Molecular identification of *Vibrio harveyi*-related bacteria and *Vibrio owensii* sp. nov., pathogenic to larvae of the ornate spiny lobster *Panulirus ornatus*

Thesis submitted by
Ana CANO-GÓMEZ
B.Sc. (University of Cádiz, Spain)
M.Appl.Sc. Biotechnology
(James Cook University, Australia)
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James Cook University

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STATEMENT OF THE CONTRIBUTIONS OF OTHERS

Contributors (affiliation):

1. Leigh Owens, main supervisor (Associate Professor Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, JCU)
2. Lone Høj, co-supervisor (Research Scientist, Australian Institute of Marine Science, AIMS)
3. Nikos Andreakis (Research Scientist, AIMS)
4. Mike Hall (Principal Research Scientist, AIMS)
5. Evan Goulden (PhD candidate, University of NSW-AIMS)
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2	Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: current methods and future prospects	x	x		x		x
3	*Vibrio owensii sp. nov*., isolated from cultured crustaceans in Australia	x	x		x	x	
4	Multilocus sequence analysis provides basis for rapid and reliable identification of *V. harveyi*-related species and confirms previous misidentifications of important marine pathogens	x	x	x			

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ANIMAL ETHICS APPROVAL

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statment on Ethics Conduct in Research Involving Human (1999)*, the Joint *NGMRC/AVCC Statement and Guidelines on Research Practice (1997)*, the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines (2001)* and the *James Cook University Statement and Guidelines on Research Practice (2001).*

Approval number A1623.

JAMES COOK UNIVERSITY AUSTRALIA

James Cook University
Townsville Qld. 4811 Australia
Noema Patterson, Animal Ethics Officer
Research Services Ph: 47814484; Fax: 47815521
email: Noema.Patterson@jcu.edu.au

Animal Ethics Committee ***APPROVAL FOR ANIMAL BASED RESEARCH OR TEACHING***		Application ID **A1623**
PRINCIPAL INVESTIGATOR	Ana Cano Gomez	Student
SCHOOL	Marine & Tropical Biology	
CO-INVESTIGATOR(S)	Leigh Owens	
SUPERVISOR(S)		
PROJECT TITLE	Diagnosis of vibrio infections in the larval rearing system of the tropical rock lobster Panulirus ornatus	
APPROVAL DATE:	**15-Dec-10**	**EXPIRY DATE: 31-Mar-11**

This project has been allocated Ethics Approval Number A1623, with the following conditions:

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2. That there is NO departure from the approved protocols unless prior approval has been sought from the Animal Ethics Committee.

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Responsible Monitor :	Schwarzkopf, Dr Lin
Email :	Lin.Schwarzkopf@jcu.edu.au

This project was approved on 15/12/2010

A/Professor Lin Schwarzkopf
Animal Ethics Committee

Approval_Form_A

Printed on 16 Dec 2010

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ABSTRACT

Vibrio harveyi and related bacteria are important pathogens responsible for severe economic losses in the aquaculture industry worldwide. The ornate spiny lobster, *Panulirus ornatus*, is a potential valuable candidate as an aquaculture species but *V. harveyi*-related disease outbreaks during the extended larval life cycle are major constraints for the development of a breeding program for the aquaculture of this species at a commercial level. Bacterial identification methods such as phenotypic tests and 16S ribosomal RNA gene analysis fail to discriminate species within the *V. harveyi* group because these are phenotypically and genetically nearly identical. Multilocus sequence analysis (MLSA) was used to identify 36 *V. harveyi*-like isolates from the larval rearing system of *P. ornatus* and to re-evaluate the identity of other important Australian pathogens. Strains DY05 and 47666-1, isolated in northern Queensland from dying larvae of *P. ornatus* and *Penaeus monodon* prawns, clustered together and apart from currently recognised species. Biochemical tests, DNA-DNA hybridization, MLSA and fatty acid analyses confirmed that the two strains represent a new species of the *V. harveyi* group, described and validated as *V. owensii* (type strain DY05^T = JCM 16517^T = ACM 5300^T = DSM =23055^T).

The phylogenies inferred from the 16S rRNA gene and five concatenated protein-coding loci (*rpoA*, *pyrH*, *topA*, *ftsZ* and *mreB*) from the 36 isolates revealed four well-supported clusters identified as *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*. Although the topological patterns corroborated 16S rRNA gene phylogeny, the latter was less informative than each of the protein-coding genes taken singularly or the concatenated dataset. Results revealed that important *V. campbellii* and *V. owensii* prawn pathogens were previously misidentified as *V. harveyi*, and also that the recently described *V. communis* is likely a junior synonym of *V. owensii*. A two-locus phylogeny based on *topA-mreB* concatenated sequences was consistent with full the five-gene MLSA phylogeny. Global Bayesian phylogenies inferred from *topA-mreB* revealed more cases of potential *V. owensii* misidentifications in global databases such as the fully sequenced 1DA3 strain, initially described as *V. harveyi*. The *topA-mreB* combined analysis provides a practical yet still accurate approach for routine identification of *V. harveyi*-related species.

A multiplex polymerase chain reaction (PCR) assay was designed to specifically detect and discriminate the highly similar species of the *V. harveyi* group (*V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii*), as relevant pathogens of marine aquaculture animals. Four sets of specific primers were designed targeting three protein-coding loci, *topA*, the *ftsZ* and *mreB*, for DNA amplification in the four species. The single tube PCR reaction contained a mix of four specific and compatible primer sets, DNA from one, two, three or all the four target vibrio species and common PCR reagents. The designed PCR protocol allows single-step, simultaneous detection and discriminative identification of *V. harveyi*-like isolates based on the amplification of different size and specific DNA regions in each of the bacterial species. Any combination of DNA templates in the multiplex PCR mix results in a two-, three- or four-band pattern visualised in agarose gels. In cases of bacterial isolation from decapod crustacea, a qualitative assessment is included in the protocol to evaluate the DNA extraction method. This consists of the addition of previously designed primers for specific amplification of the18S rRNA gene in decapod crustacean. The multiplex PCR offers rapid and accurate identification of *V. harveyi*-like clinical and environmental isolates and reliable detection of potential pathogenic strains in clinical samples.

A real-time PCR assay was also designed for detection and quantification of *V. owensii* species. The method used the SYTO9 technology for rapid and discriminative quantification of *V. owensii* by the amplification of a198-bp segment of the *topA* gene by specific primers. The detection limit was 20 fg of purified genomic DNA of *V. owensii*. Different dissociation temperatures were able to differentiate the lobster pathogen DY05^{T} (83.2ºC) from the prawn pathogen 47666-1 (83.9ºC) due to a single nucleotide difference in the PCR products of these strains. The use of SYTO9 made the real-time assay more reproducible and cost-effective than SYBR or TaqMan technologies, respectively. The design of this real-time assay will allow detection and quantification of *V. owensii* pathogens, providing the aquaculture industry with a single-day reliable decision tool depending on the level of infection. As a research tool, it will allow the study of *V. owensii* dynamics in aquaculture rearing systems and in natural habitats

Early stage *P. ornatus* lobster larvae were experimentally challenged with *V. harveyi*-related isolates by Artemia-vector oral challenge or by immersion. For

V. owensii DY05^T, oral challenge caused 90% cumulative mortality after 48 h, while immersion (~10^6 cfu ml^{-1}) caused lower (45%) and more gradual mortalities over eight days. Cell counts by serial dilutions suggested that high density of DY05^T bacteria (~10^6-10^7 cfu ml^{-1}) maintained in either the animal (by oral challenge) or the water (by immersion), were responsible for mortalities. The results suggested that Artemia delivered the pathogen directly into the larval gut where they rapidly colonised the digestive system and caused sudden mortalities. For treatments with *V. owensii* 47666-1 and a control *V. harveyi* strain (RR36), very low cell densities were found within the larvae, still healthy by the end of the experiment, suggesting that cells were not able to colonise the animal tissues. High levels of extracellular products (ECPs) from high density DY05^T cultures were also highly toxic to larvae of *P. ornatus* and caused similar symptoms as immersion treatments with live cells. Heat and digestion treatments indicated that heat-stable proteinaceous molecules secreted by DY05^T are involved in its virulence to *P. ornatus* larvae.

Protein analysis by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) of whole-cell proteins revealed identical profiles for strains *V. owensii* 47666-1 and *V. harveyi* RR36 but differences between profiles of 47666-1 and DY05^T. Comparison of SDS-PAGE band profiles between ECPs of the two strains resulted in the finding of 35 kDa OmpA_C-like protein DY05^T following OFFGEL protein fractionation, electrophoresis separation and subsequent liquid chromatography mass spectrometry (LC-MS/MS). The highest identity of this protein was with the outer membrane OmpA_C-like protein of *V. harveyi* 1DA3, a strain that has been reclassified in this study, as a potential *V. owensii* strain. This protein, which shows high homology with other previously characterised OmpA-like proteins in *V. harveyi*, *V. alginolyticus*, *V. proteolyticus* and *V. cholerae*, is expressed on the surface of bacterial pathogens and is involved in the delivery of virulence factors to eukaryotic cells via outer membrane vesicles. OmpA could be involved in the potent colonisation ability of *P. ornatus* larvae by DY05^T, which would allow proliferation and subsequent production of toxic ECPs, lethal to the animals. Future studies would further characterise toxins and OmpA-like proteins produced by DY05^T in order to understand their function and regulation during infection.

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LIST OF ABBREVIATIONS

A	adenine
AFLP	amplified fragment length polymorphism
AIMS	Australian Institute of Marine Science
ARDRA	amplified ribosomal DNA restriction analysis
BI	bayesian inference
BLAST	basic local alignment search tool
BLASTN	basic local alignment search tool nucleotide
bp	base pair
C	cytosine
CDCE	constant denaturant capillary electrophoresis
cfu	colony forming units
Ct	cycle threshold
Da	dalton
DDH	DNA-DNA hybridization
DGGE	denaturing gradient gel electrophoresis
DNA	deoxynucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECP	extracellular products
FISH	fluorescence *in situ* hybridization
FSW	filtered sea water
G	guanine
ILD	incongruence length difference test
JCU	James Cook University
kDa	kilodalton
LC-MS/MS	liquid chromatography mass spectrometry
LDC	lysine decarboxylase
LPS	lipopolysaccharide
MA	marine agar
MB	marine broth
ML	maximum-likelihood method
MLEE	multilocus enzyme electrophoresis
MLSA	multilocus sequence analysis

MLST	multilocus sequence typing
MP	maximum-parsimony method
NCBI	National Center for Biotechnology Information
NJ	neighbor-joining method
nt	nucleotide
NTC	non-target control
NTS	non-target species
OD_{600}	optical density 600 nm
ODC	ornithine decarboxylase
OMP	outer membrane protein
OMV	outer membrane vesicle
ONPG	ortho nitrophenyl-β-D-galactopyranosidase
PBS	phosphate buffer solution
PCR	polymerase chain reaction
pH	puissance d'hydrogene
pI	isoelectric point
PIS	parsimony informative sites
PY	peptone yeast
Qld	Queensland
qPCR	quantitative polymerase chain reaction
RAPD	random amplified polymorphic DNA
REP-PCR	repetitive extragenic palindromic elements PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrilamide gel electrophoresis
SEM	scanning electron microscopy
sp. nov	species nova
T	thymine
TBR	tree bisection-reconnection
TCBS	thiosulphate-citrate-bile-salts-sucrose
t-RFLP	terminal restriction fragment length polymorphism

TSA	tryptone soy agar
UV	ultraviolet

CHAPTER 1. GENERAL INTRODUCTION

The spiny lobster (*Panulirus ornatus*) is a high value but scarce seafood product in South East Asian countries and a potential candidate for aquaculture in Australia. At the Australian Institute of Marine Science (AIMS, northern Queensland) efforts focus in the microbiology and nutrition of larval stages, the two aspects that so far prevent production of *P. ornatus* at a commercial level.

Previous histology and molecular studies have shown proliferation of bacteria in the larval rearing systems coinciding with sudden mortality events, including the water, the live feeds and the digestive system of the larvae (Bourne et al., 2004; Webster et al., 2006). The lesions were similar to those reported in penaeid prawn larvae and attributed to luminous vibriosis caused by *Vibrio harveyi*-like bacteria (Lavilla-Pitogo et al., 1998). Bacterial species belonging to the *V. harveyi* group are major pathogens for reared aquatic animals, causing important economic losses in the aquaculture industry worldwide. The disease has been well described in prawns but the virulence mechanisms are not well understood. Furthermore, there appears to be considerable variability in the pathogenesis toward different host species, and also strain differences (Zhang and Austin, 2000; Conejero and Hedreyda, 2004; Austin and Zhang, 2006; Bai et al., 2007). A better understanding of virulence mechanisms and environmental factors controlling pathogenesis is of primary importance in order to develop methods for disease control.

At AIMS, several vibrio bacteria were isolated from the larval rearing system of *P. ornatus* during mass mortality events but they could only be identified as *V. harveyi*-like strains (Bourne et al., 2006). Discriminative identification of *V. harveyi*-related species is difficult since strains are highly similar in their phenotypes and genotypes and reproducibility is also limited (Gomez-Gil et al., 2004). Standard identification techniques are not suitable for this complex group of species, and evidence suggests that misidentification of environmental and clinical isolates is common. Due to the economic importance of *V. harveyi* infections, there was an urgent need to design methods for identification, typing and tracking *V. harveyi*-related populations associated with marine reared animals. The first aim of this project is to precisely identify

V. harveyi-like bacteria associated to the larval rearing system of *P. ornatus* by using multilocus sequence analysis (MLSA). MLSA is a method designed for the classification of bacterial strains at the species level. The method employs the sequencing of several single-copy housekeeping genes by polymerase chain reaction (PCR) and subsequent phylogenetic analyses of concatenated sequences to assign genotypes to the species or genus levels (Gevers et al., 2005). A combination of the latter with phenotypic identification, sequence analysis of 16S rRNA and DNA-DNA hybridization (DDH) is the strategy recommended for the description of new bacterial species (Cohan et al., 2000; Stackebrandt et al., 2002). In the case of *V. harveyi*-related species, this method presents clear advantages in resolution power and reproducibility for species identification (Thompson et al., 2005). The study is complemented with other isolates from the AIMS and James Cook University (JCU) collections and worldwide database strains are added to the study in order to offer a global report of previous *V. harveyi*-related misidentifications.

Preliminary results from the phylogenetic analysis demonstrated that two *V. harveyi*-like strains isolated from diseased lobster and prawn larvae (DY05 and 47666-1) shared some unique nucleotides in certain positions and sequences were clustering apart from those of other *V. harveyi*-related species in the phylogenetic trees. Providing that species of the *V. harveyi* group are commonly misidentified (Gomez-Gil et al., 2004; Lin et al., 2010; Pedersen et al., 1998), a detailed phenotypic and genetic characterisation was carried out for these two isolates.

Despite the need for molecular tools for refined identification of *V. harveyi*-related species, the selection of practical tools for quick detection of pathogens would have to find a balance between accuracy and other aspects such as turnaround time, ease of performance and cost. Ideally, the design of different tools would allow the selection of the most appropriate one for clinical- or research-based investigations. The aquaculture industry would also benefit from the development of molecular techniques for direct detection and quantification of *V. harveyi*-related populations without initial strain isolation. In the case of *V. harveyi*-related infections, direct quantification is essential for monitoring the pathogen in hatcheries, as well as for research into its pathogenicity and its role in natural habitats. Once optimised in terms of cost, analysis time, and technical skills required, techniques such as real-time PCR can potentially be used for

routine testing of selected control points in the farm environment, as well as for research into the ecology of these vibrio populations. Real-time PCR requires specialised equipment but the technique is becoming more routine and readily available. The second aim of this study was to design reliable molecular tools for discriminative detection of *V. harveyi*-related species, and simultaneous quantification of pathogenic strains to the larvae of *P. ornatus*. The design of these techniques was based on the previous phylogenetic information obtained by MLSA for *V. harveyi*-related species.

The pathogenicity mechanisms of *V. harveyi*-related species are well understood and species show high variability in virulence toward different host species (Austin and Zhang, 2006). Pathogenicity of *V. harveyi* has been related to a number of factors but the relationship between the presence of virulence genes and the pathogenicity of *V. harveyi*-related strains to different hosts is controversial (Zhang and Austin, 2000; Conejero and Hedreyda, 2004; Bai et al., 2007). The final aim of this project was to elucidate the pathogenicity mechanisms of *V. harveyi*-related strains to the larvae of *P. ornatus* by *in vivo* experimental challenge of pathogenic strains and characterisation of potential virulence factors at the molecular level. This research allows future refinement of pathogen tracking and reliable diagnosis as management tools to prevent disease outbreaks of vibriosis in aquaculture systems.

CHAPTER 2. REVIEW OF LITERATURE

2.1 *Vibrio harveyi*-related Species: Biology and Pathogenicity

Vibrio species belonging to the Harveyi clade represent major pathogens for aquatic animals, causing diseases responsible for severe economic losses in the aquaculture industry worldwide. The first species described in this clade, *Vibrio harveyi* (Baumann et al., 1981), is a sodium chloride dependent, curved-rod shaped, gram-negative bacterium found in marine environments (Farmer et al., 2005). First described as *Achromobacter harveyi* (Johnson and Shunk, 1936) after E.N. Harveyi, a pioneer in the systematic study of bioluminescence, *V. harveyi* was later assigned to the genus *Lucibacterium* (Hendrie et al., 1970) and *Beneckea* (Reichelt and Baumann, 1973), and finally included in the genus *Vibrio* within the family *Vibrionaceae* (Baumann et al., 1981). Other species described later, *V. carchariae* (Grimes et al., 1985) and *V. trachuri* (Iwamoto et al., 1996), were determined to be synonyms of *V. harveyi* based on molecular studies (Pedersen et al., 1998; Gauger and Gomez-Chiarri, 2002; Thompson et al., 2002).

In recent years, numerous infections by *V. harveyi* and related species have been observed and this is believed to be the consequence of globally rising temperatures (Travers et al., 2009). Higher temperatures can cause superior bacterial growth rates and increased virulence potential of marine vibrios, which may in turn alter host-pathogen relationships and enhance susceptibility of animals to epidemic diseases (Paillard et al., 2004; Gagnaire et al., 2006). Oceanic warming may also facilitate the dispersal potential of these vibrios outside their known distribution range, and was recently linked to some out-of season human infections (Sganga et al., 2009). More specifically, two cases of *V. harveyi* infections have been reported in humans: a girl infected after a shark attack off the southeast coast of the US (Pavia et al., 1989), and a child with cancer infected after swimming in the French Mediterranean Sea (Wilkins et al., 2007).

Although *V. harveyi*-related species are found in a free-living state as part of the normal flora of marine animals (Ruby and Morin, 1979), numerous strains have been recognised as the most significant pathogens in cultured marine fish and shellfish. Since the 1990s, *V. harveyi*-related infections have caused severe economic losses in large-scale prawn aquaculture in South America (Alvarez et al., 1998), Australia (Pizzuto and Hirst, 1995), and Asia (Jiravanichpaisal et al., 1994) with mortalities of 100% in larval stages of *Penaeus monodon* and *Penaeus japonicus* often encountered (Suranyanto and Miriam, 1986; Karunasagar et al., 1994; Liu et al., 1996a, b; Lavilla-Pitogo et al., 1998). In Australia and New Zealand, infections by *V. harveyi* strains have been reported to cause high mortalities of larvae and juveniles of fish and shellfish in aquaculture farms (Anderson and Norton, 1991; Owens et al, 1992; Harris, 1993; Handlinger et al., 2002). Additional cases of *V. harveyi* infections in molluscs (Pass et al., 1982; Nishimori et al., 1998), echinoderms (Morgan et al., 2001), and corals (Sutherland et al., 2004) have been described. In potential new aquaculture candidates such as lobsters and crabs, infection by *V. harveyi*-like strains is one of the factors preventing larval rearing on a commercial scale (Jawahar et al., 1996; Diggles et al., 2000; Quinitio et al., 2001; Bourne et al., 2006). The disease has been well described in prawns and named penaeid vibriosis, penaeid bacterial septicaemia, bolitas negricans, luminous vibriosis or red-leg disease. Signs of vibriosis include lethargy, tissue and appendage necrosis, slow growth, slow larval metamorphosis, body malformations, bioluminescent tissues, muscle opacity, melanisation, empty midgut and anorexia (Karunasagar et al., 1994; Roberston et al., 1998).

Vibrio harveyi has proven to be difficult to eradicate and extensive use of antibiotics in farms has resulted in the development of antibiotic resistant strains (Karunasagar et al., 1994). The effect of antimicrobial agents used on strains of *V. harveyi* and *V. splendidus* on *P. monodon* larvae was investigated by Baticados et al., (1990). Six of the antimicrobial agents used demonstrated minimum inhibitory concentrations less than 25 µg ml^{-1} and were also associated with deformities of the carapace, rostrum and the setae of *P. monodon* larvae. It was concluded from this study that the use of antibiotic therapy for luminous vibriosis in hatcheries of *P. monodon* would be unproductive and have limited application. The high dosage of antibiotics required, cost of treatment, the potential development of resistant strains and the adverse effects antibiotic exposure for larval and human health is of ongoing concern. While *V. harveyi* and close species are

not considered a risk to humans, transference of multidrug resistance from aquaculture-related vibrios in prawn farms to *V. cholerae* was speculated to exacerbate a cholera epidemic in Latin America in 1991 (Angulo, 2000). For these reasons, it was imperative that other methods of disease control were investigated.

The use of probiotics has been considered as another method of bacterial management and may provide greater broad spectrum disease control in comparison to vaccination or immunostimulation (Rengpipat et al., 2000). Probiotics are defined as entire or component(s) of microorganisms that are beneficial to the health of the host (Irianto and Austin, 2002). Wang et al., (2000) identified gram-positive bacteria such as *Bacillus spp.* as effective probiotic organisms which reduced the number of pathogens found in farmed species and improved water quality. In previous studies, Phianpark et al., (1997) demonstrated that challenge of *V. harveyi* with *Bacillus spp.* as a probiotics led to 74% survival rates of *P. monodon*. This probiotic treatment also provided disease protection by activating cellular and humoral disease defences. In more recent work, the use of *Bacillus* S11 increased the survival and growth rates of *P. monodon* species exposed to *V. harveyi* pathogens (Rengpipat et al., 2000). A strain of *V. alginolyticus* has also been identified as a successful probiotic treatment for *V. harveyi* which increased survival rates of *Litopenaeus vannamei* larvae (Garriques and Arevalo, 1995). The strain was also found to increase the mean weight of postlarvae from probiotic-treated tanks compared to other tanks by means of greater nutritional intake.

The virulence mechanisms of *V. harveyi*-related species are still not fully understood. There appears to be considerable variability in the virulence mechanisms toward different hosts (Zhang and Austin, 2000; Conejero and Hedreyda, 2004; Austin and Zhang, 2006; Bai et al., 2007). Pathogenicity of *V. harveyi* has been related to a number of factors including secretion of extracellular products (ECPs) containing substances such as proteases, haemolysins, lipases (Harris and Owens, 1999; Liu and Lee, 1999; Zhang and Austin, 2000; Teo et al., 2003), lipopolysaccharides (Montero and Austin, 1999), and bacteriocin-like substances (Prasad et al., 2005). In addition, luminescence (Manefield et al., 2000), quorum sensing (Henke and Bassler, 2004), ability to form biofilms (Karunasagar et al., 1994), bacteriophage infection (Oakey and Owens, 2000), sucrose fermentation (Alavandi et al., 2006), and capacity to bind iron (Owens et al.,

1996) have all been associated with virulence. Moreover, it has been shown that environmental factors, such as temperature and varying salinity also play a role in *V. harveyi*-mediated vibriosis (Alavandi et al., 2006).

2.2 Current Methods for Identification and Typing of *V. harveyi*-related Species

Due to the economic importance of *V. harveyi* infections, there is considerable interest in methods to identify, type and track *V. harveyi*-related populations associated with marine reared animals. Bacterial typing systems are used to distinguish genera, species or strains by detecting differences in their characteristics and provide the basis for the integration of bacterial taxonomy and epidemiology. By strain typing, taxonomists have elucidated the phylogeny and evolutionary history of *V. harveyi* and related species (Thompson et al., 2005, 2007). Typing of *V. harveyi* pathogens has also been the goal of small-scale epidemiology studies in single institutions and farms to characterise virulent subpopulations associated with certain hosts (Pizzuto and Hirst, 1995; Pujalte et al., 2003; Alavandi et al., 2006).

The characteristics of the ideal bacterial typing system for identification of *V. harveyi* in aquaculture systems would be: high discriminatory power, high typeability (proportion of strains that can be assigned a type), high reproducibility, ease of performance and low cost. However, the development of a robust discriminative identification tool for *V. harveyi*-related species is difficult since strains are highly similar in their phenotypes and genotypes (Gomez-Gil et al., 2004; Owens and Busico-Salcedo, 2006). In addition, reproducibility can be limited due to changing phenotypes and genotypes in individual strains over time (genome plasticity). This is explained by a variety of genetic events involved in evolution such as point mutations (Thompson et al., 2004a), chromosomal rearrangements (Makino et al., 2003), duplication (Zhang et al., 2001), infection by bacteriophages (Vidgen et al., 2006) and horizontal gene transfer (Tagomori et al., 2002), which might be responsible for changing phenotypes in members of these species. The best example was offered when Vidgen et al. (2006) found that infection of *V. harveyi*-like strains 645, 20 and 45 by the *V. harveyi* myovirus-like (VHML) bacteriophage resulted in modified phenotypic features, including differences in

D-gluconate utilization, γ-glutamyl transpeptidase and sulfatase activity. This study also proved that changes introduced by the phage modified the phenotypic profile of *V. campbellii* strain 642, reducing the level of assurance for their identification by biochemical tests.

Until recently, the Harveyi clade (Sawabe *et al*., 2007) included seven species: *V. harveyi, V. campbellii, V. rotiferianus, V. alginolyticus, V. parahaemolyticus, V. mytili,* and *V. natriegens*. Within this clade, the closely related ubiquitous and potentially pathogenic species of the *V. harveyi* group, form, as stated above, a tight cluster of cryptic strains characterised by highly similar phenotypes and almost indistinguishable phenotypes (Gomez-Gil et al., 2004; Owens and Busico-Salcedo, 2006). These characteristics, together with the limitation of standard identification techniques for this complex group (biochemical tests, 16S rRNA gene sequencing and specific PCR-based detection protocols), and the description of new species over the last few years suggest that several misidentifications of environmental and clinical isolates may have occurred in the past. (Pedersen et al., 1998; Gomez-Gil et al., 2004; Lin et al., 2010). For example, misclassification of *V. harveyi* and its sister species *V. campbellii* and *V. rotiferianus* is common (Gauger and Gomez-Chiarri, 2002; Gomez-Gil et al., 2004) and it has undervalued *V. campbellii* as an important pathogen of marine reared organisms. In fact, later studies confirmed pathogenicity of both *V. rotiferianus and V. campbellii* strains to marine fish and crustacea (Austin et al., 2005; Defoirdt et al., 2007b; Haldar et al., 2010).

In the following sections, currently available phenotypic and molecular methods for *V. harveyi*-related species identification and typing are presented, with a focus on the principle of each method, their advantages and limitations, and examples of their use in studies of *V. harveyi* and related species. Finally, prospects and challenges in developing molecular methods for direct detection of *V. harveyi* in complex samples are discussed.

2.2.1 Phenotypic methods

The most traditional approaches for identification and typing of vibrios are based on phenotypic methods, such as metabolic or fatty acid profiling, serological methods and profiling of antimicrobial susceptibility. These methods characterise and compare products of gene expression from different species and strains. As outlined above, the discriminatory power of phenotypic methods is limited for the *V. harveyi* group due to their highly similar phenotypes. In addition, genome plasticity may cause phenotype variability and hence affect the reproducibility of phenotypic profiling (Vidgen et al., 2006).

In many laboratories, biochemical tests are commonly used following specific keys (e.g. Alsina and Blanch, 1994a,b) for the identification of *V. harveyi*-like isolates. Commercially available standardised systems are the API 20E (bioMerieux, Inc) and Biolog GN (Biolog, Inc.), in which gram-negative bacteria isolates are identified based on carbon source utilization patterns that can be compared to a database of known organisms, assigning the best match. These tests are easily performed, readily available and their cost is relatively low. Fatty acid profiling (FAME) is an alternative biochemical approach in which fatty acid methyl ester profiles of bacterial isolates are compared. While some authors argue that *V. harveyi*-related species can be discriminated by comparison of biochemical profiles (Kita-Tsukamoto et al., 1993; Alsina and Blanch, 1994a,b; Harris et al., 1996), other studies have shown that for these species, phenotypic tests lack resolution power, cluster some type species together, and leave some isolates unclustered or unidentified (Lambert et al., 1983; Vandenberghe et al., 1999; Hisbi et al., 2000).

Serological methods offer an alternative approach to biochemical tests for identification of *V. harveyi*-related species. An enzyme-linked immunosorbent assay (ELISA) based on polyclonal antibodies was developed for rapid identification of *V. harveyi* isolates, but cross-reactivity among species has been observed (Robertson et al., 1998). Phianphak et al. (2005) developed monoclonal antibodies for detection of *V. harveyi* in prawn tissue, however this assay also showed cross-reactivity with other gram-negative bacteria and the study did not include some important closely related species as controls. A drawback of serological methods is that the necessary antibodies are not

widely available and in addition, quality control is both time-consuming and expensive. Finally, antimicrobial susceptibility profiling has been used in epidemiological studies to characterise *V. harveyi* pathogens in terms of antibiotic resistance profiles (Abraham et al., 1997; Musa et al., 2008).

2.2.2 Molecular methods

In general, molecular methods combine higher discriminatory power and higher reproducibility than phenotypic tests. These advantages are a result of their ability to detect minor genome differences and the higher stability of molecular targets compared with that of phenotypic characters for some species (Sethi et al., 1996; Tenover et al., 1997). However, for *V. harveyi*-related species, highly similar genomes or genome plasticity may also limit precise identification by molecular techniques (Thompson and Swings, 2006; Sawabe et al., 2007).

A range of different molecular methods has been used for identification and typing of *V. harveyi*-related species. The most suitable tool for a specific study will depend on the number and diversity of targeted strains, the goal of the research (identification, detection or characterisation), and the spatial and temporal scale of the study. The discriminatory power of different molecular methods varies widely, as does the ease and speed of performance and cost. The decision to use a molecular technique, as opposed to a particular phenotypic method, should be based on a comparison of simplicity, necessity for high throughput analysis, cost, and appropriateness to answer the question being asked (Riley, 2004). Under some circumstances, such as routine identification and detection in farms, speed may be another criterion to consider. Table 2.1 summarises the suitability of whole-genome and fingerprinting methods for *V. harveyi* and related species and it is based on published studies that included *V. harveyi*-related isolates.

Table 2.1: Whole-genome and molecular fingerprinting methods differentiation of *V. harveyi* related species

Technique	Principle	Advantages	Disadvantages	Reference
DNA-DNA hybridization (DDH)	Whole-genome method. Compares re-association rates of denatured genomic DNA from test bacterium to itself and related species or strain	High resolution power High reproducibility	Technically demanding Non cumulative data Expensive and slow	Gomez-Gil et al. (2004)
Amplified fragment length polymorphism (AFLP)	Whole-genome method. Fingerprint patterns for DNA fragments amplified with two primer sets after initial restriction cutting of genomic DNA	High typeability and resolution power High reproducibility Cumulative in databases Useful for epidemiology studies	Technically demanding Time consuming Expensive	Vandenberghe et al. (1999) Thompson et al. (2001) Gomez-Gil et al. (2004)
Repetitive extragenic palindromic elements PCR (REP-PCR)	Whole-genome method. Fingerprint pattern for DNA fragments amplified by primers targeting repeated interspersed sequences	High resolution power High reproducibility Relatively low cost Useful for epidemiology studies	Moderate typeability	Gomez-Gil et al. (2004)
Random amplified polymorphic DNA (RAPD)	Whole-genome method. Fingerprint patterns for random DNA fragments amplified using a single primer of arbitrary nucleotide sequences	Fast Ease of performance Relatively low cost Useful for epidemiology studies	Poor reproducibility Moderate resolution power Lack of general criteria for interpretation	Pujalte et al. (2003) Hernandez and Olmos (2004) Alavandi et al. (2006) Musa et al. (2008)
Ribotyping or ribosomal restriction fragment length polymorphism (RFLP)	Fingerprint patterns for DNA fragments after southern hybridization of restriction digested genomic DNA with ribosomal probes.	High reproducibility Resolution depends on the probe target (5.8S, 16S, 23S, IS) Automated (riboprinters) Useful for epidemiology studies	Expensive	Macian et al. (2000) Pujalte et al. (2003) Montes et al. (2006) Macian et al. (2006)
Amplified ribosomal DNA restriction analysis (ARDRA)	Fingerprint pattern generation after initial amplification of the 16S rRNA gene with subsequent digestion with restriction enzymes	Ease of performance	Poor resolution power	Urakawa et al. (1997) Hernandez and Olmos (2004) Kita-Tsukamoto et al. (2006)
Multilocus sequence analysis (MLSA)	Cluster analysis based on sequence data from multiple housekeeping genes.	Increasing resolution power with number of loci analyzed High reproducibility due to high stability of markers Cumulative in databases	Expensive Time consuming	Thompson et al. (2005, 2007)

2.2.2.1 Whole-genome analyses

DNA-DNA hybridization (DDH) evaluates the DNA similarity between two bacterial strains and has been established as the gold standard technique for bacterial species delineation. The method is also used for the definitive assignment of a strain with ambiguous properties to the correct taxonomic unit (Wayne et al., 1987). Although DDH is required for proposal of new bacterial species, it is not suitable for routine identification of isolates since the technique is complex, restricted to a few laboratories and non-cumulative, requiring the inclusion of reference strains in each identification test (Gevers et al., 2005). Some advances have however taken the technique to a microarray platform (Cho and Tiedje, 2001), and with this approach, an open database of hybridization profiles can be used if standard genome chips for bacteria are available. Modern vibrio taxonomy is defined by phenotypic characterisation and further genomic analysis, including validation by DDH experiments. While 70% DNA-DNA similarity has generally been used as a criterion for strains belonging to the same species, an 80% DNA-DNA similarity is recommended as the limit for species definition within the family *Vibrionaceae* (Thompson et al., 2004a). Gomez-Gil and coworkers (2004) made use of DDH to precisely identify 39 presumptive *V. harveyi*-related isolates as *V. harveyi*, *V. campbellii* and *V. rotiferianus*, and demonstrated that these three closely related species share > 65% DNA-DNA similarity.

Due to the labour intensive protocols and the cost involved in performing DDH analysis, several studies have evaluated the potential for using whole-genome fingerprinting techniques as alternative methods for species classification. These methods were also used in epidemiological studies for identification of constraint groups of bacteria, such as the Harveyi clade. So far, three whole-genome fingerprinting techniques have been used for analysis of *V. harveyi*-related species/strains: Amplified Fragment Length Polymorphism (AFLP), Repetitive Extragenic Palindromic Elements PCR (REP-PCR) and Random Amplified Polymorphic DNA (RAPD). AFLP is based on size separation patterns of fragments amplified with two primer sets after initial restriction cutting of genomic DNA. In general, each bacterial species has a specific AFLP pattern and since the grouping corresponds well to that obtained by DDH, the technique can be used as an alternative bacterial identification tool (Janssen et al., 1996). For vibrios, DDH similarities can be predicted from AFLP similarities, with

band pairwise similarities of around 70% corresponding to DDH similarities of ~80 to 100% (Thompson and Swings, 2006). Vibrio strains clustering at 45% AFLP pattern similarity are considered to belong to the same species (Thompson et al., 2001). REP-PCR uses PCR primers to amplify highly conserved DNA sequences present in multiple copies within the genome. After resolving the amplified fragments in a gel matrix, a REP-PCR fingerprint is created and used to differentiate bacterial isolates at the species and strain level (Versalovic et al., 1991). Finally, RAPD is based on random amplification of genomic DNA fragments using a single primer of arbitrary nucleotide sequence. Size separation of the resulting fragments allows differentiation between genetically distinct individual clones. The technique is fast, simple and inexpensive, although it suffers from non-reproducibility due to the randomness of the sites targeted by the primers (Gürtler and Mayall, 2001).

These whole-genome fingerprinting methods have been used in several studies to refine the complex taxonomy of *V. harveyi*-related species and to identify strains associated with diseases in marine animals. AFLP has several important advantages as an identification tool for *V. harveyi* related species, including the high reproducibility values of AFLP band patterns for vibrios (91 ± 3%), the ease of data accumulation in databases, and its high discriminatory power (Thompson et al., 2001). Gomez-Gil et al. (2004) reported significant correlation between DDH data and both AFLP and REP-PCR fingerprinting patterns for *V. harveyi*, *V. campbellii* and *V. rotiferianus*. In another study, the same authors used REP-PCR to identify vibrios associated with spotted rose snapper from northwestern Mexico (Gomez-Gil et al., 2006). Overall, the technique discriminated closely related vibrios such as *V. harveyi*, *V. alginolyticus*, *V. campbellii*, *V. parahaemolyticus*, and *V. rotiferianus*, and also suggested the existence of four potential new vibrio species. These studies demonstrated the value of both fingerprinting techniques for the reliable identification of *V. harveyi*-related species.

Epidemiological studies have used whole-genome fingerprinting methods to associate specific banding patterns to particular pathogenic bacterial strains or clones (Wassenaar, 2003). In vibrios, Vandenberghe and coworkers (1999) used AFLP fingerprinting to characterise bacteria associated with vibriosis outbreaks in *L. vannamei* prawns in Ecuador, and strains of *V. harveyi* being associated with diseased postlarvae, juveniles

and broodstock. RAPD fingerprinting proved useful to differentiate virulent and avirulent strains, by establishing different fingerprint patterns associated with pathogenicity in different hosts (Pizzuto and Hirst, 1995; Hernandez and Olmos, 2004; Alavandi et al., 2006). In some studies, the technique allowed clustering of strains isolated from the same animal and even of strains belonging to different outbreaks or different rearing systems (Pujalte et al., 2003; Musa et al., 2008). Hence, RAPD has often been the preferred method in epidemiological investigations for typing emerging pathogens and for source tracking of *V. harveyi* during vibriosis outbreaks. However, the method has been reported as less discriminatory for *V. harveyi* strains than some ribotyping protocols (see section 2.2.2.2) (Macian et al., 2000; Pujalte et al., 2003). The technique also suffers from non-reproducibility due to the randomness of the sites targeted by the primers, the low efficiency of the primers to initiate DNA synthesis, and the number of DNA fragment copies generated (Tenover et al., 1997). For use in taxonomical classification, RAPD should be complemented with a more accurate and reproducible identification tool (Gürtler and Mayall, 2001).

In summary, despite the usefulness of whole-genome fingerprinting methods for strain identification and characterisation, the techniques have their limitations. Compared to other molecular methods, such as PCR assays, they are relatively time-consuming, expensive, and require high technical skills to interpret band patterns. Genome plasticity of *V. harveyi* might also cause variability of band patterns, and hence affect the reproducibility of fingerprinting patterns. Furthermore, the high resolution of the techniques also means that banding patterns may not be directly comparable between different studies.

2.2.2.2 Analyses of genetic markers

Multiple loci have been investigated for their suitability as phylogenetic marker genes for detection and identification of *V. harveyi*-related species, especially for *V. harveyi*. While some authors have recommended targeting single genes (Conejero and Hedreyda, 2003; Oakey et al., 2003; Pang et al., 2005), others argue that a multilocus approach is necessary for a precise identification (Sawabe et al., 2007; Thompson et al., 2007).

a) Single-locus analyses

Most assays based on single genes for detection and identification of *V. harveyi* species rely on initial PCR amplification and in some cases, the assays also include gene sequencing or analysis of restriction enzyme digestion profiles. PCR primers that are currently available for specific amplification of *V. harveyi* genes are presented in Table 2.2.

Ribosomal genes

The 16S and the 23S ribosomal RNAs are essential to the viability of bacterial cells and hence, the genes coding for them are highly conserved. However, these genes also contain short variable sequences useful for characterisation and discrimination of microbial populations at the level of family and, in many cases, at the level of genus and species. The combination of conserved and variable sites makes these molecules ideal taxonomic markers to identify vibrios by PCR amplification and gene sequencing (Kita-Tsukamoto et al., 1993). The 16S rRNA gene is considered the standard marker for Vibrio phylogeny though since the gene evolves slowly, the differences between species are limited and often unable to resolve closely related bacterial strains (Nagpal et al., 1998; Nishibuchi, 2006).

In the case of *V. harveyi,* it is often difficult to resolve this species from other species of the Harveyi clade (*V. alginolyticus, V. campbellii, V. parahaemolyticus,* and *V. rotiferianus*) based solely on 16S rRNA gene heterogeneity. For instance, the species *V. harveyi, V. campbellii and V. rotiferianus* have more than 99% sequence identity for the 16S rRNA gene (Gomez-Gil et al., 2003). Several PCR protocols have nevertheless been designed for specific *V. harveyi* 16S rRNA gene amplification in order to detect and identify this species (Table 2.2). Oakey et al. (2003) developed a PCR protocol that yielded amplification products only for *V. harveyi* and *V. alginolyticus,* but these species could not be definitively discriminated without an additional biochemical test. It should however be noted that, although this study did not obtain amplification product for the included *V. campbellii* strains, *in silico* analysis of primer specificity showed no mismatches in the primers (VH-1, VH-2) with database sequences for *V. campbellii* (GenBank accession no. X56575) and *V. rotiferianus* (GenBank accession no. AJ316187).

Table 2.2: PCR primers available for amplification of *Vibrio harveyi* specific genes

Gene	Gene product	Primer sequence	Product length (bp)	Reference
16S rDNA	16S ribosomal RNA	VH-1: 5'-AACGAGTTATCTGAACCTTC-3' VH-2: 5'-GCAGCTATTAACTACACTACC-3'	1,300	Oakey et al. (2003)
		VHARF: 5'-CCGCATAATACCTACGGGTC-3' VHARR: 5'-ACCCGAAGTGGCTGGCAAACA-3'	967	Fukui and Sawabe (2007)
gyrB	Subunit B of DNA gyrase	A2: 5'-TCTAACTATCCACCGCGG-3' B2: 5' -AGCAATGCCATCTTCACGTTC-3'	363	Thaithongnum et al. (2006)
toxR	Transmembrane transcriptor regulator	Vh_toxR-F: 5'-TTCTGAAGCAGCACTCAC-3' Vh_toxR-R: 5'-TCGACTGGTGAAGACTCA-3'	390	Conejero and Hedreyda (2003)
toxR	Transmembrane transcriptor regulator	toxRF1: 5'-GAAGCAGCACTCACCGAT-3' toxRR1: 5'-GGTGAAGACTCATCAGCA-3'	382	Pang et al. (2005)
vvh	Haemolysin protein	VHF1: 5'-ATCATGAATAAAACTATTACGTTACT-3' VHR1: 5'-GAAAGGATGGTTTGACAAT-3'	1,257	Zhang et al. (2001)
vvh	Haemolysin gene	VhhemoF: 5'-TCAGTGCCTCTCAAGTAAGA-3' VhhemoR: 5'-GCTTGATAACACTTTGCGGT-3'	308	Conejero and Hedreyda (2004)
luxN	LuxN receptor HAI-1 system	fluxN: 5'-CTGTGTACTCACTGTTTATC-3' rluxN: 5'-GTCTAATTCGCGTTCTCCA-3'	2,048	Bassler et al. (1993)

Gauger and Gomez-Chiarri (2002) reported an error in the database for the 16S rRNA sequence of the *V. harveyi* type strain LMG4044^T (GenBank accession no. X74706). Based on this information, Fukui and Sawabe (2007, 2008) reanalyzed *V. harveyi* 16S rRNA gene sequences, designed new PCR primers for a species-specific conventional PCR, and different primers combined with a TaqMan probe for a species-specific real-time PCR assay. While the TaqMan probe discriminated between *V. harveyi* and closely related species, the primers used had no mismatches with *V. campbellii* and *V. rotiferianus*, resulting in an underestimation of *V. harveyi* abundance in the presence of high abundances of either *V. campbellii* or *V. rotiferianus* (see section 2.3.1).

Ribotyping

Ribotyping or ribosomal RNA Restriction Fragment Length Polymorphism analysis (RFLP) is based on the detection of differences in the sequences within or flanking the 16S, 5.8S and 23S ribosomal RNA genes. This technique has been extensively used for *V. harveyi*-related species identification and epidemiological studies (Macian et al., 2000, Pujalte et al., 2003; Montes et al., 2006). Depending of the target region of the probes and restriction enzymes used, the technique may have sufficient resolution to discriminate different strains of *V. harveyi*. Recently a commercially available platform for ribotyping, the riboprinter (DuPont-Qualicon, Wilmington, Delaware, USA) has prompted a new wave of interest for ribotyping. The instrument automatically performs all the steps of the procedure and generates standard electronic fingerprints that can be integrated into a common database. This platform was used by Pujalte et al. (2003) to type 47 *V. harveyi* strains, which were divided into 15 different ribotypes. Generally, ribotyping fingerprints are recognised as being very reproducible and stable over time, and the method may be useful for epidemiological studies.

Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA) is a fingerprinting method based on restriction patterns of PCR amplified ribosomal RNA genes. Urakawa et al. (1997) used this approach to type a wide range of vibrio species: however the study demonstrated that *V. harveyi*-related species, including *V. alginolyticus*, *V. parahaemolyticus*, *V. campbellii*, *V. proteolyticus* and *V. vulnificus* (biotype I) could not be resolved, even using five restriction enzymes. Similarly, Hernandez and Olmos (2004) obtained a single banding pattern when trying to identify 15 environmental

vibrio strains. In both studies the amplified fragment was limited to the 16S rRNA gene, demonstrating that ARDRA based on this gene is not useful for identification purposes or epidemiological studies of *V. harveyi* strains. It is possible however that ARDRA based on a larger part of the rRNA operon would have higher resolution, though this would need to be confirmed experimentally.

toxR gene

The *toxR* gene was first described in *V. cholerae* encoding the transmembrane transcription regulator ToxR, a regulatory gene of the *V. cholerae* toxin operon (Miller and Mekalanos, 1984). Although in some vibrio species *toxR* controls the expression of important extracellular virulence factors, the gene has also been described for non-pathogenic species and more generally it functions as a regulatory gene for expression of outer membrane protein genes (Okuda et al., 2001). The presence of the *toxR* gene or an internal portion thereof has so far been reported in a variety of vibrio species (Reich and Schoolnik, 1994; Kim et al., 1999; Okuda et al., 2001; Pang et al., 2005; San Luis and Hedreyda, 2005). The gene appears to be well conserved among vibrios though it additionally possesses a highly divergent region potentially useful to develop PCR primers for species-specific vibrio identification (Kim et al., 1999). Due to its presence in both virulent and non-virulent species, *toxR* lends itself as a potential species specific marker but not as a virulence marker.

For *V. harveyi*, two sets of PCR primers have been designed for *V. harveyi* specific amplification of the *toxR* gene (Table 2.2). Conejero and Hedreyda (2003) cloned and sequenced the gene from *V. harveyi* after initial amplification with a degenerate primer set (Osorio and Klose, 2000). Based on this sequence and publically available *toxR* sequences from other vibrio species, these authors designed primers for *V. harveyi*-specific *toxR* amplification. However, validation experiments yielded false-negative results for two *V. harveyi* strains (VIB 391 and STD 3-101). Interestingly, these two strains belong to a separate AFLP cluster of *V. harveyi* and were isolated from prawns in Thailand and Ecuador, in contrast to the rest of strains included in the study, which were all isolated from fish. Pang et al. (2005) designed alternative *V. harveyi* specific *toxR* PCR primers, which did not amplify the gene for *V. campbellii* and *V. proteolyticus* reference strains. However this study did not include *V. harveyi*

STD3-101 nor *V. rotiferianus* strains as negative controls, and further studies are needed to validate the specificity of these primers.

gyrB gene

The *gyrB* gene encodes the subunit B protein of the DNA gyrase (topoisomerase type II). Thaithongnum et al. (2006) designed a PCR protocol targeting this gene for detection of *V. harveyi*, with specific amplification for 36 out of 40 *V. harveyi* strains (as identified by biochemical profiling). The protocol was reported to be negative for all other tested vibrio species, including *V. campbellii* ATCC 25920^{T}. When used in combination with a most probable number (MPN) enrichment technique, the method enumerated as few as 15 cells ml^{-1} from artificially inoculated prawns. A more recent taxonomy study including a high number of isolates of both *V. harveyi* and *V. campbellii* reported however, that *gyrB* gene sequences lack resolution power to discriminate between these two species, clustering them together (Thompson et al., 2007). It appears therefore necessary to test the published *gyrB* primers with a larger number of strains from all species in the Harveyi clade, including *V. rotiferianus* and other recently described, before they can be accepted as being truly species specific.

lux genes

The *V. harveyi* quorum sensing system has been previously reviewed (Milton, 2006; Defoirdt et al., 2007a), and this section focuses only on aspects that are relevant for the development of identification and typing tools. It is important to remark that at the time of these studies, identification of *V. harveyi* isolates was not precise, and one of the bacteria widely used in these investigations was in fact a *V. campbellii* strain (BAA1116; Lin et al., 2010). The quorum sensing system was described as a three cell-signalling systems (LuxM/N, LuxS/PQ, and CqsA/S) that operate in parallel; each producing a distinct autoinducer (Table 2.3) involved in the expression of multiple genes in response to bacterial population densities (Bassler et al., 1993). In *V. harveyi* (and *V. campbellii*), quorum sensing has been linked to control of bioluminescence (Bassler et al., 1993; Manefield et al., 2000), biofilm formation (Hammer and Bassler, 2003), type III secretion (Henke and Bassler, 2004), protease (Mok et al., 2003) and siderophore production (Lilley and Bassler, 2000). The signalling system LuxM/N was found to be exclusive to *V. harveyi* and *V. parahaemolyticus* (Bassler et al., 1997), although it is known now that this study used *V. campbellii* strains misidentified as

V. harveyi. Under this mistaken assumption, Hernandez and Olmos (2004) investigated whether the genes coding for the autoinducer signal receptor LuxN and the the autoinducer synthase LuxM could be used as species specific marker genes for *V. harveyi*. These authors designed PCR primers that specifically amplified the *luxN* gene in environmental strains of *V. harveyi*, and surprisingly, no amplification was obtained for *V. campbellii*. However, I have successfully used these primers to amplify the *luxN* gene from two *V. campbellii* strains isolated from diseased larvae of the ornate spiny lobster *P. ornatus* (data not shown). The amplified gene fragments were sequenced (GenBank accession no. FM212935 and FM212936) and shared 96% similarity to the *luxN* gene of *V. harveyi*. These results suggest that genes involved in quorum sensing are not suitable as species specific markers for *V. harveyi*-related species.

Table 2.3: Components of the signalling quorum sensing system of *Vibrio harveyi*

System	Autoinducer	Synthase	Receptor	Common regulators
LuxM/N[1]	HAI-1 (acylated homoserine lactone)	LuxM	LuxN	$LuxR_{Vh}$ (transcriptional master regulator)[1]
CqsA/S[3]	CAI-1 (cholerae autoinducer)	CqsA	CqsS	LuxU (shared phosphotransferase)[1,4]
LuxS/PQ[1,2]	AI-2 (furanosyl borate diester)	LuxS	LuxQ	Lux O (dependent activator)[1,3]

[1]Bassler et al. (1994); [2]Chen et al. (2002); [3]Henke and Bassler (2004); [4]Freeman and Bassler (1999).

vhh genes

Haemolysins have been linked to pathogenic strains of *V. harveyi* in fish. Zhang and Austin (2000) characterised a haemolytic strain of *V. harveyi* (VIB 645) as highly pathogenic to salmonids and found two identical haemolysin genes (*vhhA* and *vhhB*). Other less pathogenic strains possessed only a single gene or alternatively, no *vvh* gene was detected (Zhang et al., 2001). Other authors have suggested however that the *vhh* gene is present in all *V. harveyi* strains and it may be suitable for species specific detection by PCR, with only 85.6% gene identity with the haemolysin gene (*tl*) of *V. parahaemolyticus* (Conejero and Hedreyda, 2004). Specific primers for *vhh* amplification designed by Conejero and Hedreyda (2004) yielded an amplification

product for all *V. harveyi* strains isolated from fish, though the PCR assay was negative for two isolates (strains VIB 391 and STD 3-101) from diseased prawn larvae. The apparent lack of *vhh* in some *V. harveyi* strains means that while primers designed for *vhh* may be useful as an additional tool in epidemiological studies, they cannot be recommended for species identification purposes. By definition, species-specific markers should be stable in the genome, and the involvement of *vhh* in virulence makes this gene susceptible to horizontal gene transfer (Waldor and Mekalanos, 1996), and thus, unsuitable as a species identification marker.

b) Multilocus sequence analysis (MLSA)

The analysis of a single locus has potentially limited resolution power for identification of closely related vibrio species as discussed above. Therefore, extensive efforts have been made to develop a multilocus sequence analysis (MLSA) approach for vibrio species delineation (Thompson et al., 2005, 2007; Sawabe et al., 2007; Pascual et al., 2010). MLSA represents the further development of multilocus sequence typing (MLST), a method originally deployed in molecular epidemiology for the classification of bacterial strains encountered within well-defined species (Maiden et al., 1998). MLSA and MLST methods are a refinement of the earlier multilocus enzyme electrophoresis (MLEE) (Selander et al., 1986), which evaluated genetic variation of metabolic enzymes based on their migration in starch gels during electrophoresis. MLEE was taken to a genomic platform with MLSA and MLST and the techniques are now considered standard typing tools for bacterial taxonomy and epidemiology studies (Wassenaar, 2003; Maiden, 2006). As opposed to MLST, MLSA is often used when species boundaries are not well known and data obtained are used to improve species descriptions. In prokaryotes for instance, although formal delineation of taxa is mainly based on traditional DDH, a combination of the latter with phenotypic identification, sequence analysis of 16S rRNA and MLSA is recommended (Cohan et al., 2001; Stackebrandt et al., 2002). The method employs the sequencing of several single-copy housekeeping genes (at least five to overcome the potential effect of recombination) and subsequent phylogenetic analyses of the concatenated dataset to assign genotypes to the species or genus levels (Gevers et al., 2005). Since the technique targets genes that are not under selective pressure, it also allows long-term classification of species and the study of long-term spread of pathogens; for example the possible evolution of *V. harveyi* from *V. campbellii* (Thompson et al., 2007).

Overall, MLSA has been described as an accurate tool for morphologically cryptic *Vibrio spp.* delineation with similar resolution power to other complex and expensive methods such as DDH, comparative genomic hybridization and AFLP (Thompson et al., 2001; Gomez-Gil et al., 2004; Lin et al., 2010). Most importantly, identification by MLSA is facilitated by a progressive accumulation of online sequences enabling data mining and exchange between researchers and diagnostic laboratories. In the case of *V. harveyi*-related species, the method presents clear advantages in aspects such as resolution power and reproducibility for species identification (Thompson et al., 2005). However, MLSA and MLST are costly and time-consuming, require a considerable amount of experience, and are not as suitable for short-term epidemiology studies of *V. harveyi* pathogens. In the latter genomic events occurring under high selective pressure, such as acquisition of virulence genes, would not be detected.

Initial work by Thompson et al. (2005) explored the usefulness of three genes for identification of vibrios by MLSA; the RNA polymerase alpha subunit gene (*rpoA*), the uridylate kinase gene (*pyrH*), and the recA recombination and DNA repair protein gene (*recA*). The results were compared with the resolution power obtained by using the 16S rRNA gene. The genus *Vibrio* was found to be both heterogeneous and polyphyletic, and vibrio species showed high gene sequence variation for these loci (19% for *rpoA*, and 27% for the *recA* and *pyrH* genes). More specifically, *V. harveyi*-related species were found to have at least 3.5%, 3%, and 5% sequence variation for *rpoA*, *recA*, and *pyrH*, respectively. A later study by Thompson et al. (2007) focused specifically on discrimination between *V. harveyi*-related species. This study was based on the analysis of seven genetic loci: *recA*, *pyrH*, *gyrB*, *gapA* (glyceraldehydes-3-phosphodehydrogenase gene), *mreB* (rod shaping protein gene B subunit), *ftsZ* (cell division protein gene), and *topA* (topoisomerase I). The authors concluded that the genes *topA*, *pyrH*, *ftsZ*, and *mreB* were suitable for discrimination between *V. harveyi*, *V. campbellii*, *V. rotiferianus* and a yet unnamed species, while more complex phylogenies were indicated for *gyrB*, *recA* and *gapA*, possibly due to slower molecular clocks for these loci. The concatenated sequences of all seven housekeeping genes provided evidence that *V. harveyi*, *V. campbellii*, and *V. rotiferianus* formed separated clusters, which might have arisen by accumulation of point mutations rather than by recombination.

Following these studies, Sawabe et al. (2007) included nine previously used housekeeping genes (16S rRNA, *rpoA*, *recA*, *pyrH*, *gapA*, *mreB*, *ftsZ*, *gyrB* and *topA*) in a broad study of the evolutionary history of vibrios. These authors identified the Harveyi clade to include seven species *V. harveyi*, *V. alginolyticus*, *V. campbellii*, *V. mytili*, *V. natriegens*, *V. parahaemolyticus*, and *V. rotiferianus* (*V. azureus* and *V. sagamiensis* and *V. owensii* were delineated later). Within this clade, the nine locus-MLSA concatenated similarity was 90.1–96.2%, and the amino acid identity was 97.2–99.4%. The sequences obtained in these three studies have been included in an online electronic taxonomy database (http://www.taxvibrio.lncc.br). Finally, Pascual et al (2010) performed MLSA with 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes from six species of the Harveyi clade and compared MLSA similarities with DDH values between species. These authors suggested that although the combination of the seven genes gave the best correlation, analysis of concatenated sequences from *ropD*, *rctB* and *toxR* offered enough resolution for identification of *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. parahaemolyticus*, *V. natriegens* and *V. alginolyticus*.

2.3 Prospects for Direct Detection of *V. harveyi* in Aquaculture Systems

Isolation of potentially pathogenic *V. harveyi* strains is essential for analysis of their genetic and phenotypic characteristics, and also for elucidation of their virulence mechanisms in relation to their specific host environment. As described above, several methods have been developed that are useful for this purpose. It is clear however that the aquaculture industry would benefit from the development of molecular techniques also for direct detection and quantification of *V. harveyi*-related populations in complex samples without initial isolation. Depending on the cost, analysis time, and technical skills required, such techniques can potentially be used for routine testing in the farm environment (Owens and Busico-Salcedo, 2006), as well as for research into the ecology of these vibrio populations. Another advantage of direct detection methods is that they can detect cells in the "viable but not culturable" (VBNC) state (Oliver, 1995). Many vibrio species enter this state when conditions for growth are poor, and potentially revert to active metabolizing cells when conditions improve. This section presents the current status for the use of direct detection techniques for vibrio communities in general, and *V. harveyi*-related populations in particular. This includes

the detection of phylogenetically defined clusters of *V. harveyi*-related strains and the specific detection of virulent strains.

2.3.1 Methods for detection of specific clusters

In the last decade, a few studies have used direct detection methods specifically targeting vibrio populations in complex samples. Thompson et al. (2004b) developed a community analysis method that combined a quantitative PCR (qPCR) specific for vibrios with quantification and separation of the amplified fragments by constant denaturant capillary electrophoresis (CDCE). This protocol was later modified for use with the more commonly available denaturing gradient gel electrophoresis (DGGE) technique (Eiler et al., 2006; Eiler and Bertilsson, 2006), in which amplified fragments are separated in a vertical polyacrylamide gel containing a denaturant gradient. These approaches were proved useful for analysing of major changes in diverse vibrio communities, but the short 16S rRNA gene fragment used in the analysis cannot resolve members of the Harveyi clade (Eiler and Bertilsson, 2006). It is possible however, that other housekeeping genes can be used to develop community profiling techniques such as CDCE, DGGE and terminal Restriction Fragment Length Polymorphism (t-RFLP) (Marsh et al., 2005) with increased resolution for vibrios.

An alternative approach for specific detection of vibrio populations in complex samples is fluorescence *in situ* hybridization (FISH), where oligonucleotide probes targeting the 16S rRNA of filter-fixed cells are visualised by epifluorescence microscopy. For instance, FISH was used to confirm the dominance of vibrios in sectioned larvae of *P. ornatus* (Webster et al., 2006). As discussed in section 2.2.2.1, there is potential to design a *V. harveyi*-specific 16S rRNA probe using the species-specific signature identified by Fukui and Sawabe (2007), though the probe specificity and signal intensity have to be empirically tested.

While methods such as CDCE, DGGE and FISH are all suitable for studying bacterial communities in complex samples, they are time-consuming and not suitable for routine monitoring purposes. In comparison, PCR-based assays can be relatively fast depending on the time needed for sample preparation and for the PCR amplification step. In real-

time PCR the fluorescently labeled amplification product is continuously detected from the very first cycles, which improves the quantification aspect of the reaction and eliminates the need for subsequent analysis of the amplification product. While real-time PCR requires specialised equipment, the technique is becoming more routine and readily available. Real-time PCR-based direct detection systems have been developed for other vibrio species (Table 2.4). For example, a real-time PCR targeting *V. vulnificus* was developed to confirm the pathogen-free status of raw oysters and also served to investigate possible methods of treatment for reducing the presence of *V. vulnificus* in seafood (Panicker et al., 2004; Panicker and Bej, 2005; Vickery et al., 2006). In an aquaculture setting, a 4 hour DNA extraction and real-time PCR amplification assay was developed for *Vibrio penaeicida* in the prawn *Litopenaeus stylirostris* and the farm environments in New Caledonia (Goarant and Merien, 2006). The single-day technique provided a decision tool for prawn farmers depending on the infection level as well as a research tool for understanding of the dynamics of the pathogen.

Fukui and Sawabe (2008) developed a real-time PCR protocol for detection and quantification of *V. harveyi*. The method discriminates between *V. harveyi* and the closely related species *V. rotiferianus* and *V. campbellii*, though high abundance of either *V. campbellii* or *V. rotiferianus* causes an underestimation of the *V. harveyi* abundance. While the development of this protocol was a major step forward for direct detection of *V. harveyi*, the detection limit of the protocol is not sufficient for an early-warning system that could detect *V. harveyi* proliferation before a potential disease outbreak (Fukui and Sawabe, 2007). There is however potential for improvement of the sample preparation step and the design of suitable primers and probes for discriminative detection of closely related species in the Harveyi clade. It seems likely that current efforts to sequence multiple housekeeping genes and the online sequence database *The Taxonomy of the Vibrios*: (http://www.taxvibrio.lncc.br/index.htm) will prove valuable for primer and probe design for direct detection methods.

To conclude, molecular methods for direct detection of phylogenetically defined clusters of *V. harveyi*-related species in complex samples are likely to be developed in the near future. Such methods may be transferable for use in aquaculture hatchery and farm environments and may even be applicable in different geographical environments.

The use of a method in any system should however always be supported by initial validation studies using isolates from the respective system.

2.3.2 Methods for detection of virulence genes

Direct detection of virulence genes in complex samples is a tool that can be used to confirm the presence of potentially pathogenic strains. It should be noted that while such a tool ideally can enable more informed risk-assessments and initiation of disease control measures, a negative result provides no guarantee that the sample is pathogen free. Molecular tools for detection of relevant virulence genes have been developed for several human pathogenic vibrios, including *V. cholerae* (Gubala, 2005; Gubala and Proll, 2006), *V. vulnificus* (Panicker et al., 2004, Panicker and Bej, 2005) and *V. parahaemolyticus* (Ward and Bej, 2005, Nordstrom et al., 2007). In each case, the tools were founded on detailed studies of the virulence mechanisms of the respective vibrio species towards the one host; human beings. In contrast, the aquaculture industry is concerned with a multitude of hosts that can be infected by *V. harveyi*-related species, which creates a more complex situation.

The relationship between the presence of virulence genes and the pathogenicity of *V. harveyi*-related strains to different hosts is still controversial (Zhang and Austin, 2000; Conejero and Hedreyda, 2004; Bai et al., 2007). In addition, the virulence of a specific *V. harveyi* strain can be attributed to a number of factors (Austin and Zhang, 2006), and several studies have shown that genomic events can drive pathogenicity in this species. For instance, non-virulent *V. harveyi* strains can become virulent after gene duplication (Zhang et al., 2001), plasmid uptake (Harris, 1993), lateral gene transfer from other bacterial species (Pizzuto and Hirst, 1995) or bacteriophage-mediated transfer of virulence genes (Oakey and Owens, 2000). In aquaculture systems, several environmental factors can serve as driving forces for such genetic exchange, including close contact between bacterial strains in mixed biofilms and animal guts, and host-pathogen interactions (Thompson et al., 2004a). As a consequence of these aspects of *V. harveyi* species virulence, any method for detection of virulence genes should preferably target a range of genes in the same assay. Recent technical advances have

made methods that target multiple genes, including multiplex PCRs (conventional or real-time) and microarrays more accessible, and rapid technological progress can be expected in this area. It is clear however that the development of techniques targeting virulence genes in *V. harveyi* require initial studies of virulence mechanisms and molecular pathogenicity of *V. harveyi* in different hosts.

2.4 Aquaculture of the Ornate Spiny Lobster *Panulirus ornatus*

Since 1970, the aquaculture industry has developed into a multi-billion dollar business and has created thousands of new jobs. According to the FAO, this industry has grown at an average rate of 7% per year from 1970 and it is set to overtake capture fisheries as a source of food fish (FAO 2005-2011). For many species such as spiny lobsters of the family Palinuridae, global demand exceeds wild harvest supply and hence there is considerable interest in the development of an aquaculture sector. In Australia spiny lobsters reach very high prices in the market and the highest production value for fishery products of around AU$400 million (ABARES, 2009). In 2010, the value of spiny lobster production dropped to AU$369 million, following a decrease in catch levels (ABARES, 2010). This year prices are expected to increase in response to lower supply in international markets but yet, meeting the increasing domestic and international consumer demand would only be possible by the development of an aquaculture sector for spiny lobster production. However, the extended larval phase of the Palinurid lobsters has so far hindered the development of a viable aquaculture sector due to the difficulties associated with the larval rearing. To date, there are no established commercial scale hatcheries for Palinurid spiny lobsters although there have been significant advances in completing the larval cycle for *Jasus edwardsii*, *Panulirus japonicus* and *P. ornatus*.

Table 2.4: Recent real-time PCRs designed for different vibrio spec

Species	Real-time PCR type	Target strains	Target genes	Samples	Reference
V. cholerae	Multiplex SYBR Green I	Pathogenic to human	Repeat in toxin (*rtxA*) Extracellular secretory protein (*epsM*) Mannose-sensitive pili (*mshA*) Toxin coregulated pilus (*tcpA*)	Pure cultures Environmental water	Gubala (2005)
	Mutiplex Molecular Beacon	Pathogenic to human	Repeat in toxin (*rtxA*) Extracellular secretory protein (*epsM*) Toxin coregulated pilus (*tcpA*)	Pure cultures Environmental water	Gubala and Proll (2006)
		Total *V. cholerae*	*V. cholerae* conserved gene (*ompW*)		
V. parahaemolyticus	Multiplexed TaqMan probes	Human pathogenic	Thermostable direct haemolysin gene (*tdh*) tdh-related haemolysin gene (*trh*)	Pure cultures Seafood (oysters)	Ward and Bej (2005)
		Total *V. parahaemolyticus*	*V. parahaemolyticys* conserved thermolabile haemolysin gene (*tlh*)		
V. vulnificus	SYBR Green I	Total *V. vulnificus*	Conserved haemolysin A gene (*vvh*)	Oyster tissue, Environm. water	Panicker et al. (2004)
	TaqMan probes	Total *V. vulnificus*	Conserved haemolysin A gene (*vvh*)	Oysters	Panicker and Bej (2005)
	FTTC-25	Pathogenic to human	16S ribosomal RNA type B	Pure cultures	Vickery et al. (2006)
V. penaeicida	SYBR Green I	Total *V. penaeicida*	16S ribosomal RNA gene (*rrs*)	Prawns (*Litopenaeus stylirostris*) Seawater, sediment pore water	Goarant and Merien (2006)
V. nigripulchritudo	SYBR Green I FRET	Total strains Pathogenic to shrimp	DNA gyrase subunit B gene (*gyrB*)	Pure cultures Seawater, sediment pore water	Goarant et al. (2007)
V. alginolyticus	SYBR Green I	Total *V. alginolyticus*	DNA gyrase subunit B gene (*gyrB*)	Seawater Seafood	Zhou et al. (2007)

The spiny or rock lobster *P. ornatus* is a high value product in South East Asian countries but the species is relatively rare in the wild. Compared to other Palinurid lobsters, this species has particularly good traits as an aquaculture candidate. It is the fastest growing of its family reaching up to one kg within 18 months after settlement, and it possesses one of the shortest larval phases on any spiny lobster species, of approximately 120-150 days 11 larval stages: I to XI (Smith et al., 2009). This lobster species is found in Indo-West Pacific waters from the Red Sea and East Africa to southern Japan, the Solomon Islands, Papua New Guinea, eastern Australia, New Caledonia and Fiji (Holthuis, 1991).

At the Australian Institute of Marine Science (AIMS, northern Qld) efforts focus on the microbiology and nutrition of *P. ornatus*. Significant advances have been made since the first production of early stage larvae at AIMS (Duggan and McKinnon, 2003). Until recently, a challenging microbial environment and poor nutrition were contributing to mass mortalities of the larvae (called phyllosoma) within 30 days of commencing a larval rearing trial (Bourne et al., 2004). Several factors of the captive larval-rearing environment contribute to high mortalities in aquaculture systems: high larval densities, excess or poor food quality, elevated water temperatures and poor water quality (Olafsen, 2001). In addition, common practices of extensive water treatment including filtering, UV radiation, chemical treatments and the use of antibiotics in procedures prior to and during larval rearing, change the microbial community of the water column. In recent years, improvements in nutrition and water treatment have considerably increased survival rates or *P. ornatus* up to mid-late stage development but production of IX and XI larval stages is still below commercial levels (Hall et al., unpublished).

As a means to understand the microbial dynamics within the larval rearing system of *P. ornatus*, the researchers at AIMS used an approach based first in initial examination by culture-based methods, histology, scanning electron microscopy (SEM), and molecular-based community studies using DGGE. The results showed proliferation of bacteria in the hepatopancreas tubule lumen associated to larval mortalities (Bourne et al., 2004). Lesions in the larvae were similar to those reported in penaeid prawn larvae and attributed to luminous vibriosis (Lavilla-Pitogo et al., 1998).

A study of the water column of the larval rearing system by flow cytometry, DGGE and clone libraries proved that the total bacterial load within the water column markedly increased in the early stages of the larval rearing trial, correlating with the beginning of the larval moult from phyllosoma stage I to stage II (Payne et al., 2006) and vibrio affiliated species were commonly retrieved in the clone libraries. This was later supported by FISH studies on phyllosoma showing vibrio proliferation in high numbers within the digestive system of the larvae a few days before mass mortality events in the tanks (Webster et al., 2006). Serious problems with vibriosis in the system were also suspected when high level of quorum sensing signal production (common for *Vibrio spp.*) were detected, coinciding with mass larval mortalities (Bourne et al., 2007). One of the potential bacterial inputs into the larval rearing system of *P. ornatus* is the common live feed organism Artemia, added to the tanks on a daily basis. Høj et al. (2009) also proved that the addition of chemically treated Artemia to the larval tanks causes a relative enrichment in vibrio cells increasing the risk of infection.

Although many different bacteria can induce mortalities within hatcheries, it is established that *V. harveyi*-related species represent the major pathogenic bacteria for penaeid larvae, juveniles and other aquatic organisms (Vandenberghe et al., 1999, 2003; Gomez-Gil et al., 2004). Diggles et al., (2000) described an outbreak of luminous vibriosis in the larvae of the New Zealand spiny lobster (*Jasus verreauxi*) and a *V. harveyi*-like strain was isolated and identified as the causative agent of the disease. Handlinger et al., (2000) also reported sporadic larval mortalities for the southern spiny lobster (*Jasus edwardsii*) due to infection with vibrio species. At AIMS, *V. harveyi*-like strains were also isolated from *P. ornatus* correlating with the beginning of the larval moult. This is a time when phyllosomas are particularly susceptible to infection as a result of the phyllosoma's external barriers to pathogen invasion being compromised (Webster et al., 2006). Bourne et al, (2006) proved that vibrios are part of bacterial biofilms formed on the walls of the larval tank and cells might slough off from these biofilms into the water a few days before mass mortality events in the tanks. Two potential pathogens were isolated from moribund larvae and biofilm during such events but they could only be identified as *V. harveyi*-like strains (Bourne et al., 2006). Finally, a study of bacterial populations of wild larvae of *P. ornatus* using clone libraries was also carried to compare bacterial populations associated with these animals in their

natural environment. Interestingly, vibrio-related sequences were rarely detected in wild caught-phyllosoma (Payne et al., 2008).

2.5 Conclusions

A range of molecular methods have been explored for use in identification and typing of the economically important marine pathogens *V. harveyi* and related species. The techniques include both whole-genome methods such as DDH, AFLP, REP-PCR and RAPD, and methods that target either individual or multiple marker genes. Promising methods for epidemiological studies of *V. harveyi*-related pathogens include RAPD and ribotyping, while MLSA is quickly emerging as the most promising identification tool. To date, little work has been done to develop molecular methods for direct detection of *V. harveyi* in complex samples. With sufficient sensitivity, such methods may be useful to monitor the levels of this organism in aquaculture systems as well as for research purposes. New molecular methods for direct detection of phylogenetically defined clusters of *V. harveyi*-related strains in complex samples are likely to be developed in the near future, driven by rapidly increasing sequence information for a range of housekeeping genes.

The design of molecular techniques for specific detection and quantification of potentially pathogenic *V. harveyi*-like strains is problematic and challenging for several reasons. First, it is important to understand the relationship between the presence of virulence genes, their expression and their virulence to different hosts, which would have to be demonstrated by a combination of traditional and molecular methods. Further studies into virulence mechanisms and molecular pathogenicity of *V. harveyi*-related species would be required; as such information is essential to develop techniques targeting multiple virulence genes. Commercial scale larval rearing of the ornate spiny lobster (*P. ornatus*) has been unsuccessful to date, due to periodic and sudden mass mortalities of the animals. During rearing, larvae are heavily colonised by bacteria, becoming susceptible to infection by opportunistic vibrio species. Internal proliferation and isolation of pathogenic *V. harveyi*-like strains coinciding with high larval mortalities have proved that vibriosis is a major constraint for successful larval rearing of *P. ornatus*.

CHAPTER 3. DESCRIPTION OF *VIBRIO OWENSII* SP. NOV.

3.1 Introduction

Several vibrio isolates from a wide range of clinical and environmental sources in Australia were being identified by the sequence analysis of several genetic markers. Preliminary results showed that two *V. harveyi*-like isolates (DY05 and 47666-1) were showing some unique nucleotides in certain positions and sequences were clustering apart from those of other *V. harveyi*-related species in the phylogenetic trees. Under the evidence that species of the *V. harveyi* group (*V. harveyi*, *V. campbellii* and *V. rotiferianus*) are commonly misidentified (Pedersen et al., 1998; Gomez-Gil et al., 2004), a detailed phenotypic and genetic characterisation was carried out for these two isolates. The strain 47666-1 was isolated from diseased larvae of *P. monodon* in a commercial prawn hatchery in northern Qld, and subsequently shown to be highly virulent to prawn larvae (Harris, 1993; Pizzuto and Hirst, 1995). Similarly, strain DY05 was isolated from diseased larvae of the ornate spiny lobster *P. ornatus* at the aquaculture facilities of AIMS (northern Qld), and subsequently shown to be highly virulent to lobster larvae (Goulden et al., 2012). At the beginning of this study, the Harveyi clade (Sawabe et al., 2007) included nine species: *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. mytili*, *V. natriegens*, and the newly described *V. azureus* and *V. sagamiensis* (Yoshizawa et al., 2009, 2010). Here, I describe the physiological, chemotaxonomic and phylogenetic characteristics of two different bacterial strains pathogenic to cultured crustacea, sharing the highest 16S rRNA gene sequence identities with *V. harveyi*, *V. campbellii* and *V. rotiferianus*.

3.2 Materials and Methods

3.2.1 Bacterial strains

The strain 47666-1 was isolated from diseased *Penaeus monodon* larvae in a commercial prawn hatchery in North Queensland, Australia, and subsequently shown to be highly virulent to prawn larvae (Harris, 1993; Pizzuto & Hirst, 1995). The strain was provided by the James Cook University. In the case of DY05^{T} the strain was isolated

from moribund stage III larvae of the ornate spiny lobster *P. ornatus* during an epizootic in the Astralian Institute of Marine Science (AIMS, North Queensland) larval rearing system. Larvae were washed and homogenized in sterile artificial seawater and plated on thiosulfate citrate bile sucrose agar (TCBS). The dominant morphotype was cultured on TCBS and cryopreserved (-80°C). Subsequently, the strain was shown to be highly virulent to lobster larvae (unpublished data).

Bacteria (strains DY05, 47666-1, *V. harveyi* LMG 4044^{T}, *V. campbellii* LMG 11216^{T}, *V. rotiferianus* LMG 21460^{T} and *V. rotiferianus* CAIM 994) were cultured on TCBS agar and MA plates at 28ºC with shaking. Stock cultures were maintained frozen at -80ºC in either marine broth (MB) with 30% (v/v) glycerol or in Microbank^{TM} cryovials (Pro-Lab Diagnostics). The authenticity of the strains was confirmed by Multilocus Sequence Analysis (MLSA; see Chapter 4).

3.2.2 Phenotypic characterisation

For morphology and physiology studies, cells were grown for 24-48 h at 28ºC on MA or in MB. Gram-staining was performed by using a Gram stain kit (Becton Dickinson, BD) according to the manufacturer's instructions. Cell morphology, size and motility were determined by light microscopy (CX31, Olympus). Luminescence was observed in the dark and measured using a 1420 Wallac Multilabel Counter (Perkin Elmer) at four h intervals. Phenotypic analyses using API 20E, API 20NE and API ZYM commercial kits (bioMérieux) were performed according to the manufacturer's instructions, except that a 2% NaCl (w/v) solution was used to prepare the inocula and the strips were incubated at 28°C for 48 h. The API 20E and 20NE were performed in triplicate, with *V. harveyi* LMG 4044^{T} and *V. campbellii* LMG 11216^{T} included as references. Salt tolerance was determined in peptone yeat (PY) broth (0.3% w/v neutralised peptone [Oxoid] and 0.1% w/v yeast extract [BD]) supplemented with NaCl concentrations between 0% and 10% w/v for 72 h at 28ºC with shaking. Growth responses to temperatures between 4ºC and 45ºC were tested in PY broth (PY) with 2% w/v NaCl for 72 h with shaking. Antibiotic sensitivity was determined using the disk susceptibility assay as described by the Clinical and Laboratory Standards Institute (CLSI, 2008a,b) for ampicillin and gentamycin (10 µg), chloramphenicol, kanamycin and

oxytetracycline (30 µg), erythromycin (15 µg), sulfisoxazole (300 µg), trimethoprim-sulfamethoxazole 1/19 (1.25/23.75 µg) and vibriostatic agent O129 (Oxoid) (10 and 150 µg). For fatty acid analyses, cells were grown for 24 h at 28ºC on Tryptone Soy Agar (TSA) medium supplemented with 1.5% NaCl (w/v). Fatty acids composition was determined by gas chromatography using the Sherlock Microbial Identification system (MIDI), according to the manufacturer's instructions (Microbial Identification Inc.)

3.2.3 16S ribosomal RNA gene and multilocus sequence analysis (MLSA)

Genomic DNA was extracted from overnight cultures grown in MB at 28°C with shaking, using the Wizard Genomic DNA Purification Kit (Promega) following manufacturer's instructions for gram-negative bacteria. The 16S rRNA genes were amplified as described by Lane et al. (1991) and sequenced using the 27f and 1492r oligonucleotides as sequencing primers. For the MLSA, the five protein-coding loci *rpoA* (RNA polymerase alpha-subunit), *pyrH* (urydilate kinase), *topA* (topoisomerase I), *ftsZ* (cell division protein FtsZ), and *mreB* (rod shaping protein MreB) were used. Genes were amplified by PCR and sequenced as described for *rpoA* and *pyrH* genes (Thompson et al., 2005), and *topA*, *ftsZ* and *mreB* genes (Sawabe et al., 2007). In addition, sequencing of 16S rRNA and *rpoA* genes was carried out for *V. rotiferianus* strain CAIM 994. Sequences of other protein-coding loci for this strain were retrieved from public databases (GenBank and http://www.taxvibrio.lncc.br/). Sequences generated in this study have been deposited in GenBank under the accession numbers GU018180-GU018182 and GU111249-GU111259 (Table 3.1). Sequences were initially aligned with those of their closest relatives available in GenBank using the BLASTN program (Altschul et al., 1990). Subsequently, sequences of the two unknown strains, close relatives, and type strains of related vibrios were aligned by ARB (Strunk et al., 2000) or Clustal_X (Thompson *et al*., 1997) for 16S rRNA and protein-coding genes, respectively. For ARB alignments, manual corrections were performed where necessary based on 16S rRNA secondary structure. Phylogenetic analyses were performed with PAUP v.4.0B10 (Swofford, 2003). Distance matrices were generated according to the Kimura-two-parameter correction (K2P) (Kimura, 1980) and phylogenies were constructed by neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony

(MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1973) methods. Stability of groupings was estimated by bootstrap analyses (1000 replications).

3.3 Results and Discussion

3.3.1 General description of *Vibrio owensii* sp. nov.

Vibrio owensii (o.wens'i.i. N.L. gen. n. *owensii* from Owens, named after the Australian microbiologist Leigh Owens, a specialist in the biology of *V. harveyi*-related species). Cells are slightly curved gram-negative rods, 1.0 µm wide by 3.1 µm long, facultative anaerobic and motile by means of at least one flagellum. After growth for 48 h at 28ºC, the strains form translucent (DY05) or opaque (47666-1), non-luminescent, non-swarming, smooth and round colonies (2-3 mm) on MA, and bright, yellow, round colonies (2-3 mm) on TCBS agar plates. Growth occurs in the presence of 1-8% NaCl (w/v) but not at 0 or 10% NaCl. Minimum temperature for growth is 12-15ºC while maximum temperature for growth is 35-37ºC. No growth occurs at 4ºC. Both strains are arginine dihydrolase-negative, lysine- and ornithine- decarboxylase-positive. Tests for citrate utilization, production of H_2S, urease, Voges-Proskauer and acid production from inositol, sorbitol, rhamnose, melobiose and arabinose are negative, while tests for nitrate reduction, indole production, tryptophane deaminase, gelatinase, oxidase, hydrolysis of esculin, assimilation of glucose, mannose, mannitol, potassium gluconate and malate, and fermentation of glucose, mannitol, sucrose and amygdalin are positive. Enzyme activities detected by API ZYM tests are alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrypsin, acid phosphatase and naphtol-AS-β1-phosphohydrolase. A difference between strains was seen for the ortho nitrophenyl-β-D-galactopyranosidase (ONPG) test, which was positive for 47666-1 and negative for DY05. Both strains were susceptible to chloramphenicol (30 µg), gentamycin (10 µg), sufisoxazole (300 µg), trimethoprim-sulfamethoxazole (1/19) (1.25-23.75 µg), and tetracycline (30 µg) and vibriostatic agent O/129 (10 and 150 µg); intermediate to erythromycin (15 µg) and kanamycin (30 µg) and resistant to ampicillin (10 µg). Major fatty acids (>1% for at least one strain) are Summed Feature 3 ($C_{16:1}$ ω7c and/or C_{15} iso 2-OH), $C_{16:0}$, $C_{18:1}$ ω7c, $C_{14:0}$, $C_{16:0}$ iso, $C_{12:0}$, Summed feature 2 ($C_{14:0}$ 3-OH, and/or $C_{16:1}$ iso I), $C_{17:0}$ iso, $C_{17:1}$ ω8c, $C_{17:0,}$ $C_{12:0}$ 3-OH, and $C_{18:0}$,. The DNA G + C content is

45.3-45.9 mol%. Strains DY05 and 47666-1 show 76% DNA-DNA hybridization values with each other and 44-55% with *V. harveyi* LMG 4044^T, *V. campbellii* LMG 11216^T and *V. rotiferianus* LMG 21460^T. The type strain is DY05^T (= JCM 16517^T = ACM 5300^T = DSM 23055^T), isolated from cultured larvae of the ornate spiny lobster *P. ornatus* in northern Qld, Australia. The description of the species was published in the journal FEMS Microbiology Letters (302, 175-181) and the species was validated by the journal International Journal of Systematic and Evolutionary Microbiology (Validation List No 132; 60, 469-472).

3.3.2 Phenotypic characteristics

Phenotypically, strains DY05 and 47666-1 (Gram negative, oxidase-positive, glucose-fermenting and grows on TCBS agar) can be clearly assigned to the genus *Vibrio* (Alsina and Blanch, 1994a,b). Characters distinguishing DY05 and 47666-1 from other strains in the Harveyi clade are presented in Table 3.2. The strains can be distinguished from most other arginine dihydrolase (ADH) negative, ornithine and lysine decarboxylase (ODC and LDC) positive vibrios by their inability to utilise citrate and their ability to produce acid from amygdalin. The latter characters are shared with *V. rotiferianus* and *V. azureus*, but DY05 and 47666-1 can be distinguished from these species by several tests including LDC (both species) and acid production from arabinose (*V. rotiferianus*), sucrose and mannitol (*V. azureus*). It should be noted that 15 out of 62 previously classified *V. harveyi* "biovar I" strains were reported to be positive for amygdalin (Carson et al., 2006) and further genotypic analyses would be useful to determine relatedness between these strains and the newly described species. Strains DY05 and 47666-1 showed similar biochemical profiles, except for the ortho nitrophenyl-*β*-D-galactopyranosidase (ONPG) test, which was positive only for strain 47666-1.

Table 3.1: *Vibrio* strains and accession numbers included in the MLSA

Species, strain	Accession number for gene				
	rpoA	***pyrH***	***topA***	***ftsZ***	***mreB***
Vibrio sp. DY05^{T}	GU111249	GU111252	GU111254	GU111256	GU111258
Vibrio sp. 47666-1	GU111250	GU111253	GU111255	GU111257	GU111259
Vibrio sp. CAIM994	GU111251	EF596721	EF596732	EF596702	EF596716
V. harveyi LMG4044^{T}	AJ842627	EU118238	DQ907488	DQ907350	DQ907422
V. campbellii LMG11216^{T}	AJ842564	EF596641	DQ907475	DQ907337	DQ907408
V. rotiferianus LMG21460^{T}	AJ842688	EF596722	DQ907515	DQ907372	DQ907445
V. alginolyticus LMG4409^{T}	AJ842558	*	DQ907472	EF027344	DQ907405
V. parahaemolyticus LMG2850^{T}	Aj842677	EU228240	DQ907509	DQ907367	DQ907440
V. natriegens LMG10935^{T}	AJ842658	*	DQ907500	DQ907359	DQ907432
V. proteolyticus LMG3772^{T}	AJ842686	*	DQ907514	EF114210	DQ907444
V. vulnificus LMG13545^{T}	AJ842737	EU118244	DQ907522	DQ907382	DQ907454
V. mytili LMG19157^{T}	AJ842657	*	DQ907499	DQ907358	DQ907431
A. fischeri LMG4414^{T}	AJ842604	EF415528	DQ907482	DQ907344	DQ907415
P. phosphoreum LMG4233^{T}	AJ842551	EF380239	DQ907495	DQ907326	DQ907393

*Sequences retrieved from the database “The Taxonomy of Vibrios” (http://www.taxvibrio.lncc.br/).

Table 3.2: Differential characters between DY05 and 47666-1 and close species

Test	1	2	3	4	5	6	7	8	9	10
Lysine decarboxylase	+	+	+	v	-	+	+	-	-	-
Ornithine decarboxylase	+	+	v	-	+	v	+	-	-	-
Urease	-	-	v	-	+	-	V	-	v	-
Voges Proskauer*	-	-	-	-	-	+	-	-	-	-
Growth at:										
0% NaCl (w/v)*	-	-	-	-	-	v	V	v	-	-
7% NaCl (w/v)	-	-	v	-	-	+	V	v	-	+
Citrate utilization*	-	-	+	+	-	+	+	+	-	+
Assimilation of:										
L-arabinose*	-	-	-	-	+	-	+	+	nd	v
D-mannitol	+	+	+	+	-	+	+	nd	nd	nd
D-mannose	+	+	+	v	-	+	+	v	nd	-
Acid from:										
L-arabinose	-	-	-	-	+	-	+	+	-	+
D-sucrose*	+	+	v	-	nd	+	V	+	-	+
Amygdalin	+	+	v	-	+	-	-	+	+	+
D-mannitol	+	+	+	nd	nd	+	+	+	-	nd
Activity of:										
Esterase (C4)	w	+	-	+	+	-	+	-	-	nd
α-chymotrypsin	+	+	-	+	+	-	-	-	-	nd
Acid phosphatase	+	+	+	-	+	+	+	+	-	nd

Strains: 1: 47666-1, 2: DY05, 3: *V. harveyi*, 4: *V. campbellii*, 5: *V. rotiferianus* (data from Gómez-Gil et al., 2003), 6: *V. alginolyticus*, 7: *V. parahaemolyticus*, 8: *V. natriegens*, 9: *V. azureus* (data from Yoshizawa et al., 2009), 10: *V. mytili*. +, positive; -, negative; v, variable; w, weak reaction; nd, no data.*Test used to differentiate ADH-, LDC+, ODC+ vibrio species (Alsina and Blanch, 1994a,b). Data of other species from Carson et al., (2006) except if otherwise indicated.

The predominant fatty acids of strains DY05 and 47666-1 were $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7 (36.6-37.5%), $C_{16:0}$ (16.6-16.7%), $C_{18:1}$ ω7 (14.6-16.4%), and $C_{14:0}$ (6.0-6.3%). For other fatty acids see description of species and Table 3.3. No clear differences from the closely related species *V. harveyi*, *V. campbellii* and *V. rotiferianus* grown under identical conditions (Gomez-Gil et al. 2003) were observed. None of the strains showed luminescence. Strain 47666-1 was originally reported as luminescent (Harris, 1993) but this was not confirmed in this study.

Table 3.3: Fatty acid composition of DY05 and 47666-1 and related species

	Strain DY05^T	Strain 47666-1	*Vibrio rotiferianus*	*Vibrio harveyi*[a]	*Vibrio campbellii*[a]
12:0	2.3	3.2	-		
14:0	6.3	6.0	9.5	4.9	4.3
16:0	16.7	16.6	25.4	13.9	17.0
17:0	1.8	1.2	-		
18:0	1.0	-	1.1		
13:0 iso	1.0	1.3	-		
15:0 iso	1.6	1.0	-		
16:0 iso	3.5	3.8	-		
17:0 iso	2.0	1.7	-		
12:0 3OH	1.0	1.6	2.9		
17:1 ω8c	1.7	1.3	-		
18:1 ω7c	14.6	16.4	10.8	21.1	22.6
Summed Feature 2[b]	2.2	3.0	7.1		
Summed Feature 3[c]	37.5	36.6	37.1		

Data reported by Gomez-Gil et al. (2003) for other related species

[a]No data is shown where the fatty acid composition was reported as similar to *V. rotiferianus* (Gomez-Gil et al. 2003); [b]$C_{14:0}$ 3-OH, and/or $C_{16:1}$ iso I; [c]$C_{16:1}$ ω7c and/or $C_{15:0}$ iso 2-OH. Data are expressed as percentages of total fatty acids. Percentages <1% are not shown. All strains were grown on TSA supplemented with 1.5% NaCl at 28°C for 24 h.

3.3.3 Phylogenetic analysis

The 16S rRNA gene sequence analysis showed that strains DY05 and 47666-1 belong to the Harveyi clade. The strains shared 99.2-99.5% 16S rRNA gene sequence identities with other species of this clade but the two strains formed a monophyletic group with 99% bootstrap support (Fig. 3.1) and 100% 16S rRNA gene sequence identity, supporting their close affiliation. The mean sequence identity for the concatenated five protein-coding loci was 98.8% between strains DY05 and 47666-1 and 94.4% between these strains and the relatives *V. harveyi*, *V. campbellii* and *V. rotiferianus*. Discrimination between these species on the basis of phenotypic and 16S rRNA gene analyses is difficult and additional molecular methods such as MLSA have become important tools for correct species delineation and identification (Sawabe et al., 2007; Thompson et al., 2007). Phylogenetic trees generated for concatenated sequences of the five protein-coding loci using NJ, MP and ML methods confirmed the clustering of *V.*

owensii strains DY05 and 47666-1 (bootstrap values of 100%, 100%, and 95%, respectively) and their distinction from close species (Fig 4.2 and 4.3a,b).

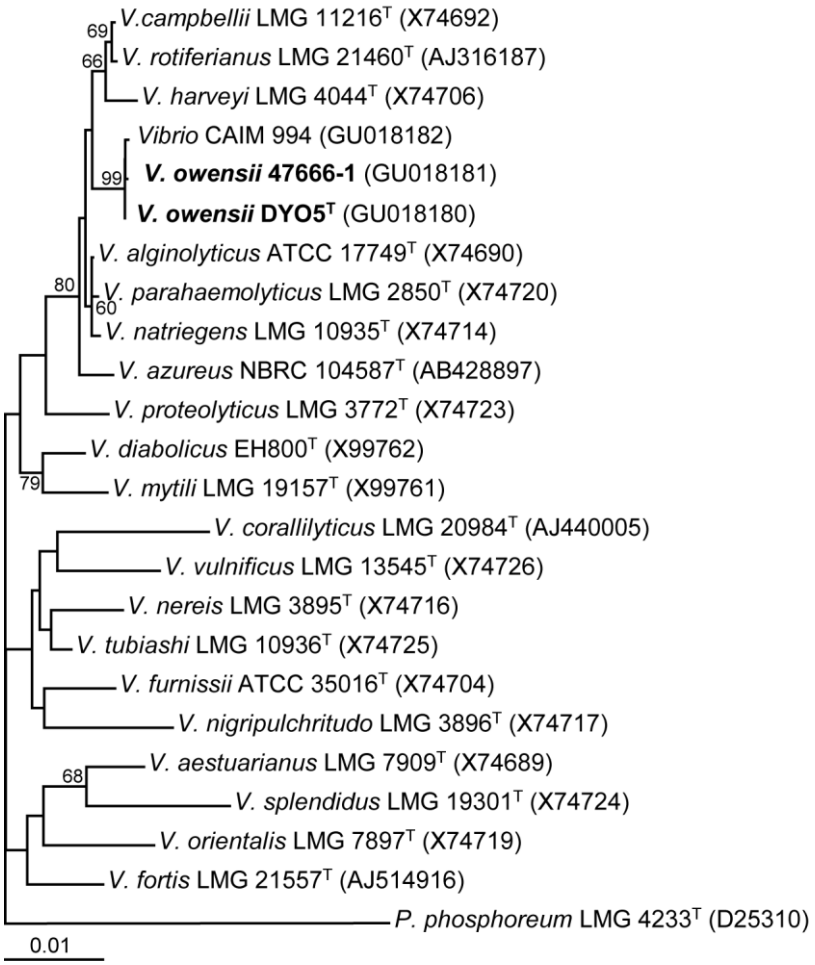

Figure 3.1: Phylogenetic analysis based on partial 16S rRNA gene sequences showing relationships between *V. owensii* strains and related species

Analysis based on the neighbor-joining algorithm and the Kimura-two-parameter correction. GenBank accession numbers provided in parentheses. *Photobacterium phosphoreum* LMG 4233^T used as an outgroup. Bootstrap support values after 1000 simulations are shown. Bar, 1% sequence divergence.

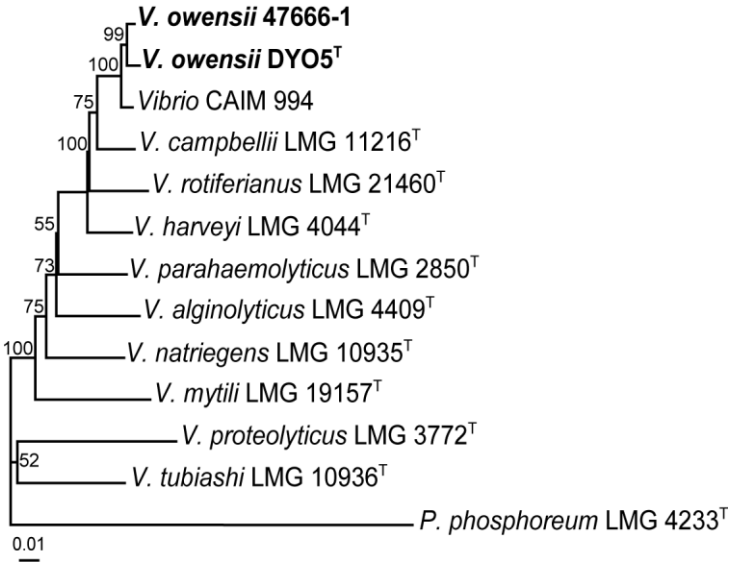

Figure 3.2: Phylogenetic analysis based on the concatenated gene sequences showing relationships between *V. owensii* strains and related species

Genes: *rpoA* (884 bp), *pyrH* (421 bp), *topA* (587 bp), *ftsZ* (443 bp), and *mreB* (507 bp) loci (total length, 2842 bp). GenBank accession numbers provided in Table 3.1. Phylogenetic analysis based on the neighbor-joining algorithm and the Kimura-two parameter correction. *Photobacterium phosphoreum* LMG 4233^T used as an outgroup. Bootstrap support values after 1000 simulations are shown. Bar, 1% sequence divergence.

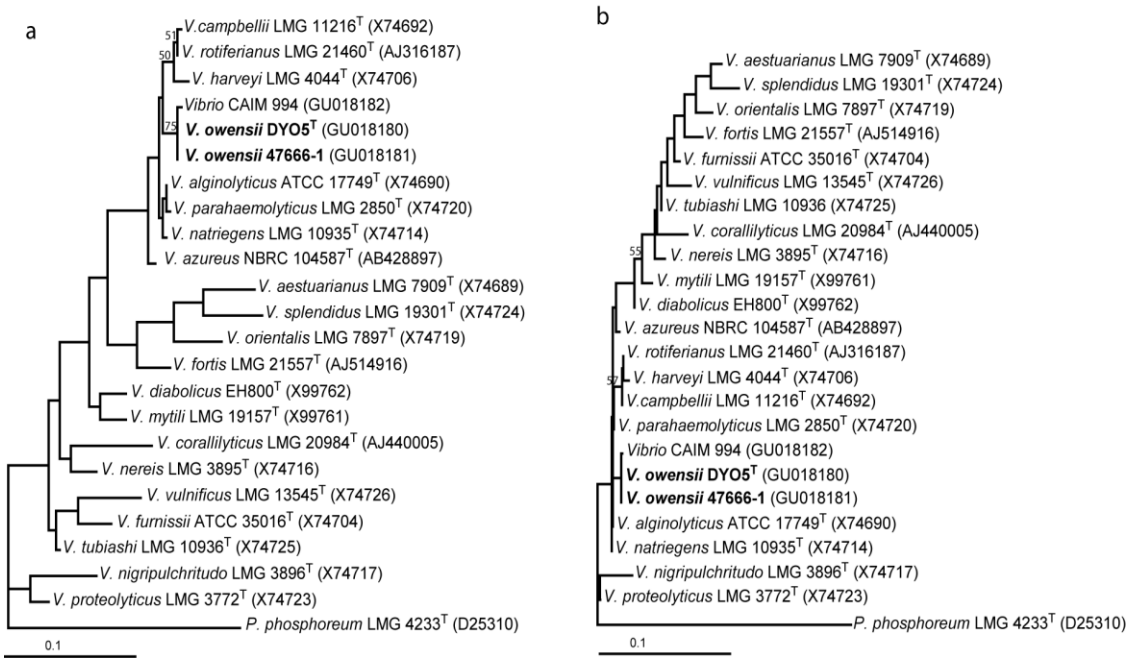

Figure 3.3: Phylogenetic analysis based on the a) maximum-parsimony and b) maximum-likelihood methods, using concatenated sequences from *V. owensii* and related species

Genes: *rpoA* (884 bp), *pyrH* (421 bp), *topA* (587 bp), *ftsZ* (443 bp), and *mreB* (507 bp) loci (total length, 2842 bp). *Photobacterium phosphoreum* LMG 4233^T used as an outgroup. Bootstrap support values after 1000 simulations are shown. Bar, 10% sequence divergence.

An extended phylogenetic analysis was undertaken to detect sequences from the public databases that could potentially belong to the same species as strains DY05 and 47666-1. Using database sequences for the *pyrH*, *topA*, and *mreB* loci, *Vibrio sp.* CAIM 994 clustered with DY05 and 47666-1 in single-gene phylogenetic analyses. Thus, this strain, which had been isolated from snapper (*Lutjanus guttatus*) in the northwest coast of Mexico, was acquired and and its 16 rRNA and *rpoA* genes were sequenced. Strain CAIM 994 was initially identified as *V. rotiferianus* but described as a possible intermediate strain according to MLSA studies (Thompson et al., 2007). Phylogenies based on 16S rRNA gene and five protein-coding loci concatenated sequences confirmed that CAIM 994, 47666-1 and DY05 formed a monophyletic group with bootstrap support values of 99-100% (Fig. 3.1 and 3.2). CAIM 994 shared 99.9% (16S rRNA) and 98.3% (five protein-coding loci) gene sequence identities with DY05 and 47666-1. These are greater than identities shared between CAIM 994 and *V. rotiferianus* LMG 21460^T (99.4% for 16S rRNA and 93.2% for five protein-coding loci). Therefore, 16S rRNA and multilocus sequence analyses supported the notion that

CAIM 994 was previously misidentified. Further studies based on phenotypic and genotypic characterisation would be required to clarify relatedness of this and other strains clustering with the *V. owensii* sp. nov. proposed here.

3.3.4 DNA-DNA hybridization and DNA base composition

Strains DY05 and 47666-1 showed 76% DDH values with each other and 44-55% with *V. harveyi* LMG 4044^T, *V. campbellii* LMG 11216^T and *V. rotiferianus* LMG 21460^T (Table 3.4). As a DDH value of 70% is generally accepted as the limit for species delineation (Wayne et al., 1987), it can be concluded that strains DY05 and 47666-1 belong to a single novel species. The DNA mol% G + C of DY05^T (45.3 mol%) and 47666-1 (45.9 mol%) support their affiliation with *Vibrio* (Baumann and Schubert, 1983).

Table 3.4: DNA-DNA hybridization values among *V. owensii* and related species

Strain	G + C content (mol%)*	DNA similarity % with:				
		1	2	3	4	5
1. *V. owensii* 47666-1	45.9	100				
2. *V. owensii* DY05^T	45.3	76	100			
3. *V. harveyi* LMG 4044^T	46-48	55	53	100		
4. *V. campbellii* LMG 11216^T	46-48	52	51	52	100	
5. *V. rotiferianus* LMG 21460^T	44.5 ± 0.01	46	44	49	47	100

* Data described by Farmer et al. (2005) and Gomez-Gil et al. (2003).

It can be concluded that strains DY05 and 47666-1 are closely related to *V. harveyi*, *V. campbelli* and *V. rotiferianus* in terms of 16S rRNA gene sequences and phenotypic profiles, but that they can be differentiated from all vibrios previously described by means of MLSA (*rpoA*, *pyrH*, *topA*, *ftsZ* and *mreB* genetic loci), DNA-DNA reassociation experiments and several biochemical characters. The strains can be identified by performing tests for lysine and ornithine decarboxylases, citrate utilization, and acid production from amygdalin, arabinose and sucrose (API 20E system). Based

on these results, strains DY05 and 47666-1 clearly represent a novel species of the genus *Vibrio*, for which the name *Vibrio owensii* sp. nov. is proposed.

3.4 Conclusions

Bacterial strains, DY05^T and 47666-1 were isolated in Qld from diseased cultured crustacea *P. ornatus* and *P. monodon*, respectively. On the basis of 16S rRNA gene sequence identity, the strains were shown to belong to the Harveyi clade of the genus *Vibrio*. A MLSA approach using five housekeeping genes (*rpoA, pyrH, topA, ftsZ*, and *mreB*) showed that the strains form a monophyletic group with 94.4% concatenated sequence identity to the closest species. DDH experiments showed that strains DY05^T and 47666-1 had 76% DNA similarity with each other but < 70% with their closest neighbours *V. harveyi* LMG 4044^T (≤ 55%), *V. campbellii* LMG 11216^T (≤ 52%) and *V. rotiferianus* LMG 21460^T (≤ 46%). Strains DY05^T and 47666-1 could be differentiated from their relatives on the basis of several phenotypic characters. Major fatty acids were $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ ω7, $C_{16:0}$, $C_{18:1}$ ω7, and $C_{14:0}$. Based on the polyphasic evidences presented here, it can be concluded that strains DY05^T and 47666-1 belong to the same novel species of the genus *Vibrio*, for which the name *Vibrio owensii* sp. nov. is proposed. The type strain is DY05^T (= JCM 16517^T = ACM 5300^T= DSMZ). The species was validated in the IJSEM, validation list No. 132 (2010).

CHAPTER 4. IDENTIFICATION OF *VIBRIO HARVEYI*-RELATED SPECIES BY MULTILOCUS SEQUENCE ANALYSIS

4.1 Introduction

Among the Harveyi clade of vibrios, species belonging to the *V. harveyi* group (*V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii*) are almost indistinguishable phenotypically and genotypically, and some strains from the same species exhibit variable phenotypic features (Alsina and Blanch, 1994a,b; Gomez-Gil et al., 2004). In clinical cases, detection and identification of *V. harveyi*-related strains used to rely on conventional biochemical tests or universal 16S rRNA gene sequencing, frequently leading to misidentification of isolates (Pedersen et al., 1998; Vandenberghe et al., 2003; Thompson et al., 2007). Furthermore, molecular techniques based on other single marker genes may occasionally not be species-specific due to the occurrence of recombination events among close species (Sawabe et al., 2007). For the aforementioned reasons, it is here hypothesised that strains belonging to the *V. harveyi*-related group, might have been misidentified as *V. harveyi* or simply classified as *V. harveyi*-like in laboratories and culture collections in the past.

In the last decade, identification of closely related bacterial strains within species has instead consisted of initial assignment to a genus or clade based on 16S rRNA gene sequencing or biochemical profiling, followed by assignment to a species via multilocus sequence analysis (MLSA). This strategy has been successfully adopted in all recent delineations of vibrio species (Beaz-Hidalgo, 2009; Yoshizawa et al., 2009, 2010). MLSA employs the phylogenetic analysis of concatenated sequences from several housekeeping genes in order to assign multi-locus genotypes to the species or genus levels (Gevers et al., 2005). Candidate loci should be single-copy genes of suitable phylogenetic content and preferentially accumulate neutral substitutions.

It is established that *V. harveyi*-related species represent the major pathogenic bacteria for penaeid larvae and juveniles and other aquaculture species (Bachère, 2003; Vandenberghe et al., 2003). At AIMS, *V. harveyi*-related infections contribute to high

larval mortalities of the ornate spiny lobster (*P. ornatus*) during experimental rearing trials (Bourne al., 2004; Hall et al., unpublished). Potentially pathogenic strains were isolated from moribund larvae and biofilm during one of these events but these could only be identified as *V. harveyi*-like isolates (Bourne et al., 2006). Initially, this study aimed to precisely identify these and other *V. harveyi*-like bacteria associated to the larval rearing system of *P. ornatus*. The study was then complemented with other isolates from the AIMS and JCU collection, which include the widest range of clinical and environmental *V. harveyi*-like isolates across Australia. Finally, worldwide database strains were added to the study in order to offer a global report of previous *V. harveyi*-related misidentification cases in culture collections around the world.

Specifically, the study makes use of 16S rRNA and five protein-coding loci (*rpoA*, *pyrH*, *topA*, *ftsZ*, and *mreB*) to produce multilocus genotypes from 36 *V. harveyi*-like strains isolated in Australia from diverse clinical and environmental sources. Thereafter, MLSA is performed to: a) identify *V. harveyi*-like strains associated with the larval rearing system of the ornate spiny lobster *P. ornatus*, b) explore the levels of genetic diversity of the *V. harveyi* group in Australia and c) evaluate the resolution power of the DNA regions employed for the discrimination of species within this cryptic bacterial group. The taxonomic status of several vibrios, including important pathogenic strains to marine cultured species is herein reconsidered. Based on global phylogenies, I propose a minimum number of genes capable of convenient yet reliable identification of *V. harveyi*-related species.

4.2 Materials and Methods

4.2.1 *Vibrio* isolates

DNA sequences were produced from 36 *V. harveyi*-like isolates obtained from clinical and environmental sources in Australia (Table 4.1). Stock cultures were maintained frozen at -80ºC in either MB with 30% (v/v) glycerol or in Microbank™ cryovials (Pro-Lab Diagnostics). Bacteria were grown overnight on thiosulfate-citrate-bile-sucrose (TCBS) agar plates and the colony morphology was recorded for each isolate after 24, 48 and 72 h. For DNA extraction, individual colonies picked after 24 h were used as inoculum for liquid cultures in MB and incubated overnight at 28ºC with shaking.

Table 4.1: List of vibrio isolates, collection sites and date of isolation

Strain	Species[a]	Origin, collection, (Year of isolation)
TC	*V. harveyi*	Sea water (Townsville), JCU (2005)
9056015	*V. harveyi*	Fish skin ulcers *Lates calcarifer*, JCU (1990)
9050405:5	*V. harveyi*	
645	*V. campbellii*	Environmental seawater, JCU (1995)
CCS02	*V. campbellii*	Diseased fish skin, *L. calcarifer*, JCU (2007)
CCS03	*V. campbellii*	
642	*V. campbellii*	Diseased prawn larva *Penaeus monodon*, JCU (1986)
A1	*V. campbellii*	Sea water (Townsville, Australia), JCU (1986)
12	*V. harveyi*	
20	*V. harveyi*	
47666-1	*V. owensii*	Diseased prawn larvae *P. monodon*, JCU (1993)
92-47426	*V. owensii*	
B2	*V. campbellii*	Healthy mudcrab larvae *Scylla serrata*, JCU (2005)
M2	*V. campbellii*	
C036	*V. harveyi*	Moribund lobster larva *P.ornatus*, AIMS (2004)
oz01	*V. campbellii*	Moribund lobster larvae *P. ornatus*, AIMS (2006)
oz08	*V. rotiferianus*	
oz09	*V. rotiferianus*	
oz11	*V. rotiferianus*	
oz12	*V. rotiferianus*	
D12	*V. harveyi*	Moribund lobster larvae *P. ornatus*, AIMS (2005)
D15	*V. harveyi*	
D16	*V. harveyi*	
D24	*V. harveyi*	
D34	*V. harveyi*	
D40	*V. harveyi*	
oz07	*V. campbellii*	Ongrown Artemia, AIMS (2006)
C071	*V. harveyi*	Aquaculture tank biofilm, AIMS (2004)
C069	*V. rotiferianus*	
H20	*V. harveyi*	Healthy lobster larvae *P. ornatus*, AIMS (2005)
H22	*V. harveyi*	
H28	*V. harveyi*	
C001	*V. campbellii*	Wild lobster larva *P. ornatus* (Coral Sea), AIMS (2004)
RR2	*V. rotiferianus*	Wild lobster larvae *P. ornatus* (Coral Sea), AIMS (2005)
RR36	*V. harveyi*	
R16	*V. campbellii*	Sea water (Coral Sea), AIMS (2005)

[a] Strains identified in this study by MLSA, initially classified as *V. harveyi* or *V. harveyi*-like.

4.2.2 DNA extraction, PCR amplification and sequencing

Bacterial DNA was extracted from overnight cultures using the Wizard Genomic DNA Purification Kit (Promega) following manufacturer's instructions for gram-negative bacteria. PCR amplification and sequencing of the 16S rRNA gene were carried out as

described in Lane et al. (1991). The *rpoA* and *pyrH* genes were amplified and sequenced as previously described by Thompson et al. (2005) and the *topA*, *ftsZ* and *mreB* loci were amplified and sequenced following Sawabe et al. (2007) (Table 4.2). All PCR amplifications were performed in a Perkin Elmer Applied Biosystems GENEAMP PCR System 9700 (Perkin Elmer, USA). PCR reactions (20 µl) contained approximately 20 ng of genomic DNA, 1X PCR buffer (Tris·Cl, KCl, $(NH_4)2SO_4$, 1.5 mM $MgCl_2$; pH 8.7) (Qiagen), 0.5 µM of each primer, 200 µM dNTPs and 0.5 units of Taq DNA Polymerase (Qiagen). PCR products were visually inspected in 1% agarose gels and finally, purified and sequenced by Macrogen Ltd (Korea) with specific primers (Table 4.2).

Table 4.2: List of amplification and sequencing primers

Gene (gene product) length	Primer name	Primer sequence (5'-3')	Annealing T (ºC)	Reference
16S rRNA (16S ribosomal RNA) 1421nt	27F	AGAGTTTGATCCTGGCTCAG	54	Lane et al. 1991
	1492R	GGTTACCTTGTTACGACTT		
rpoA (RNA polymerase alpha subunit) 1,000 nt	rpoA-01-F	ATGCAGGGTTCTGTDACAG	55	Thompson et al. 2005
	rpoA-03-R	GHGGCCARTTTTCHARRCGC		
	rpoA-05-F (seq.)	GCAGCDCGTGTWGARCARCG		
	rpoA-06-R (seq.)	CGYTGYTCWACACGHGCTGC		
pyrH (uridylate kinase) 750 nt	pyrH-02-R	GTRAABGCNGMYARRTCCA	55	
	pyrH-04-F	ATGASNACBAAYCCWAAACC		
topA (topoisomerase I) 800 nt	VtopA400F	GAGATCATCGGTGGTGATG	50	Sawabe et al. 2007
	VtopA1200R	GAAGGACGAATCGCTTCGTG		
ftsZ (cell division protein FtsZ) 750-600 nt	VftsZ75F	GCTGTTGAACACATGGTACG	50	
	VftsZ800R	GCACCAGCAAGATCGATATC		
	VftsZ700R[a]	ATCATGGCGTGACCCATTTC		
mreB (rod shaping protein MreB) 1000 nt	VmreB12F	ACTTCGTGGCATGTTTTC	50	
	VmreB999R	CCGTGCATATCGATCATTTC		

[a] The set VftsZ75F and VftsZ700R was used for amplification of the *ftsZ* locus of *V. owensii* strains (47666-1 and 92-47426) resulting in a 600 bp PCR product. The rest of the isolates were amplified with the set VftsZ75F and VftsZ800R.

4.2.3 Phylogenetic analysis

Electropherograms were assembled in Sequencher 4.9 (Gene Codes). Sequences were manually corrected and trimmed, and BLASTN searches were performed against public databases for preliminary identification. Publicly available DNA sequences of the 16S

rRNA gene and the selected five protein-coding genes were obtained for 15 and 13 type-strains respectively (Table 4.3). The former included eleven species belonging to the Harveyi clade including the recently described *V. owensii* (Chapter 3) and *V. communis* sp. nov. (Chimetto et al., 2011). Sequences were aligned using ARB (Ludwig et al., 2004) or ClustalX (Thompson et al., 1997) for 16S rRNA and protein-coding gene sequences, respectively. For the MLSA, the incongruence length difference test (ILD) (Farris et al., 1994) was employed to confirm congruence among individual loci to form a concatenated data set.

Maximum-parsimony (MP; Fitch 1971) and maximum likelihood (ML; Felsenstein, 1973) phylogenies were computed in PAUP* v.4.0B10 for Windows, and Bayesian inference (BI), for posterior probability estimates of the nodes, in MrBayes v3.1.2 (Huelsennbeck and Ronquist, 2001). Bootstrap (BT) support for individual nodes in MP and ML was calculated on 1000 replicates. Analyses engaged i) the 16S rRNA gene; ii) each of the five protein-coding loci (*rpoA*, *pyrH*, *topA*, *ftsZ* and *mreB*) separately; iii) all the five protein-coding loci in a single concatenated alignment and iv) several combinations of the five genes, in order to evaluate the potential of a simpler routine identification procedure for *V. harveyi*-related species. In the latter case, loci were added based on their resolution power and level of phylogenetic signal (proportion of parsimony informative sites, % PIS). Bayesian phylogenies were inferred from a dataset with 84 additional publicly available sequences (57 *V. harveyi* strains, 24 *V. campbellii* strains and three *V. rotiferianus* strains) to test the reliability of a potential two-locus combination of genes for global *V. harveyi*-related species identification.

For Maximum Parsimony (MP) analysis, trees were inferred using the heuristic search option, 500 random sequence additions and tree bisection-reconnection (TBR) branch swapping. Characters were unweighted and treated as unordered; gaps were treated as missing data. For ML and BI analyses, first different nested model of DNA substitution were compared in a hierarchical hypothesis-testing framework in ModelTest v3.7 (Posada and Crandall, 1998). Then the Likelihood ratio test (LRT) and the Bayesian information criterion (BIC) were used to identify the evolutionary model that fits the data best for ML and BI respectively. Model-constrained ML heuristic searches were run in PAUP* v.4.0B10 under 10 random additions and TBR branch swapping. Model-constrained BI was conducted for 5,000,000 generations, two parallel runs of four

chains each, sampling every 1,000th generation. The convergence of the parameter estimates was graphically confirmed by plotting values of likelihood against the generation time in Tracer v1.5 (Rambaut and Drummond, 2007).

For each phylogenetic reconstruction, bootstrap (BT) support for individual clades in MP an ML was calculated on 1,000 replicates using the same methods, options and constraints as used in the tree-inferences but with all identical sequences removed (Felsenstein, 1985). When topological incongruence was recovered between two phylogenies, a recombination episode was assumed and the RDP v3.44 (Heath et al., 2006) software was deployed to identify recombinant sequences and recombination breakpoints using a range of different recombination detection methods (RDP, BOOTSCAN, MAXCHI, CHIMAERA, GENECONV, SISCAN). The method is able to characterise the recombination events that are evident within a sequence alignment without any prior indication of a non-recombinant set of reference sequences. Recombination events were considered feasible only when they were identified by three out of the six aforementioned methods at $p < 0.01$.

Finally, values of sequence similarity based on the Kimura-two parameter correction were computed in PAUP* and plotted in Microsoft-EXCEL to graphically present inter- and intra-species similarities for each gene and gene-combination, following Martens et al., (2007).

Table 4.3: List of type-strains and accession numbers included in the MLSA

	Accession number for gene					
Type-strain	**16S rRNA**	***rpoA***	***pyrH***	***topA***	***ftsZ***	***mreB***
V. owensii JCM16517^T	GU018180	GU111249	GU111252	GU111254	GU111256	GU111258
V. harveyi LMG4044^T	X74706	AJ842627	EU118238	DQ907488	DQ907350	DQ907422
V. campbellii LMG11216^T	X74692	AJ842564	EF596641	DQ907475	DQ907337	DQ907408
V. rotiferianus LMG21460^T	AJ316187	AJ842688	EF596722	DQ907515	DQ907372	DQ907445
V. communis R-40496^T	GU078672	GU078697	EU251617	GU078704	AB609124	GU078686
V. alginolyticus LMG4409^T	X74690	AJ842558	[a]	DQ907472	EF027344	DQ907405
V. parahaemolyticus LMG2850^T	X74720	AJ842677	EU228240	DQ907509	DQ907367	DQ907440
V. natriegens LMG10935^T	X74714	AJ842658	[a]	DQ907500	DQ907359	DQ907432
V. mytili LMG19157^T	X99761	AJ842657	[a]	DQ907499	DQ907358	DQ907431
V. proteolyticus LMG3772^T	X74723	AJ842686	[a]	DQ907514	EF114210	DQ907444
V. vulnificus LMG13545^T	X74726	AJ842737	EU118244	DQ907522	DQ907382	DQ907454
A. fischeri LMG4414^T	X74702	AJ842604	EF415528	DQ907482	DQ907344	DQ907415
P. phosphoreum LMG4233^T	D25310	AJ842551	EF380239	DQ907495	DQ907326	DQ907393

Sequences retrieved from GeneBank and [a]"*The Taxonomy of Vibrios*" (http://www.taxvibrio.lncc.br/) *V. azureus* NBRC104587^T 16S rRNA (AB428897); *V. sagamiensis* NBRC104589^T 16S rRNA (AB428909).

4.3 Results

4.3.1 Isolate identification and single locus phylogenies

All DNA target regions were successfully amplified from the 36 vibrio isolates. Ambiguous nucleotide positions were observed in the 16S rRNA gene for some isolates. BLASTN searches of the 16S rRNA gene failed to clearly identify the 36 isolates as either *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* (99-100% sequence identities). Surprisingly, isolate R-40496^T, the type strain of the recently described

V. communis (Chimetto et al., 2011) exhibited 100% sequence identity for the 16S rRNA gene and 98 to 99% sequence identity for the rest of the loci analyzed, with the type strain of *V. owensii.*

All phylogenetic reconstruction methods reproduced similar topologies. The 16S rRNA gene phylogeny was overall congruent with the phylogenies obtained from the individual protein-coding genes (Fig. 4.1). However, the 16S rRNA topology was poorly supported. This gene showed lower phylogenetic signal and higher inter-species sequence similarity compared to the protein-coding loci (Fig. 4.1; Table 4.4). Compared to 16S rRNA and *rpoA*, gene regions *pyrH, topA, ftsZ*, and *mreB* showed higher proportion of informative sites and lower inter-species similarity values, thus higher resolution power to discriminate among species. A single topological incongruence was observed involving *V. rotiferianus* strains *oz08* and *oz11* for the *pyrH* gene (Fig. 4.1c). This result was validated by re-sequencing this gene region for the aforementioned strains. Further analyses of the *pyrH* alignment in RDP v3.44 revealed a major recombination event located in the first part of this gene region (first 116 bp) between strains *oz08*, *oz11* and a *V. owensii* strain. Interestingly, during culture all isolates identified by MLSA as *V. campbellii* formed green colonies on TCBS agar plates after 24 h, while isolates identified as *V. harveyi* and *V. owensii* formed yellow colonies that turned green within 48-72 h.

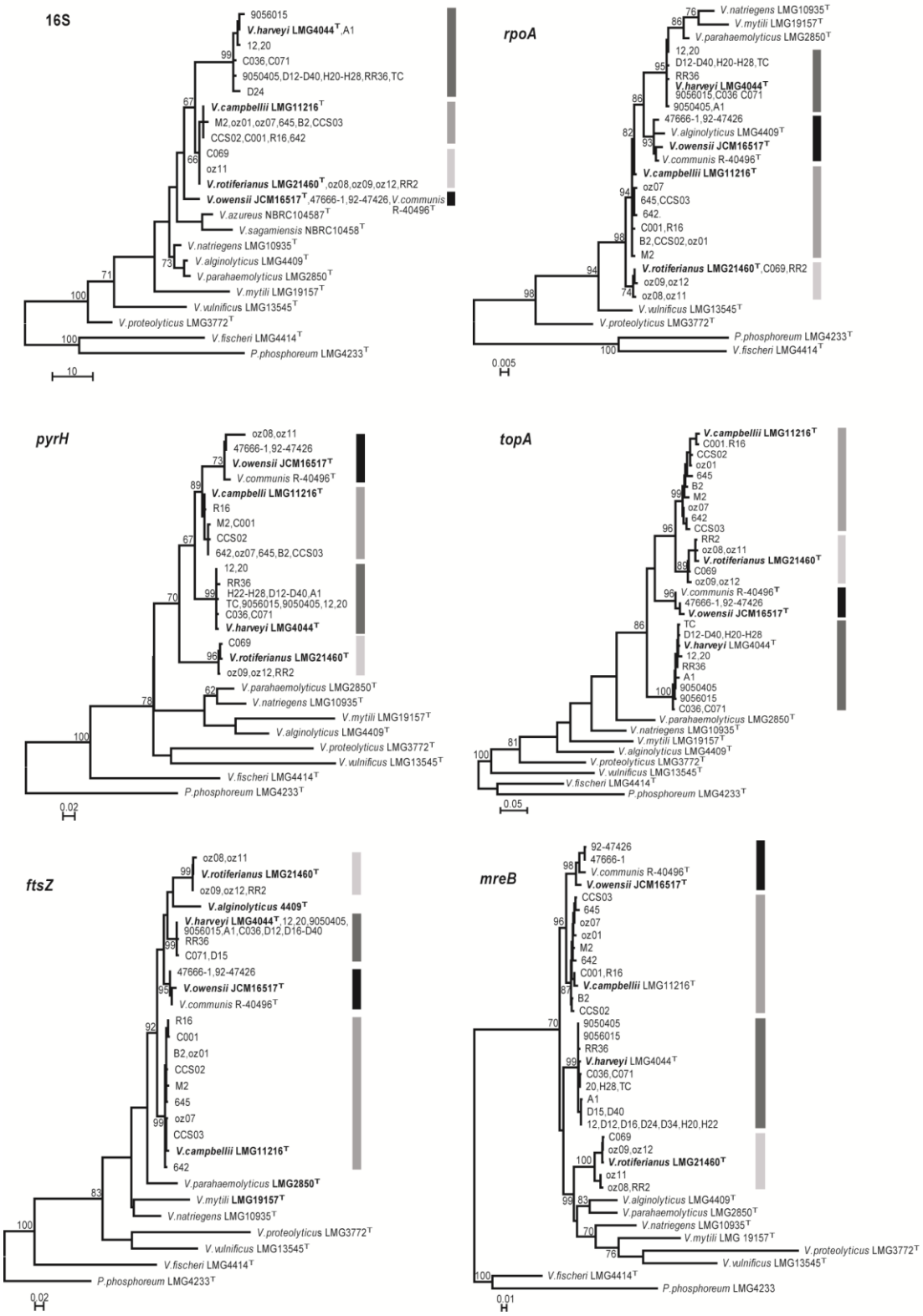

Figure 4.1: Maximum likelihood phylogenetic analysis based on partial a) 16S rRNA, b*) rpoA*, c) *pyrH*, d*) topA*, e) *ftsZ* and f*) mreB* genes

GenBank accession numbers provided below in the text. *Photobacterium phosphoreum* LMG4233^T used as an outgroup. Numbers on notes denote bootstrap support values. Bars: ■*V. owensii*; ■*V. harveyi*; ■*V. campbellii*; ■*V. rotiferianus* species clusters.

Table 4.4: Sequence analysis and statistics of single-gene and multilocus alignments

Gene sequence	Length (nt)	Model (PAUP)	% PIS[c]	%GC	%[d] Similarity	Avg Inter-*spp.* simil.(%)[e]	Intra-spo. similarity (%)[e]				Inter-*spp.* similarity(%)[e]					
							Vh	Vc	Vr	Vo	Vh/Vc	Vh/Vr	Vc/Vr	Vh/Vo	Vc/Vo	Vr/Vo
16S rRNA	1,352	HKY+I+G	1.4	53.6	99.5	99.2	99.9	99.9	99.9	100	99.1	99.1	99.8	98.8	99.3	99.4
rpoA	820	GTR+G	4.7	45.9	98.4	97.9	99.0	99.7	99.8	99.5	97.7	97.3	98.9	97.7	98.2	97.6
pyrH	467	TrN+I+G	12.4	49.4	95.6	93.7	99.9	99.6	96.5	99.8	95.5	91.2	92.7	93.5	96.1	93.1
topA	626	K80+I+G	17.9	48.3	92.4	90.1	99.4	99.8	98.7	99.6	88.5	88.0	93.9	89.9	89.9	90.6
ftsZ	468	TrNef+I+G	26.9	47.3	92.1	94.1	99.9	99.3	99.6	99.1	94.1	94.0	92.2	95.7	95.3	93.1
mreB	883	GTR+I+G	13.2	48.8	95.3	93.1	99.5	98.9	98.3	98.3	94.6	91.8	91.4	93.5	96.1	91.0
2-locus MLS[a]	1,509	HKY+I+G	15.2	48.6	94.0	91.9	99.4	99.8	99.8	99.8	92.1	90.2	92.4	92.0	93.6	90.8
3-locus MLS[b]	1,977	GTR++I+G	14.2	48.3	94.5	92.5	99.5	98.7	98.7	98.9	92.6	91.2	92.4	92.9	94.0	91.5
5-locus MLS	3,264	TVM+I+G	11.3	47.9	95.8	94.0	99.7	99.1	98.6	99.2	94.3	92.7	94.1	94.3	95.4	93.4

Gene sequences of isolates identified in the study as *V.harveyi*, *V.campbellii*, *V.rotiferianus*, *V.owensii* and each of the species type-strains.
[a] 2-locus MLS: *topA-mreB* concatenated sequences; [b] 3-locus MLS: *topA-mreB-ftsZ* concatenated sequences.
[c] % PIS= percentage of parsimony informative sites; [d] % Similarity deduced from nucleotide diversity with Kimura-two parameter (K2P)="Pi (K2P)".
[e] Intra- and inter-species similarities from nt substitutions per site between populations: Dxy (K2P). Vh:*V.harveyi*; Vc:*V.campbellii*; Vr:*V.rotiferianus*; Vo:*V.owensii*.

4.3.2 Multilocus Sequence Analysis (MLSA)

The ILD tests supported congruence in phylogenetic signal among loci and therefore a concatenated alignment was created for a five-locus MLSA (p-value <0.01; Fig. 4.2a). Four well-supported clusters of strains corresponding to *V. harveyi*, *V. rotiferianus*, *V. campbellii* and *V. owensii* were identified according to taxonomical expectations for the Harveyi clade (Fig. 4.2a and Table 4.4 for inter and intra-species genetic similarities). The five-locus MLSA reproduced the topologies obtained by single locus analyses independently of the phylogenetic reconstruction methodology, and it was statistically better supported (Table 4.4). However for the five-locus MLSA, decreased values were observed in the proportion of parsimony informative sites and nucleotide diversity compared to *pyrH*, *topA*, *ftsZ*, *mreB* single gene analyses. The type strain R-40496^{T} of *V. communis* showed 97.8% to 99.5% single locus and 98.6% five-locus sequence similarities with the *V. owensii* type strain and it was consistently recovered within the *V. owensii* cluster in all of the aforementioned phylogenetic reconstructions and the one based on the 16S rRNA gene (Fig. 4.1 and 4.2a). Based on these results, *V. communis* is considered here a junior synonym of *V. owensii*; therefore, sequences associated with *V. communis* were not used the analyses to follow.

To minimise the number of loci analysed for routine species identification, all two-locus combinations were tested. MP and ML phylogenies obtained from the *topA*-*mreB* pair of genes were consistent with the five-locus MLSs phylogenies and the four clades were retained with maximum BT support (Fig. 4.2b). This gene concatenation showed the highest proportion of informative sites (15.2% PIS) and the lowest inter-species similarity values (90.2-93.6%), compared with any other two-locus combinations (data not shown), three-locus (*topA*, *mreB* and *ftsZ*) and five-locus concatenated sequences (Table 4.4). A Bayesian phylogeny was inferred from concatenated *topA*-*mreB* genes using sequences of myisolates and additional 84 well-characterised strains of the *V. harveyi* group (Fig. 4.3). This analysis recovered each of the engaged strains in their correspondent species group, with high posterior probability support. Three exceptions were observed: strains labeled as *V. rotiferianus* CAIM994 and as *V. harveyi* D1 and PA2, were recovered within the *V. owensii* cluster.

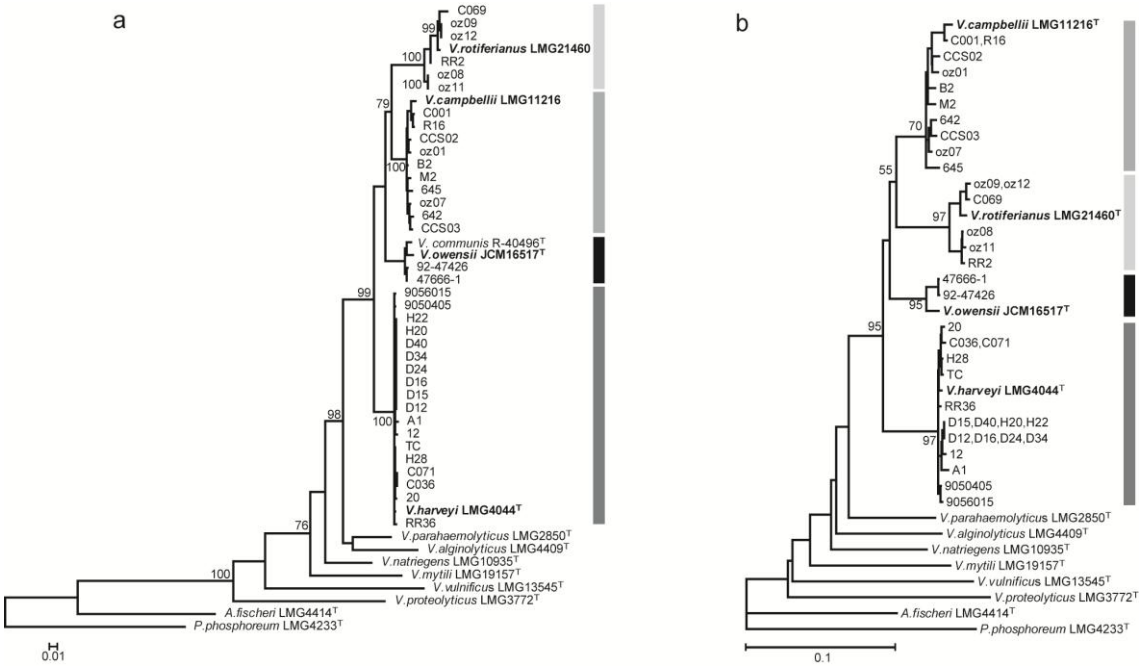

Figure 4.2: Maximum likelihood phylogenetic analysis based on partial five- and two-protein-coding loci concatenated gene sequences

a) 5 protein-coding loci *(rpoA, pyrH, topA, ftsZ* and *mreB*) and b) 2 protein-coding loci (*topA* and *mreB*) concatenated sequences. *Photobacterium phosphoreum* LMG4233^T used as an outgroup. Numbers on nodes denote bootstrap support values. Bars: ■*V. owensii;* ■*V. harveyi;* ■*V. campbellii;* ■ *V. rotiferianus* species clusters.

Accession numbers: Nucleotide sequence data reported are available in the GenBank under accession numbers HQ449743-HQ449778, HQ449779-HQ449814, HQ449815-HQ449850, HQ449851-HQ449886, HQ449887-HQ449922 and HQ449923-HQ449958 for 16S rRNA gene, *rpoA*, *pyrH*, *topA*, *ftsZ*, and *mreB*, respectively.

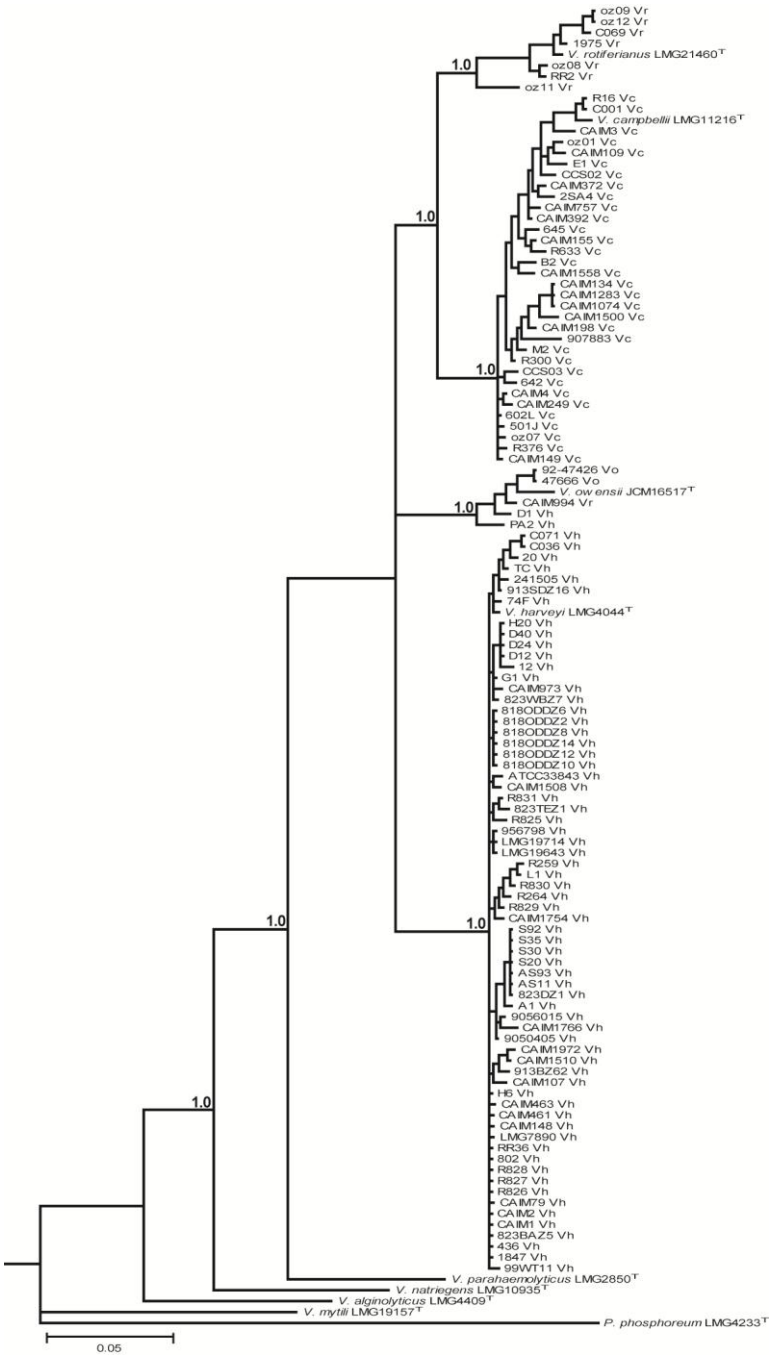

Figure 4.3: Bayesian phylogenetic reconstruction inferred from isolates under study and publicly available partial *topA*-*mreB* concatenated sequences of *V. harveyi*-related strains

Photobacterium phosphoreum 4223^{T} used as an outgroup. Numbers on nodes denote denote posterior probability support.

4.4 Discussion

Multilocus sequence analysis, cross-validated against a traditional 16S rRNA gene phylogeny, allowed precise identification of cryptic *V. harveyi*-like isolates from a wide range of Australian clinical and environmental samples as belonging to *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* species. The analysis also revealed that the newly described species, *V. communis* (deposited type-strain R-40496^{T}) (Chimetto et al., 2011) is likely a junior synonym of *V. owensii* (100% 16S rRNA and 98.6% five-locus sequence similarities between *V. owensii* and *V. communis* type strains) and this is expected to be confirmed by future DDH analysis.

Phenotypic characterisation still represents one of the most commonly used methods for *V. harveyi*-related species identification. In this study, all isolates identified by MLSA as *V. campbellii* formed green colonies on TCBS agar plates grown overnight, while isolates identified as *V. harveyi* and *V. owensii* formed yellow colonies that turned green within 48-72 h. Owens et al. (1996) suggested the existence of two major biotypes within *V. harveyi* according to different sucrose metabolism profiles. In their study, strains of *V. harveyi*, pathogenic to prawns, were sucrose-negative (green colonies on TCBS agar), whilst sucrose-positive strains (yellow colonies) were benign and even useful as probiotics. The present molecular identification suggests that *V. harveyi* and *V. campbellii* are two genetically distinct species of opposite sucrose metabolism profiles rather than two distinct biotypes belonging to the same vibrio species. Additional studies including multiple strains and cross-validation with molecular techniques are required to establish whether sucrose metabolism, responsible for colony colour, can be considered as a valid diagnostic character for discrimination between *V. harveyi* and *V. campbellii*.

Species of the *V. harveyi* group are known to share ~100% 16S rRNA gene sequence identity (Owens and Busico-Salcedo, 2006; Chapter 3). However, recalculation of this value is necessary due to the misidentification of *V. harveyi*, *V. campbellii* and *V. rotiferianus* strains and to the addition of *V. owensii* as a new member of the group. In this study, values of 16S rRNA pair-wise gene sequence similarity among these species were as high as 98.8% (*V. harveyi vs V.owensii*) and 99.8% (*V. campbellii vs*

V. rotiferianus). Although essential for bacterial taxonomy, the 16S rRNA gene appears to have insufficient resolving power if used alone for the discrimination of closely related bacteria such as those belonging to the Harveyi clade (Thompson et al., 2005; Janda and Abbott, 2007). Furthermore, the multi-copy nature of ribosomal operons and intragenomic heterogeneity reported for 16S rRNA genes in many bacteria including vibrios (Harth et al., 2007) makes necessary the use of alternative genes. The number and resolution power of suitable genes for species classification may differ given the bacterial group under study. The protein-coding loci included in this study were selected on the basis of their single copy nature, degree of conservation, ability to discriminate among *V. harveyi*-related species and availability in public databases. This combination of genes (*rpoA, pyrH, topA, ftsZ* and *mreB*) was recently successfully used to delineate the new species *V. owensii* and distinguish the latter from other species in the *V. harveyi* group (Chapter 3).

Recombination involving the *pyrH* gene was detected for *V. rotiferianus* strains *oz08* and *oz11* isolates. Recombination events in bacteria are responsible for incongruence between gene genealogies and species phylogenies. For this reason, at least five housekeeping genes in MLSA are recommended to avoid topological artefacts (Stackebrandt et al., 2002). In this study, topological incongruence due to recombination observed at the *pyrH* locus for the *V. owensii* cluster was masked following concatenation of the five protein-coding loci, and the isolates were finally assigned to the *V. rotiferianus* cluster. Although the unsuitability of *pyrH* for species identification within the Harveyi clade is already known (Pascual et al., 2010), recombination episodes have not yet been reported between and *V. rotiferianus* and *V. owensii* since the latter was described only recently. It has been argued however that *V. rotiferianus* suffered horizontal gene transfer (HGT) and that this could be responsible for species divergence (Thompson et al., 2007). These authors demonstrated topological incongruence of *V. rotiferianus* CAIM994 in phylogenies inferred from different genes and finally described this isolate as a hybrid strain. However, CAIM994 was recently identified as a potential *V. owensii* strain, sharing higher 16S rRNA and five protein-coding loci sequence identities with the *V. owensii* type strain (JCM 16517^T) than with the *V. rotiferianus* type strain (LMG21460^T) (Chapter 3).

A practical, yet accurate method for bacterial species identification can be achieved by minimizing the number of housekeeping genes following careful selection (Zeigler, 2003). The use of at least two independent loci is suggested for identification purposes whilst more genes are necessary for phylogenetic inference (Martens et al., 2007). Several studies have optimised the number of MLSA genes for taxonomic characterisation of bacterial taxa (Fargier and Manceau, 2006; Martens et al., 2007; Nzoue'a et al., 2009) including the Harveyi clade (Pascual et al., 2010). In the latter study, the number of genes was reduced from seven to three, for the identification of six species from within the Harveyi clade. More specifically, for the *V. harveyi* group, since recently described species have not been included in previous MLSA studies, a *de novo* assessment of genes exhibiting high availability in public databases and suitable discrimination power was necessary. In this study, the concatenation of *topA* and *mreB* was found to be the most discriminative for *V. harveyi*-related species identification when compared to the three and five-locus combinations (Fig. 4.4). The addition of *ftsZ* to the two-locus alignment decreased the proportion of informative sites (15.2 to 14.2%) and did not noticeably affect the nucleotide diversity (94.0 to 94.5%) or the inter-species sequence diversity (Table 4.4; Fig. 4.4).

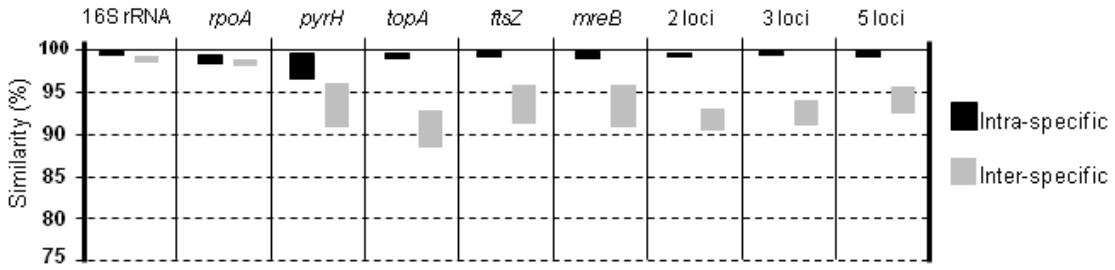

Figure 4.4: Ranges of percentage intra- (black bars) and inter-species (grey bars) similarities (%) for single loci and combinations involved in the study

*2-loci: *topA-mreB*; 3-loci: *topA-mreB-ftsZ*; 5-loci: *rpoA-pyrH-topA-ftsZ-mreB*.

The reliability of the two-locus approach was further tested by a global phylogenetic reconstruction with 84 additional *topA* and *mreB* public sequences of strains from variable sources (Fig. 4.3), all previously included in exhaustive taxonomic analyses. In the resulting phylogeny, the taxonomic position of all the strains was confirmed against

the corresponding type-strains for each species. Only the *V. rotiferianus* strain (CAIM994; Thompson et al., 2007), isolated from red snapper in Mexico, and two strains classified as *V. harveyi* (D1 and PA2; Lin et al., 2010), isolated from fish and diseased corals, were recovered within the *V. owensii* cluster, possibly due to the recent description of this species. As discussed above, strain CAIM994 was identified as a potential *V. owensii* member (Chapter 3). In addition, the strains D1, PA2 and CAIM994 clustered together with the full genome sequenced *V. harveyi*-like 1DA3 strain in Lin et al. (2010), and the cluster was suggested to represent a new unknown species. The reclassification of these strains as *V. owensii* would confirm the presence of this species in the Atlantic colonizing different hosts, and the availability of a full genome sequence of *V. owensii*.

Sequencing of *topA* and *mreB* genes could serve as a handy yet reliable strategy for routine identification of *V. harveyi*-like isolates in aquaculture systems and in environmental research studies, when simple, cost-effective but still accurate identification at the species level is required. The revelation of recombination episodes in one or both genes, following sequencing of additional isolates cannot however, be excluded hence for species delineation and taxonomic assessments a full MLSA is still recommended.

Of the 36 *V. harveyi*-related strains employed in this study, 19 were isolated from Australian diseased cultured fish and crustacea. Strain C071, potentially pathogenic to lobster larvae of *P. ornatus* (Bourne et al., 2006) was confirmed as *V. harveyi* (Table 4.1). In contrast, strain 642, pathogenic to *P. monodon* and previously classified as *V. harveyi*, was identified in this study as *V. campbellii*. Likewise, presumptive *V. harveyi* strains M2 and B2, isolated from *Scylla serrata* (mud crab) were also identified as *V. campbellii*. The species *V. harveyi* and *V. campbellii* are almost impossible to distinguish on the basis of phenotypic traits and/or 16S rRNA gene sequence analysis. Their frequent misidentification has been considered responsible of underestimating *V. campbellii* as a serious pathogen in aquaculture systems in the past (Gomez-Gil et al., 2004). The present results indicate that pathogenic strains of *V. owensii* have also previously been misidentified as *V. harveyi*. For instance, before the study carried in Chapter 3, the *V. owensii* 47666-1 strain, exceptionally pathogenic to *P. monodon* in hatcheries in northern Qld (Harris, 1993) was classified as *V. harveyi*

since 1991. The *V. owensii* isolate 92-47426 was isolated from moribund *P. monodon* larvae from the same source and Australian region.

In summnary, in this study the identity of previously misidentified *V. harveyi*-like strains was revealed. Some of the strains represent important pathogens for the Australian fish and crustacean aquaculture enterprises, Most importantly, 16S rRNA and five-locus MLSA proved the synonymy of *V. owensii* and the more recently delineated *V. communis* sp. nov. The results suggested that the 16S rRNA gene is appropriate for allocation of species to the *V. harveyi* group but its resolution is insufficient to discriminate among species, otherwise clearly resolvable by MLSA. Further, I propose a two-gene based analysis (*topA*-*mreB*) as a practical, yet accurate approach for routine *V. harveyi*-related species identification. Global phylogenies based on the latter genes also indicated previous misclassifications of *V. owensii* strains, demonstrating that this species has a much wider geographical distribution range than the one initially described.

CHAPTER 5. MULTIPLEX PCR PROTOCOL FOR DETECTION OF *VIBRIO HARVEYI*-RELATED SPECIES

5.1 Introduction

The development of fast and reliable techniques for the detection of *V. harveyi* species has been a major research area for the last ten years due to the economic impact of vibriosis in the aquaculture industry. However, the design of detection and identification methods for *V. harveyi*-related pathogens has been a difficult task due to highly similar phenotypes and genotypes among species and the recent description of new*V. harveyi*-related species. In aquaculture, costs of equipment and complex methodologies have to be balanced against precision of the results. Conventional cultured based methods such as biochemical tests and 16S rRNA gene sequence analysis are limited for precise identification of this cryptic group of species, although these techniques are still useful to provide initial assignment of unknown isolates to the Harveyi clade (as discussed in Chapters 2 and 3). Despite the need of molecular tools for discriminative detection of *V. harveyi* related species, the cost and need of specialised personnel and equipment can still be low by providing farmers with affordable techniques, such as common PCR over other expensive and complex technologies (e.g. real-time PCR, gene sequencing etc...).

As discussed in Chapter 2, molecular fingerprinting strategies and DNA sequence analysis of the 16S rRNA gene fails to achieve specificity and differentiate among *V. harveyi*-related species (Oakey et al., 2003; Fukui and Sawabe, 2007). Similarly, sequence analysis of selected gene regions: *toxR* (Conejero and Hedreyda, 2003; Pang et al., 2005); *gyrB* (Thaithongnum et al., 2006) and *vvh*/*vch* (Conejero and Hedreyda, 2004; San Luis and Hedreyda, 2006), resulted in unsatisfactory results mainly due to limited resolution power or amplification of false-negative PCR products (Thompson et al., 2007). In addition, these studies tested limited number of strains, not including the latest described species *V. rotiferianus* nor *V. owensii*. In other cases, the genes targeted were present in multiple copies in the genome or were susceptibility to horizontal gene transfer (Conejero and Hedreyda 2004).

Several studies have evaluated the potential of several housekeeping genes, selected on the basis of their stability, degree of conservation and single copy nature in the genome, to discriminate pathogen isolates from within the Harveyi clade following MLSA (Thompson et al., 2005, 2007; Sawabe et al., 2007; Chapter 4). More specifically, the latter MLSA study carried in Chapter 4 allowed the selection of suitable protein-coding loci for discriminative identification of species within the *V. harveyi* group. For this study, I have designed and tested a list of specific PCR primers targeting protein-coding loci (*topA*, *ftsZ* and *mreB*) that fulfilled the aforementioned conditions for simultaneous detection of *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*. Primer combinations were tested in monoplex and multiplex PCR assays targeting DNA from each of the four vibrio species, individually and simultaneously. An extra set of primers, amplifying a highly conserved region of the 18S rRNA gene in decapods, was included in the multiplex PCR assay as an internal control to monitor presence of PCR inhibitors in clinical samples.

5.2 Materials and Methods

5.2.1 Design of specific PCR primers

DNA sequences of the protein-coding genes: *topA*, *ftsZ* and *mreB* genes from multiple *V. harveyi*-related strains and eleven type strains of close relatives (Table 4.1 and 5.1), were obtained from results of Chapter 4 and from public databases, and subsequently aligned. In addition, another 84 publicly available sequences of *V. harveyi*-related species from the databases (gathered in Chapter 4) were added to the alignment and used to design specific oligonucleotide primers. The primers were designed and analyzed manually based on different alignments created by using Vector NTI Advance Software (Invitrogen) and BioEdit 7.0.5 (Hall, 1999) and with the aid fo AlleleID 7.7 (Primer Biosoft International) to determine the G+C content and self-dimer and hairpin structures. Allele ID software also allows the avoidance of non-target species within the alignment. The specificity of each primer sequence was evaluated using the GenBank database and BLAST (Basic Local Alignment Search Tool). Finally, the primers were custom synthesised by Sigma-Aldrich Pty Ltd. (Australia).

The designed primers targeted specific regions of the species *V. harveyi* (*topA*), *V. campbellii* (*ftsZ*), *V. rotiferianus* (*mreB*) and *V. owensii* (*topA*) and matched all sequences belonging to each of the target species in the databases (only those identified by precise molecular methods were considered). A list of primer sequences and length of target regions are presented in Table 5.2. The specificity of the primers against other vibrio species not included in the assay was determined using BLAST.

Table 5.1: List of accession numbers of target genes from type-strains

Type-strain	Accession number of target gene		
	topA	***ftsZ***	***mreB***
V. owensii JCM16517^T	GU111254	GU111256	GU111258
V. harveyi LMG4044^T	DQ907488	DQ907350	DQ907422
V. campbellii LMG11216^T	DQ907475	DQ907337	DQ907408
V. rotiferianus LMG21460^T	DQ907515	DQ907372	DQ907445
V. alginolyticus LMG4409^T	DQ907472	EF027344	DQ907405
V. parahaemolyticus LMG2850^T	DQ907509	DQ907367	DQ907440
V. natriegens LMG10935^T	DQ907500	DQ907359	DQ907432
V. fortis LMG21557^T	DQ907484	DQ907346	DQ907417
V. proteolyticus LMG3772^T	DQ907514	EF114210	DQ907444
V. corallilyticus LMG20984^T	EF114213	DQ907341	DQ907412
V. tubiashii LMG10936^T	DQ907521	DQ907381	DQ907453

Table 5.2: List of PCR primers for detection of *V. harveyi*-related species

Species detected	Target gene	Length (bp)	Primer name	Primer sequence (5'-3')
Decapods	18S rRNA	848	143-F*	TGCCTTATCAGCTNTCGATTGTAG
			145-R*	TTCAGNTTTGCAACCATACTTCCC
V. harveyi	*topA* (topoisomerase I)	121	Vh.topA-F	TATTTGTCACCGAACTCAGAACC
			Vh.topA-R	TGGCGCAGCGTCTATACG
V. owensii	*topA* (topoisomerase I)	85	Vo.topA-F	TTCATACAGACGCTGAGCCAG
			Vo.topA-R	TACCTCAACACTTCAGCAAGCG
V. campbellii	*ftsZ* (cell division protein FtsZ)	294	Vc.ftsZ-F	AAGACAGAGATAGACTTAAAGAT
			Vc.ftsZ-R	CTTCTAGCAGCGTTACAC
V. rotiferianus	*mreB* (rod shaping protein MreB)	489	Vr.mreB-F	GTGCTATCCGTGAGTCAG
			Vr.mreB-R	AGATGTCCGATGCTAGTT

*Primers designed by Lo et al., (1996).

5.2.2 Monoplex PCR detection of *V. harveyi*-related species

The PCR primers were tested on DNA of 36 *V. harveyi*-related strains obtained from Australian culture collections and from eleven type strains of related vibrios (Tables 5.1 and 6.1). All PCRs were performed in a Mastercycler Gradient (Eppendorf) or a Perkin

Elmer Applied Biosystems GENEAMP PCR System 9700 (Perkin Elmer, USA). PCR reactions (20 µl) contained approximately 20 ng of genomic DNA, 1X PCR buffer (Tris·Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7) (Qiagen), 0.5 µM of each forward and reverse primer (Table 5.2), 200 µM dNTPs, 0.5 U Taq DNA polymerase (Quiagen) and de-ionised sterile water. The thermal program consisted of (i) 5 min at 94ºC, (ii) 30 cycles of 1 min at 94ºC, 1 min at 55ºC, 3 min 72ºC, and (iii) a final 7 min at 72ºC. All thermocycle runs included a blank control with sterile water. An 8 µl sample of each PCR product was resolved by electrophoresis at 100V for 30 min in 1% agarose gels to detect amplicons of the expected sizes.

DNA samples from the eleven type strains of related vibrios were used as templates for the evaluation of the primers' specificites. The 16S rRNA gene of all isolates was previously amplified as described by Lane et al. (1991) to ensure that the DNA template was amplifiable. Repeatability of the PCR amplification was assessed by running the PCR reactions in three different ocassions. Sensitivity was assessed by simultaneous PCR runs in the same thermocycler with 10-fold serial dilutions of the DNA templates from 300 ng to 300 fg.

5.2.3 Multiplex PCR for simultaneous detection of *V. harveyi*-related species

Multiplex PCRs were performed using similar reagents (different concentrations) and equipment as for monoplex PCRs, except for the replacement of Taq DNA polymerase by HotStar Taq DNA Polymerase (Qiagen). All four forward and reverse species-specific oligonucleotides designed were combined into one PCR reaction tube and tested first in a mix, containing DNA of each *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* type strains. Primer concentration and reaction conditions are displayed in Table 5.3. This PCR reaction (40 µl) contained approximately 20 ng of genomic DNA of each type strain and the four sets of primers. An eight µl sample of each PCR product was resolved by electrophoresis at 80V for 80 min in 2.5% agarose gel to detect amplicons of the expected sizes.

DNA samples from the eleven type strains of related vibrio species were used as templates for the evaluation of the primer specificity in the multiplex PCR.

Repeatability of the PCR amplification assay was assessed running the multiplex PCRs in three different occasions. Sensitivity for detection was assessed by PCR runs with 10-fold serial dilutions of DNA templates from 300 ng to 3 pg.

Table 5.3: Multiplex PCR reaction conditions and primer concentrations

Target DNA	Primer name	Conc (uM)	Amplification conditions
Decapod 18S rRNA*	143-F 145-R	0.6	95ºC x 15 min 30 x (94ºC x 1 min; 57ºC x 1.5 min; 72ºC x 3 min) 72ºC x 10 min
V. harveyi topA	Vh.topA-F Vh.topA-R	0.3	
V. owensii topA	Vo.topA-F Vo.topA-F	0.3	
V. campbellii ftsZ	Vc.ftsZ-F Vc.ftsZ-R	0.2	
V. rotiferianus mreB	Vr.mreB-F Vr-mreB-R	0.8	

* The addition of 143-F and 145-R is optional. Reaction conditions do not change.

5.2.4 Simultaneous detection of *V. harveyi*-related species and decapod DNA

In the multiplex PCR protocol, a fifth set of primers was added to the reactions to monitor presence of PCR inhibitors in genomic preparations of clinical samples and test the efficiency of the DNA extraction method. These primers (143F and 145R) were previously designed by Lo et al. (1996) for specific amplification of an 848-bp conserved region of the 18S rRNA gene sequence in decapods. PCRs were performed using similar equipment and reagent concentrations as for multiplex PCRs except for the addition of 0.6 µM of 143-F/145-R primers (Table 5.3). PCR reactions (40 µl) contained approximately 20 ng of genomic DNA from type strains of the four targeted vibrios and from non-infected *P. monodon* larvae or *P. ornatus* larvae tissue samples. The thermal cycle was similar to that of fourplex multiplex PCR (Table 5.3).
In addition, biplex PCR protocols were designed for detection of *V. harveyi*, *V. owensii*, *V. campbellii* or *V. rotiferianus* in decapod crustacean samples. PCR reactions (20 µl) contained approximately 20 ng of decapod and vibrio genomic DNA, and similar reagent concentration as for monoplex PCR reactions. The thermal program consisted of (i) 5 min at 94ºC, (ii) 30 cycles of 1 min at 94ºC, 1.5 min at 55ºC, 3 min 72ºC, and

(iii) a final 10 min at 72ºC. After these runs, an eight µl sample of each PCR product was resolved by electrophoresis at 80V for 80 min in 2.5% agarose gel to detect amplicons of the expected sizes. Finally, samples of genomic DNA from wild and cultured decapod species in northern Qld (*P. monodon*, *Penaeus aesculentus*, *Penaeus merguiensis* and *Scylla serrata*) were tested for the presence of any of the vibrio species using the fiveplex PCR protocol described above. The genomic DNA samples were provided by Kathy LaFauce, Dean Jerry and Rusaini from JCU (Townsville).

5.3 Results and Discussion

5.3.1 Monoplex PCR detection of *V. harveyi*-related species

The four oligonucleotide sets designed in this study amplified specific DNA regions of the expected sizes for the type strains of *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* (Fig. 5.1-5.4) and for all the *V. harveyi*-like isolates included in the study (data not shown). BLAST analysis of the primers exhibited complete homology with the corresponding regions in all *V. harveyi*, *V. campbellii*, *V. rotiferianus* or *V. owensii* strains and no matches with any other vibrio species. Negative amplification was observed for non-target species included in the study. Amplification of the 16S rRNA gene for all strains tested was positive and single ~1,500-bp products were observed in agarose gels (data not shown). The monoplex PCRs produced observable bands when 300 ng to 3 pg of DNA was used as template in the reactions (Fig. 5.5), but not with 300 fg (data not shown).

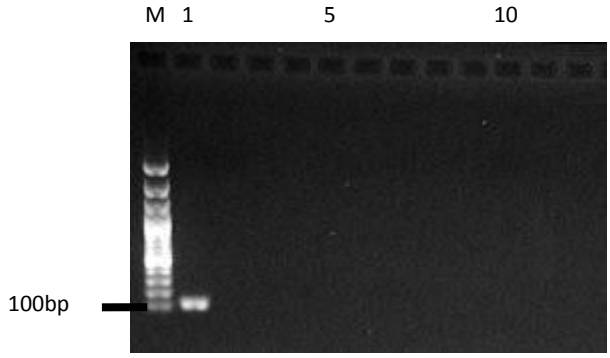

Figure 5.1: Specificity of *V. harveyi* monoplex PCR with Vh.topA-F/Vh.topA-R primers

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^{T} (121 bp); lane 2: *V. campbellii* LMG 11216^{T}; lane 3: *V. rotiferianus* LMG 21460^{T}; lane 4: *V. owensii* DY05^{T}; lane 5: *V. alginolyticus* LMG 4409^{T}; lane 6: *V. parahaemolyticus* LMG 2850^{T}; lane 7: *V. natriegens* LMG 10935^{T}; lane 8: *V. proteolyticus* LMG 3772^{T}; lane 9: *V. tubiashii* LMG 10936^{T}; lane 10: *V. fortis* LMG 21557 ^{T}; lane 11: *V. coralliilyticu*s LMG 20984^{T}; lane 12: sterile water. A 100-bp sized band (ladder) indicated as reference.

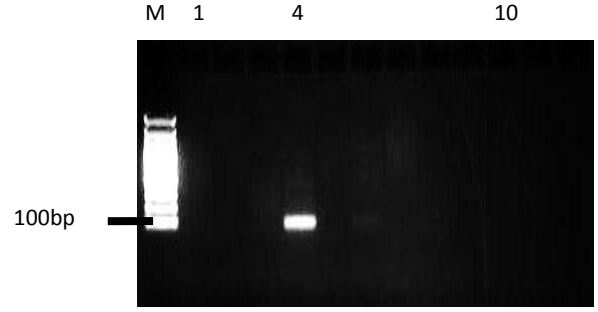

Figure 5.2: Specificity of *V. owensii* monoplex PCR with Vo.topA-F/Vo.topA-R primers

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^{T}; lane 2: *V. campbellii* LMG 11216^{T}; lane 3: *V. rotiferianus* LMG 21460^{T}; lane 4: *V. owensii* DY05^{T} (85 bp); lane 5: *V. alginolyticus* LMG 4409^{T}; lane 6: *V. parahaemolyticus* LMG 2850^{T}; lane 7: *V. natriegens* LMG 10935^{T}; lane 8: *V. proteolyticus* LMG 3772^{T}; lane 9: *V. tubiashii* LMG 10936^{T}; lane 10: *V. fortis* LMG 21557^{T}; lane 11: *V. coralliilyticu*s LMG 20984^{T}; lane 12: sterile water. A 100-bp sized band (ladder) indicated as reference.

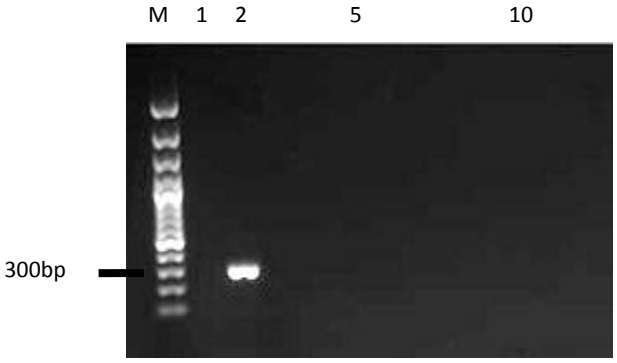

Figure 5.3: Specificity of *V. campbellii* monoplex PCR with Vc.ftsZ-F/Vc.ftsZ-R primers

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^T; lane 2: *V. campbellii* LMG 11216^T (294 bp); lane 3: *V. rotiferianus* LMG 21460^T; lane 4: *V. owensii* DY05^T; lane 5: *V. alginolyticus* LMG 4409^T; lane 6: *V. parahaemolyticus* LMG 2850^T; lane 7: *V. natriegens* LMG 10935^T; lane 8: *V. proteolyticus* LMG 3772^T; lane 9: *V. tubiashii* LMG 10936^T; lane 10: *V. fortis* LMG 21557^T; lane 11: *V. coralliilyticus* LMG 20984^T; lane 12: sterile water. A 300-bp sized band (ladder) indicated as reference.

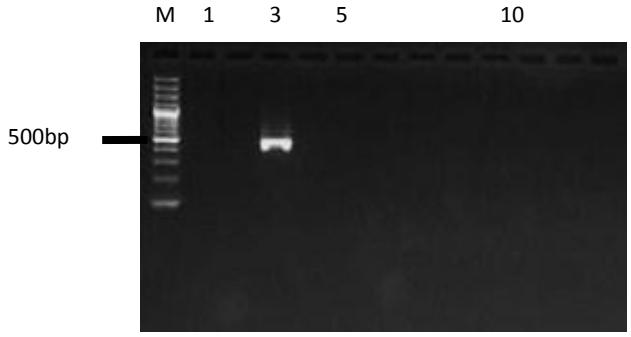

Figure 5.4: Specificity of *V. rotiferianus* monoplex PCR with Vr.mreB-F and Vr.mreB-R primers

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^T; lane 2: *V. campbellii* LMG 11216^T; lane 3: *V. rotiferianus* LMG 21460^T (489 bp); lane 4: *V. owensii* DY05^T; lane 5: *V. alginolyticus* LMG 4409^T; lane 6: *V. parahaemolyticus* LMG 2850^T; lane 7: *V. natriegens* LMG 10935^T; lane 8: *V. proteolyticus* LMG 3772^T; lane 9: *V. tubiashii* LMG 10936^T; lane 10: *V. fortis* LMG 21557^T; lane 11: *V. coralliilyticus* LMG 20984^T; lane 12: sterile water. A 500-bp sized band (ladder) indicated as reference.

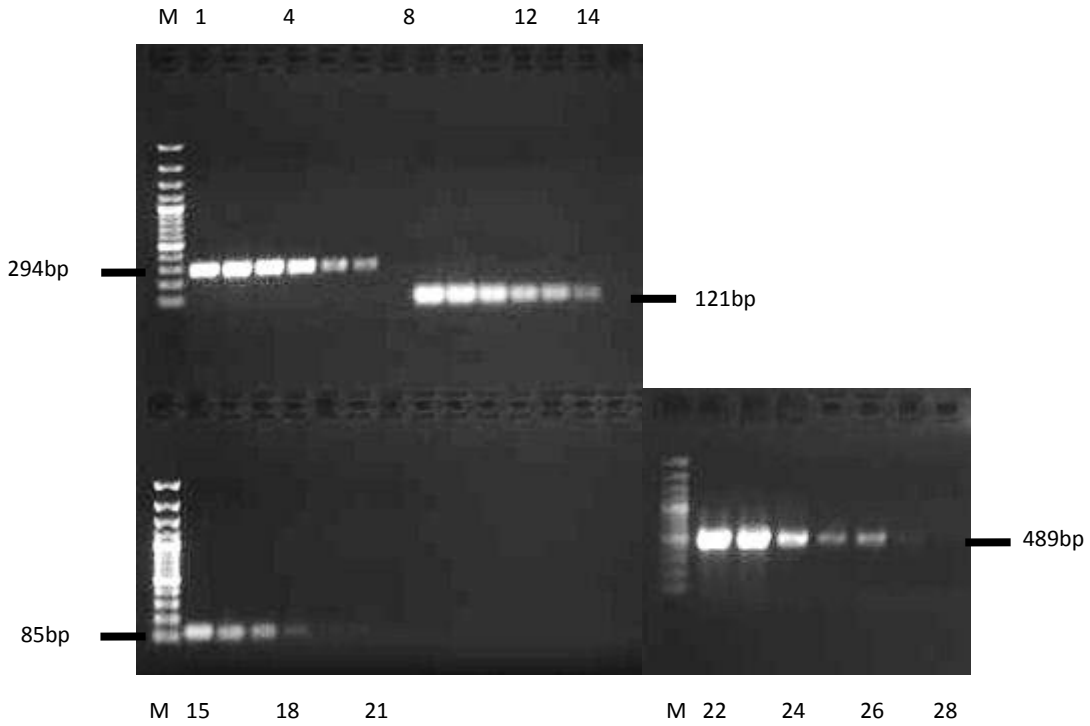

Figure 5.5: Sensitivity of the monoplex PCRs

M: 100-bp DNA ladder plus (Fermentas); lanes 1-6: 10-fold dilutions of *V. campbellii* LMG 11216^T genomic DNA from 300 ng to 3 pg amplified with Vc.ftsZ-F/Vc.ftsZ-R primers; lane 7: sterile water; lanes 8-13: 10-fold dilutions of *V. harveyi* LMG 4044^T genomic DNA from 300 ng to 3 pg amplified with Vh.topA-F/Vh.topA-R primers; lane 14: sterile water; lanes 15-20: 10-fold dilutions of *V. owensii* DY05^T genomic DNA from 300 ng to 3 pg with Vo.topA-F/Vo.topA-R primers; lane 21: sterile water; lanes 22-27: 10-fold dilutions of *V. rotiferianus* LMG 21460^T genomic DNA from 300 ng to 3 pg amplified with Vr.mreB-F/Vr.mreB-R primers; lane 28: sterile water. Sizes of PCR products are indicated.

5.3.2 Multiplex PCR for simultaneous detection of *V. harveyi*-related species

The PCR parameters including annealing temperature, annealing and extension times, choice of Taq DNA polymerase, magnesium and primer concentration were optimised for simultaneous amplification of the four DNA regions targeted in the different vibrio species. Compared to monoplex PCR reactions, multiplex PCR assays need a different cocktail of reagents and different primer concentrations for all the target regions to be amplified. In addition, the multiplex PCR conditions differ from those required by the monoplex PCRs since the primers inferfere with each other with a consequent reduction in sensitivity.

Four types of PCR products of expected sizes were visualised after completion of the multiplex PCR reactions, containing a mix of four primer sets and DNA templates from

each species, separately or in combination (Fig. 5.6 and 5.7). When DNA from the four species was combined in a single tube for the reaction to take place, four bands of the expected sizes were observed in the gels (Fig. 5.7). As usual in multiplex PCR protocols, some of the DNA template combinations containing DNA of two or three vibrio species generated an additional weak band (190 bp), although this did not interfere with the sensitivity or specificity of the PCRs.

Negative amplification was observed when other DNA from other vibrios were used as templates, except for *V. corallilyticus* LMG 20984^{T}, for which a ~1,000-bp product was obtained (Fig. 5.6, lane 11). This reaction was repeated several times, always with the same result. The specificity of this multiplex for additional detection of *V. coralillyticus,* an important coral pathogen, would have to be further tested with multiple strains of these species and close relatives and the amplified products sequenced. The multiplex PCRs produced observable bands when 300 ng to 300 pg of DNA was used as template in the reactions, while visualization was not achieved when the DNA quantity was reduced to 30 pg (data not shown). This confirms previous studies reporting that in general, the sensitivity of multiplex PCRs is reduced 10-100 times when compared with that of monoplex PCR (Tsai et al., 1994; Jackson et al., 1996).

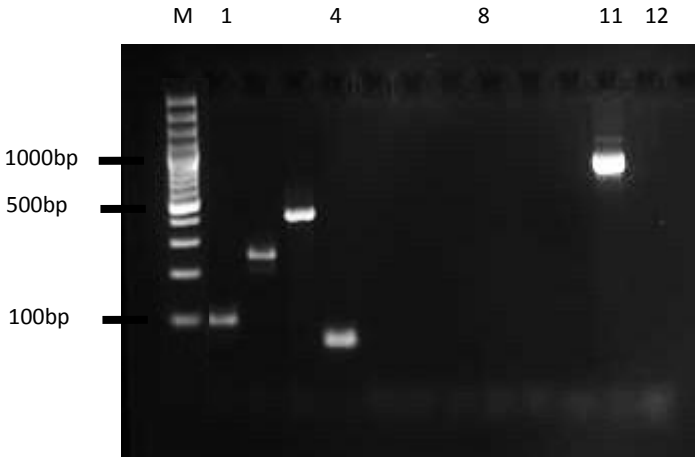

Figure 5.6: Specificity of the multiplex PCR

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^{T}; lane 2: *V. campbellii* LMG 11216^{T}; lane 3: *V. rotiferianus* LMG 21460^{T}; lane 4: *V. owensii* DY05^{T}; lane 5: *V. alginolyticus* LMG 4409^{T}; lane 6: *V. parahaemolyticus* LMG 2850^{T}; lane 7: *V. natriegens* LMG 10935^{T}; lane 8: *V. proteolyticus* LMG 3772^{T}; lane 9: *V. tubiashii* LMG 10936^{T}; lane 10: *V. fortis* LMG 21557^{T}; lane 11: *V. coralliilyticus* LMG 20984 ^{T}; lane 12: sterile water. The 100, 500, and 1,000-bp sized bands (ladder) indicated as references.

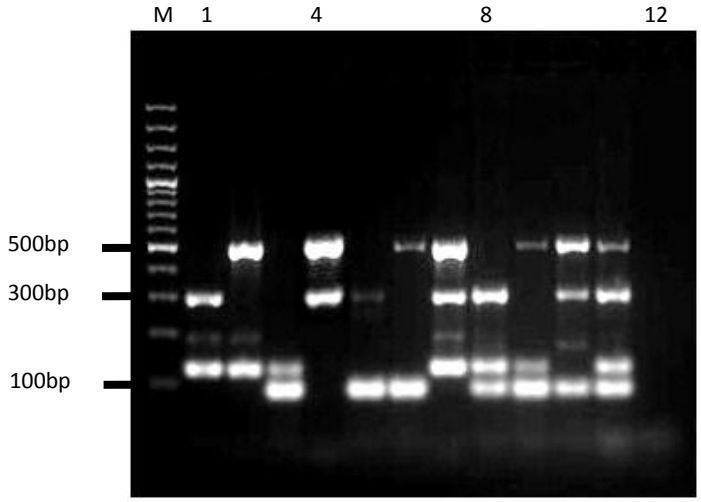

Figure 5.7: Combination of vibrio species DNA in the multiplex PCR

M: 100-bp DNA ladder plus (Fermentas); lane 1: *V. harveyi* LMG 4044^T and *V. campbellii* LMG 11216^T; lane 2: *V. rotiferianus* LMG 21460^T and *V. harveyi* LMG 4044^T; lane 3: *V. harveyi* LMG 4044^T and *V. owensii* DY05^T; lane 4: *V. rotiferianus* LMG 21460^T and *V. campbellii* LMG 11216^T; lane 5: *V. campbellii* LMG 11216^T and *V. owensii* DY05^T; lane 6: *V. owensii* DY05^T and *V. rotiferianus* LMG 21460^T; lane 7: *V. harveyi* LMG 4044^T, *V. campbellii* 11216^T and *V. rotiferianus* LMG 21460^T; lane 8: *V. harveyi* LMG 4044^T, *V. campbellii* LMG 11216^T and *V. owensii* DY05^T; lane 9: *V. harveyi* LMG 4044^T, *V. rotiferianus* LMG 21460^T and *V. owensii* DY05^T; lane 10: *V. rotiferianus* LMG 21460^T, *V. campbellii* LMG 11216^T and *V. owensii* DY05^T; lane 11: all four vibrio species DNA (20 ng each); lane 12: sterile water. The 100, 300, and 500-bp sized bands (ladder) indicated as references.

5.3.3 Simultaneous detection of of *V. harveyi*-related species and decapod DNA

Decapod DNA (*P. monodon* and *P. ornatus*) was amplified efficiently when degenerated primers 143-F/145-R (Lo et al., 1996) were included in the multiplex PCR. Bands of 848 bp from amplification of the 18S rRNA were observed in the gels when DNA from *P. monodon* or *P. ornatus* was used as the only template (e.g in hypothetical case of a healthy animal) and also when excess of decapod DNA (30-70 ng) was mixed with 20 ng DNA from any or all the four vibrio target species (e.g. in hypothetical case of infection or co-infection by these bacteria) (Fig. 5.8).

When similar or lower concentrations of crustacean DNA was combined with vibrio DNA templates, the 848-bp band was poorly or not visualised. All *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* are potential pathogens to decapod crustacea, but usually one single strain is pathogenic at a time. Therefore, PCRs targeting only single species would allow more sensitive detection and reduced costs in reagents when the aetiological agent is known. These biplex PCR reactions included

each of the vibrio primer set and the 143-F/145-R decapod primer set and produced the expected double band patterns in agarose gels (Fig. 5.8). The addition of the primer set for decapod DNA amplification in the multiplex PCR mix allowed: (1) the quality assessment of the DNA extraction method used (from optimal extractions, a PCR product would always be observed) and (2) the approximate estimation of the host/vibrio DNA proportion in the template used.

Finally, samples of genomic DNA from clinical and wild decapods in northern Qld (*P. monodon, P. aesculentus, P. merguiensis* and *S. serrata*) were negative for the presence of *V. harveyi, V. campbellii* and *V. rotiferianus* when tested in the fiveplex PCR. (Fig. 5.9). For five out of the 17 samples, the control 1,000-bp PCR product was not observed, which indicates suboptimal DNA extraction or subsequent degradation. One single sample was found positive for the presence of these vibrios. This was a *V. owensii* band (Fig. 5.9; lane 12) form DNA of cultured mudcrab larvae (*S. serrata*), obtained from the Aquaculture facilities at the JCU (Townsville) (Owens et al., 2010). Larval rearing of *S. serrata* is commercially important in many Indio-Pacific countries and remains an important source of income for many small-scale fisheries in coastal communities. One of the major constraints to further development of mud crab culture is the high mortality rates in the larval and hatchery phases of production due to vibriosis outbreaks of *V. harveyi* (Quinitio et al., 2001). It would be convenient to use recently developed identification methods such as MLSA or multiplex PCR to retest pathogenic strains *S. serrata* since it could be one more case of *V. harveyi* misidentification.

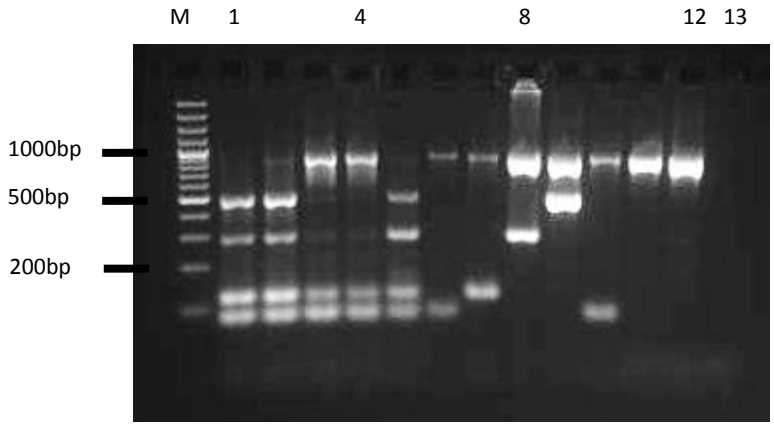

Figure 5.8: Multiplex PCR with addition of specific primers for amplification of decapod DNA

M: 100-bp DNA ladder plus (Fermentas); lane 1: all four vibrio species DNA (20 ng each) and *P. monodon* genomic DNA (20 ng); lane 2: all four vibrio species DNA (20 ng each) and excess *P. monodon* genomic DNA (30 ng); lane 3: all four vibrio species DNA (20 ng each) and excess *P. monodon* genomic DNA (50 ng); lane 4: all four vibrio species DNA (20 ng each) and excess *P. monodon* genomic DNA (70 ng); lane 5: all four vibrio species DNA (20 ng each) and *P. ornatus* genomic DNA (20 ng); lanes 6-12: biplex PCRs; lane 6: *V. owensii* DY05^{T} DNA and *P. monodon* DNA; lane 7: *V. harveyi* DNA and *P. monodon* DNA; lane 8: *V. campbellii* DNA and *P. monodon* DNA; lane 9: *V. rotiferianus* DNA and *P. monodon* DNA; lane 10: *V. owensii* DNA and *P. ornatus* DNA; lane 11: *P. monodon* DNA; lane 12: *P. ornatus* DNA; lane 13: sterile water. The 200, 500 and 1,000-bp sized bands (ladder) indicated as references.

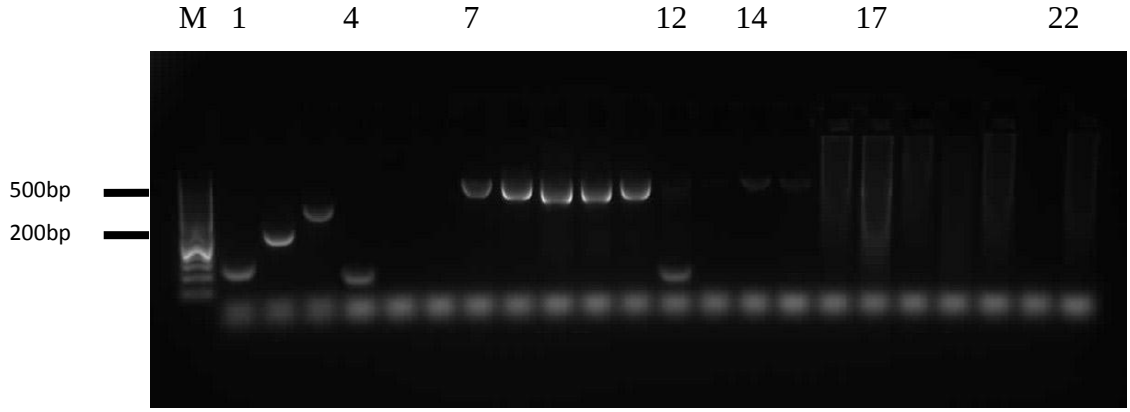

Figure 5.9: Multiplex PCR tested on DNA samples from several wild and reared decapods in northern Qld

M: 500-bp DNA ladder (Fermentas); DNA from: lane 1: *V. harveyi* (20 ng) and *P. ornatus* (20 ng); lane 2: *V. campbellii* (20 ng) and *P. ornatus* (20 ng); lane 3: *V. rotiferianus* (20 ng) and *P. ornatus* (20 ng); lane 4: *V. owensii* (20 ng) and *P. ornatus* (20 ng); lane 5: cultured larva of *S. serrata*; lane 6: wild *P. aesculentus*; lane 7: wild *P. merguiensis*; lane 8: cultured larva of *S. serrata*; lane 9-11: cultured *P. monodon*; lane 12: cultued larva of *S. serrata*; lane 13: wild *P. aesculentus*; lane 14: wild *P. merguiensis*; lane 15: cultured larva of *S. serrata*; lane 16-21: cultured *P. monodon*; lane 22: sterile water. The 200 and 500-bp sized bands (ladder) indicated as references.

This is the first method method designed to simultaneously detect and identify potential *V. harveyi, V. campbellii, V. rotiferianus* and *V.owensii* pathogens. Compared to culture-based biochemical tests (completed in 72 h) and sequencing of genetic markers, e.g 16S rRNA (completed in days or weeks), the monoplex and multiplex PCR protocols designed here offer definitive identification of *V. harveyi*-like isolates, and can be completed in three and five hours, respectively. Compared to MLSA and real-time PCR based methods, these tests are cheap, easy to perform and provide reliable, fast and cost-effective detection of *V. harveyi*-related pathogens in aquaculture systems.

In summary, this study describes a multiplex PCR assay capable of specifically detecting and discriminating the highly similar bacterial species *V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii*, as relevant pathogens of marine aquaculture animals. Four specific sets of primers were designed targeting three protein-coding genes conserved in vibrios: the *topA* gene for DNA amplification of *V. harveyi* and *V. owensii* strains, and the *ftsZ* and *mreB* genes for amplification of *V. campbellii* and *V. rotiferianus* strains, respectively. The single tube PCR reaction contains a mix of

four specific and compatible primer sets, DNA from one, two, three or all the four target vibrio species and common PCR reagents. This PCR protocol allows simultaneous detection and identification of *V. harveyi*-like isolates based on the amplification of different size and specific DNA regions in each of the bacterial species. Any combination of DNA templates in the multiplex PCR mix results in a two, three or fourplex band pattern visualised in agarose gels.

In cases of bacterial isolation from decapod crustacea, a qualitative assessment is included in the protocol to evaluate the DNA quality in genome preparations and to get an approximate estimation of the host/vibrio DNA proportion. This consists in the addition of previously designed primers for specific amplification of decapod ribosomal genes. The multiplex PCR offers fast and reliable single step detection and a discriminative identification of these highly similar vibrios. The method can be used for identification of *V. harveyi*-like clinical and environmental isolates and for direct detection of pathogens in clinical samples.

CHAPTER 6. REAL-TIME PCR PROTOCOL FOR DETECTION OF *VIBRIO OWENSII*

6.1 Introduction

The design of sensitive molecular methods for determination of Vibrio infection would greatly benefit the prawn and lobster aquaculture industry. Conventional approaches for *V. harveyi* quantification have been designed in the past, such as the most-probable-number method (MPN) combined with biochemical tests and colony blot hybridization, but these are highly time-consuming providing results after three or four days (Thaithongnum et al., 2006). The traditional and the multiplex PCR approach for the detection of pathogens have also disadvantages that have been recently overcome through the use of real-time PCR technology. A traditional PCR-based detection is time-consuming, involves a risk of contamination, as it requires visualization of PCR products in an agarose gel after amplification. Furthermore, conventional PCR does not allow accurate quantification of the bacterial density in samples being investigated such as hatchery water, food supply or complex marine samples such as sediments. The introduction of the real-time PCR platform has made detection of microbial pathogens rapid and the analysis of results simple. Accumulation of amplified DNA is measured by determining the increase in fluorescence over time, and this is followed by confirmation of specific amplification by melting curve analysis. Another advantage of the real-time approach is that they can detect cells in the "viable but not culturable" (VBNC) state.

A high proportion of *V. harveyi*-related strains have been reported as pathogenic in aquaculture environments (Nakayama et al., 2005; Alavandi et al., 2006), showing different virulence mechanism depending on the strain and the host to infect. In the case of *V. owensii*, both DY05^T and 47666-1 strains are highly pathogenic to cultured crustacea (*P. ornatus* and *P. monodon*) while CAIM 994 (Thompson et al., 2007) was isolated from fish (*Lutjanus guttatus*). Other *V. harvey*i-like strains, reclassified as potential *V. owensii* strains (PA2, 1DA3, D1; Chapter 4) were isolated from fish and diseased corals (Lin et al., 2011), and *L. vannamei* prawns (LMG 20370; Thompson et

al., 2001). If all these strains were pathogenic and commonly known genes were involved in virulence, a PCR protocol targeting a marker gene and one or two virulence genes could be considered a useful detection tool for *V. owensii* pathogens. However, the lack of knowledge about *V. owensii* pathogenicity mechanisms for aquatic animals and the known ability of vibrios to recombine under high microbial contact suggest that the presence of *V. owensii* species in any compartments of an aquaculture system would require prompt reaction to eliminate the potential for an epizootic.

In this study, the real-time PCR assay designed targeted the *topA* gene, as a suitable gene marker for *V. owensii* (Chapter 4), and used the SYTO9 technology for rapid and sensitive DNA detection and quantification. This assay would assist in the understanding of *V. owensii* infections and the development of effective methods for eradication.

6.2 Materials and Methods

6.2.1 Bacterial strains and DNA purification

The vibrio strains tested in this study are listed in Table 6.1. Bacteria kept at -80ºC were grown overnight in MB at 28ºC with shaking. Bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) following manufacturer's instructions for gram-negative bacteria.

6.2.2 Design of oligonucleotide primers

Sequences from the protein-coding gene *topA* from three *V. owensii* strains, from ten other vibrio species (Table 6.1) and from multiple *V. harveyi*-like isolates (from Chapter 4), were aligned in order to design specific primers for *V. owensii* detection. Oligonucleotide primers were designed and analyzed manually based on different alignments created by using Vector NTI Advance Software (Invitrogen) and BioEdit 7.0.5 (Hall, 1999) and with the aid fo AlleleID 7.7 (Primer Biosoft International) to determine the G+C content and self-dimer and hairpin structures. Allele ID software also allows the avoidance of non-target species within the alignment. The specificity of each primer sequence was evaluated using the GenBank database and BLAST (Basic

Local Alignment Search Tool). The oligonucleotide primers were custom synthesised by Sigma-Aldrich Pty Ltd. (Australia). The designed primers: DYA2-F: 5'-GGT AAT GTA TGG AGC AGA C-3' and DYA2-R: 5'-GGA CAT CAA CGC AAA TAC A-3', targeted 198-bp segment of the *topA* gene.

Table 6.1: *Vibrio owensii* and other type strains tested as non-target species

Strain	*topA* accession no.
V. owensii DY05^T JCM16517^T	GU111254
V. owensii 47666-1	GU111255
V. owensii CAIM994	EF596732
V. harveyi LMG4044^T	DQ907488
V. campbellii LMG11216^T	DQ907475
V. rotiferianus LMG21460^T	DQ907515
V. alginolyticus LMG4409^T	DQ907472
V. parahaemolyticus LMG2850^T	DQ907509
V. natriegens LMG10935^T	DQ907500
V. proteolyticus LMG3772^T	DQ907514
V. tubiashi LMG10936^T	DQ907521
V. corallilyticus LMG20984^T	EF114213
V. brasiliensis LMG20546^T	DQ907473

6.2.3 Real-time PCR and cycling parameters

Reactions were performed using a Rotor-Gene 6000 (Corbett Robotics) in a 72-well rotor. PCR reactions contained 1X PCR buffer (Tris·Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7) (Qiagen), 0.2 µM of DY2A-F and DY2A-R primers, 200 µM dNTPs, 0.5 units of HotStart DNA Taq Plus polymerase (Quiagen), 0.5 µl of SYTO9 dye, 20 ng of DNA template and de-ionised sterile water, to make a final volume of 20 µl. The optimised thermal program included an initial denaturation step of 95ºC for 15 min, followed by 45 cycles of amplification. Each cycle consisted of template DNA denaturation at 94ºC for 30 sec, primer annealing at 58.4ºC for 1 min and extension at 72ºC for 1 min. The increase in fluorescence was measured and recorded after the

extension step at each cycle. After the primer extension step of each amplification cycle, the increase in the fluorescence from the amplified DNA was recorded by using the green optic channel, with excitation at 470 nm and detection at 510 nm. A PCR mixture containing no DNA (PCR-grade sterile water) was always used in each run as a negative control. Following amplification, a high resolution melting curve analysis of the amplified DNA was performed between 70ºC and 99ºC, with the temperature increasing at a rate of 0.1ºC/s. Data acquisition and analysis was performed using Rotor-Gene 6000 and Microsoft Excel. The amplified DNA was further analyzed in a 1% agarose gel, and the expected molecular weight of the amplicons was confirmed by comparison to a known DNA size marker.

6.2.4 Specificity of the real-time PCR

The specificity of the primers was tested against multiple *V. harveyi*-related strains from the AIMS and JCU (Table 4.1), and against imported type strains from other related vibrios with similar *topA* gene sequences (Table 6.1). Real-time PCR was performed on DNA from *V. owensii* (DY05^T, 47666-1 and CAIM994) and all non-target species (NTS) with DYA2-F and DYA2-R primers and using the cycling conditions and reagents listed above. Amplified PCR products were sequenced by Macrogen Ltd (Korea) and sequences compared to those obtained in Chapter 3 (description of *V. owensii*).

6.2.5 Quantitation and sensitivity of detection

Standard curves and sensitivity of the real-time were determined with purified DNA from a culture of *V. owensii* (DY05^T). Purified DNA of DY05^T was serially diluted 10-fold from 20 ng to 2 fg in sterile water and subjected to real-time PCR amplification by using the cycling conditions described above. All reactions were performed in triplicate, including negative controls which contained de-ionised sterile water rather than template DNA. The minimum amount where the Ct (cycle threshold) value was within 45 for all triplicate samples was considered as the detection limit.

6.3 Results

The three tested *V. owensii* strains exhibited positive amplification of a 198-bp region of the *topA* gene with the primer set DY2A-F and DY2A-R. No amplification of *topA* occurred for any of the non-target-species or non-template control (NTS or NTC) (Fig. 6.1). BLAST analysis of the primers exhibited complete homology with the corresponding regions in all *V. owensii* strains and no matches with any other *Vibrio spp.* were obtained. A BLAST comparison of the PCR product had 98%-100% sequence identity with other potential *V. owensii* strains. These included the species *V. communis* and several *V. harveyi* (1DA3, D1, PA2) that were previously described as potential misclassified *V. owensii* strains (Chapter 4). Matches below 95% were observed for every other organism. Amplification of the 198-bp *topA* gene region in the three *V. owensii* strains was confirmed by melting temperature analysis and gel electrophoresis (data not shown). A high resolution melting curve analysis showed different melting temperatures of 83.24ºC and 83.94ºC for DY05^{T} and 47666-1 respectively, due to a 2 nucleotide (A→G) difference in the PCR products of the two strains (Fig. 6.2).

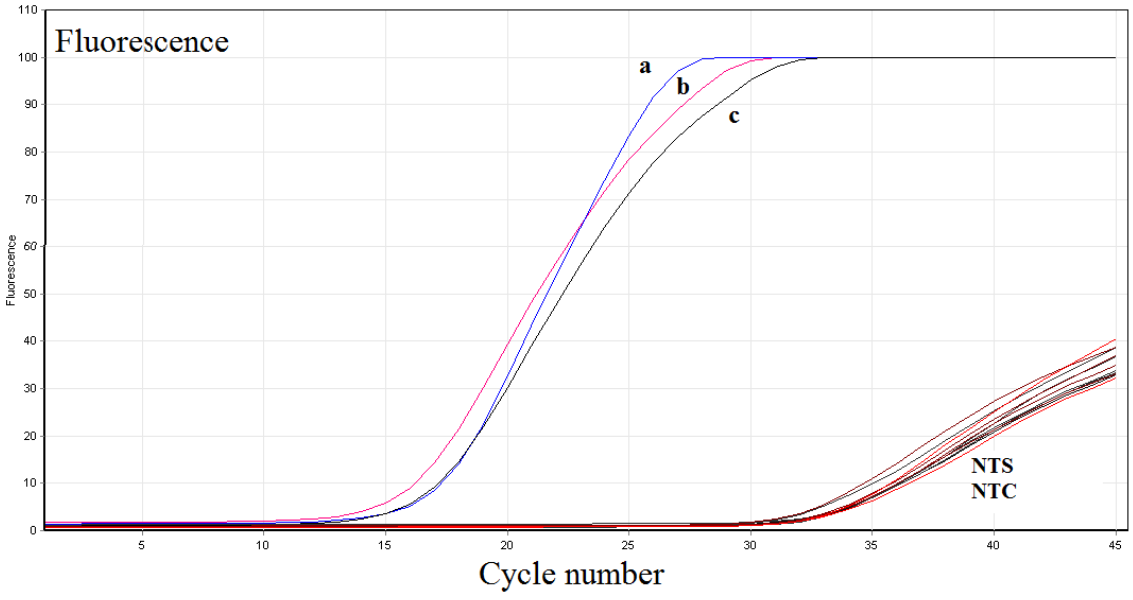

Figure 6.1: Representative results of *V. owensii* a) 47666-1, b) DY05^{T} and c) CAIM994 amplicons detection in channel Green using real-time PCR with SYTO9 technology

Negative amplification was obtained for non-target species (NTS; Table 6.1) and non template control (NTC; sterile water).

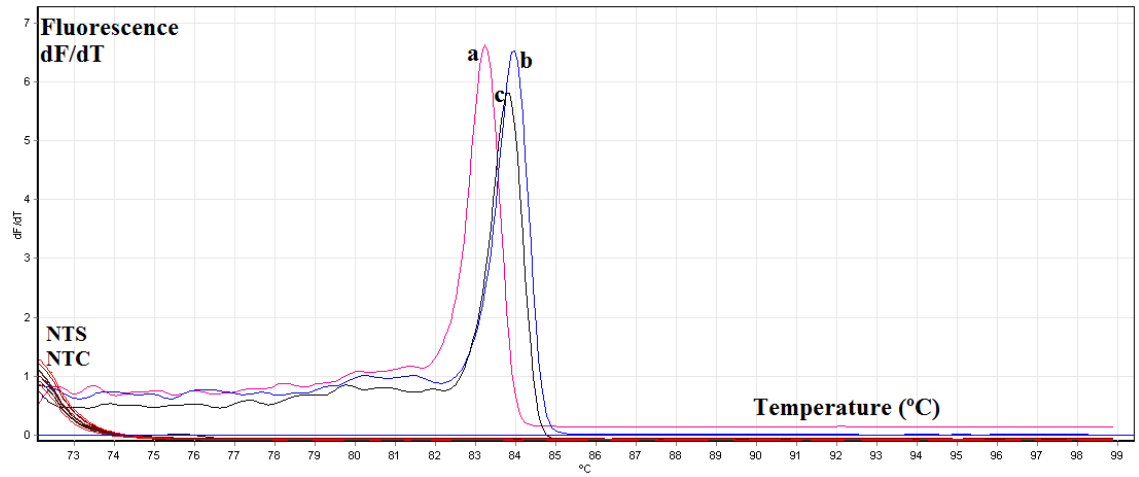

Figure 6.2: High resolution melting curve analysis after real-time amplification of *V. owensii* strains using SYTO9 technology.

Melting temperatures for a) DY05^T (83.2ºC), b) 47666-1 (83.9ºC) and c) CAIM 994 (83.8ºC). Negative amplification was obtained for non-target species (NTS; Table 6.1) and non-template control (NTC: sterile water).

The detection limit was 20 fg of purified genomic DNA of *V. owensii* DY05^T with a Ct value of 27.33 ± 2.08 (Table 6.2). The Ct values increased as the concentration of DNA decreased (Fig. 7.3A, B, C). The minimum amount of DNA detected was 2 fg (10^{-7} dilution) although in every run, at least one of the triplicate samples was not amplified, resulting in increased standard deviations in the calculated DNA concentration values (8.3 fg ± 10.42) and ct values (35.76 ± 4.95) (Table 6.2). The standard curve showed a good linear correlation between the Ct values and the concentrations of DNA template (r^2 =0.99, efficiency =1.01) (Fig 7.3B). The expected dissociation temperature was maintained at 83.2ºC for all the DY05^T DNA dilutions (Fig. 7.3D).

Table 6.2: Sensitivity of detection of purified DNA from DY05^T by real-time PCR

DNA Dilution	DNA conc.	Calculated DNA conc.*	*Ct* value*
Negative	0	0	34.78 ± 2.74
10^0	20 ng	12.89 ng ±1.47	12.39 ± 0.16
10^{-1}	2 ng	2.33 ng ± 0.29	14.89 ± 0.17
10^{-2}	200 pc	230 pc ± 20	18.26 ±0.14
10^{-3}	20 pc	25.66 pc ± 0.57	21.46 ± 0.03
10^{-4}	2 pc	1.9 pc ± 0.26	25.26 ± 0.22
10^{-5}	200 fg	236.6 fg ± 40.41	28.13 ± 0.52
10^{-6}	20 fg	27.33 fg ± 2.08	32.07 ±1.14
10^{-7}	2 fg	8.3 fg ± 10.42	35.76 ± 4.95

*The data are means ± standard deviations for three different runs. *Ct*, cycle threshold.

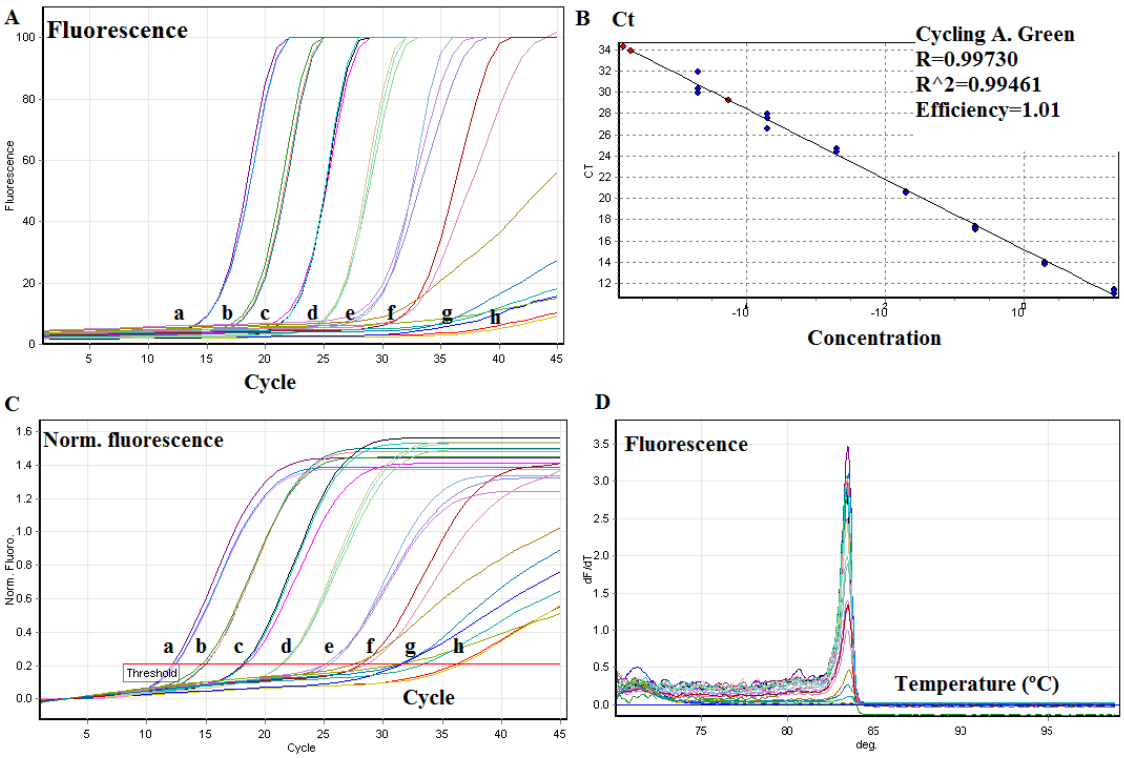

Figure 6.3: Sensitivity of real-time assay (SYTO9) for *V. owensii*

Analysis of seven 10-fold dilutions (a-g) from 20 ng to 20 fg of *V. owensii* DY05^T standard. (A) Fluorescence, (B) standard curve generated, (C) normalised fluorescence and threshold calculation and (D) high resolution melting curve analysis after real-time PCR amplification. Negative amplification for non-template control (h; NTC).

6.4 Discussion

Here I describe the first real-time assay for the rapid detection of *V. owensii* strains. Amplification with the DY2A-F and DY2A-R primer set resulted in the expected 198-bp PCR product from *V. owensii* strains DY05^T and 47666-1. The primer sequences did not show matches for any other vibrio species and no DNA amplification occurred for the ten non-target species tested, which indicated that this assay is highly specific for *V. owensii*. The specific product melting peaks with no primer-dimer of other non-specific product signals provided further evidence of its specificity. The method allows simultaneous detection and quantification of *V. owensii* cells since the primers target the single copy gene *topA* in this species genome. Although the *topA* has been used for conventional PCR of *V. owensii* (Chapter 5), this method would not provide quantitation of *V. owensii* cells unless it is used in combination with other methods, such as the time consuming most-probable-number (MPN) approach. Therefore, it was necessary to transfer this protocol to a real-time platform in order to offer a decision making tool in case of infection and to resolve research questions regarding the pathogenicity mechanisms of these strains for *P. ornatus* and *P. monodon*.

The Wizard Genomic DNA Purification Kit (Promega) was used to purify the genomic DNA of all the vibrio strains tested in this project. This kit could prepare highly pure template DNA that allowed detection of *V. owensii* in unenriched cultures without compromising the quality of the real-time PCR efficiency. Other extraction methods, such as boiling and the use of the High Pure Template Preparation Kit (Roche Diagnostics) were previously tested for DNA extraction of DY05^T, but the Wizard Genomic Purification Kit produced the best quality DNA in high concentration. By using the optimum PCR cycling parameters and reagents, it was possible to detect down to 2 fg of *V. owensii* (DY05^T) DNA. Furthermore, by high resolution melting curve analysis it was possible to discriminate between DY05^T and 47666-1 strains due to a two-nucleotide difference in their PCR products, causing a slight difference (0.7ºC) in the melting peaks showed in their dissociation curves.

The widely used SYBR Green I technology has been reported to have several limitations including limited dye stability, dye-dependent PCR inhibition, and selective detection of amplicons during DNA melting curve analysis of multiplex PCRs (Monis

et al., 2005). The SYTO9 technology was selected for the real-time PCR of *V. owensii* because this intercalating dye SYTO9 can be used over a broader range of dye concentrations without causing PCR inhibition. The improved reproducibility of DNA melting curve analysis is what makes of SYTO9 a useful dye in a diagnostic context (Monis et al., 2005). Compared to SYBR Green, used in previous tests for amplification of *V. owensii topA* gene, SYTO9 produced more robust and consistently shaped DNA melting curves and these were less affected by dye concentration (data not shown). Although melting curve analysis with dyes such as SYTO9 or SYBR Green is usually considered less specific compared to the use of fluorescent probes, i.e TaqMan PCR (Heid et al., 1996), an exhaustive optimisation of primer design and reagent concentration in this study allowed the use of SYTO9, making the real-time assay more cost-effective. This detection and quantification tool for *V. owensii* provides accurate and reliable results in real-time with no need for further analysis. The assay can be completed within 6 h, compared with days if conventional culture based methods are used. The assay has the potential to be further developed to quantify *V. owensii* from clinical samples and all aquaculture system compartments.

The procotol here described is the first real-time PCR assay designed for rapid detection and quantification of *V. owensii* pathogens. The specific primers target a 198-bp region of the *topA* gene in this species and the SYTO9 technology allows sensitive detection and quantification of *V. owensii* DNA. The detection limit is 20 fg of purified genomic DNA of *V. owensii* $DY05^T$ with a Ct value of 32.07 ± 1.14. Different dissociation temperatures in high resolution melting curve analysis were able to differentiate the lobster pathogen $DY05^T$ (83.2ºC) from the prawn pathogen 47666-1 (83.9ºC) due to a two-nucleotide difference in the PCR products of these strains. The standard curve showed a good linear correlation between the Ct values and the concentrations of purified DNA (R^2= 0.99). A refined optimisation of primer design and reagent concentration allowed the use of SYTO9, making the real-time assay more cost-effective. The real-time assay designed for detection and quantification of *V. owensii* would provide farmers with a reliable single-day decision tool depending on the level of infection. As a research tool, it will allow the study of *V. owensii* dynamics in crustacean rearing systems, the infection process in experimentally infected animals or its impact in the environment.

CHAPTER 7. EXPERIMENTAL CHALLENGE OF *PANULIRUS ORNATUS* WITH *VIBRIO HARVEYI*-RELATED STRAINS AND *VIBRIO OWENSII* EXTRACELLULAR PRODUCTS

7.1 Introduction

The ornate spiny or rock lobster (*P. ornatus*), is a potential candidate for aquaculture in Australia. It is the fastest growing species of the family Palinuridae and possesses one of the shortest larval phases of any spiny lobster. At AIMS, the aquaculture team attempts the development of a closed life cycle breeding program for *P. ornatus* at a commercial level but a challenging microbial environment still contributes to high larval mortalities in the late stages of the larval rearing (Hall et al., unpublished). During the experimental trials, signs of vibriosis are commonly observed in moribund larvae, correlating with the larval moult, when these animals are particularly susceptible to infection (Webster et al., 2006).

Numerous *V. harveyi*-like strains identified as *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* (Chapter 4) were isolated from moribund larvae, live feeds and bacterial biofilms within the larval rearing tanks of *P. ornatus*. Experimental challenges demonstrated the high virulence of *V. owensii* DY05^T against newly hatched larvae (Goulden et al., 2012) but no more isolates were tested for pathogenicity. Initial phenotypic characterisation revealed that *V. owensii* DY05^T is highly haemolytic and proteolytic (Goulden, 2012). In the delineation of the *V. owensii* species (Chapter 3), a second strain (47666-1), isolated from diseased *P. monodon* in a prawn hatchery in northern Qld (Harris, 1993) was described along with the type strain DY05^T, but the virulence of this strain to *P. ornatus* is unknown.

The aims of this study were: a) assess the virulence of several *V. harveyi*-related strains on larvae of *P. ornatus*; b) compare the virulence of *V. owensii* strain DY05^T with that of strain 47666-1 to *P. ornatus*; c) evaluate the role of Artemia as live feeds on the infection by pathogenic vibrios; and d) asses the toxicity of the extracellular products (ECPs) secreted by pathogenic strains on *P. ornatus* larvae.

7.2 Material and Methods

7.2.1 Bacterial cultures

Stock cultures of bacteria (Table 7.1) were maintained frozen at -80ºC in either MB with 30% (v/v) glycerol or in Microbank^{TM} cryovials (Pro-Lab Diagnostics). Bacteria were grown overnight on TCBS agar plates at 28ºC. Individual colonies picked after 24 h were used as inocula for liquid cultures in MB and these were incubated overnight at 28ºC with shaking.

7.2.2 Bacterial Density Measurements

Growth in bacterial cultures was determined by direct measurement of the optical density at 600 nm (OD_{600}) in a spectrophotometer (Model GeneQuant t *pro* UV/Vis Biochrom, England), from 1 ml volumes of each culture in a polystyrene cuvette. For lobster larvae *in vivo* challenges, live bacterial cells in inocula were counted with a Helber bacterial counting chamber under a light microscope. Bacterial density in water and larvae samples was determined by serial dilution. Volumes of 100 µl from each sample were diluted several times and plated on marine agar (MA) or thiosulphate-citrate-bile-salts-sucrose (TCBS) agar plates. The number of colonies and the dilution factor were used in order to determine the concentration of bacterial in the samples,

7.2.3 Oral and immersion challenge of *P. ornatus* with vibrio isolates

Healthy larvae of *P. ornatus* were exposed to several *V. harveyi*-like strains isolated from different sources in Australia and identified by MLSA (Chapter 4). Selected strains isolated from moribund larvae of *P. ornatus* (47666-1, DY05^{T}, D40 and C071) were haemolytic and/or proteolytic, while those strains isolated from healthy or wild larvae or from ongrowing Artemia were not (Table 7.1) (Hall et al., unpublished). The larvae were exposed to bacteria by Artemia-vectored oral challenge or by immersion using the experimental infection model described by Goulden et al. (2012). Briefly, oral challenge was performed by enriching instar II Artemia nauplii with ~1 x 10^{6} cells ml^{-1} of each vibrio strain in tissue culture flasks (25cm^{2}; Sarstedt) with orbital agitation at 45 rpm for 120 min. Subsequently, individual larvae, placed in 3 ml FSW volumes in 12-

well Nunc^{TM} tissue culture plates, were challenged with nine nauplii each. In immersion experiments, bacteria were inoculated directly into the wells of the culture plates at a dose of
1×10^6 cells ml^{-1}.

In all the experiments, treatments were performed in triplicate (n = 36) and survival was assessed every 24 h for seven to eight days. Three experiments (E1-E3) were performed using larvae of *P. ornatus* produced by different broodstock and genetic lineages using stage I larvae (2 days old). In the first experiment (E1), the pathogenicity of several vibrio isolates including *V. owensii* DY05^T was tested via Artemia feeding (oral challenge). Experiment E2 compared survival rates of larvae treated with *V. owensii* DY05^T and 47666-1 and *V. harvey*i RR36 by immersion (test E.2.1), as well as a passaged strain of 47666-1 (see procedure below) by oral challenge (test E.2.2). A first control treatment (fed control) consisted of Artemia cultures treated similarlye, except no bacterium was added to enrich the nauplii (blank control). A second blank control treatment consisted of non-fed larvae.

Before inoculation, all compartments of the controls (non-fed larvae, enriched and non-enriched Artemia nauplii and seawater) were examined for the presence of bacteria. This was done by homogenizing samples of one larva, 1 ml of nauplii solution and 200 µl of water in FSW. Each of these samples were then serially diluted and spread on TCBS agar and MA plates. Also, during experiment E2, moribund and healthy larvae and water volumes from oral challenge and immersion treatments were sampled similarly from several extra plates and bacterial counts recorded. All manipulations were performed in a biosafety cabinet. Cell culture plates were incubated in the dark with slow orbital agitation (45 rpm) and larval survival was assessed at approximately 24 h intervals.

Table 7.1: Vibrio strains used for experimental infection of *P. ornatus*

Species	Strain	Source, year of isolation	Characteristics
V. owensii	DY05^T	Sick *P. ornatus* larva, AIMS 2007	*H; P
V. owensii	47666-1	Sick *P. monodon* larva, JCU 1993	*H; P; pathogenic to *P. monodon*
V. owensii	92-47426	Sick *P. monodon* larva, JCU 1993	*Potential clone of 47666-1
V. harveyi	D40	Sick *P. ornatus* larvae, AIMS 2005	# H; P; QS prod
V. harveyi	C071	*P. ornatus* tank biofilm, AIMS 2004	# H
V. campbellii	oz07	Ongrown Artemia, AIMS 2006	# QS prod; luminescent
V. harveyi	H20	Healthy *P. ornatus* larva, AIMS 2005	# Not H; not P; not QS prod
V. harveyi	RR36	Wild *P. ornatus* larvae, Coral Sea 2005	# Not H; not P; not QS prod
V. campbellii	645	Environmental seawater, JCU 1995	# Not H; pathogenic to *P. monodon*

*Data from Goulden (2012) or # (Hall, unpublished). H: haemolytic; P: proteolytic; QS prod.: producer of quorum sensing molecules.

7.2.4 Passage of *V. owensii* 47666-1 in juvenile prawns of *Penaeus monodon*

In the experiments above, *V. owensii* 47666-1 showed no virulence to larvae of *P. ornatus* by oral challenge, despite of the high virulence of this strain to *P. monodon* prawns (Pizzutto and Hirst, 1995). The pathogenicity of 47666-1 could have been lost over time, and in this experiment I attempted to passage the strain by inoculation and re-isolation from its original host, *P. monodon*. Juvenile prawns were sourced from a local commercial farm in northern Qld and transported to the Aquatic Pathology Laboratory at JCU (Townsville). Twenty juvenile prawns were selected based on weight uniformity, with prawns weighing approximately 4 ± 1 g.

A total of 20 prawns were stocked in two glass aquaria (70 l) with lids, each containing 60 l of sterile seawater at a stocking density of five prawns per tank. The experimental system seawater was thermostatically maintained at 30 ± 1°C, oxygenated and filtered using the airlift corner filter system. Water salinity of 30‰ and pH 8.0 were both monitored daily. Prawns were subjected to a photoperiod of 12 h light/dark. All tanks were siphoned daily to remove waste products with 10% of the water volume removed and replaced with fresh seawater. Feeding was carried out twice daily with commercial prawn pellets. Prior to commencement of the experiment, prawns were acclimatised to the experimental system conditions for 48 h with any dead prawns replaced.

Bacterial strains were grown at 28°C in 5 ml of MB in a shaker incubator at 200 rpm. Overnight cultures were used to inoculate fresh broth and incubated for 6 h or up to OD_{600} of 0.1. Cells (1 ml) were pelleted by centrifugation at 6,000 x *g* for 10 min, washed twice in sterile 2% NaCl PBS, and stored at 4°C before being prepared for inoculation.

A total of five prawns were challenged with *V. owensii* 47666-1 to determine mortality compared to five control prawns challenged with sterile 2% NaCl PBS. Five other prawns were also challenged similarly with *V. owensii* $DY05^T$ to observe its effects. Prawns were anaesthetised on iced water prior to challenge and were subsequently inoculated via intra-muscular injection into the third abdominal segment anterior to the telson at a dose of 1×10^5 bacterial cells per prawn. Control prawns were inoculated with 100 µl PBS. Prawns which died in less than six hours following injection were discounted from experiments as it was considered more likely that these animals died of protein shock rather than from toxic effects. Animals were monitored until recovery post-inoculation and subsequently checked 1h, 2 h, 6 h post-inoculation, six-hourly for 24 h and then weekly for seven days with mortality rates recorded at each check. Anesthetised moribund animals treated with 47666-1 were sampled by exsanguation to reisolate strain. The haemolymph (2 ml) was plated on TCBS agar plates and grown at 28ºC for 24 h. Yellow colonies were selected and grown in MB for another 24 h and a subset of these cultures was stored at -80ºC for later inoculation of *P. ornatus* larvae. A second subset of these cultures was used for DNA extraction and identification of *V. owensii* 47666-1 by real-time PCR (Chapter 6).

7.2.5 Oral challenge of *P. ornatus* with passaged *V. owensii* 47666-1

To test the pathogenicity of passaged 47666-1 on healthy larvae of *P. ornatus*, those cultures re-isolated from moribund prawns and confirmed as *V. owensii* by PCR, were grown on MB overnight and introduced to the larvae via Artemia in experiment E2 (test E.2.2), as explained above (section 8.2.2).

7.2.6 Extracellular products (ECPs)

The extracellular products (ECPs) of *V. owensii* DY05^T, 47666-1 and non-pathogenic *V. harveyi* RR36 were prepared following the cellophane overlay method slightly modified (Liu, 1957). Briefly, autoclaved (121°C for 15 min) cellophane sheets were placed on the surface of MA plates, and spread with 200 µl of overnight bacterial cultures in MB. After incubation for 48 h at 28°C, each cellophane sheet was transferred to an empty Petri dish lid placed on ice. The bacterial cells were scraped into 3 ml of ice cold NaCl PBS at pH 7.2. Following centrifugation (14,000 x *g* for 15 min at 4°C), the supernatant comprising the ECPs was filtered through 0.22 µm (Millipore Millex, Watford, UK) membranes and kept on ice for immediate inoculation. The protein concentration of the ECP was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific; Rockford, IL USA), using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. Following Montero and Austin (1999), the sensitivity of ECPs to heat was examined by heating samples to 100°C for 30 min and centrifuged at 12,000 x *g* for 10 min. For protein digestion, ECPs were mixed at an ECP/proteinase K ratio of 6:1 with a solution containing 2.5 µg µl^{-1} of proteinase K (Sigma Chemical Co., Poole, UK) and incubated at 60°C for 1 h.

7.2.7 Challenge of *P. ornatus* with *V. owensii* DY05^T extracellular products

In experiment E3, larvae of *P. ornatus* were exposed by immersion to crude ECPs (test E.3.1), heat-treated ECPs (100ºC) and digested ECPs (2.5 µg µl^{-1} proteinase K) (test E.3.2) from *V. owensii* DY05^T and the *V. harveyi* control strain (RR36). Volumes of 50 µl containing 15 µg of native ECPs, heated ECPs and proteinase K-treated ECPs were added into each well of the cell culture plates, containing one larva each. Positive control larvae were orally challenged with DY05^T-fed Artemia and negative controls were treated with RR36-fed Artemia. Blank controls consisted of non-enriched Artemia-fed larvae, non-fed larvae as described for E1 (section 8.2.2) and larvae inoculated with PBS without ECPs added. For digested ECP treatments, protease inhibitors were added to the samples before inoculation to avoid any potential proteolytic digestion of larvae tissues by the proteinase K. In this case, ECP-proteinase K samples were treated with 20 µl ml^{-1} of Halt Protease Inhibitor Single-Use-Cocktail (Thermo Scientific, Rockford; IL USA). A blank control for this treatment consisted of

larvae inoculated with PBS without ECPs added and an equivalent amount of proteinase K and protease inhibitors.

Animals to be treated with ECPs and the associated controls received antibiotic treatment before inoculation to avoid the effect of any resident microbiota in larval survival. The treatment consisted of an antibiotic cocktail (25 mg l^{-1} erythromycin, 25 mg l^{-1} oxytetracycline, 10 mg l^{-1} streptomycin and 40 mg l^{-1} ciprofloxacin) added to the main rearing tank 24 h prior to collection, acclimation and challenge as outlined above. Larval survival was assessed at approximately 24 h intervals for eight days.

7.2.8 Statistical analyses

Any differences between survival curves were determined using the product-limit (Kaplan-Meier) estimator, employing log rank and Wilcoxon Chi-squared statistics, and confirmed by an Analysis of Variance (ANOVA). A post-hoc means comparison was conducted using a Dunnett's test to compare multiple sets to a defined control group. Statistical significance was standardised at $\alpha = 0.05$. All analyses were performed using the statistical software package JMP®7 (SAS).

7.3 Results

Before the beginning of each larval challenge, all system compartments (water, larvae and Artemia) were confirmed free of residual bacteria following observation of TCBS and MA plates grown overnight. To validate the observations of previous experiments showing pathogenicity of *V. owensii* DY05^T to *P. ornatus* (Goulden et al., 2012), mortality of larvae challenged with this strain was recorded in three independent experiments (E1, E2, E3).

7.3.1 Experiment E1: Oral challenge of *P. ornatus* larvae with vibrio strains

In Artemia-vectored treatments (E1) approximately 90% cumulative mortality was observed 48 h post-exposure, with the heaviest mortality rate (70%) occurring during day 1 (Fig. 7.1). Larval mortality was ~100% by day three-four, while mortality of

controls (fed and non-fed larvae) was ~0% by day five. Moribund larvae slowed down swimming, turned white and eventually sank. A few hours later, larvae had practically disintegrated on the bottom of the wells.

No significant differences (Table 7.2: Dunnett's test $p > 0.05$) were found between controls and the rest of the treatments with other vibrio strains (90-100% survival by day seven; Fig. 7.1), indicating that only *V. owensii* DY05^T was pathogenic to the larvae by oral challenge. The strain was therefore selected as a positive control for the following experiments.

Larvae challenged with *V. harveyi* RR36, a strain isolated from wild larvae, showed the highest survival rate by the end of the experiment on day seven (94.4%) compared with other vibrio treatments (75-88.8%) (Fig. 7.1). This strain was therefore selected as a second negative control (together with non-treated larvae) in the following experiments.

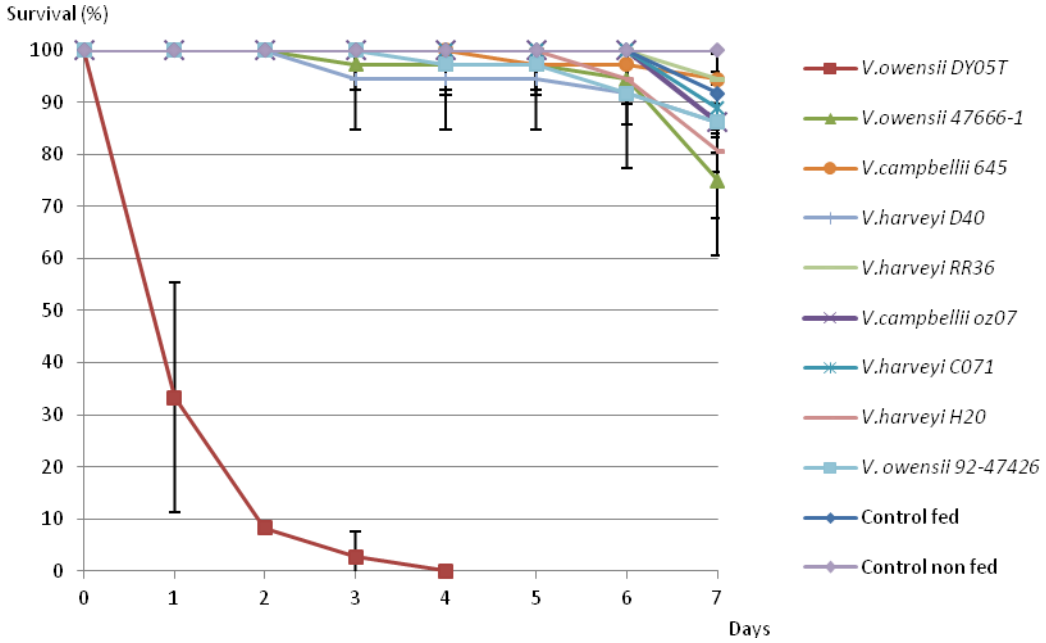

Figure 7.1: Percent survival of *P. ornatus* exposed to vibrio strains by oral challenge in experiment E1

Dose: 9 nauplii enriched with 10^6 cells ml^{-1} per larva. Controls: a) *control non-fed*: blank; b) *control fed*: negative control (larvae fed with non-enriched Artemia). Mean survival with SE bars are shown for seven days post-inculation.

7.3.2 Experiment E2: Immersion challenge of *P. ornatus* with *V. owensii* strains and oral challenge with reboosted *V. owensii* 47666-1

The survival curves in E2 differed from those in E1 when strains *V. owensii* DY05^{T} and 47666-1 isolates were administered by immersion (10^{6} cells ml^{-1}) compared to oral challenge. In test E.2.2 survival curves of DY05^{T}-Artemia-fed larvae, negative controls (RR36-Artemia, fed and non-fed larvae) and a replicate of the 47666-1-Artemia treatment showed no differences to curves in E1 (Fig. 7.2). However, for immersion treatments with DY05^{T} and 47666 (test E.2.1), survival decreased to 45% and 61.1%, respectively by the end of the experiment, demonstrating significant mortalities respective to control larvae (Dunnett's test $p = <0.0001$: Table 7.2). Survival of larvae inoculated by immersion with 10^{6} cells ml^{-1} of RR36 showed no significant differences with negative control treatments (non-fed larvae), reaching day eight of the experiment with high survival rates (75%).

Table 7.2: Comparison of means using Dunnett's method (α = 0.05) in each experiment

Control for each test indicated in italics

E1: Oral challenge		**E2: Immersion challenge and oral challenge of reboosted 47666-1**				**E3: Extracellular products (ECPs)**			
Test E.1		**Test E.2.1**		**Test E.2.2**		**Test E3.1**		**Test E.3.2**	
Control fed		*Control non-fed*		*Control fed*		*Control PBS*		*Proteinase K control*	
Treatment	p-value	Treatment	p-value	Treatment	p-value	Treatment	p-value	Treatment	p-value
V. campbellii oz07-Art+	0.9822	PBS control	1.0000	Non-fed control	0.9767	Not inoculated	0.9936	PBS	0.7918
V. campbellii 645-Art+	0.8404	Not inoculated	0.7918	RR36-Art+	1.0000	DY05^{T} ECP	<0.0001*****	Not inoculated	0.1828
V. harveyi H20-Art+	0.9338	RR36 immersion**#**	1.0000	47666-1-Art+	0.9936	DY05^{T} ECP 100ºC	<0.0001*****	RR36-protK	0.9989
V. harveyi D40-Art+	0.3136	47666-1 immersion**#**	<0.0001*****	*Passage*d 47666-1-Art+	1.0000	RR36 ECP	<0.0001*****	DY05^{T}-protK	0.9936
V. harveyi C071-Art+	1.0000	DY05^{T} immersion**#**	<0.0001*****	DY05^{T}-Art+	<0.0001*****	RR36 ECP 100ºC	<0.0001*****		
Control non fed	0.9822					DY05^{T}-Art+	<0.0001*****		
V. harveyi RR36-Art+	0.9999					RR36-Art+	0.9767		
V. owensii 47666-1-Art+	0.4342								
V. owensii DY05^{T}-Art+	0.0000*								

* Significant differences to control treatments (n=36)
\+ Vibrio oral challenge using Artemia as vector (9 nauplii per larva previously enriched with 10^6 cells ml^{-1})
\# Vibrio immersion challenge by bath inoculation (10^6 cells ml^{-1})
ProtK: proteinase K

Sampled moribund larvae fed on DY05^T-enriched Artemia contained ~1.5 x 10^7 cfu ml^{-1} two days post-inoculation while a few remaining healthy larvae contained only ~2.7 x 10^3 cfu ml^{-1} by day three of the experiment. Water sampled from the wells of these larvae contained only ~1.75 x 10^5 cfu ml^{-1} (moribund) and ~2.7 x 10^4 cfu ml^{-1} (healthy) (Table 7.3). Larvae treated fed with 47666-1 and RR36-enriched Artemia were not sampled since these treatments did not cause significant mortality to the animals.

Sampled moribund larvae inoculated by immersion with DY05^T and 47666-1 live cells contained only ~2.3 x 10^4 and ~1.8 x 10^4 cfu ml^{-1} respectively by five days post-inoculation and healthy larvae inoculated with RR36 cells contained ~1.5 x 10^3 cfu ml^{-1} (Table 7.3). In contrast to oral challenge in E1, the water sampled from the wells of bath inoculated moribund larvae by day five-six contained the highest bacterial load: ~6.3 x 10^6 cfu ml^{-1} (DY05^T) and ~4.1 x 10^6 cfu ml^{-1} (47666-1), while water sampled from wells of healthy RR36-treated larvae contained only ~9.1 x 10^4 cfu ml^{-1}.

In the prawn inoculation experiments, four of the five prawns injected with 47666-1 showed signs of disease two days post-inoculation and they were sampled on day three to re-isolate the *passag*ed strain from the haemolymph. Those prawns injected with DY05^T did not show any signs of disease and survived the experiment. Oral challenge with the *passag*ed 47666-1 strain (test E.2.2) did not cause significant larval mortality compared to controls (fed larvae) (Dunnett's test $p = 1.000$) (Table 7.2), with a similar survival curve to that of larvae treated with the original 47666-1 strain (Fig. 7.2).

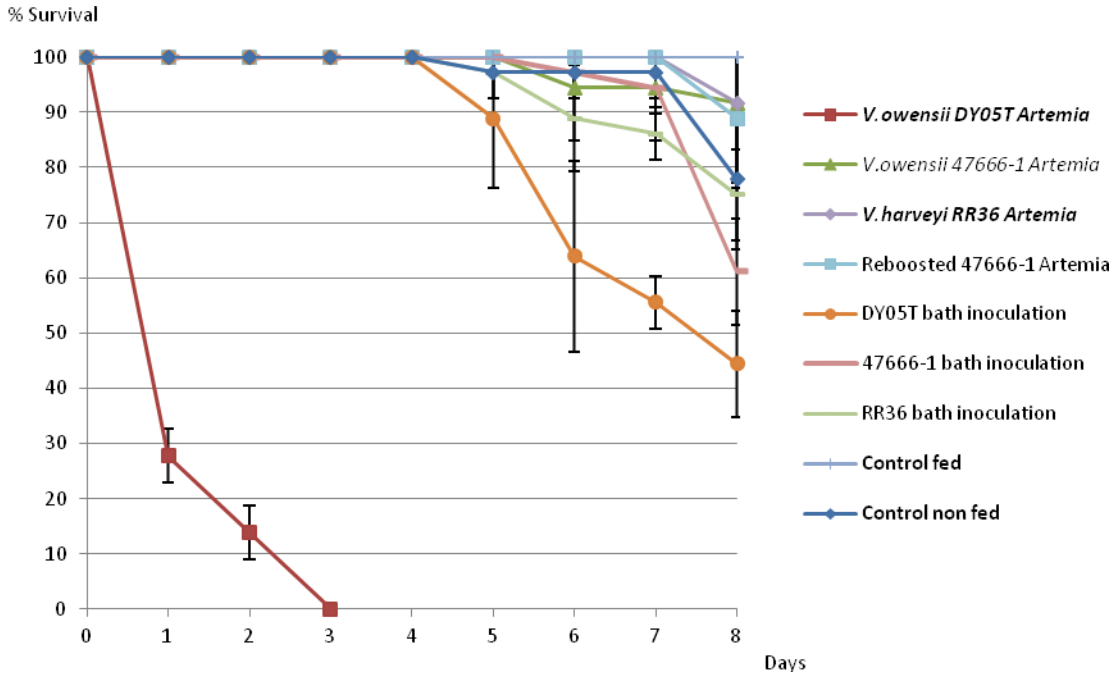

Figure 7.2: Percent survival of *P. ornatus* larvae exposed to *Vibrio owensii* strains by oral challenge and immersion in experiment E2

Doses: Artemia: 9 nauplii enriched with 10^6 cells ml^{-1} per larva; Bath inoculation/immersion: 10^6 cells ml^{-1}. Controls: a) *control non-fed*: negative control; b) *control fed*: negative control (larvae fed with non-enriched Artemia); c) *V. owensii DY05^T Artemia*: positive control; d) *V. harveyi RR36 Artemia*: negative control. Mean survival with SE bars are shown for eight days post-inculation.

Table 7.3: Bacterial counts in larvae and water post-inoculation of bacteria

Treatment		Bacterial load cfu ml^{-1} (serial dilutions TCBS agar)			
		Moribund		Healthy	
		Larvae	Water	Larvae	Water
***V. owensii* DY05^T**	Artemia*	1.5×10^7 $\pm 2.4 \times 10^6$	1.7×10^5 $\pm 9.7 \times 10^4$	2.7×10^3 $\pm 1.2 \times 10^3$	2.7×10^4 $\pm 4.5 \times 10^3$
	Immersion+	2.3×10^4 $\pm 7 \times 10^3$	6.3×10^6 $\pm 1.2 \times 10^6$	-	-
***V. owensii* 47666-1**	Immersion+	1.8×10^4 $\pm 4 \times 10^3$	4.1×10^6 $\pm 1.1 \times 10^6$	-	
***V. harveyi* RR36**	Immersion+	-		1.5×10^3 ± 682	9.1×10^4 $\pm 6.6 \times 10^4$

Mean ± SE (n= 6 for each sample type).
*Artemia: ~9 nauplii per larva previously enriched with 10^6 cells ml^{-1}.
+Immersion: bath inoculation of 1×10^6 cfu ml^{-1}.

7.3.3 Experiment E3: Challenge of *P. ornatus* with vibrio extracellular products

Protein concentrations in ECPs obtained by the cellophane overlay method were ~300 µg ml^{-1} for $DY05^T$ and RR36 strains. In experiment E3 (test E.3.1), larvae challenged with 50 µl (15 µg) ECPs of $DY05^T$ and RR36 strains showed 40-60% survival by day five of the experiment and < 10% survival by the end of the experiment (day eight) compared to control larvae inoculated only with PBS (80.5% survival by day eight) (Dunnett's test $P < 0.0001$) (Table 7.2; Fig. 7.3). Similar curves and survival rates were obtained in test E.3.2 tests with treatments of heat treated-ECPs of the strains compared to PBS controls (Dunnett's test $P < 0.0001$). However, digested ECPs (proteinase K-treated) of both strains did not affect larval survival significantly compared to control larvae (proteinase K-treatment). By day eight, mortalities were 66.6% and 66.8% for digested $DY05^T$- and RR36-ECP treatments respectively, similar values to those values obtained with the proteinase K control treatment (61.1%) (Fig. 7.3).

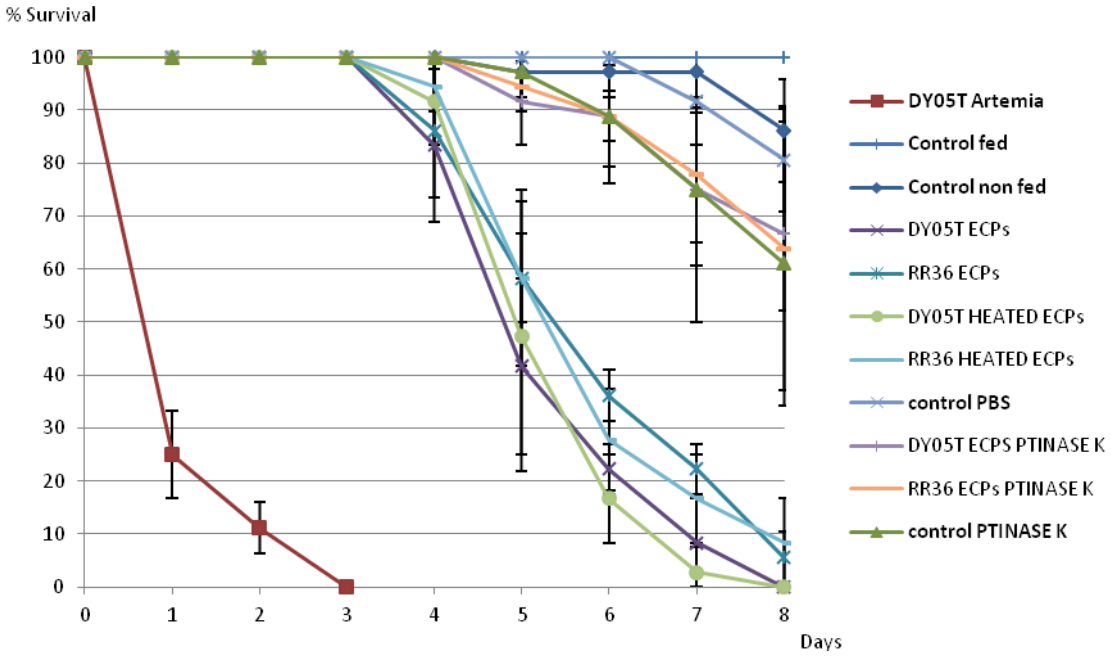

Figure 7.3: Percent survival of *P. ornatus* larvae exposed to ECPs of *V. owensii* $DY05^T$ and *V. harveyi* RR36 in experiment E3

Dose of ECPs: 50 µl per larva. Controls: a) *control non-fed*; negative control; b) *control fed*: negative control (larvae fed with non-enriched Artemia); c) *V. owensii* $DY05^T$ *Artemia*: positive control; d) *control PBS*: negative control; e) *control Ptinase K*: negative control (PBS/protease inhibitors). Mean survival with SE bars are shown for eight days post-inoculation.

7.4 Discussion

The results of experiments reported here support previous studies showing high virulence of *V.owensii* strain DY05^T to larvae of *P. ornatus* (Goulden et al., 2012). Several other *V. harveyi*-related strains were also tested and the results indicated the specific pathogenicity of DY05^T for *P. ornatus* larvae. This strain, administered to the larvae via enriched Artemia (10^6 cells ml^{-1}), caused 90% mortality in 48 h, while other isolates including *V. campbellli*, *V. harveyi* and other *V. owensii* strains had no effect on survival rates (90-100% by the end of the experiment). The *V. harveyi*-related strains have been identified as disease agents in larvae of lobsters but recorded mortality rates in previous studies were not as dramatic as those observed for *P. ornatus* with DY05^T (Goulden et al., 2012). Diggles et al. (2000) found 75% mortality of spiny lobster larvae (*Jasus verreauxi*) over four weeks after *V. harveyi* inoculation. However, compared to my experiments, inoculation doses (10^4 cells ml^{-1}) and water temperature (24ºC) were lower, and larvae were in their IV or V instar stage. In a more similar experiment, Bourne et al., (2006) inoculated newly hatched larvae of *P. ornatus* with a *V. harveyi* strain (10^6 cells ml^{-1} at 28ºC) but mortality rates only reached 72% by day six of the challenge.

Recent studies have proved that not all *V. harveyi* strains are pathogenic to crustacea and for those pathogenic strains, there is a considerable variability in the virulence mechanism toward different host species (Zhang and Austin, 2000; Conejero and Hedreyda, 2004; Austin and Zhang, 2006; Bai et al., 2007). In larvae of *P. ornatus*, signs of infection including lethargy, slow swimming activity and white body opacity, were similar to those signs observed in previous descriptions of vibriosis in penaeid prawns and other lobsters by *V. harveyi* (Karunasagar et al., 1994; Lavilla-Pitogo et al., 1998; Diggles et al., 2000). In contrast to most pathogenic *V. harveyi*, *V. owensii* strains are not luminescent (Chapter 3) and therefore, infected animals do not glow in the dark. Immediately after death, larvae started decomposition and were found completely disintegrated 48 h later, suggesting strong proteolytic activity by the still live bacteria within the animal.

The experiment included not only potentially pathogenic strains of *V. harveyi*-related species, as indicated by their haemolytic, proteolytic or quorum sensing activity (Table

7.1), but also other strains (*V. harveyi* H20 and RR36), isolated from healthy and wild larvae of *P. ornatus*. As expected, H20 and RR36 strains caused no detrimental effect to the larvae. Furthermore, survival rates for RR36-treated larvae were even higher (94.5% survival by day six) than for control treatment larvae (91.6% survival by day six). Due to its wild origin and the high survival rate observed for *V. harveyi* RR36, the strain was used as a negative control in the following experiments. The strain 47666-1 (previously classified as *V. harveyi*) was not isolated from lobster larvae but it was also included in this study because it was proven to be a missclasified *V. owensii* strain (Chapter 3). The strain had been isolated from diseased *P. monodon* larvae in a commercial prawn hatchery in northern Qld, and it was described as highly virulent to prawn larvae (Harris, 1993). In this study however, 47666-1 was not pathogenic to larvae of *P. ornatus* when administered via Artemia (94.4% survival by day six). It is known that changes in environmental conditions are capable of retarding and abolishing the virulence of *V. harveyi* pathogens (Prayitno and Latchford, 1995; Robertson et al., 1998). Since both DY05^T and 47666-1 strains were isolated from rearing systems with similar culture conditions to those used in the experiments, it is unlikely that environmental changes are the cause of virulence loss. Alternatively, the strain 47666-1 might have lost its virulence spontaneously during long storage. In these cases, it is a common practice to passage the strain through its host of origin in order to restore its pathogenicity (Yurchenco et al., 1953). However, after the passage of 47666-1 through prawn (10^6 cells per prawn) and reisolation of the strain from moribund individuals, the *passage*d 47666-1 strain did not show virulence to *P. ornatus* (100% survival by day 7). Interestingly, five prawns injected with the same dose of DY05^T did not show signs of diseased and survived the experiment. Considering that DY05^T and 47666-1 are members of the same species and share similar phenotypes and genotypes, these results were further pointing out a highly specific pathogenicity of DY05^T for *P. ornatus*.

The sudden mass mortality caused by DY05^T was suggesting that high bacterial densities were responsible for some toxic activity on larvae of *P. ornatus*. In order to have control over the number of cells inoculated, the next experiment (E2) included immersion treatments by bath inoculation of bacteria. Also, in order to test the toxicity of potential secreted toxins, larvae were treated with bacterial ECPs in experiment 3 (E3). In experiment E2, bacteria inocula (10^6 cells ml^{-1}) caused mortality with a four days delay compared to oral challenge treatments via Artemia. Decreased survival rates

were observed not only for DY05^T (44.5%) but also for 47666-1 (61.1%) by day eight of the experiment. Although mortality with 47666-1 showed significant differences with control treatments (PBS and RR36), this strain only caused 5.5% mortality by day seven, increasing to 38.9% by the end of the experiment. Therefore the pathogenicity of 47666-1 by immersion to larvae of *P. ornatus* was questionable. The delayed effect of *V. owensii* DY05^T on larval mortality in immersion treatments led to a hypothesis that accumulation of bacteria in each plate well was a slow process, compared to direct oral challenge administration. Furthermore, a certain bacterial density within the animal or the water seemed to be the trigger to toxicity and larval death. Comparison of cell counts in moribund and healthy larvae showed high bacterial densities within moribund larvae three days post-inculation of DY05^T via Artemia (10^6 cells ml^{-1}). It seems that Artemia-vectored cells attached to the gut and proliferated within the animal in the following hours after inoculation. As expected, since cells were delivered directly into the gut of the larvae via Artemia, low bacterial densities were found in the surrounding water (~1.75 x 10^5 cfu ml^{-1}). In contrast, a few healthy animals, which probably did not get to feed on any Artemia, contained low bacterial loads within the body (~2.7 x 10^3 cfu ml^{-1}). The lack of control over the number of inoculated cells was a disadvantage of the Artemia-vector dosage form.

In immersion treatments, similar bacterial loads were found for DY05^T and 47666-1 treated larvae by day five, but in this case, cells were mainly found within the surrounding water (~10^6 cfu ml^{-1}). In contrast, sampled moribund larvae contained lower bacterial loads (~2 x 10^4 cfu ml^{-1}); similar to those levels found within the water and larvae of control RR36 treatments. The results of these experiments showed that while high densities of DY05^T cells were found within oral challenged larvae, bacteria proliferated mainly in the water if the larvae were inoculated via immersion. This indicated that high cell densities, achieved within the animal (oral challenge) or in the water (immersion), were responsible for DY05^T virulence and larval mortality. Artemia and other live feeds are widely used in larval rearing aquaculture systems (Leger et al., 1987) but this study suggest that the effects of DY05^T and other potential pathogens on lobster larvae would be more sudden and detrimental if grazing live feeds are used, as opposed to different type of diets.

The evidence of potential toxicity of high dense DY05^T populations on *P. ornatus* was further supported by experiment E3. The ECPs of DY05^T and control RR36 were both toxic to the animals causing ~90% mortalities seven days post-inoculation. The ECPs to inoculate were obtained from DY05^T and control RR36 strains grown *in vitro* to very high densities. Subsequently, low doses of these ECPs were inoculated (50 µl per larva), but since these had been obtained from high density cultures, the samples were probably highly concentrated in potential toxic molecules. It has been hypothesised that production of toxic ECPs by *V. harveyi*-related species is regulated by a density dependent quorum sensing communication system (Mok et al., 2003). This would explain why both DY05^T and RR36 ECP samples were lethal to the larvae. It is likely that *in vitro* obtained ECPs of all *V. harveyi*-related species are toxic to *P. ornatus* at high doses, but only those strains with the ability to colonise the host, such as DY05^T, are able to produce such ECP levels *in vivo*.

Experiment E2 proved that only DY05^T was able to colonise the larvae and proliferate to such density levels responsible for toxicity to the animals. Sample moribund larvae contained high DY05^T loads if they had been fed on DY05^T- enriched Artemia, although bacteria were mainly found free living in the surrounding water for moribund animals from immersion treatments. Similar to these immersion treatments with live cells, survival rates started to decay four days after ECP inoculation, which would be explained by the progressive accumulation potential toxins within the larvae. The ECP-treated larvae shared the signs observed for live bacteria-infected larvae (lethargy, slow swimming activity), and differed in that animals did not turn white and opaque when moribund, due to the lack of bacteria. In these larvae, the cytotoxic effect was delayed (4 days) compared to larvae treated with enriched-Artemia live cell treatments (1 day). Artemia might be delivering bacteria directly into the larvae guts and thus, produced toxins would be highly concentrated in the proximity of intestinal cells.

Finally, experiment E3 also proved that toxic molecules contained in the ECPs were heat-stable since 30 min of incubation at 100ºC did not decrease virulence toxicity compared to native ECP treatment. In contrast, proteolytic digestion of ECPs (proteinase K) did abolish its virulence completely, indicating that proteinaceous components (and not lipopolysaccharide) were involved in the ECP toxicity. For a

better understanding of the virulence mechanisms of DY05^T to *P. ornatus* larvae, this study was followed by a molecular characterisation of DY05^T ECPs (Chapter 8).

In summary, the reported high virulence of *V. owensii* type strain DY05^T for larvae of *P. ornatus*, was confirmed in this study. Interestingly, no other *V. harveyi*-like strain isolated from diseased larvae including *V. owensii* 47666-1 was pathogenic to *P. ornatus*. Delayed larval mortalities when DY05^T was administered by immersion compared to oral challenge led to the hypothesis that high bacterial densities achieved within the animal or the water were triggering toxicity and larval death. The experiment also suggested that in cases of DY05^T being present in the rearing systems, live feeds such as Artemia would be detrimental to the larvae compared to other artificial diets, because Artemia delivers the pathogen directly into the gut of the larvae where they rapidly colonise tissues causing disease and death. The ECPs from *V. owensii* DY05^T were highly toxic to the larvae of *P. ornatus*, but those from control *V. harveyi* RR36 were lethal to the animals too. This can be explained considering that the ECPs were obtained from *in vitro* cultures at high densities, when it is hypothesised that production of toxic substances occurs. *In vivo*, however, only DY05^T was able to colonise the larvae and proliferate to such high density levels, lethal to *P. ornatus*. Heat and digestion treatments indicated that heat-stable proteinaceous molecules secreted by DY05^T are involved in virulence to *P. ornatus* larvae.

CHAPTER 8. PROTEIN PROFILE ANALYSIS OF *VIBRIO OWENSII* AND IDENTIFICATION OF AN OMPA_C-LIKE PROTEIN FROM STRAIN DY05^{T}

8.1 Introduction

The development of the aquaculture industry requires the typing of microbes associated to the most common diseases in order to design pathogen detection methods and control strategies such as vaccination. This process is facilitated by the comparison of phenotypic and molecular profiles between different species and between virulent and avirulent strains. Two phenotypically and genetically similar strains have been described so far belonging to the *V. owensii* species. Strain DY05^{T} and its extracellular products (ECPs) are highly pathogenic to *P. ornatus* larvae. In contrast, the *V. owensii* strain 47666-1, a well known *P. monodon* pathogen, was avirulent to *P. ornatus* larvae in preliminary experiments, but high doses of its ECPs were toxic to the larvae (Chapter 7). Previous *in vitro* assays have shown that DY05^{T} produces amylases, haemolysins, proteases and phospholipases (Goulden, 2012) and 47666-1 showed a similar enzymatic profile (not reported results). It is well known that while enzymatic and phenotypic tests provide profiles of metabolic capabilities useful for strain differentiation and offer an insight into potential virulence mechanisms, these methods have disadvantages such as lack of discrimination power and reproducibility (Sethi et al., 1996; Tenover et al., 1997), as discussed in Chapter 2. For the purposes of this study, only genomic methods (MLSA and high resolution melting curve analysis: Chapter 4 and 6) were available for discrimination of DY05^{T} and 47666-1 strains.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell soluble proteins has been described as a useful typing method for bacterial identification (Kersters, 1985; Priest et al., 1993), including vibrios (Benediktsdottir et al., 2000) or for determination of pathogenic strains (Krech et al., 1988; Huey and Hall, 1989). Bacterial SDS-PAGE profiling generates complex and stable patterns that are easy to interpret and compare. In the case of *V. harveyi*-related species, the existing evidence suggests that this technique is useful for taxonomic typing since protein profile

grouping seems to have a phylogenetic basis only and there would be no association between certain protein patterns and virulence (Maiti et al., 2009).

Other microbial typing studies have focused on secreted ECPs as important virulence determinants of *V. harveyi*-like strains for different aquaculture species (Liu et al., 1996; Harris and Owens, 1999; Montero and Austin, 1999; Zhang and Austin, 2000; Zorrilla et al., 2003). These authors demonstrated a significant correlation between virulence of *V. harveyi*-like isolates and the production of proteases, phospholipases, haemolysins and/or LPSs in their ECPs. Previous infection trials proved that *V. owensii* DY05^T was highly pathogenic to *P. ornatus* by proliferation to high density levels, either within the larvae, if these were orally challenged via Artemia feeding or in the surrounding water, if cells were directly inoculated into the wells (Chapter 7). Similarly, low doses of DY05^T ECPs (50 µl per larva) obtained from high density *in vitro* cultures produced similar symptoms and larval mortalities to immersion treatments with live cells. Heat and proteinase K treatment of ECPs suggested that heat-stable proteinaceous molecules, and not LPS, were responsible for toxicity of native ECPs. The next step was to further characterise these secreted ECPs at the molecular level. Molecular characterisation of *V. owensii* has been carried out previously for 47666-1 ECPs (Harris and Owens, 1999), but the present study represents the first detailed characterisation of the ECP produced by the *V. owensii* type strain, DY05^T.

The aim of this study was to find an association between expression profiles of *V. owensii* DY05^T and virulence to larvae of *P. ornatus* by: 1) comparing whole protein and LPS profiles of DY05^T from those of *V. owensii* 47666-1 and *V. harveyi* RR36, and 2) finding potential molecules associated with virulence by comparing ECP protein profiles of DY05^T with those of less or non-virulent strains.

8.2 Materials and Methods

8.2.1 Bacterial cultures and whole-cell lysates

Bacterial stocks of *V. harveyi* RR36 and *V. owensii* 47666-1 and DY05^T were kept at -80ºC were cultured in MB in aerated cell culture flasks and incubated at 28ºC with shaking at 45 rpm. Cells were grown to late exponential (OD_{600}=1.4; 19 h) or stationary

(OD_{600}=2; 43 h) phase under these conditions. Aliquots (2 ml) of these cultures were centrifuged at 14,000 x *g* for 3 min and cells washed in NaCl PBS twice. Optical densities (OD_{600}) were adjusted to 0.6 and pelleted cells resuspended in 100 µl of lysis buffer (2% SDS, 4% 2-mercaptoethanol; 10% glycerol; 0.002% bromophenol blue; 1M Tris pH 6.8). Fresh whole-cell lysate samples were used immediately for the consecutive analyses.

8.2.2 Protein and lipopolysaccharide from whole-cell lysates

For total protein, a subsample (50 µl) of whole-cell lysate preparation was heated at 100ºC for 5 min and centrifuged at 7,000 x *g* for 10 min at 4ºC (Dijkshoorn, 2001). A 15 µl aliquot was mixed with 15 µl of TruSep 2X SDS-sample buffer (NuSep, Lane Cove, NSW, Australia), boiled for 5 min, centrifuged at 6,000 x *g* for 3 min and kept on ice for immediate SDS-PAGE.

For LPS, a subsample (50 µl) of whole-cell lysate preparation was treated with proteinase K (Sigma Chemical Co., Poole UK; 2.5 µg μl^{-1} prepared in SDS-sample buffer) and incubated at 60ºC for 1 h (Apicella, 2008). A 15 µl aliquot was mixed with 15 µl of TruSep 2X SDS-sample buffer, boiled for 5 min, centrifuged at 6,000 x *g* for 3 min and kept on ice for immediate SDS-PAGE. Culture of strains and subsequent SDS-PAGE of whole-cell protein and LPS were repeated on three different days.

8.2.3 Extracellular products (ECPs)

The ECPs of $DY05^T$, 47666-1 and RR36 were prepared using the cellophane overlay method (Liu, 1957). Briefly, autoclaved (120°C for 15 min) non-plastified cellophane sheets were placed on the surface of MA plates, and spread with 200 µl of overnight bacterial cultures grown in MB. After incubation at 28°C for 48 h, each cellophane sheet was transferred to an empty Petri dish lid placed on ice. Bacteria were then scraped into 3 ml of ice cold phosphate buffered saline (PBS; Oxoid) at pH 7.2. Following centrifugation (14,000 x *g* for 15 min at 4°C), the supernatant comprising the ECPs was filtered through a 0.22 µm pore-size Millipore Millex (Watford, UK) porosity filter and kept on ice. The protein concentration of the ECPs was determined

with the Pierce BCA Protein Assay Kit (Thermo Scientific; Rockford, IL USA), using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. For SDS-PAGE preparation, protein concentration in the ECPs was adjusted to ~250 mg ml^{-1}. An aliquot (40 µl) of ECP sample was mixed with 40 µl of NuSep 2X SDS-PAGE sample buffer, boiled for 5 min, centrifuged at 6,000 x *g* for 3 min and kept on ice for immediate SDS-PAGE. Culture of strains and subsequent SDS-PAGE of ECPs was repeated on three different days.

8.2.4 Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE)

Prepared samples with whole-cell protein (15 µl), LPS (15 µl) and ECPs (40 µl) were loaded on precast Tris-Hepes 4-20% acrylamide gels (NuSep) and run as described by Laemmli (1970) in a mini Protean II electrophoresis cell (Bio-Rad). The gels were run for 40 min at a constant voltage of 150 V using Tris-Hepes SDS running buffer (NuSep) according to the manufacturer's instructions. For protein and LPS visualization, gels were stained with Coomassie blue or silver, respectively. A PageRuler Prestained Protein Ladder (Thermo Scientific; Rockford, IL USA) was used as the molecular weight marker.

8.2.5 OFFGEL electrophoresis of protein from DY05^T extracellular products

Two crude ECP samples from DY05^T obtained as per section 9.2.3 were prepared by TCA (trichloroacetic acid)/acetone precipitation for OFFGEL electrophoresis and subsequent spectrometry analysis at the Biomedical Proteomics Facility (Monash University, Melbourne, Australia). Volumes of 1 ml (500 µg protein in PBS) were centrifuged at 20,000 x *g* for 30 min. The pellets were washed in 300 µl of ice cold acetone and further centrifuged at 20,000 x *g* for 5 min. This step was repeated twice and the final pellets were air dried. Samples were analysed by OFFGEL fractionation and SDS-PAGE according to Chenau et al., (2008). Briefly, a sample was reconstituted in 1.8 ml rehydration buffer (thiourea, DTT, glycerol and OFFGEL buffer pH 3-10) and loaded on to a rehydrated 13 cm IEF strip pH 3-10 via a strip of 12 sample cups (150 µl sample/cup). The sample was then focused overnight using an Agilent OFFGEL system (Agilent Technologies, Santa Clara, CA, USA). After focussing, subsamples

were collected from each well individually (tubes labelled F1-F12; F=fraction; # = well number) with a total volume per fraction of ~150 µl. From each fraction 30 µl aliquots were mixed with 10 µl of 4X sample buffer and run on a 12% Tris-Glycine gel (40 mA for 3 h). The Novex Sharp prestained marker Invitrogen) was used as the ladder for molecular weight reference. The gel was stained with InstantBlue Coomassie overnight and destained with water for 1 h.

8.2.6 In-gel trypsic digestion of DY05^{T} extracellular proteins

Gel bands were manually excised from the gel with a scalpel and destained with a solution of 50 mM ammonium bicarbonate and 50% acetonitrile (Coomassie stain). The bands were washed with 100 mM ammonium bicarbonate for 30 min. The proteins were reduced in 2.5mM DTT at 60ºC for 30 min, then alkylated with 10 mM iodoacetamide for 30 min in darkness at room temperature. The gel pieces were washed and dehydrated with alternating washing cycles of 50 mM ammonium bicarbonate and acetonitrile. After complete dehydration, the gel piece was rehydrated with a solution containing 0.5 µg trypsin (Promega corp., Madison, WI, USA) in 20 mM ammonium bicarbonate. The gels pieces are incubated at 37ºC overnight and sonicated for 2 min prior to analysis.

8.2.7 Liquid chromatography-mass spectrometry (LC-MS/MS) analysis

Tryptic digests were analysed by LC-MS/MS using a the HCT ULTRA ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled online with a 1200 series capillary high-performance liquid chromatography (HPLC; Agilent technologies, Santa Clara, CA, USA). Samples were injected onto a Zorbax 300SB reversed phase column (3.5 µm x 7.5 µm x 150 mm) with buffer A (5% acetonitrile 0.1% formic acid) at a flow rate of 10 µl min^{-1}. The peptides were eluted over a 30-min gradient to 55% buffer B (90% acetonitrile, 0.1% formic acid). The eluant was introduced into the Bruker electrospray source (positive ion mode) via low flow electrospray needle with a capillary voltage of 4,000V dry gas at 300ºC, flow rate of 8 L min^{-1} and nebuliser gas pressure at 1,500 mbar. Peptides were selected for LC-MS/MS analysis in autoMSn mode with smart parameter settings selected and active exclusion released after 1 min.

Data from LC-MS/MS run was exported in Mascot generic file format (*.mgf) and searched against the National Center for Biotechnology Information (NCBI) non-redundant and Swiss-Prot databases using the Mascot search engine (version 2.1, Matrix Science Inc., London, UK) with all taxonomy selected. The following search parameters were used: missed cleavages, 1; peptide mass tolerance, ± 0.4 Da; peptide fragment tolerance, ± 0.2 Da; peptide charge, 2+ and 3+; fixed modifications, carbamidomethyl; variable modification, oxidation (Met).

8.3 Results

8.3.1 Sodium dodecyl sulphate-polyacrilamide gel electrophoresis of protein and lipopolysaccharide from whole-cell lysates and extracellular prodcuts

The protein profiles of whole-cell lysates from *V. owensii* 47666-1 and control strain *V. harveyi* RR36 did not show significant differences, showing similar patterns and band intensities in the gels (Fig. 8.1). However, the protein profile of *V. owensii* DY05^T showed a slightly different band pattern compared with the other two strains, especially in the size range of 20-50 kDa. The LPS profiles for the three strains were similar but these varied for each strain if cultures were grown to the late exponential (clear lanes) or stationary (protein smears, the result of degradation) phases (Fig. 8.2 lanes 2, 4, 6, 9). Finally, several differences were observed for protein profiles of the ECPs from DY05^T (20-45 kDa) compared to those from 47666-1 and RR36, which were almost identical (Fig. 8.3).

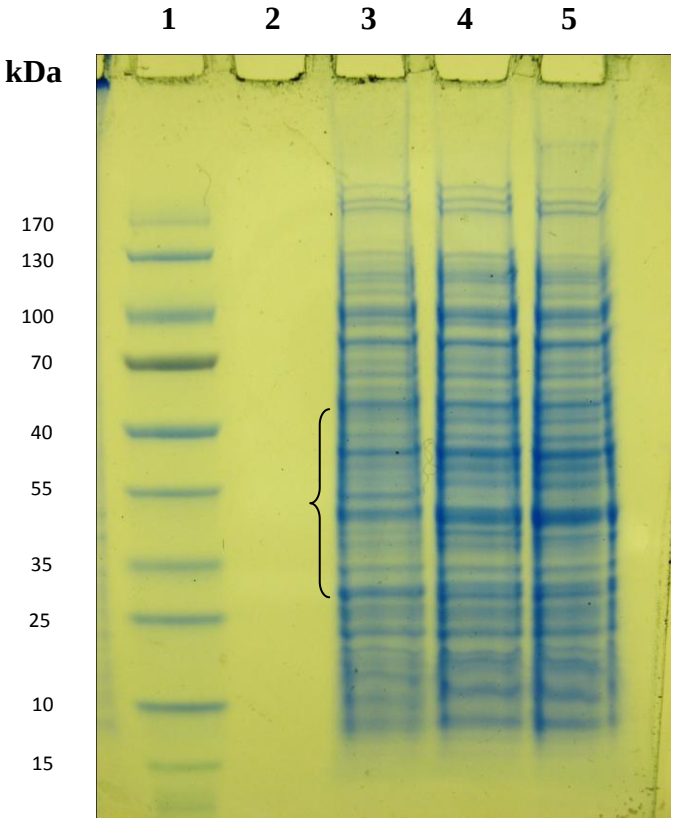

Figure 8.1: SDS-PAGE of whole-cell protein from vibrio strains

Acrylamide gel 4-20%, Coomassie-stained. Lane 1: PageRuler prestained protein ladder; lane 2: blank (lysis buffer); lane 3: *V. owensii* DY05^{T}; lane 4: *V. owensii* 47666-1; lane 5: *V. harveyi* RR36. Bracket indicates a region with band differences between DY05^{T} and two other strains.

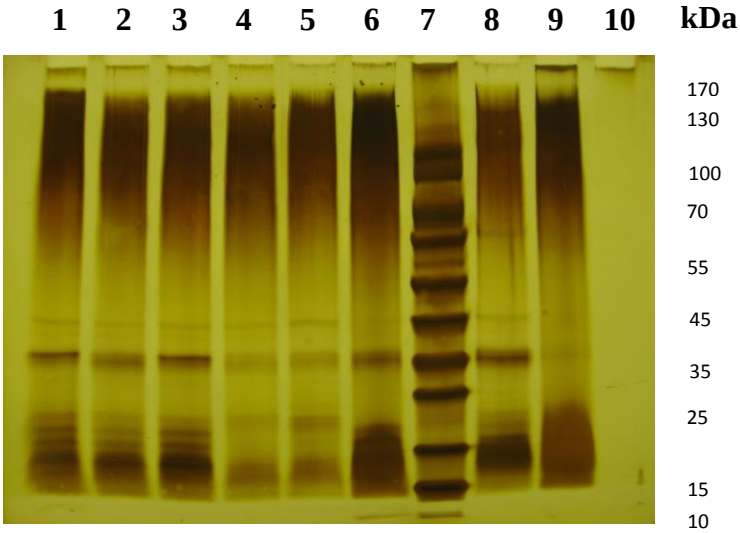

Figure 8.2: SDS-PAGE of LPS from vibrio strains

Acrylamide gel 4-20%, silver-stained. Lane 1, 3: *V. owensii* DY05^{T} late-exponential phase culture; lane 2, 4: DY05^{T} stationary phase culture; lane 5: *V. owensii* 47666-1 late-exponential phase culture; lane 6: 47666-1 stationary phase culture; lane 7: PageRuler prestained protein ladder; lane 8: *V. harveyi* RR36 late-exponential phase culture; lane 9: RR36 stationary phase culture; lane 10: blank (lysis buffer).

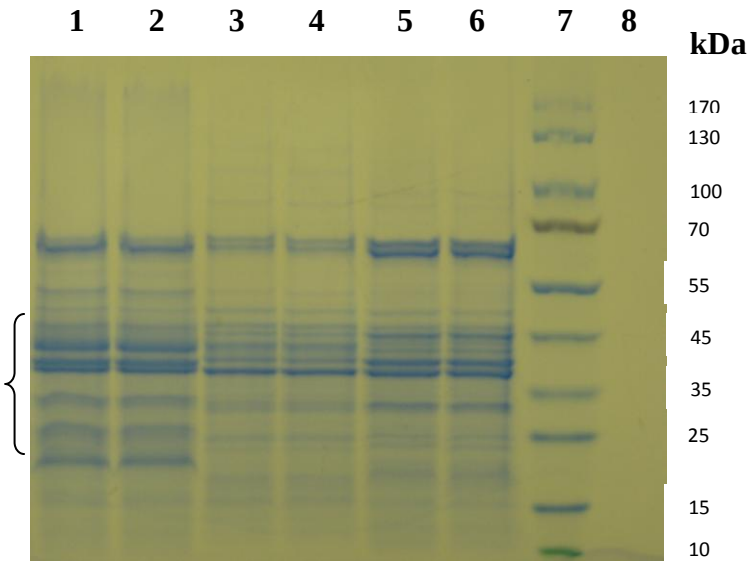

Figure 8.3: SDS-PAGE of protein from ECPs of vibrio strains

Acrylamide gel 4-20%, Coomassie-stained. Lane 1-2: *V. owensii* DY05^T; lane 3-4: *V. owensii* 47666-1; lane 5-6: *V. harveyi* RR36; lane 7: PageRuler prestained protein ladder; lane 8: blank (PBS). Bracket indicates a región with band differences between DY05^T and two other strains.

8.3.2 Protein fractionation of DY05^T extracellular proteins and liquid chromatography-mass spectrometry

The SDS-PAGE profiles following OFFGEL fractionation of 500 µg of protein in DY05^T ECPs are shown in Fig. 8.4. Seven protein bands with molecular weights ranging from 20-50 kDa (labeled 1-7) were selected for trypsin digestion and LC-MS/MS analysis. The digested peptides matched several proteins in the NCBI protein database.

A list of protein matches in the NCBI database and conserved domains is shown in Table 8.1. Of particular relevance are bands #1 and #2 (~36 and 32 kDa) which both matched, with high scores, a 35.8 kDa/326 amino acid conserved hypothetical protein (Acc.no: EEZ89332) from the *V. harveyi* strain 1DA3 (probably a misclassified strain of *V. owensii*, see Chapter 4). The protein sequence and the matching peptides (seven for band #1; five for band #2) are shown in Fig. 8.5. This molecule is an OmpA_C-like protein, a peptidoglycan binding domain similar to the C-terminal domain of the outer-membrane protein OmpA from *E. coli.* A non-specific hit for these bands in the database was a component of Surface antigen-2 superfamily.

For the rest of the molecules analyzed (bands 3-7 in Fig. 8.4), hits in the database included metabolic and immunogenic receptors, antioxidant, amino acid transporter, and periplasmic peptide binding proteins from *V. harveyi* 1DA3 (*V. owensii*) or *V. harveyi* HY01, a strain recentrly reclassified as *V. campbellii* in Lin et al. (2010) (Table 8.1). A detailed Mascot search result report with sequences and functions of these proteins is shown in Appendix A.

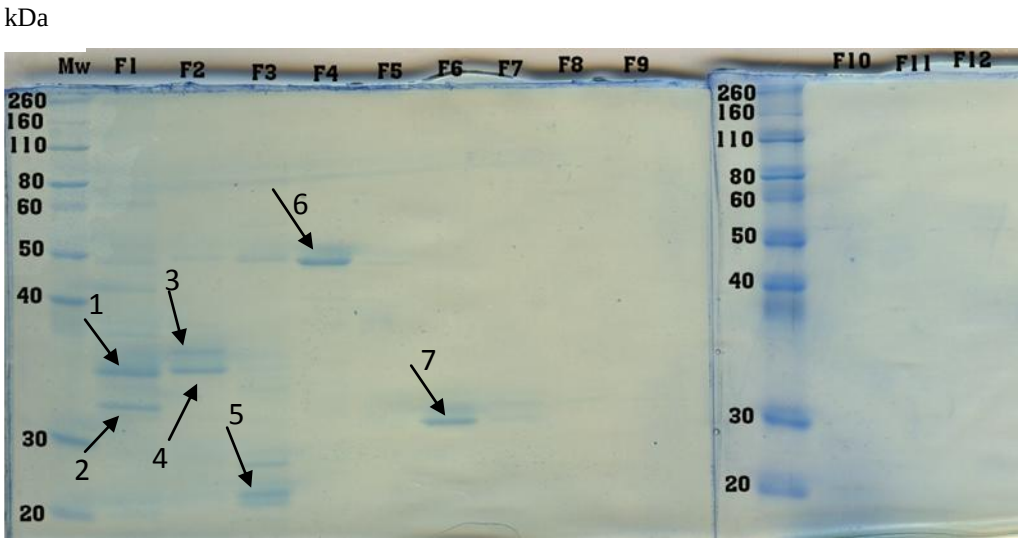

Figure 8.4: Protein fractionation of ECPs from *V. owensii* DY05^T

Coomassie blue-stained 12% SDS-polyacrylamide tris-glycine gel of 12 fractions (20-50kDa) obtained by Agilent OFFGEL fractionation. Each lane was loaded with 40 µg total protein from *V. owensii* DY05^T ECPs. Lane 1: Sharp prestained MW ladder (Invitrogen). Lane F1-F12: fractions from the first dimension (pH 3-10; 13 cm strip).

MKKVAIAVAA VVAGGLINSA QAEMYIGGKV GMTTLDDACY LNSPCDDDAF GAGMHIGYDF
TDIIGLEYGV DYLGNYEANF K**SGANTANTI DGDLWALTLA PK**FNWHLNDT WNLFAKVGGA
YMISGDEK**DI VPTGSLGAEY TIDR**NWSVRA EYQRYQDISD DVLDDMDANF FGIGVNYK**FA**
AAPVVAAVVT EEVMEEEPVM MTKTHKEEYG TGTFEFDSAT LTDSVSERLD NFVNFLNEYP
QAQVEITGYT DSSGPAAYNQ KLSERR**AQSV ADYLIAAGID ADR**FTVKGMG EENPVADNST
HEGREKNR**RV EVVVPEFQYE ELVQPE**

Figure 8.5: Peptide matches of DY05^T protein bands #1 and #2 and OmpA_C-like protein Acc.no: EEZ89332

EEZ89332 (NCBI) = *OmpA_C-like protein Vibrio strain 1DA3*:= EEZ89332; (38.5 kDa). The matching peptides are highlighted in grey (band 1) or written in red (band 2). *V. harveyi* 1DA3 is a potential *V. owensii* strain as suggested in Chapter 4.

Table 8.1: List of top BLAST similarities in the NCBI database with DY05^T ECP protein bands

Gel band	Estimated MW (kDa)	Protein ID (top hit)	Species, strain	Accession number	MW (kDa)	Conserved domain regions	% seq. coverage
1	36	Conserved hypothetical	*V. harveyi* 1DA3[a]	EEZ89332	35.8	▪ OmpA_C-like peptidoglycan binding domain ▪ COG3637 surface antigen	44.1
2	32	Conserved hypothetical	*V. harveyi* 1DA3[a]	EEZ89332	35.8	▪ OmpA_C-like peptidoglycan binding domain ▪ COG3637 surface antigen	29.7
3	37	Conserved hypothetical	*V. harveyi* 1DA3[a]	EEZ86398	36.2	▪ COG3637 surface antigen ABC-type proline/glycine betaine transport system ▪ Tripartite tricarboxylate transporter receptor	26.9
4	36	Antioxidant, AhpC/Tsa family	*V. harveyi* HY01[b]	EDL68011	22.2	▪ Peroxiredoxin (PRX) family	28.7
5	22	Aminoacid ABC transporter, periplasmic amino acid-binding	*V. harveyi* 1DA3[a]	EEZ88278	28.4	▪ Periplasmic binding protein (PBP) family	44.5
6	50	Peptide ABC transporter, periplasmic peptide-binding	*V. harveyi* 1DA3[a]	EEZ89999	57.7	▪ Substrate-binding domain of ATP-binding cassette (ABC) typnickel/ dipeptide/ oligopeptide-like transporter	32.7
7	31	Immunogenic	*V. harveyi* 1DA3[a]/HY01[b]	EEZ86026	35	▪ Tripartite tricarboxylate transporter family receptor, TctC	40.5

[a]*V. harveyi* 1DA3 is a potential *V. owensii* strain as suggested in Chapter 4.
[b]*V. harveyi* HY01 is a *V. campbellii* strain as reclassified in Lin et al. (2010)

8.4 Discussion

In this study, SDS-PAGE of whole-cell protein from *V. owensii* DY05^T showed a unique and different pattern compared to that of *V. owensii* 47666-1 and *V. harveyi* RR36, especially in the size range of 25-50 kDa. The different profiles between the two *V. owensii* strains and the identical profile for *V. owensii* 47666-1 and *V. harveyi* RR36 do not support previous reports about the usefulness of whole-cell protein profiling for measurement of genome relatedness (Kerserts, 1985). Only a few research groups have used SDS-PAGE protein typing of *V. harveyi*-like strains and the conclusions of these studies are misleading. In a recent study, Maiti et al. (2009) found identical protein-banding patterns for 65 Indian isolates of *V. harveyi*. Previously, Pizzutto and Hirst (1995) examined whole-cell protein profiles of 17 *V. harveyi* strains isolated from Australian *P. monodon* prawns and found a variety of different protein patterns. To understand the evidences provided in these studies it is important to consider the date of the publications. In Maiti et al. (2009) the isolates had probably been identified as *V. harveyi* by using modern identification tools, but these methods were not available at the time of Pizzutto and Hirst (1995). In the latter study, the authors identified two major protein profile groups and strains 20, 645, 642 and 47666-1 were allocated to group I. Using MLSA, I confirmed the identity of strain 20 as *V. harveyi* but the rest of the isolates were reclassified as *V. campbellii* (645 and 642) and *V. owensii* (47666-1) strains (Chapter 4). Therefore, what the results of Pizzuto and Hirst were indicating was that a single protein profile was shared by three different species: *V. harveyi*, *V. owensii* and *V. campbellii*. In my analysis, the results obtained for *V. owensii* also suggest that whole protein pattern grouping is independent of the taxonomy of the isolates at the species level. However, only three strains belonging to two species were included and further test of more strains are necessary to establish if this method is useful for species identification.. In any case, the SDS-PAGE protein profiles obtained provided clear discrimination between DY05^T and 47666-1, for which two different and easily distinguishable patterns were repeatedly observed. Compared to expensive and time consuming biochemical characterisation and MLSA (can take from two days to weeks), SDS-PAGE of whole-cells can be performed in 2 h with minimal expense if high resolution melting curve technology is not available.

The evidence from previous infection trials of *P. ornatus* with *V. owensii* DY05^T (Chapter 7) was pointing to some colonisation factor as an essential pathogenicity mechanism of the strain, and the ECPs produced by proliferating cell populations as the toxicity determinant. As for whole-cell protein analysis, the ECP protein profile of DY05^T was compared with those of *V. owensii* 47666-1 and *V. harveyi* RR36, in order to identify regions where it would be more likely to find molecules associated to virulence factors. Rico et al., (2008) showed that all six *V. harveyi* strains virulent to farmed Senegalese sole (*Solea senegalensis*) showed a common electrophoretic protein pattern in their ECPs, while other eleven avirulent strains showed a different pattern type. This strategy, followed in previous studies about *V. harveyi* pathogenesis, succeeded in the characterisation of toxins responsible for virulence of different strains to crustacea and fish. These included proteases (Liu et al., 1996, 1999; Harris and Owens, 1999), LPS (Montero and Austin, 1999) and haemolysin (Zhong et al., 2006). Harris and Owens (1999) isolated an exotoxin in the cell culture supernatant of *V. owensii* 47666-1 strain, highly pathogenic to *P. monodon*. This protein comprised two subunits of 55 and 45 kDa as the major bands observable in SDS-PAGE gels. Similarly, the protein profile obtained here for 47666-1 showed two bands of 55 and 45 kDa (Fig. 8.3) but these were not the strongest bands in the gels. A different protocol for the preparation of ECPs could explain these differences as evidenced in Montero and Austin (1999). These authors obtained different protein profiles in the gels if the ECPs were obtained by the cellophane overlay method compared to filtration, dialysis and concentration of cell culture supernatants. In the case of DY05^T ECPs, the gels showed a different protein pattern to those observed for strains 47666-1 and RR36, in the size range of 20-50 kDa, but the presence of close multiple bands in this region was making their isolation complicated. Proteins were therefore analyzed by OFFGEL electrophoresis in order to get better band discrimination and purity before LC-MS/MS analysis for protein identification.

OFFGEL™ electrophoresis is a relatively new development in proteomic research where molecules are focussed in solution according to their isoelectric point (pI). The separated components are recovered in liquid fractions, which greatly facilitates downstream processing, allowing multi-dimensional separations of complex samples (Chenau et al., 2008). Following LC-MS/MS of proteins (20-50 kDa) from DY05^T ECPs, a 36 kDa and a 32 kDa band were found to match the OmpA_C-like protein of

the vibrio strain 1DA3. The complete genome sequence of this strain is available in the databases as *V. harveyi* (Thompson et al., 2009). However, 1DA3 was included in the MLSA identification study (Chapter 4) and results showed that it is possibly a misclassified strain of *V. owensii*. The identity of this strain as *V. owensii* is now supported by the match of its OmpA_C-like protein with that of the type strain of *V. owensii* (DY05^T).

OmpA is one of the major Omp that assembles in the outer membrane of gram-negative bacteria and it is highly conserved among the Enterobacteriaceae family (Poolman, 1996). Functions attributed to OmpA include roles in bacterial conjugation, in bacteriophage binding and in cell growth (Jeannin et al., 2002). It also functions as a porin and contributes to the ability of gram-negative bacteria to invade host cells (Sugawara and Nikaido, 1979; Jeanin et al., 2002). For example, OmpA-deficient *E. coli* exhibited reduced invasive capacity and attenuated virulence (Weiser et al., 1991) and the OmpA-like protein of *Leptospira interrogans* has been proved essential for virulence of this bacterium (Ristow et al., 2007). For *V. cholerae*, high levels of OmpA have been associated with an increased colonisation ability of intestinal mouse cells by the bacterium (Song et al., 2008). Only one outer membrane protein (OMP) has been characterised for the *V. harveyi* type strain LMG 4044^T (the porin VhOmp), which caused haemolysis of human red blood cells (Schulte et al., 2009), although this strain has been described as avirulent for many marine species (Pujalte et al., 2003; Hernandez and Olmos, 2004; Won and Park, 2008).

The OmpA-like protein characterised in this study for *V. owensii* DY05^T shares high homology with that of *V. alginolyticus*, *V. proteolyticus*, and *V. cholerae* species (Appendix A). The protein was isolated from the ECPs of DY05^T; therefore it is likely a secreted protein rather than a membrane linked molecule. It is known that OMPs are sometimes secreted as a component of outer membrane vesicles (OMVs), produced by a wide variety of gram-negative bacteria (Beveridge, 1999) including vibrios (Kondo et al., 1993) during growth. In pathogenic bacteria, OMVs are considered virulence factors, playing roles in the establishment of a colonisation niche, the delivery of virulence factors to host cells and the modulation of host defense (Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006).

Some defining characteristics of outer membrane material as OMVs are that these are composed of LPS, contain OMPs including OmpA (Kuehn and Kesty, 2005) and are a product of growing cells, not from dead or lysed cells (Kuehn and Kesty, 2005). From the methods used and the results obtained, it is likely that most protein contained in DY05^T ECPs were actively secreted as free molecules or as components of secreted OMVs. Firstly, cell lysis or death in the cultures was not likely, since bacteria were grown for 48 h and gently removed by centrifugation. Secondly, any live or dead cell would have been removed by filtration of ECPs through 0.22 µm filters. Thirdly, most proteins identified in the ECPs of DY05^T had functions associated with OMPs or secreted molecules (OmpA-like protein, immunogenic receptors, antioxidant, amino acid transporter, and periplasmic peptide binding proteins, while no cytoplasmic components were identified (Table 8.1). Any LPS component of the ECPs in DY05^T was intentionally removed TCA precipitation since previous experiments proved that LPS present in proteinase K-treated ECPs was non-toxic to larvae of *P. ornatus* (Chapter 7).

Other proteins characterised in DY05^T ECPs were antigens belonging to the Surface antigen-2 superfamily, commonly expressed on the surface of pathogens. In addition to specific virulence factors, OMVs are known to contain compounds that are recognised by eukaryotic cells in the immune response pathways. For example, mice infected with *Salmonella typhimurium* generate CD4* T cells that recognise antigens in *Salmonella* OMVs (Ernst et al., 2001). *Salmonella* cells however can coordinately reduce recognition by the immune system by regulating carried antigens and LPS in the OMVs (Bergman et al., 2005). An overstimulated inflammatory response to OMVs is likely in response to high levels of OMPs, LPS or lipoprotein, all biologically active molecules present in most bacteria that can activate immune systems (Galdiero et al., 1999). Therefore, bacterial vesicles that do not carry exotoxins can nevertheless cause damage due to the host inflammatory response as long as they are able to proliferate and produce high levels of OMVs (Kuehn and Kesty, 2005).

I hypothesise that *V. owensii* DY05^T is a potential producer of OMVs and the heat-stable OmpA_C-like protein found in its ECPs is a potential virulence factor of the bacterium to larvae of *P. ornatus*. The difference between the pathogen DY05^T and other non-pathogenic *V. harveyi* related strains associated *P. ornatus* seems then to

reside in its colonisation ability (possibly regulated by OmpA) and the subsequent high levels of toxic ECPs achieved after host invasion and proliferation. The potential exotoxin(s) of DY05^T are unknown since ECPs were obtained from *in vitro* cultures; i.e, with no presence of the host as a potential factor influencing toxin production. Furthermore, not all ECP fractions were analysed in this study. Future studies will aim at the characterisation of potential toxins produced by DY05^T and the understanding of OMV and OmpA-like protein production and regulation.

In summary, the SDS-PAGE protein profiles from whole-cells obtained in this study provided clear discrimination among DY05^T and 47666-1, for which two stable, different, and easily distinguishable patterns were observed for each strain. Two protein bands of 36 and 32 kDa were found in the ECPs of DY05^T and these were identified as an OmpA_C-like protein, closely related to that of the *V. harveyi* strain 1DA3 available in the databases. The identity of this strain as a misclassified member of *V. owensii* was suggested by MLSA (Chapter 4) and it is now further supported in this proteomic study. The OmpA protein has been proved to contribute to the ability of gram-negative bacteria to invade host cells and when secreted within OMVs, these are considered potent virulent factors. OmpA could be involved in the potent colonisation ability of the digestive system of *P. ornatus* larvae by DY05^T, which would allow proliferation and subsequent production of toxic ECPs, lethal to the animals. Mores studies are necessary to understand the role of the OmpA_C-like protein of DY05^T in the infection process.

CHAPTER 9. GENERAL DISCUSSION

Based on a global phylogenetic analysis of *V. harveyi*-related species by MLSA, the aims of this project were: 1) precisely identify *V. harveyi*-like isolates associated to the larval rearing system of ornate spiny lobster *P. ornatus*, a good candidate for aquaculture in Australia; 2) design reliable methods for identification and detection of *V. harveyi*-related species; and 3) investigate the virulence mechanisms of *V. owensii* strains for *P. ornatus*. The access to a wide range of environmental and clinical *V. harveyi*-related strains from the AIMS and JCU collections and the presence of multiple vibrio DNA sequences available in the databases, allowed the upgrade of the MLSA-based study not only to a national, but to a global context. Under the hypothesis that numerous isolates remain misidentified or misclassified in laboratories and databases across the world, the MLSA approach was useful for precise identification of important *V. harveyi*-like strains. In Australia, cases of misidentification included the *V. campbellii* strain 642, previously *V. harveyi*, pathogenic to *P. monodon* and carrying a bacteriophage (Oakey and Owens, 2000), and the delineation of *V. owensii* sp. nov., pathogenic to larvae of *P. ornatus* (DY05^{T}) and *P. monodon* (47666-1). Strain 47666-1 was isolated from diseased prawn larvae in a commercial prawn hatchery in northern Qld in 1991, and since then, it has been extensively characterised as a highly pathogenic strain of *V. harveyi* (Harris, 1993; Pizzuto and Hirst, 1995; Harris and Owens, 1999).

The ease of data accumulation in databases provided by MLSA allows the ongoing description of new *V. owensii* strains in different hosts and locations, such as those found in starfish and corals across the Pacific (Rivera et al., 2011; Wilson et al., unpublished). Global MLSA phylogenies showed clustering of several strains, previously classified as *V. harveyi*, *V. campbellii* and *V. rotiferianus*, with those of *V. owensii*. This is the case of strains CAIM 994, D1, PA2 and 1DA3 isolated from fish and corals in the Atlantic (Lin et al., 2010). At the beginning of this project, the Harveyi clade (Sawabe et al., 2007) included seven species: *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. mytili*, and *V. natriegens*. Only in the last three years, five new species have been delineated within this clade: *V. azureus*, *V. sagamiensis* (Yoshizawa et al., 2009, 2010), *V. owensii*, *V. communis* (Chimetto et al., 2011) and *V. jasicida* (Yoshizawa et al., 2011). The MLSA also

suggested that the recently described species *V. communis* is possibly a junior synonym of *V. owensii* (100% 16S rRNA and 98.6% five-locus sequence similarities between *V. owensii* and *V. communis* type strains). It is likely that some of the strains characterised in these recent publications belong to the same species, due to the simultaneity of the studies. In the next few years, several reports amending the taxonomic status of these species are expected. The possible reclassification of more strains as *V. owensii* would confirm the suspected wide geographical distribution of the species and its ability to colonise a high variety of marine host species. Finally, if strain 1DA3 is reclassified as *V. owensii*, a full genome sequence would be availabl for this species, for convenient use in further studies.

Different virulence mechanisms have been described for *V. harveyi*-related species in aquaculture environments, depending on the strain and the host to infect (Nakayama et al., 2005; Alavandi et al., 2006). In addition, the genome plasticity of vibrio genomes under different conditions and the potential gene transfer via bacteriophage infection (Oakey and Owens, 2000), make these characters transferrable within closely-related species, complicating the task of finding a single mechanism responsible for virulence. The *V. owensii* strain DY05^{T} is highly pathogenic to larvae of *P. ornatus* but a preliminary experiment suggested that it might not be virulent to *P. monodon*. In contrast, *V. owensii* 47666-1 is highly pathogenic to cultured *P. monodon* but not to larvae of *P. ornatus*. It seems that highly specialised virulence factors are involved in the pathogenesis of *V. owensii* strains for different hosts, supporting the general hypothesis above for *V. harveyi*-related pathogens. In the MLSA study, none of the clusters formed could be unambiguously associated with pathogenicity. In general, the clusters included strains from diseased or moribund animals as well as strains from healthy animals and seawater. It has been hypothesised that particular conditions in aquaculture rearing systems promote the growth of opportunistic bacteria and unpredictably trigger mechanisms for virulence in avirulent strains (Olafsen, 2001). These unknown mechanisms and the limited knowledge in regard to the virulence factors used by *V. harveyi*-related strains, suggest that the detection of any of these species in aquaculture rearing systems should be considered a risk factor for vibriosis outbreaks.

Due to the severe economic losses caused by vibriosis in the aquaculture industry, the design of techniques for detection of *V. harveyi* pathogens has been the aim of many research groups in the last years. Early detection of pathogens at specific control points would benefit the development and sustainability of hatcheries and farms by allowing managers to take appropriate action in case of an outbreak. The MLSA work carried out in this project allowed the selection of suitable genes for discriminative identification of species within the *V. harveyi* group. The genes *topA*, *ftsZ* and *mreB* fulfilled the required conditions for the design of a multiplex PCR tool that allowed simultaneous detection of *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii* in a single PCR reaction. Compared to MLSA-based identification and other sequencing-based methods, this technique does not require specialised personnel or equipment, and an internal control monitors the presence of PCR inhibitors in clinical samples. This technique is highly suitable for farmers and microbial diagnosticians since it provides fast, cost-effective detection and discriminative identification of *V. harveyi*-related species from clinical samples.

The development of *P. ornatus* and *P. monodon* breeding programs will also benefit from the real-time PCR designed in this study, since it provides direct, fast detection and quantification of *V. owensii* as a major pathogen of these two important aquaculture species. In addition, high resolution melting curve analysis allows discrimination between DY05^{T} and 47666-1 without the need of complex and time-consuming MLSA or protein profiling. Furthermore, the technique has the potential to provide accurate quantification of the bacterial density in more complex samples such as hatchery water, food supply or complex marine samples such as sediments. The real-time assay would also assist in the understanding of *V. owensii* infections by studying the dynamics of the pathogen during host invasion, and after the implementation of control treatments for eradication. In summary, the multiplex and real-time PCR technologies designed are proposed as practical management tools to prevent disease outbreaks in potential hosts such as *P. ornatus* and *P. monodon*, and as research tools to understand the impact of *V. owensii* infections in aquaculture systems and the environment.

Results from experimental infection of *P. ornatus* larvae with DY05^{T} and the finding of a secreted OmpA_C-like protein in the extracellular products (ECPs), suggested that the strain is a potential producer of OMVs, a recognised virulence factor in many bacterial

species. OmpA could be involved in the high colonisation ability of the digestive system of *P. ornatus* larvae, facilitating proliferation and subsequent production of toxic ECPs, lethal to the animals. Vesiculation is a ubiquitous process for bacteria grown in a variety of environments including solid and liquid culture and biofilms (Beveridge, 1999). The maximum rate of vesicle production occurs during the end of log phase of growth, as documented for *E. coli* and *V. cholerae* (Chatterjee and Das, 1967; Hoekstra et al., 1976). Under these circumstances, bacterial OMVs can mediate co-aggregation of cells enabling biofilm formation and colonisation (Grenier and Maryand, 1987). Schooling and Beveridge (2006) found that OMVs from biofilm contained more proteolytic activity than those from planktonic cells. Recent experiments have proved that DY05^T grows in a biofilm if appropriate surfaces, such as glass, are provided (Høj et al., unpublished). The strain is also able to form biofilms in the cell culture plates where experimental challenge of *P. ornatus* was carried out (Høj et al., unpublished). In this study, cells of *V. owensii* DY05^T, 47666-1 and *V. harveyi* RR36 were grown on MA plates to their late exponential phase before ECP preparation for *in vivo* experiments and SDS-PAGE analysis. Similarly, for infection trials inocula were prepared by growing the strains in cell culture flasks with slow orbital agitation (45 rpm). It is likely that under these conditions, the cells grow forming biofilms, supporting the previous suggestion that biofilms forming in *P. ornatus* larval rearing tanks represent a reservoir for *V. harveyi*-related phyllosoma pathogens (Bourne et al., 2006).

Several studies have demonstrated that OMVs could aid in securing a niche in competitive bacterial environment such as during the colonisation of a host (Kadurugamuwa et al., 1998; Li eta l., 1998). During infection trials, DY05^T was able to colonise and proliferate within the gut of experimentally infected *P. ornatus* larvae, while 47666-1 and RR36 control strains did not, and cell counts eventually dropped. These experiments showed that although ECPs of both DY05^T and RR36 (obtained *in vitro*) were toxic to the larvae, only live cells of DY05^T were able to colonise and proliferate within the animal, possibly producing lethal levels of ECPs. Further studies are necessary to understand the role of the OmpA_C-like protein and potential toxins of DY05^T in the infection process. The OMPs contained in OMVs are difficult to purify without contamination by lipoprotein or endotoxin; therefore its interaction with innate cells remains poorly investigated for many bacterial species. Thanks to the advent of genetic engineering tools, experiments such as the design of OmpA or toxin mutants

would provide further evidence of the role of these molecules in the infection of *P. ornatus* by *V. owensii* DY05^{T}.

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APPENDIX A: MASCOT AND NCBI RESULTS

Protein bands identified in DY05^{T} ECPs (Chapter 7)

a) Band 1 (~ 36 kDa):

Top hit: gi|269835250|gb|EEZ89332.1|; Conserved hypothetical protein [*Vibrio harveyi* strain 1DA3] ; **Score:** 673

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 35,816 Da ; **Number of amino acids:** 326

Peptides matched: 7 ; **Theoretical pI:** 4.23

Query coverage: 44.1%

Sequence (match peptides: **bold red**):

MKKVAIAVAA VVAGGLINSA QAEMYIGGKV GMTTLDDACY LNSPCDDDAF GAGMHIGYDF
TDIIGLEYGV DYLGNYEANF KSGANTANTI DGDLWALTLA PKFNWHLNDT WNLFAK**VGGA**
YMISGDEKDI VPTGSLGAEY TIDRNWSVR**A EYQRYQDISD DVLDDMDANF FGIGVNYKFA**
AAPVVAAVVT EEVMEEEPVM MTKTHKEEYG TGTFEFDSAT LTDSVSERLD NFVNFLNEYP
QAQVEITGYT DSSGPAAYNQ KLSER**RAQSV ADYLIAAGID ADRFTVKGMG EENPVADNST**
HEGREKNR**RV EVVVPEFQYE ELVQPE**

Conserved domains:

Specific hit: OmpA_C-like peptidoglycan binding domain

Non-specific hits: OmpA-like transmembrane domain; COG3637 surface antigen

b) Band 2 (~32 kDa): different fragment of same protein as in Band 1

Top hit: gi|269835250|gb|EEZ89332.1|; Conserved hypothetical protein [*Vibrio harveyi* strain 1DA3] ; **Score:** 361

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 35,816 Da ; **Number of amino acids:** 326

Peptides matched: 5 ; **Theoretical pI:** 4.23

Query coverage: 29.7%

Sequence (match peptides: **bold red; additonal matches in Band 1: **bold black**):

MKKVAIAVAA VVAGGLINSA QAEMYIGGKV GMTTLDDACY LNSPCDDDAF GAGMHIGYDF
TDIIGLEYGV DYLGNYEANF K**SGANTANTI DGDLWALTLA PK**FNWHLNDT WNLFAK**VGGA**
YMISGDEKDI VPTGSLGAEY TIDRNWSVR**A EYQRYQDISD DVLDDMDANF FGIGVNYKFA**
AAPVVAAVVT EEVMEEEPVM MTKTHKEEYG TGTFEFDSAT LTDSVSERLD NFVNFLNEYP
QAQVEITGYT DSSGPAAYNQ KLSER**RAQSV ADYLIAAGID ADRFTVKGMG EENPVADNST**
HEGREKNR**RV EVVVPEFQYE ELVQPE**

c) Band 3 (~ 37 kDa):

Top hit: gi|269832276|gb|EEZ86398.1|; Conserved hypothetical protein [*Vibrio harveyi* 1DA3]; ; **Score:** 300

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 36,207 Da ; **Number of amino acids:** 330

Peptides matched: 5 ; **Theoretical pI:** 4.67

Query coverage: 26.9%

Sequence (match peptides: **bold red**):

MTYKTPLLLA IGALAATNAN ASECGTVTIA DMNWNSATLI ANVDRFILEH GYGCDAELIP
GDTMPTGTSM IEK**GQPDVAP ELWSNSLK**DA LDKGVEEKRL RYAGK**ALVDG GEEGFWIPAY**
LVKQYPEMKT IEGVKKHAKL FSHPEDKAKS AFYSCPAGWN CQISAGNLFK **AMDLADSGFD**
IIDPGSSAGL SGSIAKAYER EQAWFGYYWA PTAVLGKYDM VKVDFGSGVN EEEFVSCTTQ
ADCEAPK**ATM YPPSPVHTIT TEEFASRSPA AYDYFTK**RGF TNADMNQLLA WMEDNQADGE
ETMFHFLENY PQIWTAWVPQ DVAKKVQGAL

Conserved domains:

Non specific hit: ProX (ABC-type proline/glycine betaine transport systems, periplasmic components involved in amino acid transport and metabolism)

d) Band 4 (~36 kDa):

Top hit: gi|148868957|gb|EDL68011.1|; Antioxidant, AhpC/Tsa family [*Vibrio harveyi* HY01 ; **Score:** 213

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 22,183 Da ; **Number of amino acids:** 202

Peptides matched: 5 ; **Theoretical pI:** 4.85

Query coverage: 28.7%

Sequence (match peptides: **bold red**):

MVLVGRQAPD FTAAAVLGNG EIVENFNFAE FTKGKKAVVF FYPLDFTFVC PSELIAFDNR
LADFQAKGVE VIGVSIDSQF SHNAWR**NTAI EDGGIGQVKY PLVADVK**HEI CK**AYDVEHPE**
AGVAFRGSFL IDEDGLVRHQ VVNDLPLGRN IDEMLRMVDA LNFHQKHGEV CPAQWEEGKA
GMDASPKGVA AFLSEHADDL SK

Conserved domains:

Specific hit: PRX Typ2cys

PRXs are thiol-specific antioxidant (TSA) proteins, which confer a protective role in cells through its peroxidase activity by reducing hydrogen peroxide, peroxynitrite, and organic hydroperoxides.

e) Band 5 (~22 kDa):

Top hit: gi|269834188|gb|EEZ88278.1| Amino acid ABC transporter, periplasmic amino acid-binding protein [*Vibrio harveyi* 1DA3] ; **Score:** 650

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 28,444 Da ; **Number of amino acids:** 258

Peptides matched: 8 ; **Theoretical pI:** 5.09

Query coverage: 44.5%

Sequence (match peptides: **bold red**):

MEMKKWLLAA TLAATAVSGM AQAKEWKTVR **FGIEGAYPPF SWTETDGSIK** GFDVDMANAL
CEEMQVKCQI VAQDWDGIIP SLLARKYDAI IAAMSITEER KKKVDFTGKY AQIPNKFIAK
K**GAGLDFDNL K**DVKVGVQRA TTHDK**YLTDN YGDDVEIVRY GSFDEAYLDL ANGRIAAVLG**
DASALEEGVL NKAGGEAYEF VGPSLTDPKW FGEGMGIAVR KQDKDLTKKL DAAIKALREK
GVYQEIAGKY FNYDVYGD

Conserved domains:

Specific hit: PBPb

Bacterial periplasmic transport systems use membrane-bound complexes and substrate-bound, membrane-associated, periplasmic binding proteins (PBPs) to transport a wide variety of substrates, such as, amino acids, peptides, sugars, vitamins and inorganic ions.

f) Band 6: (~50 kDa):

Top hit: gi|269835923|gb|EEZ89999.1| peptide AB transporter, periplasmic peptide-binding protein [*Vibrio harveyi* 1DA3] ; **Score:** 851

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 57,725 Da ; **Number of amino acids:** 519

Peptides matched: 13 ; **Theoretical pI:** 5.46

Query coverage: 32.7%

Sequence (match peptides: **bold red**):

MQMKTMKSKL AVALMAAGLS FNAMAADIKV GYAADPVSLD PHEQLSGGTL QMSHMVFDPL
VR**FTQDMDFE PR**LAESWERV NDTTVRFKLR QGVK**FHSGND MTADDVVWTF ER**LQSSPDFK
AIFDPYEKIV K**VDDYTVDLV TK**GPYPLVLQ TATYIFPMDS KFYSGKTEDG KDKSELVKHG
NSFASTNVSG TGPFIVTSRE QGVKVEFERF KDYWDKESKG NVDK**LTLVPI KEDATR**VAAL
LSGGVDMIHP VAPNDHKRVK DAEGIDLVTL PGTR**IITFQL NQNSNEALK**D VRVR**QAIVHA**
INNEGIVKKI MK**GFATAAGQ QSPAGYVGHD DK**LVPRYDLK KAKELMK**EAG YEDGLTLTMI**
APNNRYVNDA K**VAQAAAAML SKIGIKVDLK** TMPK**AQYWPE FDK**CAADMLM IGWHSDTEDS
ANFNEFLTMT RNEDTGR**GQY NCGYYSNPEM DKVVEAANVE TDPAK**RAEML KGVEATLYND
AAFVPLHWQS EAWGAKSNVK AADIVNPMVM PYFGDLVVE

Conserved domains:

Specific hit: PBP2_ NikA_DppA_OppA_like_2

The substrate-binding component of an uncharacterised ABC-type nickel/dipeptide/oligopeptide-like import system contains the type 2 periplasmic binding fold. This CD represents the substrate-binding domain of an uncharacterised ATP-binding cassette (ABC) type nickel/dipeptide/oligopeptide-like transporter. The structural topology of these domains is most similar to that of the type 2 periplasmic binding proteins (PBP2), which are responsible for the uptake of a variety of substrates such as phosphate, sulfate, polysaccharides, lysine/arginine/ornithine and histidine. Besides transport proteins, the PBP2 superfamily includes the ligand-binding domains from ionotropic glutamate receptors, LysR-type transcriptional regulators, and unorthodox sensor proteins involved in signal transduction.

g) Band 7 (~ 31 kDa):

Top hit: gi|269831896|gb|EEZ86026.1| immunogenic protein [*Vibrio harveyi* 1DA3]; ; **Score:** 390

Taxonomy: Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; *Vibrio*

Nominal mass (M): 34,919 Da ; **Number of amino acids:** 323

Peptides matched: 10 ; **Theoretical pI:** 6.61

Query coverage: 40.5 %

Sequence (match peptides: **bold red**):

MLKLKQIAVA ASLAALSASF SVSAKDTFVT IGTGGVTGVY YPTGGAICRL VNKSRADHGI
RCSVESTGGS IYNINTIR**AG ELDLGIAQSD WQYHAYNGTS K**FEDKGPFKE LRAVFSVHPE
PFTVVARKDA NINTFDDLKG KRVNIGNPGS GQR**GTMEVLM KEYGWTNDDF K**LVSELKASE
QSKALCDNKI DAMIYTVGHP SGAIK**EATTS CDSNIVTVAG PQVEK**LVSDN SFYR**IANVPG
GMYRGSESDV QTFGVGATFV SSTAVPDEVV YNVVKAVFEN FDDFR**RL**HPA **FANLK**KEEMV
K**DGLSAPLHP GAEK**YYKEVG LIK

Conserved domains:

Non-specific hit: TRAP transporter solute receptor, TAXI family

This family is one of at least three major families of extracytoplasmic solute receptor (ESR) for TRAP (Tripartite ATP-independent Periplasmic Transporter) transporters. The others are the DctP (TIGR00787) and SmoM (pfam03480) families. These transporters are secondary (driven by an ion gradient) but composed of three polypeptides, although in some species the 4-TM and 12-TM integral membrane proteins are fused. Substrates for this transporter family are not fully characterised but, besides C4 dicarboxylates, may include mannitol and other compounds.

Table 9.1: List of significant BLAST alignments with DY05^{T} OmpA_C-like protein

Accession	Description	Max score	Total score	Query coverage	E value
ZP_06174328.1	conserved hypothetical protein [Vibrio harveyi 1DA3] >gb|EEZ89332.1| c	672	672	100%	0.0
ZP_01985891.1	outer membrane protein OmpA [Vibrio harveyi HY01] >gb|EDL69410.1| ou	588	588	100%	0.0
YP_001448381.1	hypothetical protein VIBHAR_06262 [Vibrio harveyi ATCC BAA-1116] >gb|	568	568	100%	0.0
ZP_04921185.1	outer membrane protein OmpA [Vibrio sp. Ex25] >ref|YP_003287650.1| o	550	550	96%	0.0
ZP_01260138.1	outer membrane protein OmpA [Vibrio alginolyticus 12G01] >gb|EAS7669	546	546	96%	0.0
ACE00211.1	OmpA [Vibrio alginolyticus]	545	545	96%	0.0
EGF41850.1	outer membrane protein OmpA [Vibrio parahaemolyticus 10329]	539	539	96%	0.0
NP_800696.1	outer membrane protein OmpA [Vibrio parahaemolyticus RIMD 2210633] >	538	538	96%	0.0
ACJ23322.1	outer membrane protein OmpA [Vibrio parahaemolyticus]	514	514	91%	0.0
ZP_08912575.1	outer membrane protein A precursor [Vibrio rotiferianus DAT722]	461	461	96%	5e-162
ZP_06182366.1	outer membrane protein OmpA [Vibrio alginolyticus 40B] >gb|EEZ81348.1	430	430	76%	1e-150
ZP_08911638.1	hypothetical protein VrotD_16305 [Vibrio rotiferianus DAT722]	410	410	98%	6e-142
ZP_01259478.1	putative outer membrane protein OmpA [Vibrio alginolyticus 12G01] >ref|	407	407	100%	1e-140
ACX48919.1	cellulase [Streptomyces sp. GS11] >gb|ADN52664.1| cellulase [unculture	406	406	98%	3e-140
ZP_04921394.1	outer membrane protein OmpA [Vibrio sp. Ex25] >ref|YP_003287906.1| o	394	394	100%	3e-135
ZP_02197015.1	putative outer membrane protein OmpA [Vibrio sp. AND4] >gb|EDP57865.	382	382	98%	4e-131
NP_799758.1	putative outer membrane protein OmpA [Vibrio parahaemolyticus RIMD 22	372	372	100%	1e-126
EGF40995.1	putative outer membrane protein OmpA [Vibrio parahaemolyticus 10329]	370	370	100%	4e-126
ZP_01989775.1	outer membrane protein OmpA [Vibrio parahaemolyticus AQ3810] >gb|ED	370	370	100%	5e-126
ZP_01984388.1	outer membrane protein OmpA [Vibrio harveyi HY01] >gb|EDL71048.1| ou	369	369	98%	2e-125
YP_001447027.1	hypothetical protein VIBHAR_04892 [Vibrio harveyi ATCC BAA-1116] >gb|	367	367	98%	5e-125
NP_762516.1	outer membrane protein A [Vibrio vulnificus CMCP6] >ref|NP_937163.1| o	311	311	99%	1e-102
YP_004191243.1	outer membrane protein A [Vibrio vulnificus MO6-24/O] >gb|ADV89040.1|	310	310	99%	6e-102
ZP_01869029.1	outer membrane protein OmpA [Vibrio shilonii AK1] >gb|EDL52390.1| oute	263	263	99%	4e-84
ZP_01987835.1	outer membrane protein [Vibrio harveyi HY01] >gb|EDL67484.1| outer me	262	262	97%	1e-83
ZP_05716070.1	outer membrane protein OmpA [Vibrio mimicus VM573] >ref|ZP_0603403	254	254	98%	1e-80
ZP_06038490.1	outer membrane protein A precursor [Vibrio mimicus MB-451] >gb|EEY37	254	254	98%	1e-80
EGU21153.1	outer membrane protein OmpA [Vibrio mimicus SX-4]	254	254	98%	1e-80
ZP_06177825.1	conserved hypothetical protein [Vibrio harveyi 1DA3] >gb|EEZ85929.1| c	248	248	56%	5e-80
EGF44315.1	outer membrane protein OmpA [Vibrio parahaemolyticus 10329]	252	252	98%	8e-80
NP_797143.1	outer membrane protein OmpA [Vibrio parahaemolyticus RIMD 2210633] >	252	252	98%	1e-79
ZP_06175948.1	conserved hypothetical protein [Vibrio harveyi 1DA3] >gb|EEZ87724.1| c	251	251	98%	2e-79
ZP_05720172.1	outer membrane protein OmpA [Vibrio mimicus VM603] >gb|EEW07278.1|	251	251	98%	2e-79
ZP_04411579.1	outer membrane protein A precursor [Vibrio cholerae TM 11079-80] >gb|I	251	251	98%	3e-79

*The Expect (E) value is an estimate of how many sequences would score well by chance in the database searched.

APPENDIX B: LIST OF CONFERENCES

Cano-Gomez et al. (2010). Oral presentation. "*Vibrio owensii sp. nov*., isolated from cultured crustaceans in Australia". ASM 2010 **Sydney Annual Scientific Meeting and Exhibition of the Australian Society for Microbiology**. Sydney (Australia) 4-8 July 2010.

Goulden, E.F., et al. (2010) "Pathogens and probionts of the ornate spiny lobster (*Panulirus ornatus*) phyllosoma". Oral presentation. 9th **International Marine Biotechnology Conference**. Qingdao (China) 8-12 October 2010.

Goulden, E.F., et al. (2010) "Pathogens and probionts of the ornate spiny lobster (*Panulirus ornatus*) phyllosoma". Oral presentation. 13th **International Symposium on Microbial Ecology ISME**. Seattle, Washington (USA) 22-27 August 2010.

Cano-Gomez et al. (2009). Poster presentation. "Molecular identification of *Vibrio harveyi* related pathogens in Australia". **World Association of Veterinary Laboratory Diagnosticians-14th International Symposium**. Madrid (Spain) 17-20 June 2009.

Cano-Gomez et al. (2008). Oral presentation. "Molecular diagnosis of vibrio infections in the tropical rock lobster". **Skretting Australasian Aquaculture** 2008 International Conference and Trade Show. Brisbane (Australia). 3-6 August 2008.

Cano-Gomez et al. (2008). Poster presentation. "Identification of vibrios from the larval rearing system of the ornate spiny lobster by MLSA" 12th **International Society for Microbial Ecology conference**. Cairns (Australia) 17-22 March 2008.

Cano-Gomez et al. (2007). Poster presentation. "Multilocus sequence analysis as a tool for identification of pathogenic vibrios from the larval rearing system of the tropical rock lobster *Panulirus ornatus*". **World Association of Veterinary Laboratory Diagnosticians-13th International Symposium**. Melbourne (Australia) 12-14 November 2007.

APPENDIX C: PUBLISHED MANUSCRIPTS DURING PHD CANDIDATURE

Directly related to the project

1. **Cano-Gomez, A**., Høj, L., Owens, L., Andreakis, N. (2011). Multilocus Sequence Analysis provides basis for rapid and reliable identification of *Vibrio harveyi*-related species and confirms previous misidentifications of Australian pathogens. Systematic and Applied Microbiology 34, 561-565.
2. **Cano-Gomez, A**., Goulden, F.E., Owens, L, Høj, L. (2010). *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. FEMS Microbiology Letters 302, 175-181.
3. **Cano-Gomez, A**., Bourne, D.G., Hall, M.R., Owens, L., Høj, L. (2009). Molecular identification, typing and tracking of *V. harveyi* in aquaculture systems: current methods and future prospects. Aquaculture 287, 1-10.

Other publications during the PhD Candidature

1. Rivera-Posada, J.A., Pratchett, M., **Cano-Gomez, A**., Arango-Gomez, J.D., Owens, L. (2011). Injection of *Acanthanster planci* with Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS). I. Disease Induction. Diseases of Aquatic Organisms 97, 85-94.
2. Rivera-Posada, J.A, Pratchett, M., **Cano-Gomez, A.**, Arango-Gomez J.D., Owens, L. (2011). Refined identification of *Vibrio* bacterial flora from *A. planci* based on biochemical profiling and sequence analysis of housekeeping genes. Diseases of Aquatic Organisms 96, 113-126.

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