAGAAAAG
cpsH1        ................................................................................
cpsH2        ................................................................................

             161       171       181       191       201       211       221       231
consensus    CATTACTAAAACTTAATCCTAATGTATTTTCAAAAAGTACCGAAAAATATTTTGATTTGATTTTTAATAATGTTTCAGAT
cpsH1        ................................................................................
cpsH2        ................................................................................

             241       251       261       271       281       291       301       311
consensus    ATTTGTTTTGATTATGTCTTTTTTTTGAAGTGTGAAACTCCAACATTAAAAGTATTAAGGAAATATAGAGCATATTTTAA
cpsH1        ................................................................................
cpsH2        ................................................................................

             321       331       341       351       361       371       381       391
consensus    GAATGCGAAATTTTGCTTATATATGTGGGATTCGATATCAAATGTAAAAAATATTGAAAAAAAATTAATTTATTTTGATA
cpsH1        ................................................................................
cpsH2        ................................................................................

             401       411       421       431       441       451       461       471
consensus    TTATATCTTCATTTGATAAAAAAGACAGTGAAGAGCGAGGATTTAACTTTAGACCGTTATTTTATAGTGATGAGTATGCG
cpsH1        ................................................................................
cpsH2        ................................................................................

             481       491       501       511       521       531       541       551
consensus    AAACCATATAAGAAACAATTTTATAAATATGATATTTGTTCATTTGGCACAATTCATTCAGATAGATATAGTATCTTGAC
cpsH1        ................................................................................
cpsH2        ................................................................................

             561       571       581       591       601       611       621       631
consensus    AAAATTTGTTAATTATTCAAAAAAAAATAATCTTAAATTTTATTTTTTTAATTTTCTTCAAGGGAAGTTTATGTTTTATT
cpsH1        ................................................................................
cpsH2        ................................................................................

             641       651       661       671       681       691       701       711
consensus    TTTACAAAATTGTAAAAAAAGATTTTTTWAAAGCAAACATTTCAGAATTTAGTTTTGTAAAGAAAAATAGTCAAGAAATT
cpsH1        ............................A...................................................
cpsH2        ............................T...................................................

             721       731       741       751       761       771       781       791
consensus    ATTAAAACAATTCTAGATTCAAAAGTTGTTTTAGATATTCAACATCCAAATCAAACTGGCTTAACGATGCGAACAATTGA
cpsH1        ................................................................................
cpsH2        ................................................................................

             801       811       821       831       841       851       861       871
consensus    AATGATTGGATTGAATAAAAAAATAATTACGACTAATAATTCAATCGTAAACTATGATTTTTACAATAAAAACAATATTT
cpsH1        ................................................................................
cpsH2        ................................................................................

             881       891       901       911       921       931       941       951
consensus    TAATTATTGATCGACATAATATTGAAATTGATAGAGAATTCTTTGAAACAGAATATTCTGCATTAAATCAAGATGTTTAT
cpsH1        ................................................................................
cpsH2        ................................................................................

             961       971       981       991       1001      1011      1021
consensus    AAAAAATATAGTTTAGAATTTTGGTTATACGATACTTTAGGGATTAAACAGGATGATGAATAA
cpsH1        ............................................................
cpsH2        ............................................................
```

APPENDIX 3
Suppliers

Takara polymerases and InTron Biotechnology products

Scientifix Pty. Ltd
PO Box 18 Southland Centre
Cheltenham
Victoria 3192
Tel: 1800 007 900
Fax: 03 9548 7177
www.scientifix.com.au

Oligonucleotide Primers and Fine chemicals

Sigma-Aldrich Pty. Ltd.
PO Box 970 Castle Hill,
NSW 1765
Tel: 1800 800 097
Fax: 02 9841 0500
www.sigmaaldrich.com

Invitrogen Australia Pty Limited
Level 3, 501 Blackburn Road
Mount Waverley VIC 3149
Australia
Phone: 03 8542-7400
FAX: 03 9544-5622
www.invitrogen.com

Eppendorf equipment

Quantum Scientific Pty. Ltd.
1/31 Archimedes Place
Murarrie, Qld 4172.
Tel: 1800 777 168
Fax: 1800 625 547
www.quantum-scientific.com.au

Hoeffer, Amersham and GE Healthcare equipment and reagents

GE Healthcare Biosciences Pty Ltd.
Bldg 48, 21 South Street
Rydalmere NSW 1701
Tel: 1800 150 522
Fax: 02 8820 8200
www1.gelifesciences.com/

ISBN: 978-0-646-53019-2