THE ROLE OF PODOVIRUS-LIKE BACTERIOPHAGE IN THE VIRULENCE OF *VIBRIO HARVEYI* STRAIN 47666-1

Thesis submitted by

NANCY BUSICO- SALCEDO, DVM

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ABSTRACT

Two studies were conducted to demonstrate the transfer of virulence between the
strains infected with phage VHPL and the same strains uninfected with the phage.
The first study was to determine if a bacteriophage isolated from the more virulent
strain (47666-1) would show the same virulence effects as bacteriophage VHML.
The second study was to develop polyclonal and monoclonal antibodies specific to
the toxic protein subunits of *V. harveyi* 47666-1. These antibodies were then used to
detect this specific exotoxin from the previously naïve strains of *V. harveyi* infected
with the phage VHPL.

SDS-PAGE analysis showed an up regulation and production of extracellular
proteins in previously naïve *V. harveyi* strains receiving this phage compared with
uninfected strains. Haemolysin assays indicated a significant increase ($P<0.001$) in
both halo of clearing and colony diameter in bacteriophage-infected strains of *V.
harveyi* compared to the same individual strains uninfected with the phage.
However, siderophore production was not significant as all of the inducible strains
did not respond positively on Chrome Azurol-S (CAS) agar. Chitin degradation, on
the other hand, resulted in significantly greater zones of clearing ($P<0.001$) in strains
infected with bacteriophage from *V. harveyi* 47666-1 than the same strains receiving
no phage. In bath challenge assay, the results indicated that as a group, there was a
significant difference in mortality rate among strains infected with bacteriophage
from *V. harveyi* 47666-1 ($F=82.824$, $DF=9,40$, $P<0.001$) than strains of *V. harveyi*
without the bacteriophage.

Polyclonal (PAbs) and monoclonal (MAbs) antibodies to the specific toxic subunits
45 and 55 kDa of *V. harveyi* 47666-1 were produced and used to detect specific toxic
subunits from previously naïve strains of *V. harveyi* challenged with the phage. In
western blot assay, PAbs produced only one specific toxin subunit having molecular
weight of approximately 55kD in strains 30, 12 and 20 while no bands where
obtained from strain 643. Two MAbs (3A1-9 and 5A2-1) were characterised and
both detected toxic protein subunits in all naïve strains of *V. harveyi* infected with

the phage. However, cross reactions were observed in naïve strains 30, 12 and 20 uninfected with the phage.

In conclusion, the presence of bacteriophage VHPL from *V. harveyi* 47666-1 probably enhanced the virulence using assays with four naïve strains of *V. harveyi* as a model. There was significant up-regulation of proteins based on SDS-PAGE, up-regulation of haemolysins, chitinases and greater mortality to larvae of *P. monodon*. It is therefore suggested that the presence of this bacteriophage may either partly or fully confers virulence to *V. harveyi* strain 47666-1.

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LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{600}	Absorbance at 600 nm
ABTS	2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid)
ACMM	Australian Collection of Marine Microorganisms
ANOVA	Analysis of variance
APS	Ammonium Persulphate
BDS	Bovine donor serum
BSA	Bovine serum albumin
CAS	Chrome Azurol-S
CFSE	Cell-free supernatant extract
CFU	Colony forming units
DAB	3,3'-diaminobenzidine tetrahydrochloride
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal bovine serum
HAT	Hypoxanthine-aminopterin-thymidine
HR	Halo ratio
HRPO	Horseradish peroxidase
HT	Hypoxanthine-thymidine
IP	Intraperitoneal
JCU	James Cook University
kb	Kilobases
kDa	Kilodalton
LD_{50}	Mean lethal dose
LSD	Least significance difference
MAbs	Monoclonal antibodies
mm	Millimeter
MW	Molecular weight

nm	Nanometer
NSW	New South Wales
OD	Optical density
OD$_{600}$	Optical density at 600nm
OPI	Oxaloacetate-pyruvate-insulin media supplement
PAbs	Polyclonal antibodies
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Polyvinylidene fluoride
PYSS	Peptone, yeast, synthetic sea salt
RNA	Ribonucleic acid
SDS	Sodium-dodecyl-sulphate
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
VHML	*Vibrio harveyi* Myovirus-like
VHPL	*Vibrio harveyi* Podovirus-like

CHAPTER 1

GENERAL INTRODUCTION

Vibrio harveyi is a Gram-negative, luminous bacterium which is ubiquitous in marine environments. It is found free-living in the water column and in the gut of some marine animals (Nealson and Hastings, 1992). It affects a wide variety of marine vertebrates and invertebrates such as cultured penaeid shrimp (Harvey, 1961; Sunaryanto and Mariam, 1986; Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 1994; Pizzutto and Hirst, 1995; Ruangpan, 1998; Vandenberghe *et al.*, 1998) and fish (Kraxberger-Beatty *et al.*, 1990; Alvarez *et al.*, 1998). There were at least two strains of *Vibrio harveyi* shown to cause devastating disease to larvae of *Penaeus monodon* in northern Australia (Harris and Owens, 1999)These two strains (47666-1 and 642) produced exotoxins lethal in *P. monodon* and mice. It was suggested that these exotoxins were the probable virulence determinants for the respective strains of *V. harveyi*. Other toxic extracellular proteins such as proteases, phospholipase, haemolysins (Liu *et al.*, 1996), and cysteine protease (Liu and Lee, 1999) have been implicated in the pathogenicity of this organism. Virulence-related products such as siderophores (Owens *et al.*, 1996), bacteriocins (McCall and Sizemore, 1979), resistance plasmids (Harris, 1993), and chitinases (Svitil *et al.*, 1997) have also been identified to contribute to the virulence of *V. harveyi.*

Many virulence factors have been implicated in vibriosis as the cause of mortality in prawns. It was suggested that virulent isolates within *V. harveyi* are uncommon and that virulence may be explained by a genetic transfer of virulence factors (Pizzutto and Hirst, 1995). Virulence factors encoded on bacteriophage may confer a wide range of traits to their hosts including toxin production and immunity to superinfection (Paul *et al.*, 2002). Phage thrive in bacterial populations where they constantly transfer their genetic material in a horizontal fashion (Lindqvist, 1998). Phage can multiply by two alternative mechanisms: the lytic cycle or the lysogenic cycle. The former ends with the lysis and death of the host cell, whereas the latter results to survival of the host cell (Tortora *et al.*, 2000). The global significance of lysogenic conversion is the spread of toxin and

virulence genes to susceptible hosts (Paul *et al.*, 2002). In support of this concept, there is a number of bacteria where a variety of bacterial toxins are coded for by bacteriophage include *Corynebacterium diphtheriae* which transmit genes coding for the diphtherial toxin (Freeman, 1951), pathogenic strains of *Shigella* producing shigella toxin (Hale, 1991), *Staphylococcus* enterotoxin A encoded for by a prophage (Betley and Mekalanos, 1985), *Clostridium botulinum* toxins (Johnson *et al.*, 1980), *Pseudomonas aueroginosa* cytotoxins (Hayashi *et al.*, 1990), *Escherichia coli* verotoxins and cytotoxins (Takeda and Murphy, 1979), *Vibrio cholerae* enterotoxin (Waldor and Mekalanos, 1996) and *Mycoplasma arthritidis* bacteriophage (MAV1) causing enhanced arthritogenicity in rats (Voelker *et al.*, 1995).

The involvement of bacteriophage in the virulence of *V. harveyi* has been reported in various studies (Ruangpan *et al.*, 1999; Oakey and Owens, 2000; Munro *et al.*, 2003). Studies on the virulence of *V. harveyi* strain 642 have been done by Munro *et al.* (2003) using a haemolysin assay, a protein assay and bioassay challenge with larvae of *Penaeus monodon*. The results revealed that virulence was enhanced by the presence of bacteriophage, *Vibrio harveyi* Myovirus-like (VHML) which caused previously naïve strains of *V. harveyi* to be virulent upon infection. It was confirmed that the VHML phage confers virulence to *V. harveyi* strain 642 (Munro *et al.*, 2003).

The current study was geared towards identifying if other virulent strain, *V. harveyi* strain 47666-1, which was infected with a bacteriophage, was virulent due to infection with the phage. *V. harveyi* strain 47666-1 has been identified as more virulent than strain 642 causing 100% mortality to the larvae of *P. monodon* at 10^4 CFU ml^{-1} (Harris and Owens, 1999). It has been shown to be infected with *Vibrio harveyi* Podovirus-like (VHPL) bacteriophage, classified under the *family Podoviridae* (Oakey, 2000). Accordingly, this bacteriophage was characterised as having an icosahedral head (~70nm diameter), short stumpy tail, and has a linear double stranded DNA genome.

In order to assess the virulence of bacteriophage from *Vibrio harveyi* strain 47666-1, various virulence assays were conducted to investigate whether infection of this

bacteriophage to previously naïve strains of *V. harveyi* will cause these strains to become virulent. Optical density (OD) readings comparing the response of previously known naïve strains of *V. harveyi* (Oakey and Owens, 2000) upon infection to bacteriophage from *V. harveyi* 47666-1 was undertaken as a presumptive measure whether bacteriophage was able to infect these strains. A method employing SDS-polyacrylamide gel electrophoresis was applied to visualize the presence of extracellular proteins and possible up regulation of proteins from bacteriophage of *V. harveyi* 47666-1 when infected the four naïve strains of *V. harveyi*. The use of dual-layered agar in detecting the zone of haemolysis as a result of exotoxin production was carried out to determine the haemolytic activity of bacteriophage-infected strains of *V. harveyi* as compared to uninfected strains. The production of siderophores was investigated using the universal assay of Schwyn and Neilands (1987) to assess the ability of the infected strains to sequester iron from host fluids which is one of the virulence determinants. An assay for chitin degradation was used to determine the ability of the bacterial strains to excrete chitinase which is regarded as virulence determinant. A bath challenge assay with nauplii of *Peneaus monodon* using approximately $10^6 - 10^7$ cells/µl of bacterial strains infected and uninfected with bacteriophage was used to measure the pathogenicity of the bacteriophage.

The production of polyclonal (PAbs) and monoclonal (MAbs) antibodies served as a tool in testing the presence of antigens and studying cross-reactivity among antigens.   Polyclonal antibodies are derived from preparation containing many kinds of cells. They contain multiple antibodies reacting to different parts of the cell thus invariably give a stronger reaction than a monoclonal antibody (Zola, 1995). Monoclonal antibodies on the other hand, contain a single type of antibody specific to the target antigen thus give a more conclusive result than PAbs. The PAbs raised against *V. harveyi* strain 47666-1 CFSE was used to test the presence of these antigens in previously naïve strains of *V. harveyi* infected with the phage. The MAbs was used in detecting specific toxic sub-units from *V. harveyi* 47666-1 and in determining if the previously naïve strains of *V. harveyi* challenged with the phage VHPL would produce the same toxic subunits as *V. harveyi* strain 47666-1.

The identification of the virulence determinants of *V. harveyi*, which are possible bacteriophage-mediated, is essential in the design of prophylactics and/or therapeutics for the prevention and treatment of vibriosis in prawns. This information could also provide an insight into the pathogenesis of *V. harveyi* infections in prawns.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Classification and Identification of *Vibrio harveyi*

2.1.1 History of classification

Vibrio harveyi was first described as a species of *Acromonobacter* by Johnson and Shunk (1936). Studies in classification of luminous bacteria reported three major groups. The first group contains *Photobacterium fischeri*; the second group consists of *Photobacterium leiognathi* and *Photobacterium phosphoreum* and the third group contains *Beneckea harveyi, Beneckea splendida,* and *Vibrio cholerae* biotype *albensis* (Hendrie *et al.*, 1970; Reichelt and Baumann, 1973; Baumann and Baumann, 1977). Later, this bacterium was registered in "Approved Lists of Bacterial Names" under the denominations of *Beneckea harveyi* and *Lucibacterium harveyi*. In 1980, Baumann and others abolished *Beneckea* and *Lucibacterium* and transferred the various species into *Vibrio*. The phenotypic characters of *Vibrio harveyi* are very close to *Vibrio carchariae*. Pedersen *et al.* (1998) concluded that *Vibrio carchariae* is a junior synonym of *Vibrio harveyi* which was confirmed by Gauger & Gomez-Chiarri (2002). Thompson *et al.* (2002) also reported another *Vibrio* species, *Vibrio trachuri*, as a junior synonym of *Vibrio harveyi* based on phylogenetic analysis and polyphasic approach using 16S rDNA sequencing, fluorescent amplified length polymorphisms (FAFLP), DNA-DNA hybridization experiments and G+C content of DNA.

The general morphology, characteristics and biochemical traits of *Vibrio harveyi* are shown in Table 2.1.

Table 2.1 Morphological, physical and nutritional characteristics of *V. harveyi* [modified from Baumann *et al.* (1984) as cited by Munro (2001)].

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2.1.2   Identification of *Vibrio harveyi*

The great diversity of *V. harveyi* poses certain difficulties in the biochemical determination and identification of environmental *Vibrio* species. It was concluded that biochemical criteria are not always sufficient to distinguish between some *Vibrio* species because of their variable characters (West *et al.*, 1986). This is not the case for clinical isolates where a standard characterisation is applied (Alsina and Blanch, 1994). In the biochemical characterisation test, *V. harveyi* and *V. campbellii* exhibited uniform characteristics, because of their close phenotypic and genotypic relationship (Bryant *et al.*, 1986). To identify and differentiate environmental marine bacteria, it was suggested that API 20NE should be coupled with additional tests beyond those provided in the API kit to produce a more conclusive and realistic result (Breschel and Singleton, 1992).

The API style of biochemical test kits comprise only of twenty reactions and profile only the medically important bacteria like *V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* (API Analab Products, Plainview, USA). The profiles of isolates of *V. harveyi* from disease outbreaks in the Philippines matched those of *V. alginolyticus* and *V. parahaemolyticus* using the API 20NE and 20NFT systems (Lightner *et al.*, 1992). Out of sixteen *V. harveyi* strains, five isolates were positively identified as *V. alginolyticus* and *V. parahaemolyticus* using API 20NE kits. The other eleven isolates returned less definitive matches to either *V. alginolyticus, V. parahaemolyticus,* or *V. vulnificus*. Identification of *Vibrio* isolates using the Biolog GN technique is not accurate when subjected to AFLP genomic fingerprinting (Vandenberghe *et al.*, 1998). The AFLP patterns of these strains showed only 30% similarity with the cluster containing the type strain and reference strains of *V. harveyi.*

Thiosulfate-citrate-bile salts-sucrose (TCBS) agar is a widely used medium for isolating and enumerating *Vibrio* species, however, it is not a differential medium for *V. harveyi* due to its variability to utilise sucrose and can not be distinguished from other sucrose-positive or sucrose-negative species (Harris *et al.*, 1996). A selective medium was developed for the culture of *Vibrio harveyi*. This medium, called VHA (*Vibrio harveyi* Agar) has been shown to have definitive potential as a *Vibrio* specific, primary differential medium on which it is possible to differentiate *V. harveyi* colonies from colonies formed by other *Vibrio* species (Harris *et al.*, 1996). It is characterised by a pH of 9, a strong NaCl concentration (30 g/l) and by the presence of ornithine and cellobiose (Harris *et al.*, 1996). The decarboxylation of ornithine alkalises the medium which contains indicators of pH whereas the acidification of cellobiose lowers the pH. After 48 hours of incubation to 28^0C, colonies of *Vibrio harveyi* have a diameter from 2 to 5 mm, they are generally with regular contour and their colour is blue or slightly green with a dark green centre and is sometimes surrounded by yellow halation.

Identification of *Vibrio* species using the classical numerical taxonomy methods had been used by a number of authors (Baumann and Schubert, 1984; Bryant, 1991; Austin and Lee, 1992). These still represent the best means for proper identification and differentiation of *V. harveyi*. Once an isolate has been

characterised as a straight or curved rod, Gram-negative, oxidase-positive and facultatively anaerobic, taxonomic methods of identifying and differentiating *V. harveyi* from other *Vibrio* species usually rely on ornithine and lysine decarboxylase and arginine dihydrolase test as a major means of grouping bacterial isolates (Alsina and Blanch, 1994). Alsina and Blanch (1994) reported a fast and presumptive identification for environmental isolates of *Vibrio* spp. This set of biochemical keys can be used for strains that are Gram-negative, oxidase positive, grow on TCBS medium and are facultative anaerobes. This method can deliver presumptive identification of *Vibrio* species using a maximum of ten tests with 90% confidence. This system was designed for routine environmental screening and cannot resolve some of the more complex issues of the taxonomy of Vibrionaceae. However, it is still an excellent method for preliminary identification of environmental species of *Vibrio harveyi*.

To screen for bacteria pathogenic to cultured tiger prawns, *Penaeus monodon*, such as *V. harveyi* through their haemolytic activity, Chang *et al.* (2000) studied a newly developed prawn blood agar consisting of 1 ml of tiger prawn hemolymph in medium containing 200 ppm Rose Bengal. It was noted that this blood agar was faster and more accurate for determining prawn haemolytic activity of bacterial isolates compared to sheep blood agar which is the conventional method of hemocytolytic assay. For detection of subclinical vibriosis, indirect immunodot blot assay using avidin-biotin complex (ABC) proved to be the best method (Song *et al.*, 1992). This method can detect 10^3 organisms per 100 μl of hepatopancreatic homogenates. The monoclonal antibodies in enzyme immunoassays were shown to react with 24 *V. harveyi* strains including the type strain ATCC14126. In addition, this method would be suitable for both confirmation of the identification of *V. harveyi* isolates and also for field monitoring of *V. harveyi* levels in aquaculture farms (Song *et al.*, 1992). The monoclonal antibodies developed by Hanna *et al.* (1991) showed adequate specificity against pure bacterial suspensions. However only one strain of *V. harveyi* was used for developing antibodies against this species and there has been no further report of these antibodies being screened against a greater number of isolates.

Identification and typing of *Vibrio* strains using genomic approaches such as DNA-DNA hybridization, polymerase chain reaction (PCR) and ribotyping are useful for taxonomic studies and identification to the subspecies level (Austin *et al.*, 1995). For example, the use of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has a potential to differentiate genus *Aeromonas* at the genomospecies level (Oakey *et al.*, 1996). Protein profile analysis and M13 DNA fingerprinting in *V. harveyi* showed that this species of *Vibrio* is genetically diverse (Pizzutto and Hirst, 1995) and studies using random amplification of polymorphic DNA observed genetic heterogeneity of this species (Karunasagar *et al.*, 1998). Based on DNA-DNA hybridisation experiments, Pedersen *et al.* (1998)concluded that *V. carchariae* is a junior synonym of *V. harveyi* which showed 88% DNA binding between these type strains. On the other hand, arbitrarily primed PCR (AP-PCR) proved to be a reliable tool for strain differentiation since it generates fingerprints that can be used to compare microorganisms at the species level and within species with high discriminating power (Welsh and McClelland, 1990). It also allows identification of isolates at the genospecies level and studies of inafraspecific population structures of epidemiological interest (Goarant *et al.*, 1999). Recently, a PCR using 16S ribosomal DNA (16S rDNA) sequences can be used to confirm the identification of this organism (Oakey *et al.*, 2003). This technique was more rapid and economical than the conventional biochemical and morphological testing thus making diagnosis faster. However, its use was limited to confirmation of identification of a bacterial isolate and therefore not applicable as a single tool for the detection of *V. harveyi* in a mixed population.

2.2   Bacterial Luminescent Disease

2.2.1   Epizootics

Outbreaks of vibriosis have been reported worldwide. A minority of *V. harveyi* strains have been shown to cause disease in a variety of aquatic animal hosts including marine fish, bivalves and crustaceans (Table 2.2). Studies have shown that *V. harveyi* has been isolated from diseased but not healthy larvae and often

from the water in the rearing facilities (Jiravanichpaisal and Miyazaki, 1994; Karunasagar *et al.*, 1994).

In general, the infections associated with *V. harveyi* in fish are more opportunistic in nature than reports of disease in invertebrates. It was reported as an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch) (Kraxberger-Beatty *et al.*, 1990). It was also isolated from diseased seahorse, *Hippocampus* sp. (Alcaide *et al.*, 2001); from pearl oyster, *Pinctada maxima* (Pass *et al.*, 1987); from rock lobster, *Jasus verreauxi* (Diggles *et al.*, 2000); from diseased marine fish such as cultured silvery black porgy, *Acanthopagrus cuvieri* (Saaed, 1995), brown-spotted grouper, *Epinephelus tauvina* (Rasheed, 1989), sea bream, *Sparus aurata* (Balebona *et al.*, 1995), dentex, *Dentex dentex* (Company *et al.*, 1999); from cage-cultured seabass, *Lates calcarifer* Bloch (Tendencia, 2002) and sunfish, *Mola mola* (Hispano *et al.*, 1997). In a study by Owens *et al.* (1996), *V. harveyi* strains were pathogenic by intraperitoneal inoculation with 10^3 to 10^5 cells in Atlantic salmon (*Salmon salar*) however, this was an artificial route of infection and there have been no published reports of disease outbreaks caused by *V. harveyi* in this fish species. Skin infections in barramundi (*Lates calcarifer*) associated with *V. harveyi* isolates have only been reported in fish thought to be stressed in a captive environment or already infected with other agents (Anderson and Norton, 1991). Tendencia (2002) reported *V. harveyi* infection in seabass, *Lates calcarifer,* in the Philippines. The disease usually infects fish when they are stressed and signs exhibited include anorexia and darkening of the whole fish. There are also local haemorrhagic ulcers on the mouth or skin surface, focal necrotic lesions in the muscle or eye opacity.

An outbreak of disease in *Pinctada maxima* (pearly oyster) with 80% mortality following resettlement on leases after collection from wild sites was shown to be caused by *V. harveyi* (Pass *et al.*, 1987). These oysters were thought to be predisposed to infection by cold temperatures, overcrowding and inadequate water circulation during transport from wild sites. Lavilla-Pitogo and Dela Pena (1998) also reported an epizootic of luminescent, non-sucrose fermenting *Vibrio harveyi* in larvae of *P. monodon* in the Philippines. The exposure of the larvae to 10^2 *V. harveyi* cells/ml resulted in significant mortality within 48 h. It is noteworthy to

include the contribution made by environmental conditions or variation in the susceptibility of the host animals particularly when raised under intensive culture when outbreak of disease is concerned.

2.2.2   Sources of *V. harveyi*

Studies on the sources of *V. harveyi* by Lavilla-Pitogo, *et al* (1992)reported that the midgut contents of *P. monodon* spawners which are shed into the water almost simultaneously with the eggs during spawning are the main source of the luminescent bacteria. It was also found as a minor component of the microflora on the exoskeleton of female black tiger prawns, *P. monodon*, in Thailand (Jiravanichpaisal and Miyazaki, 1994). Moreover, this species of *Vibrio* seems to dominate the luminous bacterial population in nearshore seawater. Lavilla-Pitogo *et al.* (1990) found 5-7 CFU ml^{-1} of *V. harveyi* in nearshore water in the Philippines. Similarly, Makemson *et al.* (1992) reported 26-58 CFU ml^{-1} of *V. harveyi* from surface seawater samples in the Arabian Gulf. Ramesh *et al.* (1989) reported levels of 10^{3} CFU g^{-1}, of which 70% were *V. harveyi* in estuary sediments from southern India. It was elucidated that the ability of this species to utilise a wide variety of organic compounds as carbon and energy sources contributed to the survival of this species when competing for the scarce nutrients present in seawater (Ramesh *et al.*, 1989).

Table 2.2 Hosts in which *V. harveyi* has been implicated as a pathogen, and the associated disease (cited in Harris 1998[a], Vandenberghe 1998[b], Diggles 2001[c] Alcaide *et al.* 2001[d]).

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2.2.3 Luminous vibriosis

Luminous vibriosis is the term describing the disease caused by luminescent *V. harveyi*. Dead and dying animals visibly luminesce during a serious outbreak of this disease syndrome and have been observed to collect in a luminescent mat at the bottom of larval rearing tanks (Lavilla-Pitogo *et al.*, 1992). In larval prawns, it was probable that virulent luminous bacteria initiate infection by entering through the mouth and feeding apparatus. Scanning electron micrographs revealed colonisation of the bacteria specifically on the feeding apparatus and oral cavity of the larvae (Lavilla-Pitogo *et al.*, 1990). External signs in diseased prawns were brittle shells, brown or black spots on the shell, darkened or red body surface, pink or brown gills, murky whitish muscle, a lack of food in the midgut, and a folded base of the tail (Lavilla-Pitogo *et al.*, 1990). Affected larvae of penaeid shrimp developed luminescence, reduced feeding and had poor development, sluggish swimming, reduced escape mechanisms, degeneration of hepatopancreatic tissue with resultant formation of necrotic bundles, and increased mortality (Robertson *et al.*, 1998). These infections were usually septicaemic, with pure cultures of the causative strain isolated from the haemolymph and hepatopancreas (Jiravanichpaisal and Miyazaki, 1994; Liu *et al.*, 1996).

It has also been suggested that exotoxins may be involved in the disease process of luminous vibriosis. Muir (1991) reported that the histopathology of *P. monodon* larvae experimentally infected with *V. harveyi* showed changes suggestive of toxicity. The ganglionic neuropiles of infected larvae showed extensive vacuolation and granulation compared to uninoculated controls. Owens *et al.* (1992) reported extensive colonisation of the connective tissue of experimentally infected *Penaeus esculentus*. Connective tissue infection was also characteristic of experimentally infected oyster in the study of Pass *et al.* (1987). Histopathology of moribund shrimp showed extensive hepatopancreatic tubular necrosis and replacement by bacterial-haemocytic nodules, often melanized and marked haemocytic enteritis (Nithimathachoke *et al.*, 1995).

2.2.4 Contributing factors to luminous vibriosis

Many factors have been associated with luminous vibriosis. The increased amount of organic matter in the pond water particularly in the nursery reservoir

pond and tanks and contaminated equipment used between ponds were the probable factors (Nithimathachoke *et al.*, 1995). Cultures of marine algae are prone to contamination with *Vibrio* spp. due to either aerosol transmission or from cross contamination from the hands of workers or equipment (Harris and Owens, 1997). *Artemia* cysts are known to carry a residual flora consisting primarily of Gram-positive bacteria such as *Bacillus, Micrococcus* and coryneforms (Igarashi *et al.*, 1989). However, upon hatching in seawater of 35 ppt salinity, the microflora associated with *Artemia* nauplii rapidly becomes dominated by members of the autochthonous microflora of seawater such as *Vibrio* and *Pseudomonas* species (Igarashi *et al.*, 1989).

The seasonality of outbreaks of luminous vibriosis has been reported in some countries (Sunaryanto and Mariam, 1986; Ramaiah *et al.*, 2000). The level of *V. harveyi* in sites affected by run-off from heavy rainfall can rise as much as 100 fold as a consequence of nutrient run-off (Ramesh *et al.*, 1989; Harris and Owens, 1997). Also, during the rainy season, the salinity of seawater fell as low as 10 ppt (Prayitno and Latchford, 1995) which triggers *V. harveyi* strains to increase to a high level thus increasing their virulence. However, growth of these strains in media of pH 5.5 prior to challenge of *Penaeus monodon* had an opposite effect, significantly decreasing the virulence of these strains (Prayitno and Latchford, 1995). These effects probably indicate the expression of virulence genes being affected in response to bacterial processing of environmental signals.

2.3 Control of Luminous Vibriosis

2.3.1 Reducing the bacterial load

Harris and Owens (1997) identified seven critical control points in a commercial *P. monodon* hatchery in continuous or semi-continuous production where bacterial levels should be monitored and controlled to avoid contamination of the hatchery with undesirable levels of virulent *V. harveyi* strains. These were: (1) incoming waters; (2) spawners; (3) algal cultures; (4) *Artemia* cultures; (5) larval and post-larval rearing water; (6) tank and pipe surfaces; and (7) hatchery workers. The first four of these critical control points are the major sources of importance for the

initial introduction of virulent bacterial strains into the hatchery environment (Lavilla-Pitogo *et al.*, 1990; Lavilla-Pitogo *et al.*, 1992; Harris and Owens, 1997). The last three critical control points are of more importance for avoiding cross contamination or persistence of virulent strains within the hatchery (Karunasagar *et al.*, 1996; Harris and Owens, 1997). Sterilisation of incoming seawater, the reduction of cross- contamination of tanks within the hatchery and particularly the separation and sterilisation of eggs as soon as possible from spawners and the spawners faeces were points of hygiene addressed by Lavilla-Pitogo *et al.* (1992). The persistence of virulent strains of *V. harveyi* on tank surfaces was shown to be an important point of contamination in an Indian hatchery (Karunasagar *et al.*, 1996). In this study, *V. harveyi* living in a biofilm on high density polyethylene or concrete slabs survived treatment with 100 ppm chlorine and 50 ppm chloramphenicol and tetracycline. It was suggested that scrubbing the tank surfaces with strong cleansing agents or dry-out were the best measures to remove biofilms on these surfaces in order to decrease the population of pathogenic *V. harveyi* strains.

Abdel-Aziz (2001) recommended some measures to prevent the occurrence of *V. harveyi*-associated mortalities. These were: (1) maintenance of good husbandry practices and proper nutrition to reduce stress; (2) quarantine practices for live and newly acquired shrimp; (3) strict sanitation procedures prior to and during rearing of shrimp larvae; (4) control of pathogenic vibrios using ultraviolet-irradiated water as well as employing a series of good quality filtration systems; (5) the use of previously-chlorinated water during spawning and rearing; (6) periodical siphoning-out of sediments and debris from the bottom of rearing tanks; (7) disinfection of infected stock prior to discarding followed by cleaning and disinfection of hatchery after each larval rearing period and; (8) daily water exchange of 80-90%.

2.3.2 Antibiotics

The use of antibiotics as prophylactic agents to prevent bacterial infection of penaeid larvae had been employed in many shrimp hatcheries (Baticados and Paclibare, 1992). However, the development of antibiotic resistance is one of the

major consequences resulting from prophylactic antibiotic use. Indeed, there have been several reports of antibiotic-resistant pathogens isolated from both Indonesia and the Philippines (Baticados and Paclibare, 1992; Supriyadi and Rukyani, 1992). Baticados *et al*. (1990) determined the effect of 24 commonly used chemotherapeutic agents on strains of *V. harveyi*, *V. splendidus* and on *P. monodon* larvae. Of the used chemotherapeutics, only six of these antibiotics showed minimum inhibitory concentrations less than 25 µg/ml and even at these concentrations these antibiotics caused deformities in the carapace, rostrum and setae of larvae. The conclusions of this study were that chemical treatment of luminous vibriosis would be limited and ineffectual in long- term application to hatcheries for *P. monodon*. This was based on the relatively high dosage necessary to exert an effect on the target bacteria, the corresponding cost of treatment, the risk of development of resistant strains and the damaging effects of antibiotic exposure to both larval and human health. The biofilm formation of *V. harveyi* on surfaces like high density polyethylene plastics causes resistance to sanitisers (Karunasagar *et al.*, 1996). Also the use of antibiotics as prophylactics to control bacteria led to the persistence of antibiotic resistant *V. harveyi* in hatchery tanks ((Karunasagar *et al.*, 1994). These results suggest the need for physical removal of biofilm on tank surfaces and periodic drying of tanks to reduce the chance of infection by organisms such as *V. harveyi* (Karunasagar *et al.*, 1996).

In addition, many of the most commonly used antibiotics such as erythromycin, oxytetracycline and chloramphenicol are dangerous to human health. The use of chloramphenicol as antibiotic for food animals is banned in Australia because of the risk this agent poses to human health. Therefore, other methods of disease control are urgently needed, and an understanding of virulence mechanisms and environmental factors controlling pathogens is of primary importance in developing such alternative source (Prayitno and Latchford, 1995).

2.3.3 Probiotics

The use of probiotics is another approach to bacterial control to maintain a beneficial balance of bacteria and other microorganisms in the culture systems

(Maeda, 1988). Effective probiotic treatments may provide broader-spectrum and greater non-specific disease protection than vaccination or immunostimulation (Rengpipat *et al.*, 2000) and are more desirable and environmentally benign than antibiotics and chemicals. In aquaculture, Verschuere, *et al.* (2000) defined probiotics as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value by enhancing the host response towards disease, or by improving the quality of its ambient environment.

Gram-positive bacteria such as *Bacillus* spp. have been evaluated as probiotic by improving the water quality and reducing the number of pathogens in the vicinity of the farmed species (Wang *et al.*, 2000). There have been several reports of aerobic heterotropic bacteria being successfully used as probiotics for the culture of invertebrate larvae. A bacterial strain named strain CA2 was used as a food supplement for rearing larval cultures of the Pacific oyster *Crassostrea gigas* (Douillet and Langdon, 1994). Relative to untreated control cultures, strain CA2, when added to larval cultures at 10^5 CFU/ml, resulted in increased larval growth, a greater rate of larval settlement and a subsequent increase in the size of oyster spat (Douillet and Langdon, 1994). Another bacterial strain, PM-4, originally isolated from a rearing pond was shown to be beneficial for the survival and growth of larvae of the crab *Portunus triberculatus* (Nogami and Maeda, 1992). Strain PM-4 was shown to be vibriostatic against a strain of *Vibrio anguillarum* and a negative correlation between the growth of strain PM-4 and *Vibrio* spp was observed in rearing water (Nogami and Maeda, 1992). Addition of strain PM-4 increased the survival of larvae to the crab I instar such that the survival rate in seven treated tanks averaged 27.2% compared to 6.7% survival in nine tanks which did not receive probiotic treatment. A strain of *V. alginolyticus* has also been successfully used as a probiotic to increase survival of *Penaeus vannamei* post larvae (Garriques and Arevalo, 1995). This strain was added in high density to post larval rearing tanks in a 22-day trial which resulted in mean survival from the four treated tanks of 90.1%. Probiotic treatment in this case also resulted in an increased mean weight of post larvae from probiotic treated tanks compared to other tanks, suggesting that this *V. alginolyticus* strain also enhanced larval nutrition in this trial (Garriques and Arevalo, 1995). Chythanya *et al.,* (2002)

reported a probiotic, *Pseudomonas* I-2, that inhibited the growth of shrimp pathogenic vibrios including *Vibrio harveyi, V. fluvialis, V. parahaemolyticus, Photobacterium damsela,* and *V. vulnificus.* *Vibrio alginolyticus* has been shown to have antagonistic effects against pathogenic bacteria such as *V. harveyi.* However the properties of this probiotic still need further testing (Vandenberghe *et al.,* 1999). Beneficial microorganisms found to be effective for growth inhibition of *V. harveyi* on a laboratory scale include *V. alginolyticus* (Ruangpan, 1998), *Chlorella* sp. (Direkbusarakom *et al.,* 1997) and *Skeletonema costatum* (Panichsuke *et al.,* 1997). Challenge of *V. harveyi* with *Bacillus* sp. as probiotic feed achieved 74% relative survival (Phianpark *et al.,* 1997). The use of *Bacillus* S11 increased the survival and growth of *P. monodon* exposed to *V. harveyi* (Rengpipat *et al.,* 2000). This probiotic provided disease protection by activating both cellular and humoral immune defenses.

The precise mechanism of action of probiotics is largely unknown and it was elucidated that caution should be exercised in choosing probiotics as some apparently harmless organisms may regain virulence, or are pathogenic to aquatic animals (Irianto and Austin, 2002). Fuller (1989) recommended some properties of an ideal probiotic. Probiotics should provide actual benefit to the host, be able to survive in the digestive tract, capable of commercialisation and should be stable and viable for prolonged storage conditions and in the field.

2.3.4   Bacteriophage therapy

The use of bacteriophage in the control of vibriosis seems very promising. An experiment with bacteriophage from diseased ayu, *Plecoglossus altivelis*, provide protection against infection by *Plecoglossus plecoglossicida*, a pathogen of ayu. These bacteriophages are representatives of Myoviridae and Podoviridae that reduced the number of bacterial cells in the kidneys of affected ayu and underlying water environment (Park *et al.,* 2000). A follow-up study in ayu *Plecoglossus altivelis* by Park and Nakai (2003) showed that phage-impregnated feeds administered to ayu pond resulted to a decreased mortality rate and the appearance of phage in the kidneys. The successful effect of phage in phage therapy can be

shown in an increase presence of phage in survivors and death of host bacterial cells.

2.4 Virulence Factors of *Vibrio harveyi*

Most *V. harveyi* strains are not harmful to larvae of *P. monodon* however, some strains are extremely pathogenic. Highly virulent strains of *V. harveyi* cause acute, devastating disease outbreaks with 50 to 100% mortality from inocula as low as 100 cells/ml (Harris, 1998). Despite extensive studies on this species, the virulence mechanism of this bacterium is not well understood.

2.4.1 Siderophores

One of the virulence factors associated in vibriosis is the ability to utilise iron from the host fluids by means of an efficient iron-sequestering system (Crosa, 1984). Since iron acquisition in microbial pathogens is limited due to strong binding capacity of this element to the high-affinity iron-binding proteins of animal hosts (Neilands, 1981), many bacteria have complex systems to transport iron into the cell in the form of siderophores (Aznar *et al.*, 1989). Most microorganisms, including fungi and bacteria, use siderophores to fulfil their iron requirements (Winkelmann, 1991). A siderophore is a low molecular weight, Fe (III)-specific ligand (Neilands, 1981). The possession of a siderophore-mediated iron transport system is related to the increased virulence of some bacterial pathogens (Griffiths, 1987). In addition, siderophores act as growth or germination factors, as potent antibiotics, have a role in receptor-dependent iron transport and function as virulence factors in animal and plant diseases (Neilands, 1981). They are molecules designed to trap traces of iron under the form of very stable complexes. Considerable evidence exists for the influence of iron on microbial growth. Indeed, a soluble iron fed or when injected into an infected animal may greatly increase the virulence of some pathogens (Madigan *et al.*, 2000).

Siderophores were reported as a major virulence determinant for pathogens such as *Aeromonas salmonicida* (Hirst *et al.*, 1994), *Vibrio anguillarum* (Sigel and

Payne, 1982), *Shigella flexneri* (Crosa, 1989), *Vibrio cholerae* non-01 (Amaro *et al.*, 1990), *Aeromonas hydrophila* (Barghouti *et al.*, 1989) and *Salmonella typhimurium* (Payne, 1983). The presence of siderophores or other specific systems for using host iron sources may be advantageous for the survival and growth of the pathogen (Biosca and Amaro, 1991). Siderophore activity appeared to be linked to pathogenicity in vertebrates but not in invertebrates (Owens *et al.*, 1996). In vertebrates, high affinity iron-binding proteins such as transferring and lactoferrin present in serum and secretions are tightly bound (Crosa, 1989). However, invertebrates seem to lack iron-binding compounds such as lactoferrin and transferrin (Owens *et al.*, 1996). Moreover, it has been suggested that the lack of competition for iron between invertebrates and invertebrate pathogens may have resulted in a decreased necessity for these bacteria to produce siderophores (Harris, 1993; Owens *et al.*, 1996). In *V. harveyi,* siderophore production seems to be related to strain origin such that bacterial strains infecting vertebrates showed enhanced siderophore production than strains infecting invertebrates (Owens *et al.*, 1996). These authors concluded that siderophores must be considered a virulence factor in piscine infecting *V. harveyi* but not in strains infecting invertebrates.

2.4.2 Chitinases

Chitin is one of the most abundant biopolymers in nature and is produced in the marine environments by many marine organisms such as zooplankton and phytoplankton (Gooday, 1990). The great diversity of chitin structures present in the environment necessitates bacteria to produce different chitinases to efficiently hydrolyse the different forms of chitin (Svitil *et al.*, 1997). The presence of chitinases presumably aids the invasion of the pathogen and provide nutrients directly in the form of amino acids or indirectly by exposing other host material to enzymatic degradation (Gooday, 1990). Chitinases consists of a group of hydrolytic enzymes that are able to break down polymeric chitin to chitin oligosaccharides, diacetylchitobiose, and *N*-acetylglucosamine (Thompson *et al.*, 2001). There are two enzymes encoded by genes involved in chitin degradation. The first enzyme is chitiobiase, which cleaves the bond joining the two *N*-acetylglucosamine units in chitobiose (Jannatipour *et al.*, 1987) and the other

enzyme chitinase, encoded by gene *chiA*, was tentatively identified as the main chitinase of *V. harveyi* (Soto-Gil, 1988). Typically, bacteria produce more than one type of chitinase. For example, *Bacillus circulans* WL-12 secretes at least six major chitinases which vary in enzyme activity (Watanabe *et al.*, 1990). *Streptomyces lividans* produces three separate chitinase genes (Miyashita *et al.*, 1991).

In natural marine systems, most bacteria attached to chitinaceous particles are vibrios (Nagasawa *et al.*, 1987). Baumann *et al.,* (1980) reported that all pathogenic *Vibrio* species elaborate an extracellular chitinase. This was shown by Baumann and Schubert (1984) in which chitinase-positive *Vibrio* isolates were pathogenic to animals. *Vibrio harveyi* excretes about 10 chitinases when grown on chitin and that the composition of the excreted chitinases varies when cells are exposed to different chitins, some of which are always present while others are excreted only with particular chitins (Svitil *et al.*, 1997). Based on restriction analysis, immunological data, and enzymatic properties, it has also been shown that six genes are responsible for the coding a total of 10 excreted chitinases in this species (Svitil *et al.*, 1997). Montgomery and Kirchman (1993) showed that the attachment of *V. harveyi* to chitin was specific and was mediated by at least two peptides associated with the outer membrane of the cell. One of these peptides that appeared to mediate initial attachment to chitin was a 53 kDA peptide whilst the other peptide that was induced by chitin and presumably involved in time-dependent attachment was a 150 kDA chitin-binding peptide.

2.4.3 Role of plasmids in virulence and antibiotic resistance

Bacterial plasmids are molecules of double-stranded DNA and are responsible for virulence of the bacteria causing plague, dysentery, anthrax and tetanus as well as many other diseases of man, animals, fish and plants (Hardy, 1984). Bacteria that contain antibiotic resistance plasmids have been shown to exhibit higher rates of survival in aquatic environments (Baya *et al.*, 1986). Plasmids may act as a vector to quickly spread genetic information throughout a bacterial population (Richmond, 1973). The genes that encode resistance are the resistance determinants or an R factor. These are products that inactivates the antibiotics or

prevent the antibacterial drug from contacting its target within the cell (Richmond, 1973). The increase in resistance can be attributed to the selection of resistant strains which have the ability to exchange plasmids encoding resistance (Baya *et al.*, 1986).

Higher prevalence of plasmid-bearing strains in marine vibrios of the Gulf of Mexico was found in a polluted site rather than in an unpolluted site (Hada and Sizemore, 1981). Bacteria containing plasmid DNA that demonstrate antibiotic resistance are more frequently encountered in isolates acquired from toxic chemical waste than from sewage-impacted waters or from uncontaminated open ocean sites (Baya *et al.*, 1986). In a study by Pedersen *et al.* (1998), antibiotic-resistant *Vibrio anguillarium* carried plasmids that exhibited resistance against macrolides, spiramycin and lincomycin. However no conjugation experiments were conducted to detect possible R factors. Schmidt *et al.* (2001) showed antibiotic resistant plasmids contributed substantially to the horizontal spread of antimicrobial resistance within species of *Aeromonas salmonicida*.

A conjugative R factor in a certain strain of *V. harveyi* virulent to *P. monodon* was reported by Harris (1993). This R factor confers resistance to erythromycin, streptomycin, kanamycin, sulphafurazole and co-trimoxazole. Karunasagar (1994) also reported a highly virulent antibiotic resistant strain of *V. harveyi* that colonised larval tanks in a hatchery in India. This strain showed resistance against co-trimoxazole, chloramphenicol, streptomycin and a vibriostatic agent 0/129.

2.4.4 Bacteriocins

Bacteriocins are plasmid-derived proteins produced by bacteria that exhibit an antimicrobial mode of action against sensitive, usually closely related bacterial species (Tagg *et al.*, 1976). Bacteriocins are named in accordance with the species of organism that produces them such as colicin in *Escherichia coli* coded by Col plasmids and subtilisin in *Bacillus subtilis* (Madigan *et al.*, 2000). McCall and Sizemore (1979) reported a bacteriocin-like substance in *Vibrio harveyi* (formerly classified in the genus *Beneckea*) mediated by a plasmid.   This lethal substance termed harveyicin was the first well-documented bacteriocin in marine bacteria

reported. The likely role for *V. harveyi* bacteriocin is to confer a competitive advantage on bacteriocin-producing strains over closely related, non-bacteriocinogenic strains in the enteric environment (Hoyt and Sizemore, 1982).

2.4.5 *N*- (*β*-hydroxybutyryl) homoserine lactone: a possible role for bioluminescence

Expression of the luminescence system in many bacteria is controlled by a cell density-dependent induction, called autoinduction (Sun *et al.*, 1994). Autoinducer serves as a pheromone which signals cells in a light organ symbiosis to luminisce (Greenberg *et al.*, 1979). Quorum sensing probably enhances bioluminescence, virulence factor expression, antibiotic production and biofilm development (Chen *et al.*, 2002). The *lux* genes encode the enzymes for bioluminescence, luciferase, and these are expressed only when the autoinducer signal accumulates to a critical external concentration or when the cells reach a high population density (Von Bodman and Farrand, 1995). The enzyme luciferase, consisting of two different subunits (α and β), catalyzes the oxidation of a reduced flavin and a long-chain aldehyde (Ziegler and Baldwin, 1981).

Pathogenic bacteria use acyl-homoserine lactone-mediated autoinduction to control the expression of virulence functions. For example, *Erwinia stewarti*, the aetiological agent of Stewart's wilt of sweet corn requires induction by acly-HSL-mediated activation for invasion and pathogenicity (Von Bodman and Farrand, 1995). Virulence and exoenzyme production of the plant pathogen, *Erwinia carotovora*, is controlled by a small diffusible signal molecule, *N* - (3-oxohexanoyl) homoserine lactone (Pirhonen *et al.*, 1993). In *Pseudomonas aeruginosa, N* (3-oxohexanoly) homoserine lactone is involved in pathogenesis during infection (Jones *et al.*, 1993). The marine bacterium *Vibrio fischeri* also possesses autoinducer to signal cells in a light organ symbiosis to luminescence (Eberhard, 1972; Greenberg *et al.*, 1979). This autoinducer is similar in structure to the *V. harveyi* autoinducer which suggests that the regulation of luminescence induction in these bacteria may be related in spite of their differences in *lux* gene

organization. *Vibrio anguillarum* also uses acyl-homoserine lactone to regulate virulence gene expression (Milton *et al.*, 1997).

*N-(β-*hydroxybutyryl) homoserine lactone is the autoinducer molecule regulating bioluminescence in *V. harveyi* (Cao and Meighen, 1989). This autoinducer enables the bacterium to monitor its own population by quorum sensing. A recently discovered autoinducer, AI-2, is found to be produced by a large number of bacterial species including *Vibrio harveyi* hence proposed to serve as a "universal" signal for inter-species communication (Chen *et al.*, 2002). The autoinducer structure and the development of light emission in *V. harveyi* is similar to *V. fischeri*. Thus it was suggested that both have a common mechanism in the regulation of autoinduction of the *lux* system (Cao and Meighen, 1989) However, the regulatory system which operates in *V. harveyi* is very different and more complex than in *V. fischeri* (Bassler *et al.*, 1997). Luminescence in *V. harveyi* is thought to be affected by two autoinducer molecules. Only one of these molecules, N-(3-hydroxybutanoyl) homoserine lactone (HBHL), as been characterised (Cao and Meighen, 1989). The production of these molecules is encoded by genes which are not contiguous with the luminescence operon, comprising of *lux*CDABEGH. The *V. harveyi* autoinducers interact with separate sensor proteins produced by the *lux* N and *lux* PQ loci respectively to alter the activity by phosphorylation, of the regulatory protein *lux* O (Bassler *et al.*, 1997).

It has been shown that halogenated furanones produced by marine microalga *Delisea pulchra* interfere with AHL-regulated gene expression. Manefield *et al.* (2000) investigated the ability of a halogenated furanone to inhibit the quorum sensing regulated luminescence phenotype of the pathogenic *V. harveyi* strain 47666-1 and found that this inhibited luminescence as well as extracellular toxin production.

2.4.6 Exotoxins and other exoenzymes

Virulence is reported to be associated with the presence of extracellular products (ECPs). This extracellular protein is produced during the mid exponential phase

of growth and has sequence similarity to virulence-associated proteins in *Salmonella, Shigella,* and *Bacillus* species (Manefield *et al.*, 2000).

Some *Vibrio* species produced exotoxins and exoenzymes that play a role in their pathogenicity. For example, a thermostable direct haemolysin, as a major virulence factor of *V. parahaemolyticus* (Raimondi *et al.*, 2000) has an enterotoxigenic effect on rabbit small intestine and could be responsible for exhibiting a watery diarrhea (Nishibuchi *et al.*, 1992). An extracellular cytolytic toxin has been reported in *V. vulnificus* that possess cytolytic, lethal and vascular permeability factor activities in the guinea pig and the mouse (Gray and Kreger, 1985). Fouz *et al.* (1993) reported extracellular products of *Photobacterium damsela* that exhibit phospholipase, haemolytic and cytotoxic activities in diseased fish. The production of proteinase by *V. alginolyticus* is involved in the pathogenesis of bivalve vibriosis (Nottage and Birkbeck, 1987). The extracellular products (exotoxins) in *V. penaeicida,V. alginolyticus* and *V. nigripulchritudo* demonstrated toxic effects in shrimp but not on a fish cell line (Goarant *et al.*, 2000). A shellfish-pathogenic *Vibrio* sp. isolated from moribund Americal oyster larvae produced a heat-labile, extracellular toxin that caused malformations and mortalities in developing oyster embryonic culture (Brown and Roland, 1984).

V. harveyi was shown to produce proteases, phospholipase, haemolysins or exotoxins which are important for pathogenicity (Liu *et al.*, 1996). Liu and Lee (1999) also reported that a cysteine protease was the major exotoxin lethal to tiger prawn, *Penaeus monodon*. This cysteine protease may markedly interfere with haemostasis leading to the occurrence of unclottable hemolymph, thus significantly contributing to the pathogenicity of *V. harveyi* (Lee *et al.*, 1999). A thermostable exotoxin in *V. harveyi* recovered from diseased post larval *Penaeus vannamei* was also reported lethal to Dublin Bay prawns (Montero and Austin, 1999). These exotoxins were shown to have proteolytic, haemolytic and cytotoxic activities. In a study by Zhang and Austin (2000) *V. harveyi* strain 645 produced extracellular protein with a maximal effect on salmonids. These extracellular proteins contain caseinase, gelatinase, phospholipase, lipase and haemolysins with the highest titre of haemolytic activity towards *Salmo salar* (atlantic salmon) and *Oncorhynchus mykiss* (rainbow trout) erythrocytes. In addition, the possession of

double haemolysin genes has been associated in the virulence of *V. harveyi* (Zhang *et al.*, 2001). Marine toxins such as tetrodotoxin and anhydrotetrodotoxins were produced by *Vibrio harveyi* (Simidu *et al.*, 1987) however this remained to be confirmed as the role of *Vibrios* in their production is unclear. Two luminous strains of *Vibrio harveyi* (642 and 47666-1) were shown to produce proteinaceous exotoxins that caused mortality in CBA mice and juvenile *P. monodon* at low doses (Harris and Owens, 1999). Toxin, T1 produced by *V. harveyi* strain 47666-1 had an LD_{50} of 2.1 $\mu g\ g^{-1}$ by intra-peritoneal injection in CBA mice and 1.8$\mu g\ g^{-1}$ by intramuscular injection in juvenile *P. monodon*. Toxin, T2 produced by *V. harveyi* strain 642 had an LD_{50} of 3.1 $\mu g\ g^{-1}$ by intraperitoneal injection in CBA mice and 2.2 $\mu g\ g^{-1}$ by intramuscular injection in juvenile *P. monodon* (Harris and Owens, 1999).

2.4.7 Bacteriophages

Bacteriophages are infectious agents made up of DNA or RNA and a protein capsid which protect their genetic material and display structural components necessary for phage to infect its bacterial host (Lindqvist, 1998). Phages thrive in bacterial populations where they constantly transfer their genetic material in a horizontal fashion. A phage infection may lead to death or survival of the cell. If the cell survives, the phage genome may become established as a prophage in the form of an integrated copy in the host chromosome or maintained as a plasmid or the phage infection may simply abort leading to cell survival (Lindqvist, 1998). Phages can multiply by two alternative mechanisms: the lytic cycle or the lysogenic cycle. The former ends with the lysis and death of the host cell, whereas the latter results to survival of the host cell (Tortora *et al.*, 2000). The lysogenic cycle can confer virulence to bacteria (Oakey and Owens, 2000). Many bacterial virulence determinants are encoded by accessory element-borne bacteriophage. For example, β prophage of *Corynebacterium diptheriae* which transmit genes coding for the diphtherial toxin (Freeman, 1951), pathogenic strains of *Shigella* producing shigella toxin (Hale, 1991), *Staphylococcus* enterotoxin A encoded for by a prophage (Betley and Mekalanos, 1985), *Clostridium botulinum* toxins (Johnson *et al.*, 1980), *Pseudomonas aueroginosa* cytotoxins (Hayashi *et al.*,

1990), *Escherichia coli* verotoxins and cytotoxins (Takeda and Murphy, 1979), *Vibrio cholerae* enterotoxin (Waldor and Mekalanos, 1996) and *Mycoplasma arthritidis* bacteriophage (MAV1) causing enhanced arthritogenicity in rats (Voelker *et al.*, 1995).

Ruangpan *et al.* (1999) found a bacteriophage and bacterial cells of *Vibrio* morphology which have lethal toxicity to cultivated *Penaeus monodon*. They suggested that the presence of bacteriophage may sometimes mediate toxicity of *V. harveyi* by the transfer of a toxin gene or a gene controlling toxin production. A temperate phage in *Vibrio harveyi* VH1039 isolated from tea brown gill syndrome in black tiger prawn, *P. monodon,* is a lysogenic bacteriophage and morphologically identified as siphoviruses (Pasharawipas *et al.*, 1998). Lysogenic VH1039 caused no symptoms in the shrimp but when combined with *Vibrio harveyi* VHN1 lead to shrimp death in several hours after intramuscular injection. At least two bacteriophages have been isolated from two different strains of *V. harveyi* that have been shown to produce toxin (Harris and Owens, 1999). The first isolated phage from strain 47666-1 classified under *family Podoviridae* (Oakey, 2000) and the second, isolated from strain 642 classified into the *family Myoviridae* (Oakey and Owens, 2000). Oakey and Owens (2000) isolated a bacteriophage from a toxin-producing strain of *Vibrio harveyi* (strain 642) in tropical Australia. This bacteriophage is classified into the *family Myoviridae* based upon morphological characteristics such as an icosahedral head, a neck/collar region and a sheathed rigid tail, nucleic acid characteristics (double-stranded linear DNA) and was termed bacteriophage VHML (*Vibrio harveyi* Myovirus-Like). Munro *et al.*, (2003) further studied this bacteriophage and confirmed that infection of bacteriophage to previously naïve *V. harveyi* strain 12, 20, 45 and 645 (Oakey and Owens, 2000) confers virulence to *V. harveyi* strain 642. Results from SDS-PAGE gels showed that the bacteriophage VHML caused an up-regulation of certain extracellular proteins from *V. harveyi* strain 12, 20, 45 and 645. With regards to haemolysin production, the halo size and colony size from strains of *V. harveyi* that were bacteriophage infected were significantly larger as a group (P<0.001) from the strains without the bacteriophage VHML. Moreover, the bioassay experiment using bath challenge method demonstrated that strains of *V. harveyi* infected with the bacteriophage VHML caused significantly

(P<0.001) higher mortality (LD50) in larval prawns than the same strains without the bacteriophage. Monoclonal antibodies (MABs) confirmed that the infection of the bacteriophage VHML resulted in proteins being synthesised and excreted by the previously naïve bacteria as shown by the production of bands that were recognised by the MABs while no bands were detected in the strains uninfected with the bacteriophage (Munro *et al.*, 2003).

2.5 Conclusion

The marine bacterium *Vibrio harveyi* occurs naturally in tropical marine waters. It can be found as a free-living organism or as part of the gut flora of marine animals, as an opportunistic pathogen and as both a primary and an opportunistic pathogen of marine invertebrates. Many virulence factors have been implicated in the severity of outbreaks caused by luminous vibriosis. It was suggested that virulent isolates within *V. harveyi* are rare and that virulence is likely to be due to a mobile genetic element such as plamids or bacteriophages (Pizzutto and Hirst, 1995). The possession of toxic extracellular proteins such as proteases, phospholipases, haemolysins and cysteine proteases can increase the virulence of this marine bacterium. In addition, virulence factors such as siderophores, chitinases, bacteriocins and resistance plasmids also contribute to the pathogenicity of *V. harveyi*.

The involvement of bacteriophage in the virulence of *V. harveyi* has been documented previously by several authors (Ruangpan *et al.*, 1999; Oakey and Owens, 2000; Munro *et al.*, 2003). At least two strains of *V. harveyi* (strains 47666-1 and 642) were shown to cause devastating disease in prawns in northern Australia and that these strains carry a genetic mobile element responsible for its virulence. A bacteriophage VHML from *V. harveyi* strain 642 was confirmed to confer virulence to previously known naïve strains of *V. harveyi* (Munro *et al.*, 2003). While it was reported that *V. harveyi* strain 47666-1 was more virulent than strain 642 causing close to100% mortality to *P. monodon* larvae at 10^4 CFU ml^{-1}, there is no information yet to confirm whether the bacteriophage in this strain confers virulence in *V. harveyi* strain 47666-1. The better understanding of the

mechanisms by which *V. harveyi* causes disease could provide a basis for the design of more effective and permanent method to prevent and control luminous vibriosis in prawns in northern Australia and SE Asia. The information will also provide an insight into the pathogenesis of *V. harveyi* infection in prawns and other marine animals.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Source of Isolates of *Vibrio harveyi*

All isolates used in this study were from the Microbiology and Immunology culture collection at James Cook University. The strain numbers, study numbers and source of the *V. harveyi* isolates are given below in Table 3.1.

Table 3.1 The strain number, study number and source of isolates of *V. harveyi* used in this study.

STRAIN NUMBER	STUDY NUMBER	SOURCE
#47666-1	#47666-1	*P. monodon larvae*
#12	#12	Environmental: seawater
#20	#20	Environmental: seawater
#30	#30	Environmental: seawater
ACMM 643	#643	Environmental: seawater

ACMM (Australian Collection of Marine Microorganisms)

3.2 Storage and Propagation of Stock Isolates of *Vibrio harveyi*

A stab of frozen culture from a bacterial suspension in a cryotube was used to inoculate PYSS (bacteriological peptone, yeast, sea salt) agar plates. The culture was incubated overnight at 28 $^{\circ}$C. The culture was then examined for purity and if pure, was used for experimental purposes. Several isolated colonies from an agar plate were suspended in PYSS broth. The suspension was incubated in an orbital incubator shaken at 110 rpm, with a temperature of 28 $^{\circ}$C until appropriate cell density was achieved (section 3.3).

Storage preparation for *V. harveyi* isolates was done by the addition of 10% glycerol (w/v) to a late exponential phase broth culture. One millilitre aliquots of this bacterial suspension were then dispensed into cryotubes (Nunc, Sydney,

Australia). The working stocks were stored at –20 $^{\circ}$C and the long term storage stock were stored at –70 $^{\circ}$C.

3.3 Spectrophotometric Analysis and Estimation of *Vibrio harveyi* Cell Density

An ultrospec III spectrophotometer (Pharmacia, Sydney, Australia) with a 1 cm path length was used to measure the cell density of *V. harveyi* in broth culture. One millilitre of bacterial suspension was dispensed into a 2ml plastic cuvette (Sarstedt, Numbrecht, Germany) and the absorbance at 600nm (A_{600}) was measured against sterile PYSS (blank).

The growth curve for strain 47666-1 was plotted through an absorbance at 600 nm in spectrophotometer and was correlated with cell density values. For a desired experimental procedure, a mid to late exponential phase culture was used throughout the study. This correlated with A_{600} values in the range 0.95 to 1.05, corresponding to a viable cell density of approximately 10^{9} colony forming units (CFU) ml^{-1}.

CHAPTER 4

CHANGES TO *VIBRIO HARVEYI* STRAINS EXPERIMENTALLY INFECTED WITH BACTERIOPHAGE FROM 47666-1

4.1 Introduction

Vibrio harveyi is a marine species known to be a primary and opportunistic pathogen that is ubiquitous in marine environments. It has been identified as the main cause of luminous vibriosis that caused great economic losses to prawn farmers throughout the world. While there are strains of *V. harveyi* that cause no effect on prawns, at least two pathogens were known to cause devastating disease in northern Australia (Harris and Owens, 1999). These strains are *V. harveyi* strain 47666-1 and ACMM 642. Studies on the virulence of *V. harveyi* ACMM 642 had been done by Munro *et al.* (2003) using haemolysin assay, protein assay and bioassay challenge with *Penaeus monodon* larvae. The results revealed that virulence was mediated by the presence of bacteriophage, *Vibrio harveyi* Myovirus like (VHML) which caused naïve strains of *V. harveyi* to be virulent upon infection.

While it was reported that *V. harveyi* 47666-1 was more virulent than ACMM 642 causing 100% mortality to *P. monodon* larvae at 10^2 CFU ml^{-1} (Muir, 1991), there is no information yet to confirm whether a bacteriophage in this strain confers virulence. This strain has been shown to be infected with *Vibrio harveyi* Podovirus-like (VHPL) bacteriophage, classified under the *family Podoviridae* (Oakey, 2000). Accordingly, this bacteriophage is characterised by an icosahedral head (~70nm diameter), short stumpy tail, and has a linear double stranded DNA genome.

This study aimed to determine the effect of bacteriophage VHPL from *V. harveyi* 47666-1 when infected to previously naïve strains of *V. harveyi*.   Various virulence assays were undertaken to confirm the effect of bacteriophage infection

on the virulence of *V. harveyi* 47666-1. SDS-PAGE analysis was used to show the transfer of exotoxins by the production of extracellular proteins and up regulation of these proteins in naïve strains of *V. harveyi* challenged with the phage. Haemolysin production in dual layered agar was used to determine the haemolytic activity of bacteriophage-infected strains. Production of siderophores in avirulent strains of *V. harveyi* infected with bacteriophage from a virulent strain, *V. harveyi* 47666-1, was investigated using the universal assay of Schwyn and Neilands (1987). An assay for chitin degradation was undertaken to show the production of the enzyme chitinase in naïve strains of *V. harveyi* challenged with phage VHPL. The pathogenicity of the bacteriophage VHPL was also assessed using a bioassay challenge with nauplii of *Penaeus monodon*.

A better understanding of the role of bacteriophage mediated virulence to bacteria could provide an insight into the pathogenesis of vibriosis and assist in the design of more effective and permanent method to prevent and control this disease in prawns and other marine animals.

4.2 Materials and Methods

4.2.1 Bacterial isolates and growth conditions

All isolates used in this study were from the Microbiology and Immunology culture collection at James Cook University, Australia. After original isolation, strains were cultured in PYSS (bacteriological peptone, yeast, and sea salt) agar and incubated overnight at 28°C. Several isolated colonies from an agar plate were suspended in PYSS broth (Appendix 1.1). The suspension was incubated at 28°C in an orbital incubator shaken at 110 rpm until the appropriate cell density was achieved. The isolate was then prepared for storage by the addition of 10% glycerol (w/v) and were stored at −70°C for long-term storage and at −20ºC for working stock.

4.2.2   Bacteriophage extraction and concentration

A loop of *Vibrio harveyi* strain 47666-1 from the –20°C working culture was used to inoculate PYSS broth (Oakey and Owens, 2000). The culture was incubated overnight at 28°C to give a mid to late exponential phase culture corresponding to an optical density of 0.95 – 1.05 at 600 nm. This corresponded to a cell density of approximately 2 x 10^9 CFU ml^{-1}. The culture was induced into the lytic cycle by the addition of 100ng ml^{-1} mitomycin C (Sigma-Aldrich, Castle Hill, NSW, Australia). Induced cultures were returned to incubation for 15-18 hours on an orbital shaker at 28°C at 110 rpm. Cells were centrifuged at 5000 *x g* for 10 min to pellet bacterial cells. The supernatant was harvested and filtered using a 0.45μm disposable millipore filter (Millipore, North Ryde, NSW, Australia). The filtered supernatant was then placed in polycarbonated centrifuge tubes (Beckman, Palo, Alto, CA). The cell free supernatant were pelleted by ultracentrifuging at 200 000 *x g* for 4 hours. The pelleted phage was resuspended in 0.01 original volume sterile SM buffer (Appendix 4.2) and was stored at 4°C.

4.2.3   Infecting the phage from *Vibrio harveyi* 47666-1 to naïve bacteria

Those bacterial strains which were shown to have no prophage by Oakey and Owens (2000) were challenged with the bacteriophage from *V. harveyi* strain 47666-1 using methods described therein. Bacteria were cultured in 100 ml aliquots of PYSS and incubated as stated previously until OD$_{600}$ was approximately 0.2. The cultures were aseptically divided into four equal aliquots. A volume of 500μl of concentrated VHPL phage extract from *V. harveyi* 47666-1 was added to two of the aliquots and 500μl SM buffer added to the other two. All tubes were returned to incubation for 2 h to allow potential infection to occur. All infected strains were stored as previously described.

4.2.4   Detecting infection of bacteriophage in the previously naïve bacteria

All tubes with added extract and one of the 'blank' tubes were induced with 100ngml^{-1} mitomycin C and further incubated. Optical density readings were taken periodically through the experiment to a maximum of 30 h post-induction.

A decrease in the OD_{600} which was not evident in either the control or the induced control was taken as a presumptive infection from the 47666-1 phage extract.

4.2.5 Preparation of cell-free supernatant

The procedure for the preparation of cell-free supernatant extract was modified from Harris and Owens (1999) and Munro *et al.* (2003). Sterile PYSS was inoculated with a stab from a strain of *V. harveyi* from an overnight culture. This culture was grown to a density corresponding to an absorbance of 0.95-1.05 at 600 nm. Disposable cuvettes (2 ml) (Sarstedt, Adelaide, Australia) were used to measure absorbance in an Ultrospec III spectrophotometer (Amrad Pharmacia Biotech, Boronia, Australia). Each culture of bacteria with and without the phage VHPL was lysed using mitomycin C at a dose of 100ng ml^{-1}. They were returned to incubation for 5 hours prior to pelleting by centrifugation at 12 200 x g for 20 min. The supernatant was harvested and filtered through a 0.45 μm filter followed by 0.22μm membrane filter. To ascertain that the bacteria had been completely removed from the lysates, a loopful of supernatant was streaked onto PYSS agar plate and was observed for bacterial growth. The lysates that showed no growth on plates were further processed with the concentration method.

4.2.6 Concentration of the supernatant

A 50 to 300 times concentration of the supernatant was achieved by membrane filtration. Two membranes and filter devices were used:

(1) For the preparation of small volumes of cell-free supernatant extract (CFSE), the supernatant was spun through a Centriprep-30 concentrator filter (Amicon, Beverly, USA) at 1500 x g using a swinging bucket rotor in a Clements 2000 benchtop centrifuge (Clements, Sydney, Australia). In this method, a 15-ml supernatant was concentrated to approximately 500 μl in 90 min.

(2) For the preparation of larger volumes, approximately three litres of supernatant was concentrated by filtration through a 68 mm YM 50 (50 kDa) (Amicon, Beverly, USA) in an Amicon (Millipore, Sydney, Australia) model 8200 ultrafiltration stir-cell at 4°C, under pressure of

nitrogen gas applied at 100 psi. In this method, a 3000-ml supernatant was concentrated to approximately 15 ml in 2 days.

4.2.7 Analysis of CFSE protein profiles by SDS-PAGE

Each CFSE was analysed by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Equal amounts of each CFSE was mixed with phosphate buffered saline (PBS) (Appendix 4.1) to achieve a uniform protein concentration. The mixed protein samples were then diluted with equal volume of SDS reducing buffer (Appendix 4.4.1), mixed, boiled at 100°C for 5 minutes and used immediately in SDS-PAGE.

4.2.8 SDS Poly-acrylamide gel electrophoresis (SDS-PAGE)

One dimensional poly-acrylamide gels of 7.5 cm length, using 1mm spacers, with a 12% separating gel (Appendix 4.4.6) and 4% stacking gel (Appendix 4.4.7) were used and run by the method of Pizzutto & Hirst (1995) and Munro (2001), being a modification of the method of Laemmli (1970).

A Bio-Rad Mini PROTEAN 3 Electrophoresis Cells (Bio-Rad, Regents Park, NSW, Australia) was used to electrophorese the gels with 1 x SDS running buffer (pH 8.3) (Appendix 4.4.5). Samples were prepared by mixing equal volumes of concentrated CFSE with reducing buffer (Appendix 4.4.1). Twenty four microliters of each sample was loaded into the wells. Broad range SDS-PAGE molecular weight markers (Bio-Rad) were included in each run to indicate the protein size of samples. Electrophoresis was performed at 125 V until the tracking dye left the bottom of the gel. Once finished, gels were removed from glass plates and placed in Coomassie blue stain (Appendix 4.4.8) for at least one hour at room temperature. Gels were destained for 3 hours or overnight using destaining solution (Appendix 4.4.9). Where gels from Coomassie blue staining produced unclear bands, a more sensitive staining technique, silver-stain plus, (Bio-Rad, #161-0449) was used. A digital image of the resulting protein bands was then captured using Olympus digital camera (Olympus Optical Co. Ltd.).

4.2.9 Protein estimation

Estimation of total protein in solution was performed by the Pierce BCA method (Progen Industries, Darra, Australia).

4.2.10 Haemolysis production for the strains infected with the phage from *Vibrio harveyi* 47666-1

Dual layered agar plates (Appendix 1.3) enriched with 5% sterile sheep blood were used to demonstrate haemolysin production in the four naïve strains infected and uninfected with phage from *V. harveyi* 47666-1. Individual strains were plated onto PYSS agar using isolation streaks and were incubated overnight (section 3.2). Individual colonies were transferred from PYSS agar plates onto dual layered agar plates using sterile toothpicks and were incubated overnight. Both the colony diameter and the area of haemolysis were measured in cm.

4.2.11 Screening of *Vibrio harveyi* strains for siderophore production

4.2.11.1   Preparation of chrome azurol S (CAS) agar plates

The CAS agar plates (Appendix 2) used in this study were prepared by the method of Drew (1992), being a modification of the method described by Schwyn and Neilands (1987).

4.2.11.2   Inoculation of CAS agar plates

Individual colonies from overnight cultures of *V. harveyi* strains on PYSS agar were transferred onto a CAS agar plate using a sterile wooden toothpick. The plates were divided into halves. In each half plate, at three well-separated positions, the agar was inoculated with a *V. harveyi* strain. The plates were sealed with parafilm and incubated at 28°C for two days before results were assessed.

4.2.11.3　　　Detection of siderophore production

Both the diameter of the halo around each colony and the colony diameter were measured. The difference between the halo and colony diameter was used to quantify the relative activity (mm) of siderophore production *V. harveyi* on CAS agar. Siderophore production was considered positive when the halo diameter divided by the colony diameter exceeded 1.3 (Amaro *et al.*, 1990).

4.2.12　Chitin degradation assay for the strains infected with the VHPL phage from *Vibrio harveyi* 47666-1

Chitin overlay agar (Appendix 3) was used to demonstrate chitin degradation in the four naïve strains infected with phage from *V. harveyi* 47666-1. Individual strains were plated onto PYSS agar plates using isolation streaks and were incubated overnight (section 3.2). Individual colonies were transferred from PYSS agar plates onto chitin overlay agar plates using sterile toothpicks and were incubated overnight. Both the colony diameter and the area of clearing were measured in mm.

4.2.13　Challenge of larvae of *P. monodon*

The modified method used by Muir (1991), Harris and Owens (1999) and Munro *et al.* (2003) was performed in bath challenge experiments with nauplii of *P. monodon*. The seawater where the nauplii came from in the prawn hatchery was used instead of autoclaved seawater. Nauplii were challenged with 10^6- 10^7 CFUml^{-1} of bacteria per flask of 15 larvae in 150 ml seawater. These were replicated five times. An equivalent volume of PYSS broth was used as a broth control. The larvae were then assessed for survival after 48 hours from initial challenge.

4.2.14　Statistical analysis

All data were checked for normality via a Q-Q plot. Haemolysin and chitin assays were not normal, therefore a $\log_{10}$ transformation was conducted. Once the data

was normalised, analysis between groups was performed by One-way Analysis of Variance (ANOVA) and comparison of individual means was performed using Least Significance Difference (LSD) multiple comparison using the computer program SPSS^{TM} version 11.

4.3 Results

4.3.1 Phage infection to naïve strains of *V. harveyi*

The experiments using optical density (OD) indicated that out of 29 naïve strains (Oakey and Owens, 2000) challenged with bacteriophage from *V. harveyi* strain 47666-1, only 3 strains (strain 643, 30 and 12) could be infected.

Table 4.1 Results of analyses showing strains of test bacteria which have no prophage (Oakey and Owens, 2000) and strains which could be infected with the phage from *Vibrio harveyi* 47666-1 using optical density (OD) as an indicator.

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The induction of mitomycin C in *V. harveyi* strain 47666-1 (Fig. 4.1) caused a decreased in OD values as noted from about two hours after induction and onwards, indicating lysis.
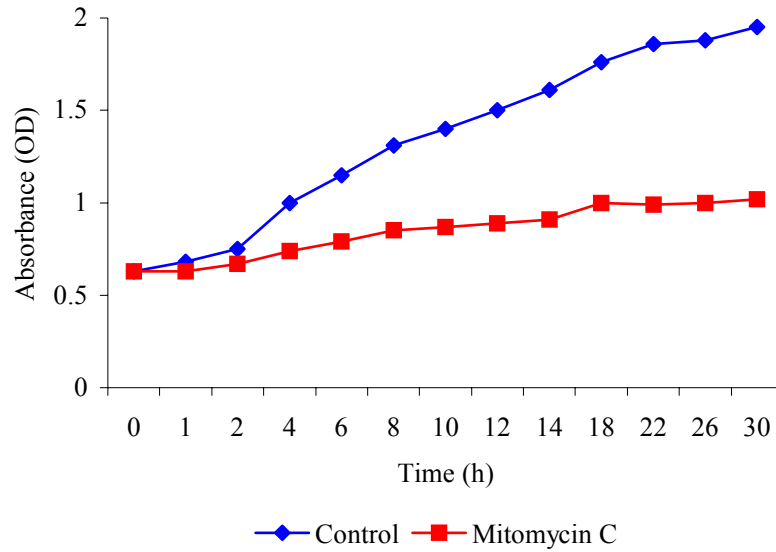
Figure 4.1. Optical density changes of *V. harveyi* strain 47666-1 over time after induction with mitomycin C.

Naïve strains 643, 30 and 12 were used in the virulence assay as strains able to be infected with the phage from *V. harveyi* 47666-1 as shown by a decline in OD readings a few hours after induction with mitomycin C. Naïve strain *V. harveyi* 20 was used as a representative of negative strains as it did not show a decline in OD value indicating no infection with the phage. The growth profiles of strains uninfected and infected with the bacteriophage, VHPL, are shown in Graphs 4.2a-4.2d. These growth curves showed the differences of OD values among bacteria with no stressor, bacteria with stressor (mitomycin C) and bacteria with the stressor and bacteriophage. The pronounced decreased in growth curve indicated lysis of bacterial cells due to the stressor and phage. The cultures receiving mitomycin C but no bacteriophage did not show cell death in any of the naïve strains tested which indicated that they harboured no prophage.
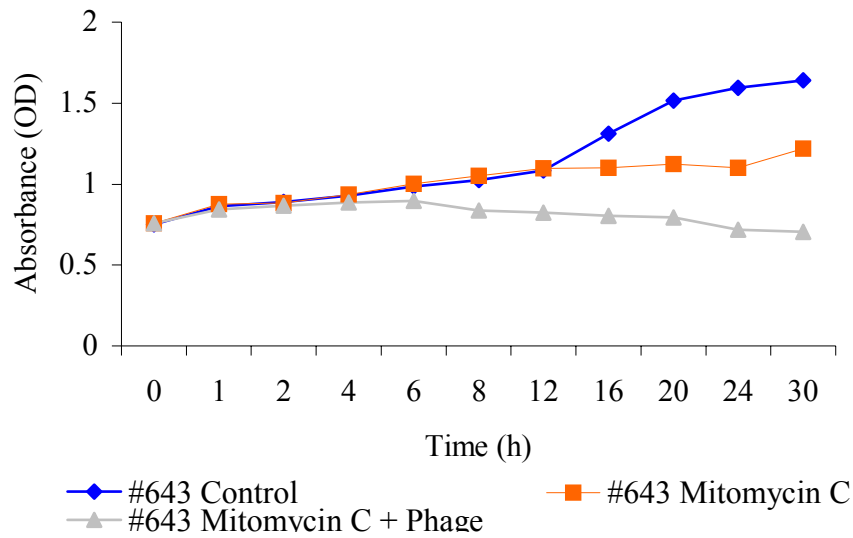
Figure 4.2a. Optical density changes of *V. harveyi* strain 643, infected and uninfected with phage VHPL from *V. harveyi* strain 47666-1 over time after induction with mitomycin C.
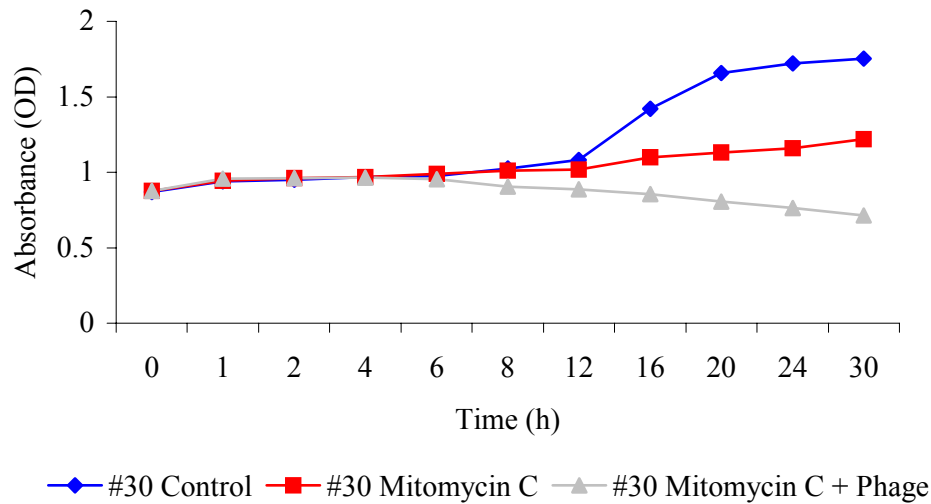
Figure 4.2b. Optical density changes of *V. harveyi* strain 30, infected and uninfected with phage VHPL from *V. harveyi* strain 47666-1 over time after induction with mitomycin C.
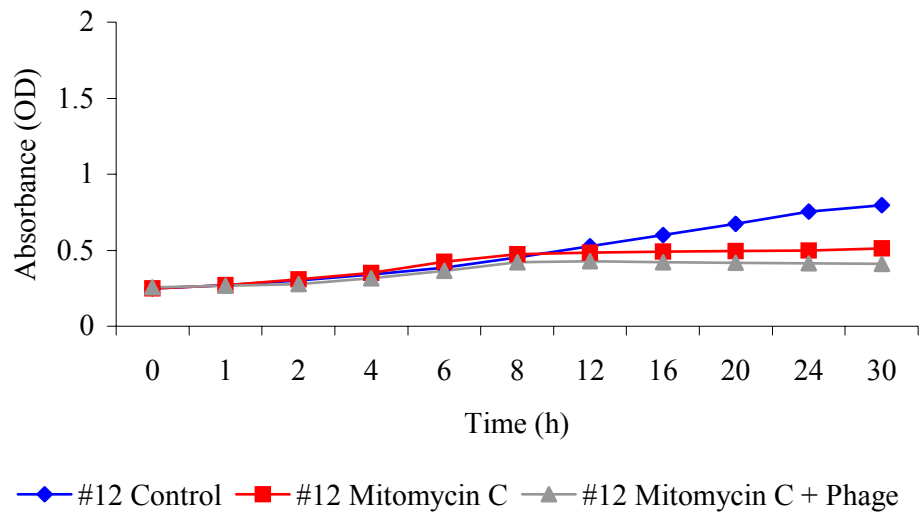
Figure 4.2c. Optical density changes of *V. harveyi* strain 12, infected and uninfected with phage VHPL from *V. harveyi* strain 47666-1 over time after induction with mitomycin C.
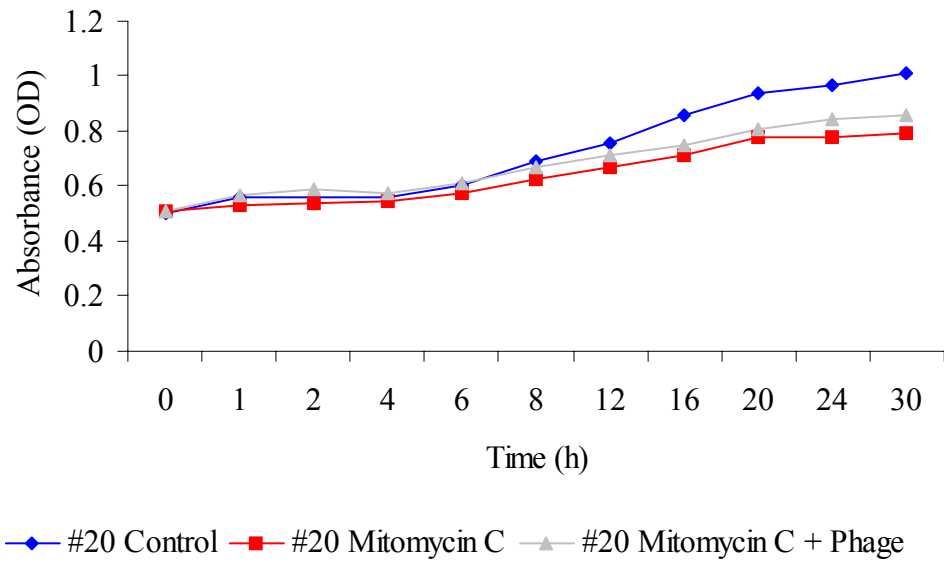
Figure 4.2d. Optical density changes of *V. harveyi* strain 20 infected and uninfected with phage VHPL from *V. harveyi* strain 47666-1 over time after induction with mitomycin C.

4.3.2 Detection of bacteriophage infection in previously naïve host by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Generally, the presence of bacteriophage caused an up-regulation of certain extracellular proteins of bacterial cultures (Fig. 4.3a-4.3b).
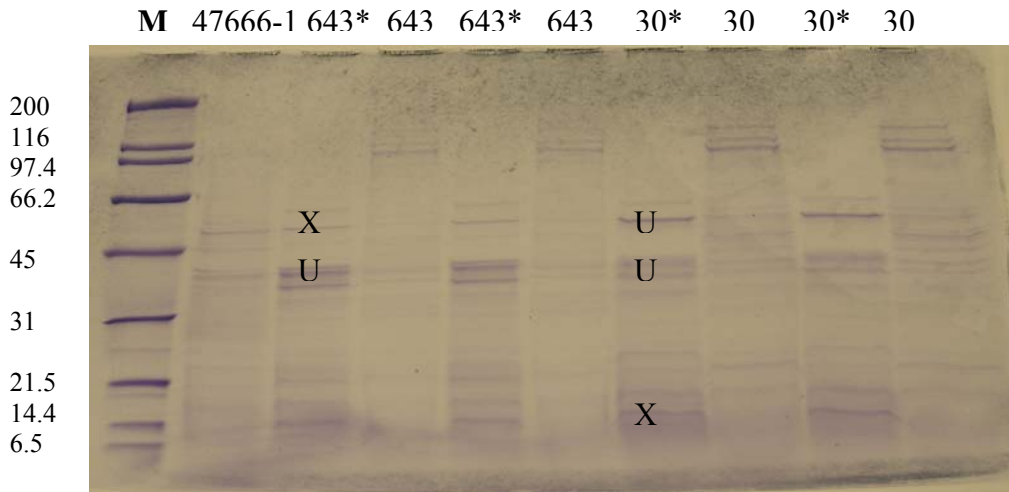
Figure 4.3a. Protein profile of *V. harveyi* strain 643 and 30 (SDS-PAGE 12%, Coomassie blue-stained) showing cell free supernatant extract (CFSE).
Note: (*) bacteriophage infected strain, (X) extra protein band and (U) up-regulation of protein band.
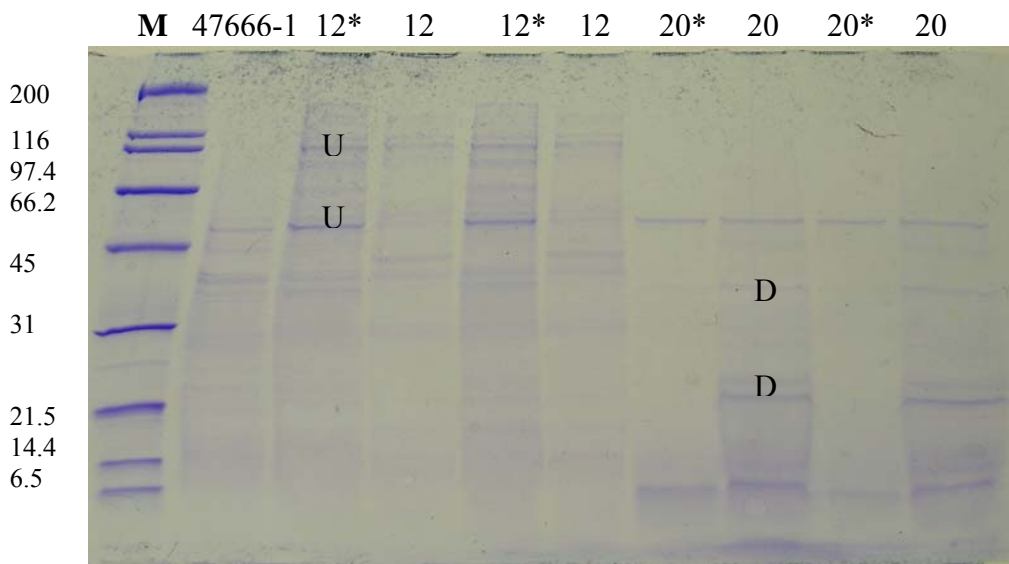
Figure 4.3b. Protein profile of *V. harveyi* strain 12 and 20 (SDS-PAGE 12%, coomassie blue stained) showing cell free supernatant extract (CFSE).
Note: (*) bacteriophage infected strain, (X) extra protein band, (U) up-regulation of protein band and (D) down-regulation of protein band.

These results showed that *V. harveyi* strains 643, 30 and 12 infected with bacteriophage from *V. harveyi* 47666-1 showed an up-regulation of protein with each having acquired a specific protein band of 55kDa from the phage.

Based from these SDS-PAGE gels it was evident that the presence of phage from *V. harveyi* strain 47666-1 caused an increase in band intensity and some additional protein bands in strains 643, 30 and 12 that was not apparent in the same strain without the bacteriophage.

4.3.3 Haemolysin Production Results

The haemolysis production from *V. harveyi* strains 47666-1, 643, 30, 12 and 20 are shown in Figure 4.4a-4.4e.
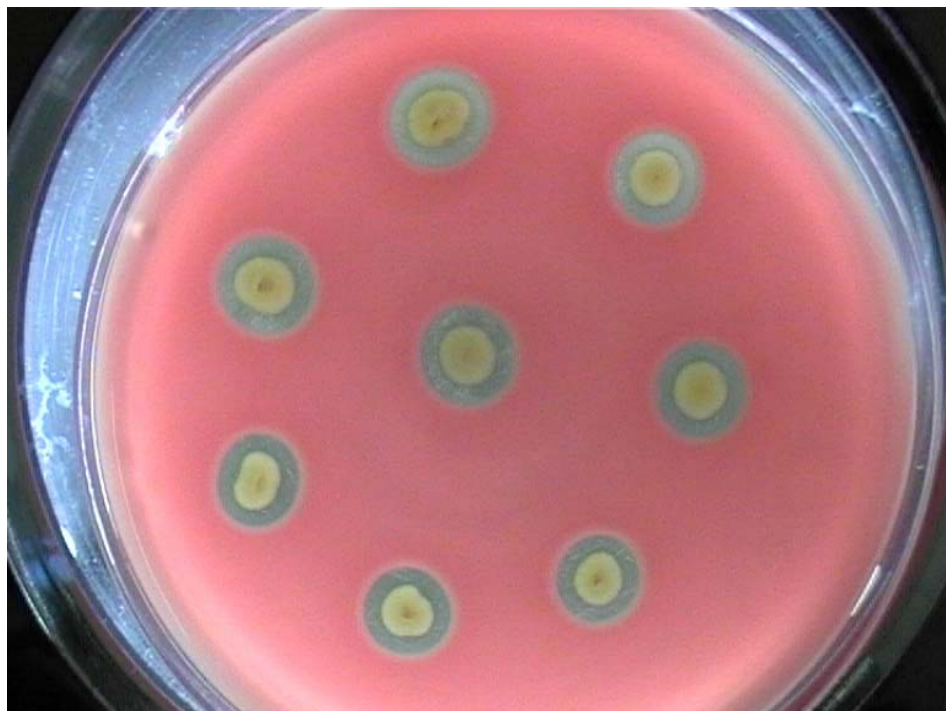
Figure 4.4a. Haemolysis production in *V. harveyi* strain 47666-1 (arrow). This strain is infected with bacteriophage VHPL, so all colonies in this picture have phage.
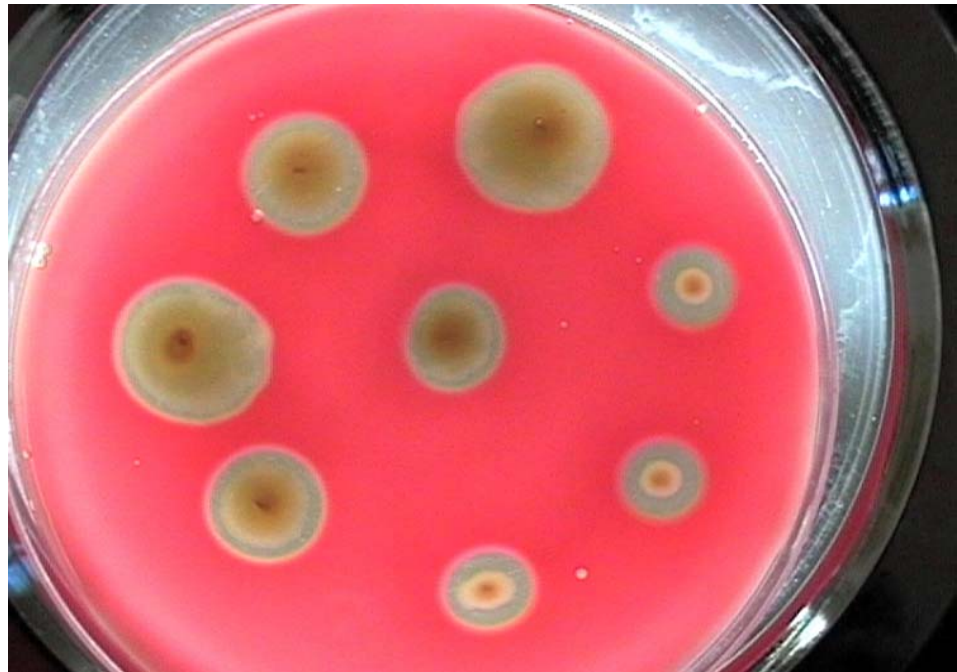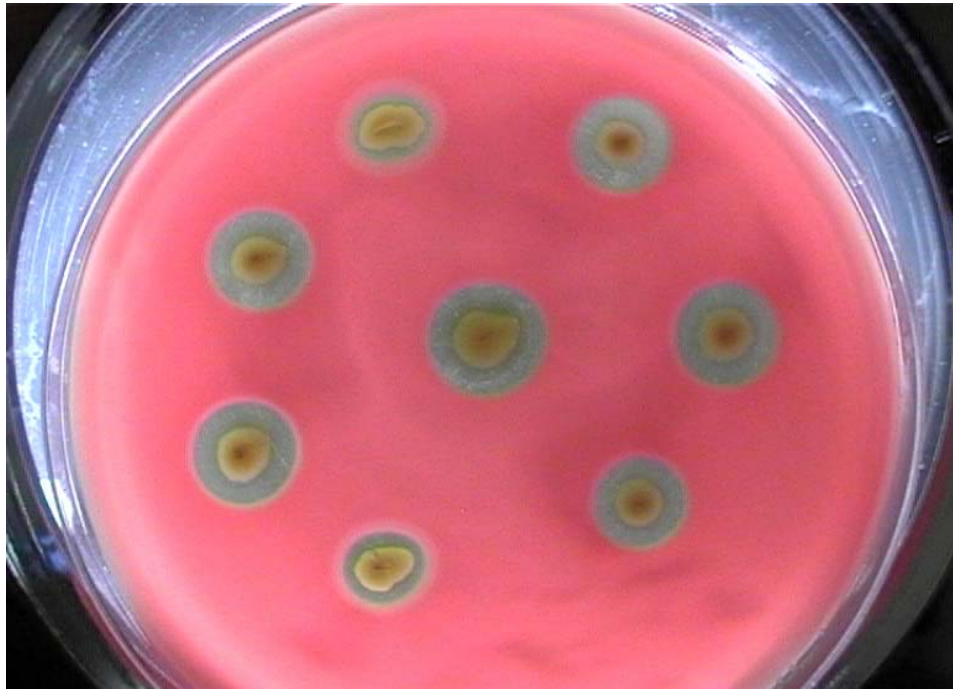
Figure 4.4b. *Vibrio harveyi* strain 643 showing up regulation of haemolysin (arrow) with phage VHPL (top photo) compared to the same strain without the phage (bottom photo).
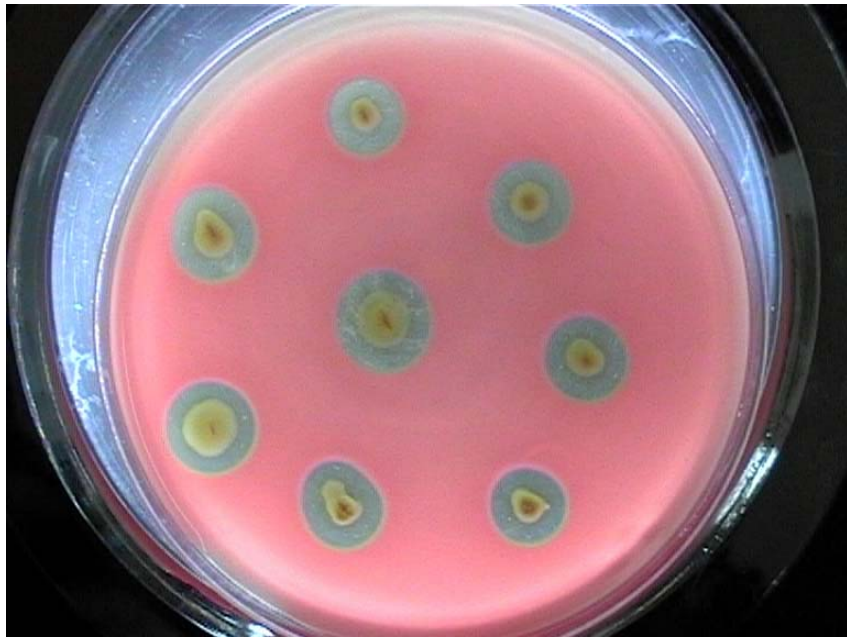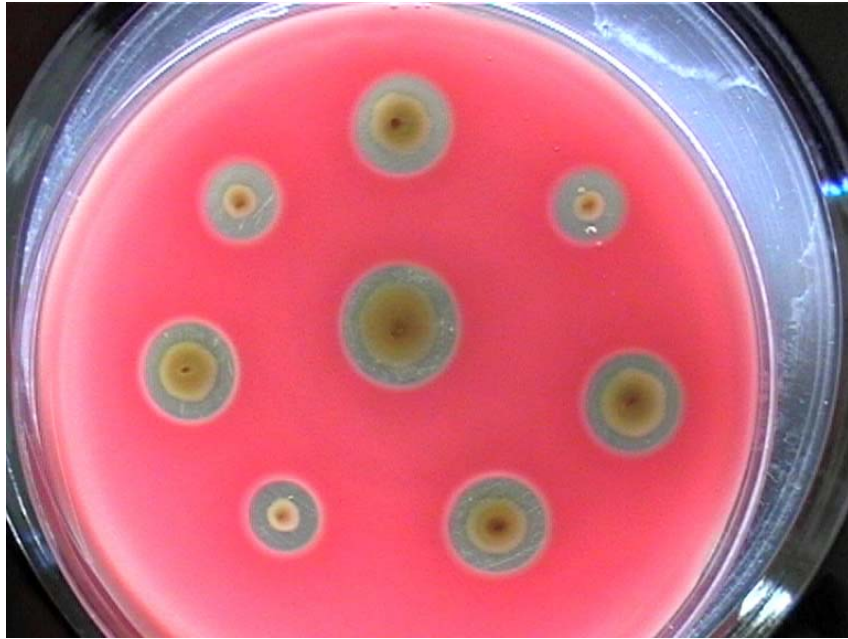
Figure 4.4c. *Vibrio harveyi* strain 30 showing up regulation of haemolysis (arrow) with phage VHPL (top photo) compared to the same strain without the phage VHPL (bottom photo)
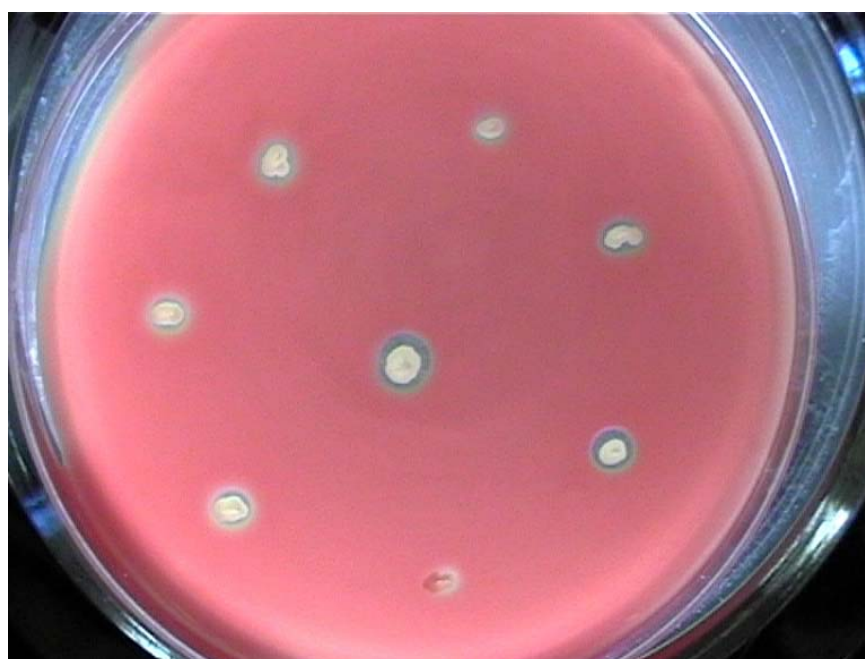
Figure 4.4d. These are the photos of *Vibrio harveyi* strain 12 with the phage (top) and without the phage (bottom). An up regulation of haemolysis is evident in the phage-infected strain compared to uninfected strain (arrow).
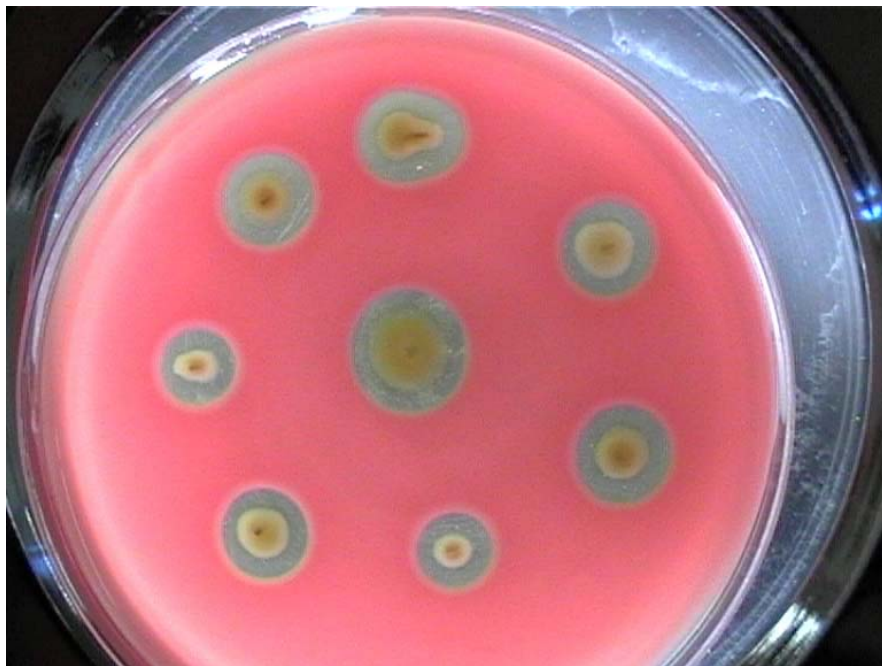
Figure 4.4e. *Vibrio harveyi* strain 20 with phage (top) and without phage (bottom) showing haemolysin production (arrow).

The presence of bacteriophage enhanced the haemolytic activity of bacterial cultures. The mean colony and haemolysin halo diameter of four naive strains of *V. harveyi* infected and uninfected with phage from *V. harveyi* strain 47666-1 are shown in Fig. 4.5.
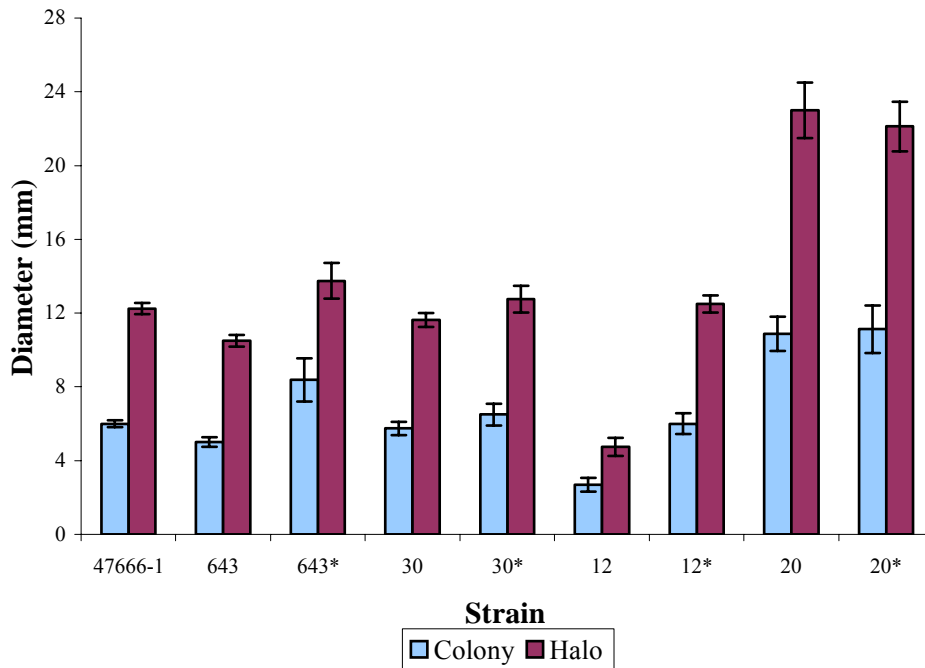
Figure 4.5. Mean colony diameter and haemolysin diameter of the previously stated strains of *V. harveyi* with and without the bacteriophage on sheep blood agar. Note: (*) bacteriophage-infected strain.

Table 4.2. Significant differences (P value) of the log of haemolysin halo diameter from strains infected and uninfected with bacteriophage on sheep blood agar. Note (*) bacteriophage infected strain.

	47666-1	643	643*	30	30*	12	12*	20
47666-1								
643	0.065							
643*	0.231	**0.003**						
30	0.518	0.225	0.068					
30*	0.711	0.028	0.405	**0.311**				
12	0.000	0.000	0.000	0.000	0.000			
12*	0.829	0.040	0.324	0.389	0.877	**0.000**		
20	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
20*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.661**

Table 4.3. Significant differences (P value) of the log of colony diameter from strains infected and uninfected with bacteriophage. Note (*) bacteriophage infected strain on sheep blood agar.

	47666-1	643	643*	30	30*	12	12*	20
47666-1								
643	0.190							
643*	0.069	**0.002**						
30	0.705	0.348	0.030					
30*	0.718	0.096	0.143	**0.461**				
12	0.000	0.000	0.000	0.000	0.000			
12*	0.846	0.263	0.045	0.854	0.579	**0.000**		
20	0.000	0.000	0.033	0.000	0.001	0.000	0.000	
20*	0.000	0.000	0.029	0.000	0.000	0.000	0.000	**0.954**

As a group, the log of haemolysin halo and colony diameter of naïve *V. harveyi* strains infected with phage from *V. harveyi* 47666-1 differed significantly (F=28.84, DF=8,72, P<0.001 and F=20.542, DF=8,72, P<0.001 respectively) from strains without the phage (Table 4.2 and 4.3). In *V. harveyi* strains 643 and 12, a significant difference in terms of log of halo diameter and colony diameter (P<0.001) was observed. However, this was not evident in strain 30 which showed no significant difference in both halo and colony diameter from the same individual strain without the phage. The same observation was seen in *V. harveyi* strain 20 which did not elicit a significant difference (P>0.05) in either measurement. Using Pearson correlations, a correlation exists between an increased colony diameter and an increased halo haemolysin production (Pearson correlation, n = 72, P <0.001).

4.3.4 Siderophore Production Results

Most of the *V. harveyi* strains infected and uninfected with phage VHPL were negative for siderophore production having a mean of less than 1.3 except strain 20 that gave the strongest reaction in CAS agar plates of 1.4 (Table 4.4).

Table 4.4. Siderophore production, measured by halo ratio (HR) values, for *Vibrio harveyi* strains with or without the bacteriophage VHPL calculated after 48h.

Strains of *V. harveyi*	HR values
#47666-1	1.2
#643	1.1
#643 + phage VHPL	1.1
#30	1.1
#30 + phage VHPL	1.1
#12	1.1
#12 + phage VHPL	1.1
#20	1.4
#20 + phage VHPL	1.4

4.3.5 Chitin Degradation Assay Results

The ability of the bacteria to secrete chitinase in chitin-enriched medium was tested with phage infected and naïve *V. harveyi* strains and results are depicted in Fig. 4.6. The levels of secreted chitinase activity in chitin-induced cultures with and without the phage were measured after 3 days of incubation.
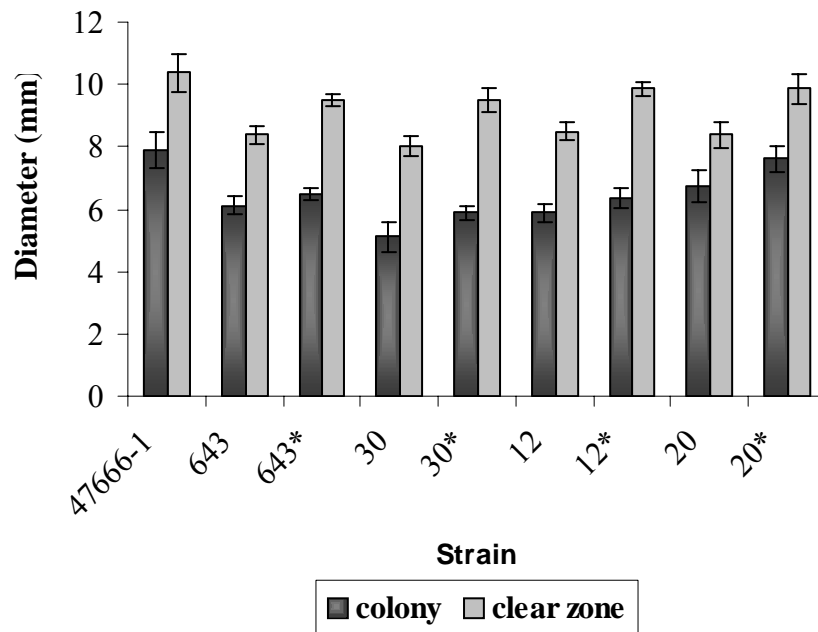
Figure 4.6. Mean colony diameter and clear zone diameter of the previously stated strains of *V. harveyi* with and without the bacteriophage on chitin agar. Note: (*) bacteriophage-infected strain.

Table 4.5. Significant differences (P value) of the log of the clear zone from *V. harveyi* strains infected and uninfected with bacteriophage on chitin agar. Note (*) bacteriophage infected strain.

	47666-1	643	643*	30	30*	12	12*	20
47666-1								
643	0.001							
643*	0.190	**0.029**						
30	0.000	0.400	0.003					
30*	0.168	0.035	0.944	**0.004**				
12	0.002	0.798	0.053	0.274	0.062			
12*	0.513	0.005	0.507	0.000	0.463	**0.010**		
20	0.001	0.924	0.023	0.455	0.028	0.725	0.004	
20*	0.445	0.007	0.580	0.001	0.532	0.014	0.912	**0.005**

Table 4.6. Significant differences (P value) of the log of colony diameter from *V. harveyi* strains infected and uninfected with bacteriophage on chitin agar. Note (*) bacteriophage infected strain.

	47666-1	643	643*	30	30*	12	12*	20
47666-1								
643	0.008							
643*	0.052	**0.460**						
30	0.000	0.026	0.004					
30*	0.002	0.660	0.240	**0.070**				
12	0.002	0.631	0.225	0.076	0.968			
12*	0.027	0.652	0.772	0.008	0.375	**0.354**		
20	0.083	0.339	0.826	0.002	0.165	0.153	0.611	
20*	0.807	0.016	0.087	0.000	0.005	0.004	0.047	**0.135**

From Figure 4.6, it can be seen that as a group, the log of the zone of chitin clearance (mm) from *V. harveyi* strains with the phage was significantly greater (F=5.160, DF=8,63, P<0.001) than the strains of *V. harveyi* without the phage. The clear zone of the naïve *V. harveyi* strains 643, 30, 12 and 20 infected with the phage were significantly different (P<0.05) from the same strains without the phage.

The colony sizes of all strains tested did not show significant difference between phage-infected and uninfected strains (Table 4.6). Whilst the ANOVA on colony size was significant, none of the comparisons of a strain with and without the phage were significant. It was other cross-strain comparisons that were significant e.g. strain 643 vs strain 47666-1. Whilst interesting, this information does not contribute to the analysis of data on the phage and therefore not examined further.

4.3.6 Bath challenge of larvae of *P. monodon* with bacterial strains of 643, 30, 12 and 20 with and without the phage.

The bioassay experiment with nauplii of *Penaeus monodon* was undertaken to determine the lethal effect of bacteriophage VHPL when infected to the four previously known naïve strains of *V. harveyi* (Fig. 4.7 and Table 4.7).
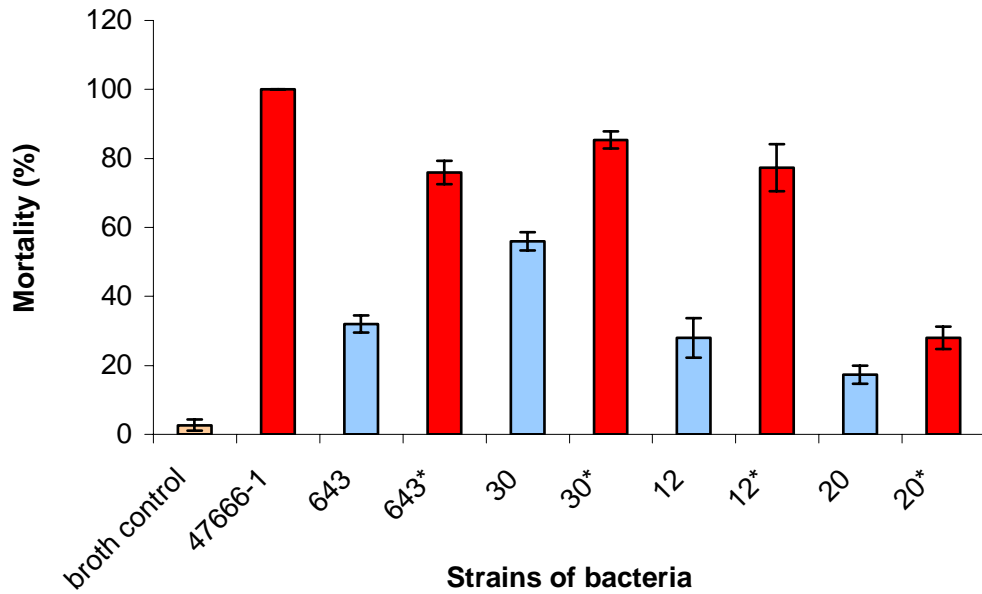
Figure 4.7. Mean mortality and standard error from five replicate flasks of fifteen nauplii of *Penaeus monodon* 48 hours after being bath challenged with strains of *V. harveyi*. Note: (*) bacteria with bacteriophage.

Table 4.7. Significant differences (P value) of mortality in nauplii of *P. monodon* from individual strains compared to other individual strains. Note: (*) bacteria with bacteriophage.

	Broth	47666-1*	643	643*	30	30*	12	12*	20
Broth									
47666-1*	0.000								
643	0.000	0.000							
643*	0.000	0.000	**0.000**						
30	0.000	0.000	0.000	0.000					
30*	0.000	0.007	0.000	0.076	**0.000**				
12	0.000	0.000	0.440	0.000	0.000	0.000			
12*	0.000	0.000	0.000	0.796	0.000	0.127	**0.000**		
20	0.007	0.000	0.007	0.000	0.000	0.000	0.044	0.000	
20*	0.000	0.000	0.440	0.000	0.000	0.000	1.000	0.000	**0.044**

The results indicate that as a group, there was a significant difference in mortality rate among strains infected with bacteriophage from *V. harveyi* 47666-1 (F=82.824, DF=9, 40, P<0.001) than strains of *V. harveyi* without the bacteriophage. It can be gleaned in Fig. 4.7 that *V. harveyi* 47666-1 differed

significantly in terms of mortality rate among other strains under study. Table 4.7 shows that all the bacterial strains under study infected with the phage VHPL showed significant difference in mortality rate as compared to the same strains uninfected with the phage. The presence of phage as compared to uninfected strains contributed to significantly greater mortality in nauplii of *P. monodon* (P<0.001).

4.4 Discussion

Mitomycin C was used to examine inducible bacterial isolates. The decrease in optical density (OD) values after induction with mitomycin C was taken as a presumptive indication of infection by bacteriophage. The results show that 3 out of 29 naïve bacterial strains could be infected with phage extracted from *V. harveyi* 47666-1 and mimic the results of Oakey and Owens (2000) when they infected bacteriophage VHML from *V. harveyi* strain 642 to 36 naïve bacterial strains. They found out that bacteriophage VHML has a narrow range of host that could only infect four of the 36 strains tested. Of note was that the strains of *V. harveyi* involved were mostly different between the two *V. harveyi* bacteriophages. VHML infected 12, 20 45 and 645 whilst VHPL infected 12, 30 and 643. This suggests that the two phages may have different receptor sites as modes of infection. Suttle (2003) cited that cyanopodoviruses (T3-like morphology) typically have a much narrower host range, and that the host range of isolates is typically restricted to very closely related hosts. Strain 20 did not exhibit a decrease in optical density as compared to the three other strains tested. Therefore it was used herein as a representative negative strain unable to be infected with the phage. However, OD readings are only used as a presumptive infection, hence further tests need to be undertaken to confirm bacteriophage infection to naïve strains of *V. harveyi* under study.

A method employing SDS-polyacrylamide gel electrophoresis was applied to detect the presence of extracellular proteins and possible up regulation of proteins from VHPL infection of the four naïve strains of *V. harveyi*. In this study, it was confirmed that the presence of bacteriophage in naïve strains caused these strains to produce additional extracellular proteins compared to the same individual

strains receiving no phage. These results are similar to the findings of Munro *et al.* (2003) with a bacteriophage VHML from a moderately virulent bacterium, *V. harveyi* 642. The infection of VHML caused up-regulation of proteins which was not evident in the same individual strains uninfected with the phage.

Strain 643 infected with the phage exhibited an increase in band intensity at molecular size of 55 kDa, the same toxic sub unit as strain 47666-1 which has been previously identified by Harris and Owens (1999). The same picture of protein up-regulation was seen in phage-infected strain 30 where a band intensity of approximately 55kDa molecular size was observed. This means that the toxic sub-unit from strain 47666-1 had entered and altered the protein synthesis of the tested strains. Strain 12 exhibited up-regulation of all protein bands with the addition of three extra protein bands with molecular weight of approximately 190, 97 and 55 kDa respectively. The lowest protein band was similar to the toxic sub unit band in strain 47666-1. Strain 20 infected with phage showed a down-regulation of protein bands, with the uninfected strain having extra protein bands of molecular masses of approximately 97 and 15 kDa respectively.

Haemolytic activity has been considered as an important virulence property of a pathogen (Liu *et al.*, 1996). This was shown in a study by Zhang and Austin (2000) using pathogenic isolate, *V. harveyi* VIB 645. It produced exotoxin with the highest titre of haemolytic activity towards *Salmo salar* (Atlantic salmon) and *Oncorhynchus mykiss* (rainbow trout) erythrocytes. In our study, infection with the bacteriophage from *V. harveyi* 47666-1 of naïve *V. harveyi* strains resulted in a significant increase (P<0.001) in both halo of haemolysis and colony diameter as compared to the same individual strains uninfected with the phage. Using Pearson correlation, there was an association (P<0.05) between an increase in halo diameter and an increase in colony diameter of the bacteria under study. However, bacteriophage-infected strain 30 did not show any difference in either halo or colony size compared to the uninfected strain. However, although it was suggested that haemolysin might contribute to the pathogenicity of a bacterium (Liu *et al.*, 1996), not all pathogenic bacteria possess this character. A study by Sudhesh and Xu (2001) found that pathogenic *V. parahaemolyticus* to be a poor producer of haemolysin but highly virulent to *P. monodon* with an LD$_{50}$ value of

1 X 10^5 CFU/prawn. Toranzo *et al.* (1983) also demonstrated that haemolysins were able to be produced by both pathogenic and non-pathogenic marine vibrios from striped bass (*Morone saxatilis*) thus, it is not yet possible to determine conclusively what role the haemolysins play in the pathogenesis of vibriosis.

The ability to sequester iron from the host fluids has also been suggested as one of the virulence factors associated in vibriosis (Biosca and Amaro, 1991). This can be achieved by means of iron-sequestering systems such as siderophore production. Production of siderophores in avirulent strains of *V. harveyi* infected with bacteriophage from a virulent strain, *V. harveyi* 47666-1, was investigated using the universal assay of Schwyn and Neilands (1987). The pathogenic strain, *V. harveyi* 47666-1, showed no significant siderophore production in this study which coincides with the results obtained by Harris (1993).   An avirulent strain, *V. harveyi* 20, showed much larger halo ratio (HR) value and hence higher levels of siderophore production. However, there was no siderophore enhancement observed as it produced the same HR value when challenged with the phage. There are possible reasons why siderophore production in strains of *V. harveyi* under study did not elicit significant differences. One might be because most of the avirulent strains used in this study were slow growers in the agar except strain 20 which spread quickly throughout the plate in just few hours of incubation. Schwyn and Neilands (1987) stated that the halo size was dependent on growth conditions where slowly growing cultures produced smaller zones than faster growing ones. Another possible reason is the host group from where the bacteria were isolated. Owens *et al.* (1996) demonstrated that invertebrate isolates produced the lowest level of siderophore activity as compared to vertebrate isolates. In this study, strain 47666-1 and its phage and other strains were from an invertebrate host (seawater isolates, most of them), hence the mechanisms of iron uptake were less pronounced.

The use of chitin as nutrient source is quite widespread among microbes (Gooday, 1990). It has been reported that *V. harveyi* produces chitinase for the degradation of chitin to biologically useful soluble oligosaccharides (Tarsi and Pruzzo, 1999). Further, chitinases probably aid the invasion of the pathogen as well as provide a source of nutrients in the form of amino acids (Gooday, 1990).   The up-

regulation of chitinase production in bacteriophage-infected strains of *V. harveyi* was shown in this study. It was apparent that cultures induced with phage had caused striking increases in chitinase activity as compared to uninduced strains of *V. harveyi*. However, the colony diameter of neither infected nor uninfected strains did not differ significantly. This might be because all the tested strains grow well in this medium thus having uniform colony growth. It is interesting to note that previously naïve strain 20 infected with phage showed up-regulation of chitinase the same as all other three inducible strains.

The virulence of *V. harveyi* to larvae of *P. monodon* has been studied by several authors (Pizzutto and Hirst, 1995; Harris and Owens, 1999; Soto-Rodriguez *et al.*, 2003). Toxic doses of 10^4 cells ml^{-1} of *V. harveyi* 47666-1 have shown to cause 100% mortality after 48 hours of incubation (Harris and Owens, 1999). In this study, we used approximately 10^6 cells ml^{-1} bacterial culture of *V. harveyi* 47666-1 and 100% of the infected larvae died. All of the naïve strains of *V. harveyi* infected with *V. harveyi* 47666-1 phage caused a significantly greater mortality rate than the same individual strains uninfected with the bacteriophage. These results were similar to those of Munro *et al.* (2003) where naïve strains of *V. harveyi* were infected with bacteriophage VHML from *V. harveyi* ACMM 642 and mortality rate was higher than strains uninfected with the phage. It can therefore likewise be suggested that the presence of bacteriophage VHPL causes or at least enhances the pathogenicity of *V. harveyi* 47666-1 to prawn larvae.

In this study, *V. harveyi* strain 20 was chosen as a negative strain unable to be infected with the phage as shown in the mitomycin C assay. However, results from SDS-PAGE, chitinase assay and prawn bioassay suggest that this strain has been infected with the phage VHPL. The result of SDS-PAGE analysis showed that infection with phage might have changed the biochemical activity of this strain resulting to the down-regulation of proteins. Experiments on chitin degradation and the bioassay have demonstrated virulence with high chitinase excretion on chitin overlay agar and high mortality to prawn nauplii when challenged with the phage. This strain might have been lysogenised by phage upon infection but failed to lyse probably due to relatively low dose of mitomycin C used. During the course of the experiment, different doses of mitomycin C were

used according to the methods of different authors: doses of 30 ngml^{-1} (Oakey and Owens 2000) and 50 ngml^{-1} (Munro *et al.* 2003). However, these methods failed to show a decrease in OD values after the 30-hour observation period. The author herein used a 100 ngml^{-1} dose and this lead to a considerable fall in OD in *V. harveyi* strains 12, 30 and 643 but not in strain 20; hence it was presumed that strain 20 was uninfected with the phage. Different types of bacteriophage may respond variably to inducing agent such that some phages can be induced in lower dose e.g 30ngml^{-1} (Oakey and Owens, 2000) and some in higher dose e.g 1µgml^{-1} (Weinbauer and Suttle, 1996). Further work is required if reasons for induction failure or confirmation of VHPL infection of strain 20 are required, but this is not in the scope of this manuscript.

In conclusion, the presence of bacteriophage VHPL from *V. harveyi* 47666-1 probably enhanced the virulence using several assays with four naïve strains of *V. harveyi* as experimental models. There was significant up-regulation of proteins based on SDS-PAGE, up-regulation of haemolysins, chitinases and greater mortality to larvae of *P. monodon*. It is therefore suggested that the presence of this bacteriophage may either partly or fully confers virulence to *V. harveyi* strain 47666-1.

CHAPTER 5

PRODUCTION OF MONOCLONAL AND POLYCLONAL ANTIBODIES FROM *Vibrio harveyi* 47666-1

5.1 Introduction

Vibrio harveyi is a marine bacterium responsible for the devastating luminous vibriosis in prawns worldwide. Its virulence had been attributed to the production of exotoxins each having a specific subunit of 45 and 55kDa (Harris and Owens, 1999). The possession of a genetically mobile element such as a bacteriophage was thought to be associated in conveying this virulence to susceptible hosts. Recent studies on bacteriophage transduction to previously naïve strains of bacteria confirmed that phage conferred virulence to *Vibrio harveyi* (Munro *et al.*, 2003). In order to detect the specific toxic subunits in *V. harveyi* strain 47666-1, monoclonal antibodies were produced to determine if experimental phage-infected strains of *V. harveyi* were able to produce the same toxic subunits as strain 47666-1.

The use of monoclonal antibodies (MAbs) is a valuable tool in testing the presence of specific antigens and studying cross-reactivity among antigens. These antibodies are produced by the fusion of B cells from the immunised animal with myeloma cells (Kohler and Milstein, 1975). The result of this cell fusion is a hybridoma, which, under an appropriate growth conditions, will produce two desired qualities: the ability to grow continually, and the ability to produce large amounts of pure antibody. To produce the desired monoclones, the cells must be grown in either two ways: by injection into the peritoneal cavity of a suitable mouse (mouse ascites method) or by *in vitro* tissue culture (Zola, 1995).

Polyclonal antibodies (PAbs), on the other hand, are derived from preparation containing many kinds of cells. This conventional method involves the immunisation of a laboratory animal with an antigen and then, after antibodies

have been produced, these are collected from the blood serum. They are less desirable than MAbs because of a very small amount of usable antibody they can provide. However, because a polyclonal preparation will contain multiple antibodies reacting to different antigens, they invariably give a stronger reaction than monoclonal antibody (Zola, 1995).

The production of monoclonal antibodies for the detection and identification of antigens, diagnosis and treatment of diseases such as in cancer and infectious diseases had been widely practised. For example, the use of monoclonal antibodies for rapid detection and identification of *Listeria monocytogenes* in foods (Erdenlig *et al.*, 1999), development of MAbs that identify *Vibrio* species from infections of humans, fish and shellfish (Chen *et al.*, 1992), development of MAbs for specific detection of human enteric adenoviruses (Singh-naz and Naz, 1986), the production and characterisation of MAbs to salmon pancreatic disease virus (Todd *et al.*, 2001) and the production of MAbs and PAbs for detection of specific subunits in *V. harveyi* 642 (Munro *et al.*, 2003). This phase of the study was to produce monoclonal and polyclonal antibodies from phage extract of *V. harveyi* 47666-1. The PAbs raised against this extract was used to test the presence of these antigens in previously naïve strains of *V. harveyi* infected with the phage. The MAbs were used in detecting specific toxic subunits from *V. harveyi* 47666-1 and in determining if the previously naïve strains of *V. harveyi* challenged with the phage VHPL would produce the same toxic subunits as *V. harveyi* strain 47666-1.

5.2 Materials and Methods

5.2.1 Identifying toxic protein sub-units from *V. harveyi* strain 47666-1

The toxic protein subunits from cell-free supernatant extract (CFSE) from *V. harveyi* strain 47666-1 were identified in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The molecular weight of each subunit is known to be 55 and 45 kDa respectively (Harris and Owens, 1999). Each toxic sub-unit was identified through the use of broad range standard marker.

5.2.2 Obtaining and isolating toxic protein sub-units from *V. harveyi* strain 47666-1

Each specific toxin band was identified by running CFSE through SDS-PAGE. A two-well comb gel was used to electrophorese the proteins. In a small well, five microlitres of pre-stained broad range SDS-PAGE markers were placed while 400µl of concentrated CFSE was placed in the large well. After the running dye had run off the bottom of the gel, the gel was removed from the glass plates. The marker lane was used to determine the size of each toxic protein bands.   The gel with the corresponding bands was then removed with a gel cutter and was stored at 4 ^{0}C. This gel served as the antigen for polyclonal and monoclonal antibody production.

5.2.3 Animal ethics approval

All experimentation on animals was performed under ethics approval number A780-02.

5.2.4 Production of polyclonal antibodies (PAbs) in chickens

5.2.4.1   Immunisation of chickens

Two semi-adult chickens were vaccinated with a multiple emulsion of antigen (section 5.2.2) mixed with PBS. A multiple emulsion was made by homogenising the antigen in 1:1 ratio with PBS using a 25-gauge syringe. When completely homogenised, one ml of the mixture was injected into the breast muscle of the chicken.

On day 14 and 28 after the initial injection, a booster dose was administered using the same technique as the original injection. On day 7 after the booster, three millilitres of blood was taken from the chicken via the wing vein. The blood was allowed to clot at room temperature. The serum was then removed and centrifuged at 1500 x *g* for 10 minutes. Titres were monitored using indirect ELISA (section 5.2.4.2) and specificity monitored via western blotting. On day 8, a further ten ml. of blood was removed from each chicken via the wing vein. The

blood was then processed as before. The serum was subsequently stored in aliquots of 200 µl at –20 ºC.

5.2.4.2   ELISA

5.2.4.2.1   Preparation of CFSE antigen

The concentrated cell-free supernatant from lysed naïve and experimentally phage-infected strains of *V. harveyi* were produced using a similar process as discussed in Chapter 4.

5.2.4.2.2   Optimisation

Different antigen concentrations and serum dilutions were evaluated by checkerboard titration to determine the optimal conditions for the assay procedure. The optimal dilution was defined as the reciprocal of the highest dilution that gave 0.5 absorbance in one hour. The antigen was serially diluted across the plate with steps of 1 in 2; 1 in 4; 1 in 8; 1 in 16; 1 in 32; 1 in 64; 1 in 128; 1 in 256; 1 in 512; 1 in 1024; 1 in 2048 and 1 in 4096. The antigen in the ELISA wells was in volumes of 100 µl. The concentrated CFSE (antigen) was allowed to bind to the plate overnight at 37ºC. The plates were then blocked for 1 hr with post-coating buffer (TropBio Pty., Townsville, Queensland, Australia) prior to adding the PAbs. The PAbs were serially diluted down the plate starting at a 1 in 2 dilution and finishing at 1 in 1024 with steps of 1 in 2; 1 in 4; 1 in 8; 1 in 16; 1 in 32; 1 in 64, 1 in 128; 1 in 512 and 1 in 1024. The PAbs were also in aliquots of 100 µl. Dual absorbances (at 414 and 492 nm) were read using the ELISA plate reader (Genesis Version 3.00, Life Sciences, United Kingdom).

Dilutions of concentrated CFSE

→

Dilutions of
antiserum

↓
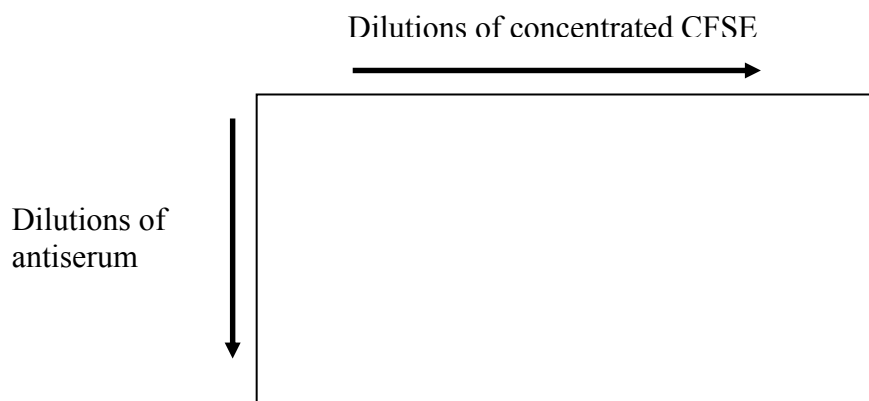
Figure 5.1. The checkerboard system used to determine the optimum coating concentration for concentrated CFSE, with consideration for antiserum concentration.

5.2.4.2.3   General procedure

All buffers for ELISA, including coating, post-coating, wash and diluting buffer, were obtained from Tropbio (Tropbio Pty., Townsville, Queensland, Australia). A 1:512 dilution of pure CFSE and carbonate coating buffer were dispensed in an IWAKI plastic microtitre plate (Crown Scientific, Australia) at 100 µl well^{-1}. The coated plates were incubated overnight at 37ºC and washed three times with PBS the following day. After this, 150 µl of post-coating buffer was added in each well and incubated for 1 h at room temperature. Post-coating buffer was flicked from the wells, and rinsed with wash buffer three times. This was then followed by adding 100 µl of the PAbs (1:256) or MAbs (neat supertanant) to the ELISA wells and further incubated for 1 h. The wells were then rinsed thrice with ELISA wash buffer. Rabbit anti-chicken IgG (H+L) – horse radish peroxidase (HRPO) conjugate (secondary antibody) (TropBio, Townsville) or goat anti-mouse HRPO-conjugated antibody was then added to each well (100 µl) at a dilution of 1:120 using ELISA diluent buffer and incubated for 1 h at room temperature. Excess solution was removed and the wells were rinsed three times with ELISA wash buffer. A 100 µl of the colorimetric substrate 2,2'-azino-di-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) was then added.   Plates were read after an hour of incubation at dual absorbance (412 and 492 nm). Each serum sample was tested in triplicate.

5.2.5 Production of monoclonal antibodies (MAbs) in Balb/c mice

5.2.5.1 Immunisation schedule

Six male BALB/c mice (8-9 weeks old) were immunised against specific toxic subunits of *V. harveyi* 47666-1. Each mouse was injected intraperitoneally (i.p) with a 300 μl dose containing an emulsion of either 55 or 45 kDa protein subunit from *V. harveyi* strain 47666-1 CFSE and PBS. The mice were reimmunised three more times at 2-week intervals using the same dose and route of administration.

One week after the final injection, tail bleeds were carried out on each mouse. Blood was collected onto filter paper discs (TropBio, Townsville) and each disc was placed into 500 μl PBS. Antibody titres were assessed by indirect enzyme-linked immunosorbent assay (ELISA) (Section 5.2.4.2.2). The immune mouse showing the highest titre was used as a spleen cell donor in hybridoma production. Five days before the cell fusion, the immune mouse was given with the usual 300 μl of the specific protein band, plus 50 μl of undiluted CFSE from *V. harveyi* strain 47666-1 into the tail vein.

5.2.5.2 Culture of myeloma cells

The myeloma cells (Sp2/0) were obtained from culture collection of Microbiology and Immunology, School of Biomedical Science at James Cook University, Townsville, Australia. They were cryopreserved in liquid nitrogen placed in cryotubes. The day prior to fusion, the myeloma cells were cultured in flat-bottomed 25 cm^2 polystyrene flasks in a CSL MDM medium (TropBio, Townsville) and supplemented with 10% Bovine Donor Serum (BDS) (TropBio, Townsville). The cultures were incubated at 37°C in a humid, 5% CO_2-enriched atmosphere. Prior to fusion, the myeloma cells were counted (Section 5.2.5.3) and divided into 75 cm^2 flasks with 50 ml CSL + 20% Foetal Bovine Serum (FBS) (TropBio, Townsville) + 0.5 ml of 1 x oxaloacetate-pyruvate-insulin media supplement (OPI) to provide a monolayer of approximately 80% confluence with 90% viability on the day of the fusion. The number of myeloma cells used was determined by the splenocyte count, to give a ratio of one myeloma cell per ten splenocytes.

5.2.5.3 Cell count

Equal volumes of cell suspension and 0.2% trypan blue solution were mixed, loaded into the counting chamber of the haemocytometer and were examined under a phase contrast optics inverted microscope. Trypan blue is excluded from living cells but stains dead cells blue. The number of viable cells per ml of the medium was determined by counting the unstained cells in the corners of the haemocytometer and applying the following formula:

$$\text{Concentration of viable cells (cells/ml)} = \frac{\text{Number of cells counted}}{\text{Number of corners counted}} \times 2 \times 10\ 000$$

5.2.5.4 Preparation of spleen cells

The spleen cells were prepared using the method of Zola (1995). The immunized mouse was killed by CO_2 inhalation and the spleen was removed aseptically by soaking the carcass in 70% ethanol before opening the peritoneum. Any excess fat was carefully removed. The spleen was then placed in a Petri dish containing approximately 10 ml of CSL MDM media maintained at 37ºC. The dead mouse was removed and the area was resterilised. The spleen cells were purged gently by repeatedly injecting CSL MDM media from the Petri dish into the spleen from a three ml syringe with a 23-gauge needle, taking care not to rupture the outer spleen capsule. Large particles were disrupted by gently pipetting the cells several times. The spleen capsule and other visible lumps were then removed. The cell suspension were then put into a sterile 50 ml centrifuge tube (Falcon) and the Petri dish was washed with approximately 15 ml of CSL MDM at 37ºC and was added to the 50 ml centrifuge tube. One ml of BDS was used to underlay the cell suspension. This was left to stand for about five minutes so any large aggregates could come out of suspension. The cells above the BDS were then transferred into another 50 ml centrifuge tube. The cells were counted in a haemocytometer before centrifuging at 200 x g for five minutes.

5.2.5.5 Fusion protocol

Ten grams of polyethylene glycol (PEG) was autoclaved (15 p.s.i./15 min) and while the PEG was liquid, 14 ml of 15% DMSO in phosphate-buffered saline (PBS) was added (3 ml DMSO to 17 ml PBS). The solution was mixed and was divided into one ml aliquots and stored at –20ºC. Prior to use, it was warmed at 37ºC.

Before fusion, the splenocytes were washed and resuspended in a small volume (approximately 4 ml). Myeloma cells were added at a ratio of spleen cells to myeloma cells of 10:1 into a 50 ml centrifuge tube (Falcon). The cells were then centrifuged at 300 x g for 5 min. After centrifugation, one ml of warm PEG solution was added to the cells and quickly resuspended. The cells were gently mixed for one minute by tapping the centrifuge tube. After one minute, the pellet was then diluted with the 3 ml of warm CSL MDM medium and this was added over a 10-min period drop-wise. During this time the cells were continually mixed by gently tapping the tube. After the 10-min period, another 7 ml of warm medium was added again over a 10-min period. The cells were centrifuged at 200 x g for five min and resuspended in HAT medium (containing100 ml of CSL MDM + 20% FBS + 2% hypoxanthine-aminopterin-thymidine (HAT) + 1% OPI media). The cells were allowed to stand for one hour before finally plating out the cells. The resulting hybridoma solution was transferred into 10, 96-well cell culture plates (Nunc, Denmark) at 100µl well^{-1} and incubated at 37ºC in a humid 5% CO_2 incubator.

On day seven, the wells were examined for colony development, under an inverted phase contrast microscope, and then a further 100 µl of fresh media containing HT (hypoxanthine-thymidine) instead of HAT was added carefully into the wells without disturbing the cells.

5.2.5.6 Screening for antibody production

On day 14, colonies were screened for antibody production to the specific antigen. Screening was carried out by indirect ELISA (Section 5.2.4.2.2). For each

screening, 100 µl of PAbs was first coated in the cell culture plate and incubated overnight at 37ºC. This was followed by rinsing once using TEN buffer (Appendix 4.3) then coated with post-coating buffer (TropBio, Townsville) at 150 µl well^{-1} and incubated for 2 h at room temperature. The plate was then rinsed three times with PBS and dried in 37ºC for 2 h.   A neat supernatant from monoclones under study as the primary antibody was transferred from a well in the cell culture plate into a well in the ELISA plate. The secondary antibody was goat anti-mouse IgG (H + L) – HRP conjugate. Another well without antigen (negative control) was also used to compare with the positive clones. The cell culture plates were topped up with 100 µl of fresh medium per well.

5.2.5.7 Cloning of monoclones

Single colonies that were reactive to the specific antigen were taken out and placed into a 24-well cell culture plate when 80% confluency was reached. The medium used was CSL MDM, 20% FBS, 2% HT. After removing the cells for cloning, the remaining cells in the well were fed with HT medium and then were allowed to proliferate and frozen into liquid nitrogen.

Cloning was started from 24-well plates, with the remaining cells grown up in 25 cm^2 flasks for cryopreservation (Section 5.2.6). The cells in the well were counted and a suspension containing 30 cells ml^{-1} was placed in 10 ml HT medium. This was then plated into 96-well cell, flat-bottomed culture plates at 100µl well^{-1}. After seven days, 100 µl of HT medium was gently added to each well and the supernatant that contained single colonies was screened by indirect ELISA (section 5.2.4.2.2) for production of MAbs. These hybridomas were re-cloned until all wells containing single colonies tested positive against the specific antigen.

5.2.5.8 Isotyping of MAbs

Monoclonal antibody isotyping was carried out on selected hybridoma supernatants using a self-indicating dipstick (Sigma Immunochemicals Immunotype^{TM} kit, Sigma) according to manufacturer's protocol. Briefly, one

isotyping strip was placed in a sterile tube and was then added with 2-3 ml of neat hybridoma supernatant. This was incubated for 30 min before adding the biotinylated secondary antibody. This was then followed by the addition of ExtrAvidin^R-Peroxidase and after which a mixture of substrate buffer, substrate chromogen and 2% H_2O_2 was added. In each step, the strip was washed once with PBS-T-BSA (phosphate buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin) for five min. The assays were carried out at room temperature with shaking in orbital shaker platform. The isotype signal (either immunoglobulin (Ig) G1, G2a, G2b, G3, A and M) appeared approximately 20 min. after the addition of the last mixture. The strip was then removed from the tube and immersed in 0.1 M NaOH for 1-2 minutes, and then rinsed with distilled water.

5.2.5.9   Western blotting

To check the specificity of polyclonal and monoclonal antibodies, the technique of western blotting was performed. Protein samples mixed with SDS-reducing buffer at 1:1 ratio (24µl well^{-1}) were separated on 10% acrylamide pre-cast gels (Gradipore Ltd., Monarch Medical, Stafford City, Qld.) by SDS-PAGE technique (Section 4.2.11). The procedure for wet electrophoretic transfer was adapted from Towbin *et al.* (1979). The gels were transferred into gel cassettes (Bio-Rad Laboratories, Sydney) in direct contact with polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Australia). Care was taken to ensure that all components were completely saturated with transfer buffer and no air bubbles were trapped. The holder was then loaded into the transfer chambers ensuring that the gels were on the cathode side of the holders. A cooling module was inserted into each chamber. The chambers were then filled with transfer buffer and the complete unit was placed in a water bath at a constant 4ºC. The blots were run at 47 V for 3.5 hours. Kaleidoscope pre-stained broad range molecular weight standards (Bio-Rad, Australia) were used to monitor transfer efficiency. Once the membranes (blots) were thoroughly dry, they were incubated at room temperature with either MAbs or PAbs (primary antibody) for 1 hour. The membrane was washed twice for 5 minutes each time using fresh PBS. The secondary antibody, either rabbit anti-chicken IgG (H+L) – HRP conjugate (TropBio, Townsville) or

goat anti-mouse IgG (H+L) – HRP conjugate (TropBio, Townsville) was added and blots were incubated for a further 1 h, followed by washing three times for 5 minutes each time using fresh PBS. Detection of the antigen was visualised using the substrate diaminobenzidine (DAB-fast tablets, Sigma Aldrich) and incubated at room temperature until bands were clear. All the incubation steps were performed with shaking in orbital shaker platform

5.2.6   Cryopreservation of cell lines

5.2.6.1   Freezing cultured cells

Cells that were reactive to specific antigen were cryopreserved for future use. Prior to crypreservation, a confluence of 80% was obtained prior to freezing. Cells were aspirated off the surface of the flask. The cell suspension was centrifuged at 300 x g for 5 minutes. The cell pellet was resuspended in a one ml solution of 70% CSL MDM, 20% BDS and 10% dimethyl sulfoxide (DMSO) if it was from a 25 cm^2 flask or into a 3 ml solution if it was from a 75 cm^2 flask. One ml aliquots were transferred into 1 ml cryopreservation tubes (Nunc, Denmark) and placed in a storage cane which was then inserted into a cardboard sleeve and placed into a
−70ºC freezer for 24 hours. This was then placed into liquid nitrogen for storage.

5.2.6.2   Thawing cells out

Frozen cells were removed from liquid nitrogen and were placed into a 37ºC water bath. The vial was then removed and diluted by the drop wise addition of an equal volume of HT medium. The suspension was allowed to stand for five minutes and then added to an equal volume of HT medium. The suspension was left to stand for a further five minutes before pelleting at 300 x g for 5 min. The cells were then resuspended in one ml of HT medium and transferred to a 25cm^2 cell culture flask with ten ml of 37ºC HT media.

5.3 Results

5.3.1 Optimisation of indirect ELISA
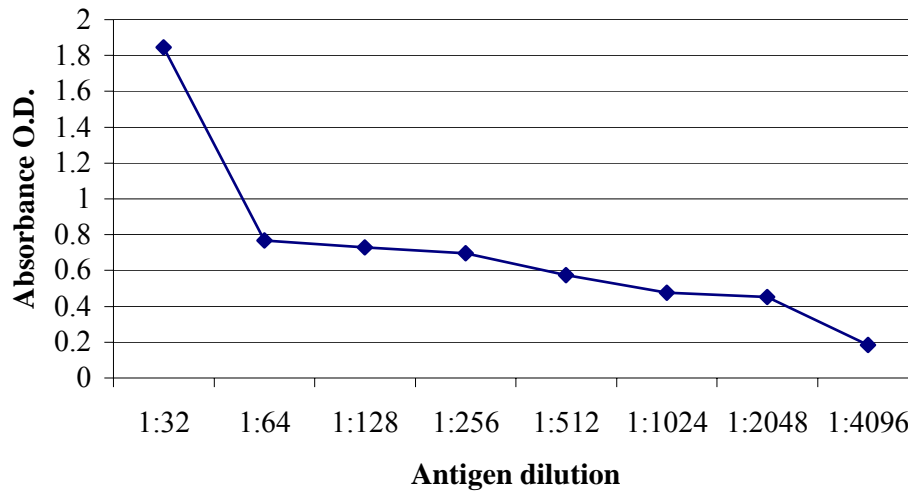
Figure 5.2. The determination of the optimum coating concentration of *V. harveyi* 47666-1 CFSE antigen

The optimal coating concentration of the antigen was determined to be 1:512 (Fig. 5.2). This was the largest dilution that produced an absorbance above 0.5 OD while still maintaining acceptable reading.
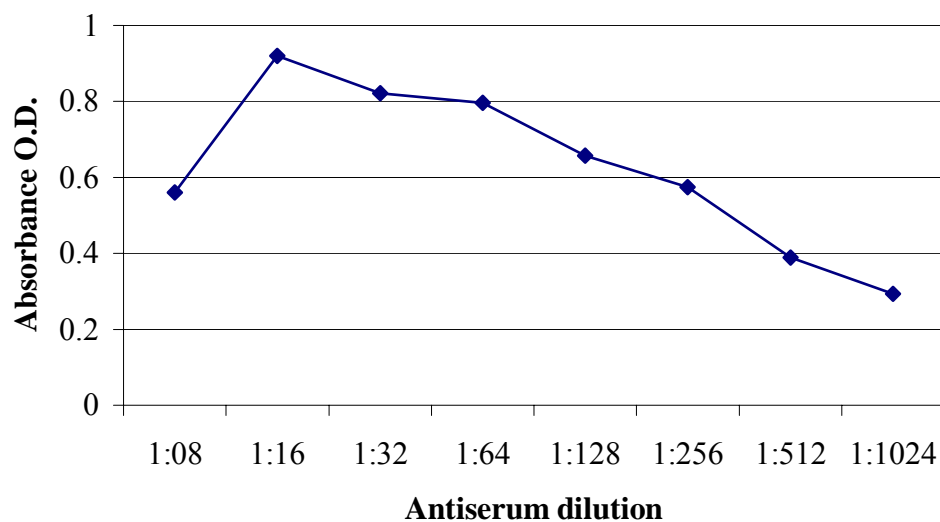
Figure 5.3. The determination of the optimum coating concentration of polyclonal antiserum from chicken against *V. harveyi* CFSE antigen.

The polyclonal antibodies raised in chickens against *V. harveyi* 47666-1 concentrated cell-free supernatant extract resulted in a reasonable titre at 1:256 before showing a more rapid decline in activity (Fig. 5.3). This is the largest dilution that can conserve PAbs while still staying above the absorbance of 0.5.

5.3.2   Polyclonal antibodies from chickens on a western blot

Polyclones raised against emulsified gel containing the toxic protein from *Vibrio harveyi* strain 47666-1 resulted in an increase antibody titre using horse radish peroxidase-labelled conjugate (Fig. 5.4) compared to well without antigen. However, there was no significant difference in antibody titre from phage infected strains compared to uninfected strains subjected to antigen-capture ELISA ($P > 0.05$). Confirmation test by western immunoblot analysis revealed a 55kDa molecular weight toxic protein, a specific toxin sub-unit from *V. harveyi* strain 47666-1. This specific band was also found in *V. harveyi* strains 30, 12 and 20 with and without the phage. No band was seen in *V. harveyi* strain 643 (Fig. 5.5).
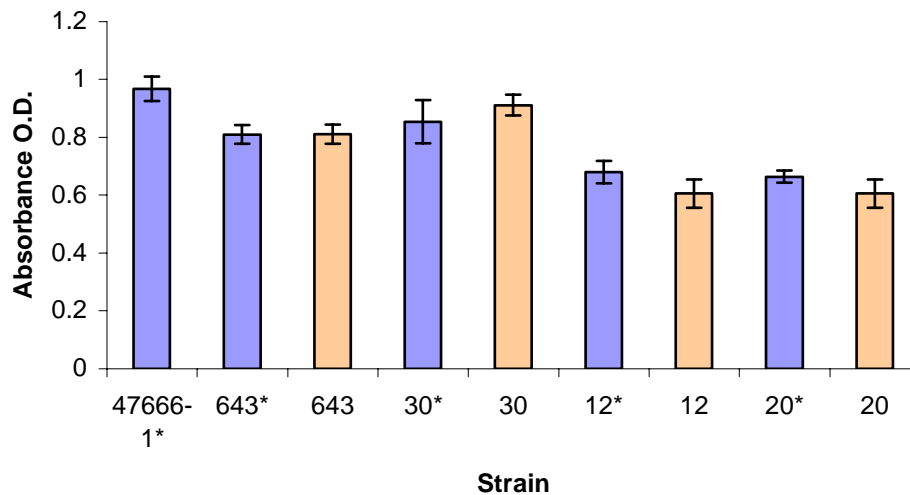
Figure 5.4. Antibody titre of chicken polyclonal antibodies from the two major toxic protein bands of *V. harveyi* 47666-1 reacting to different strains of *V. harveyi.* Note (*) bacteriophage-infected.

20 20* 20 20* 12 12* 12 12* 30 30* 30 30* 643 643* 643 643* 47666-1 M

195
112
59
30
24
13
6
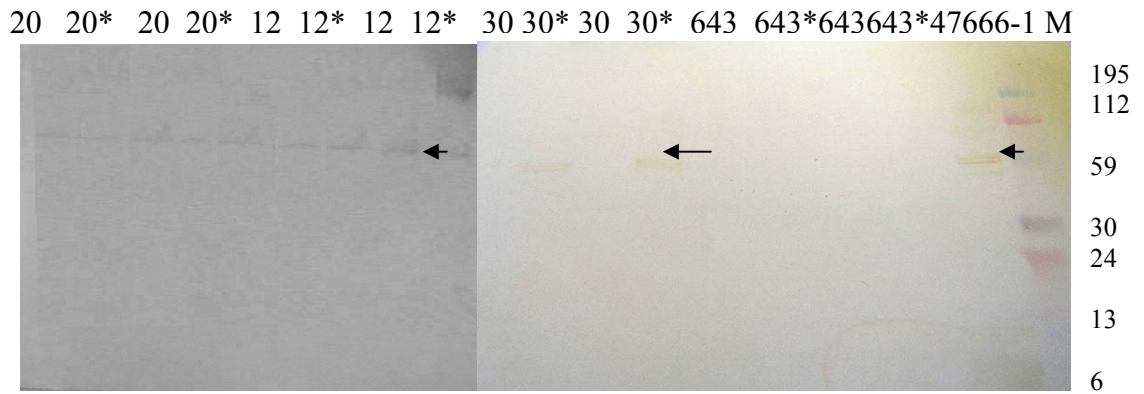
Figure 5.5. Western blot of PAbs responding to *V. harveyi* strain 47666-1 and other strains of *V. harveyi*. Note (*) bacteriophage-infected strain, arrows stand for the specific toxic protein band of 55kDa. Note: strain 643 shows no toxin bands.

5.3.3   Screening for antibody production from monoclones

Two fusions were performed, each for 55kDa and 45kDa molecular weight toxic protein band. The screening of the 55kDa protein band resulted in the production of 48 positive wells that were expanded into 24 well plates. They were tested by ELISA against the negative control (CFSE of naïve, lysed *V. harveyi* strain 643) once the cells had an 80% confluency in the well. The clones that gave the highest response were selected by 9 cycles of limiting dilution on the basis of their ELISA reactivity. This resulted to 4 stable hybridoma colonies designated as 3A7-3, 3E9-3, 3E1-6 and 3A1-9.

For the screening of the 45kDa toxic protein band, a total of 36 positive wells were produced and expanded into 24 wells. The 15 wells that gave the highest response to ELISA were re-cloned and re-screened. After cloning by 9 cycles of limiting dilution, 2 stable hybridoma colonies were produced designated as 5A1-7 and 5A2-1 and were used throughout the experiment.

Results from the indirect ELISA detecting MAbs against the *V. harveyi* 47666-1 antigen is shown in Fig. 5.6.
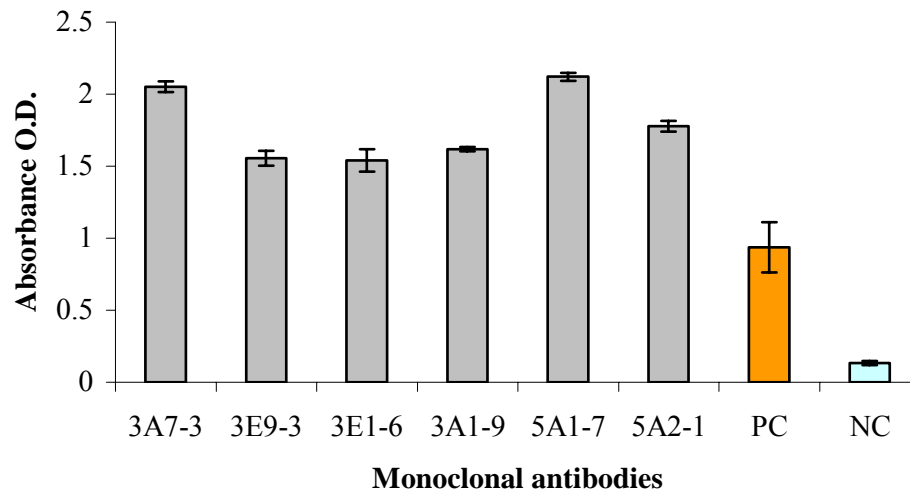
Figure 5.6. MAbs 3A7-3, 3E9-3, 3E1-6 and 3A1-9 are specific to the 55 kDa molecular weight toxic protein band whilst MAbs 5A1-7 and 5A2-1 are specific to 45 kDa toxic protein band. PC stands for the positive control for PAbs and NC for the negative control (naïve strain 643)

It can be gleaned from Figure 5.6 that MAbs 3A7-3 and 5A1-7 had the highest affinity towards the antigens. These were followed by the other four MAbs which also showed considerable affinity against the antigen since they were approximately10 times stronger than the negative control.

5.3.4   Isotyping of monoclonal antibodies

Based on the results of dipstick isotyping kit (Sigma), all MAbs (5A2-1, 5A1-7, 3A1-9, 3A7-3, 3E9-3 and 3E1-6) belong to the IgM sub-class.

5.3.5   Monoclonal antibodies from mice on a western blot

Six hybridoma clones producing antibodies (3A7-3, 3E9-3, 3E1-6, 3A1-9, 5A1-7 and 5A2-1) were used to detect the specific toxic protein subunits. Two of these MAbs (3A1-9 and 5A2-1) reacted with the 55kDa and 45kDa protein bands (Fig. 5.7-5.10). However, both MAbs cross-reacted with those antigens from naïve strains of *V. harveyi*. Other MAbs, 3A7-3, 3E9-3, 5A1-7 and 3E1-6, were not able to recognise these two major toxic protein bands and thus were not further characterised.

Results from western blot analysis using MAb 3A1-9 against *V. harveyi* strain 643 indicate specific toxin subunits having bands of approximately 55kDa and 45kDa molecular weights that were recognised in the infected strain and were absent in the uninfected strain. Further, the CFSE of the uninfected strain has an epitope in the 50kDa molecular weight sub-unit that is missing in VHPL-infected strain (Fig. 5.7).
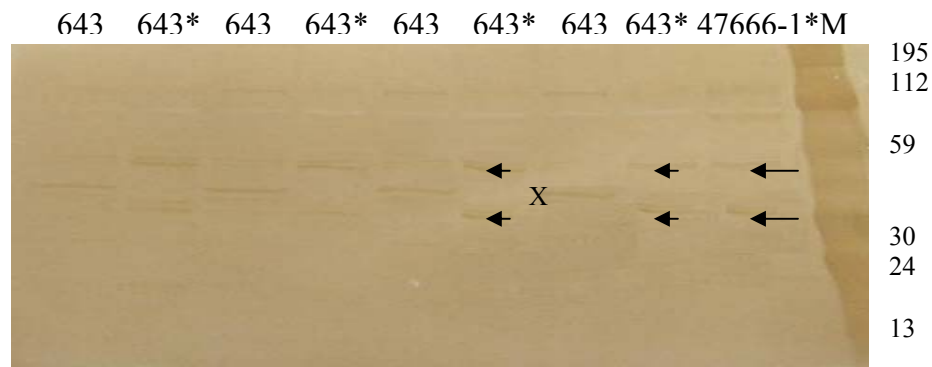
Figure 5.7. Western blot of MAbs from monoclone 3A1-9 responding to *V. harveyi* strain 47666-1 and 643 protein bands. Note (*) bacteriophage-infected strain, arrows stand for the specific toxic protein bands of 55kDa and 45kDa, respectively. X stands for an extra-protein band of 50kDa in uninfected strain 643.

Cross-reactions were noted in both infected and uninfected strains 30 subjected to MAb 5A2-1 (Fig. 5.8). However, phage-infected *V. harveyi* 30 produced a heavy band intensity in 45kDa molecular weight protein compared to uninfected strain. Both the two major protein bands were observed in both infected and uninfected strain 12.

30   30*   30   30*   30   30*   30   30* 47666-1* M

195
112
59
30
24
13

X
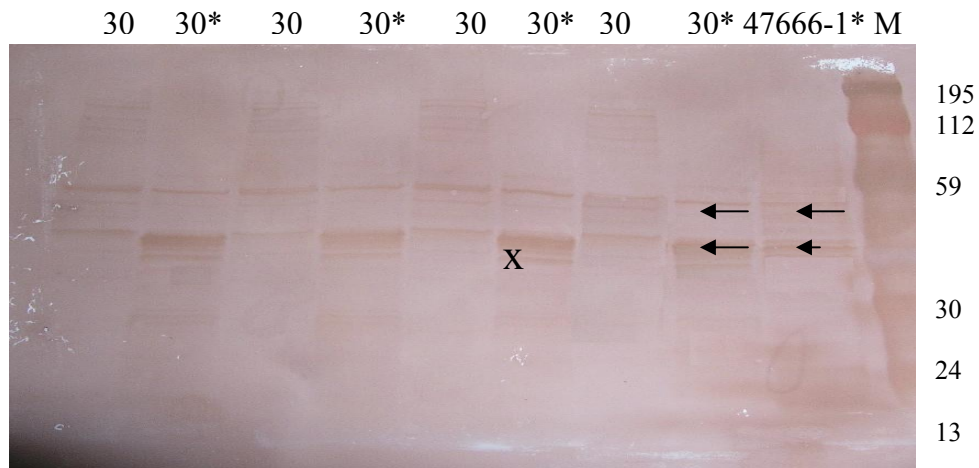
Figure 5.8. Western blot of MAbs from monoclone 5A2-1 responding to strain 47666-1 and 30 protein bands. Note (*) bacteriophage-infected strain, arrows stand for the specific toxic protein bands of 55kDa and 45kDa. X stands for up-regulation of protein band in molecular weight of approximately 45kDa in phage-infected strain.

In *V. harveyi* strain 12 using MAb 3A1-9, only a protein band of approximately 55kDa molecular weight was observed in both phage-challenged and unchallenged strains. However, extra protein bands of approximately 30 and 20kDa were missing in CFSE of phage-infected strain and were present in the uninfected strain 12 and infected strain 47666-1 (Fig. 5.9).

12   12*   12   12*   12   12*   12   12* 47666-1* M

195
112
59
30
24
13
6

X
X

Figure 5.9. Western blot of MAbs from monoclone 3A1-9 responding to strain 47666-1 and 12 protein bands. Note (*) bacteriophage-infected strain, arrows stand for the specific toxic protein band of 55kDa. X stands for an extra-protein band of approximately 30 and 20kDa molecular weight.

Western blot analysis of *V. harveyi* strain 20 using MAb 5A2-1 resulted to specific protein bands of 55 and 45kDa molecular weights (Fig 5.10). However, non-specific bands were also observed in both phage-infected and uninfected strains. A band of approximately 50kDa in naïve strain 20 was missing in phage- infected strain. Cleaving of protein bands of molecular weights of approximately 45kDa and 43kDa was observed in phage-infected strain whilst only one band of 45kDa was seen in the uninfected strain. An up-regulation of protein band of approximately 20kDa molecular weight was likewise seen in phage-infected strain.
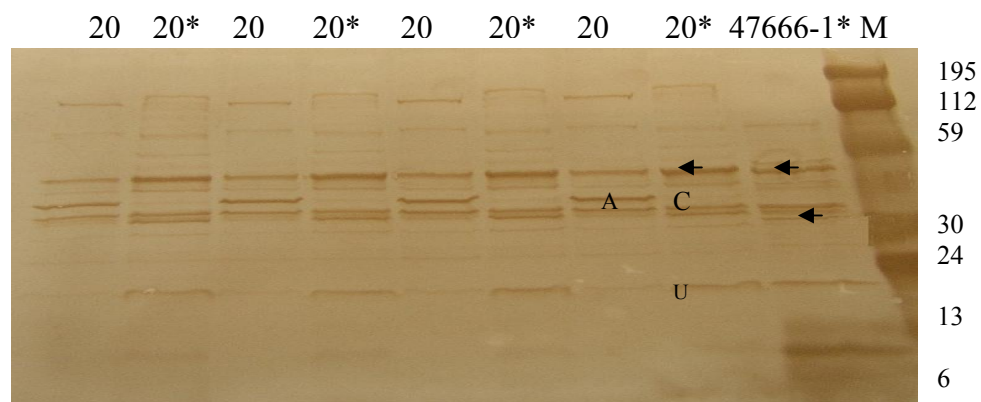
Figure 5.10. Western blot of MAbs from monoclone 5A2-1 responding to strain 47666-1 and 20 protein bands. Note (*) bacteriophage infected strain, arrows stand for the specific toxic protein band of 55kDa, C stands for cleaving of protein bands of molecular weight of approximately 45kDa and 43kDa, A means additional protein band of approximately 50kDa in uninfected strain 20 and U for an up-regulation of protein band of 20kDa in phage-infected strain.

The summary of results of the PAbs and the two strongest MAbs against specific protein bands in *V. harveyi* 47666-1 and the four naïve strains of *V. harveyi* are shown in Table 5.1.

Table 5.1   A summary table showing protein band sizes stained in *V. harveyi* 47666-1, 643, 30, 12 and 20 as recognised by PAbs and MAbs against specific toxin subunits from strain 47666-1. Note (*) bacteriophage infected strains. Molecular protein band sizes are in kiloDaltons (kDa)

	47666-1	643*	643	30*	30	12*	12	20*	20
PAbs	55, 45	-	-	55	-	55	55	55	55
3A1-9	55, 45	55, 45	50	-	-	55	55, 30, 10	-	-
5A2-1	55, 45	55	-	55, 45	55, 45	45, 30	45, 30, 10	55, 45, 43, 20	55, 50, 45

The PAbs and MAbs detected the two major toxic protein bands in *V. harveyi* 47666-1 (Table 5.1). The MAbs from monoclone 3A1-9 detected the two major bands in strain 643 infected with the phage and only one major band in strain 12 infected and uninfected with phage. No bands were observed in both strains 30 and 20. The MAbs from monoclone 5A2-1 detected one major protein band in strain 643 infected with the bacteriophage, the two major protein bands in strain 30 infected and uninfected with phage, protein bands of 45kDa and 30kDa molecular weights in strain 12 infected and uninfected with phage, and both the major protein bands in strain 20.

5.4   Discussion

Virulence in *V. harveyi* has been linked to the possession of a bacteriophage. In Chapter 4, a bacteriophage *Vibrio harveyi* Podovirus-like (VHPL) from *V. harveyi* strain 47666-1 conferred virulence to the four naïve strains through haemolysin assay, chitinase assay and prawn bioassay. The transfer of virulent properties in *V. harveyi* e.g. exotoxins was further analysed by the production of polyclonal and monoclonal antibodies using antigen specific to the toxic protein subunits. The specific toxic subunits, having molecular weights of 55 and 45kDa respectively, were shown as the major protein bands in *Vibrio harveyi* 47666-1 cell free supernatant extract (CFSE) by Harris and Owens (1999).

Polyclonal antibodies (PAbs) raised against emulsified mixture of two major toxic protein bands recognised only one specific subunit having molecular weight of 55kDa. This specific band was found consistently in SDS-PAGE analysis (Chapter 4) in previously naïve strains of *V. harveyi* challenged with the phage. Using PAbs in western blot assay, the same protein band was found in *V. harveyi* strain 30, 12 and 20 infected with phage. However, no band was recognised in strain 643. This finding suggests that the polyclones had low affinity against the antigen from strain 643. This antigen may not always be released in the extracts made with SDS and hence not recognised by PAbs on western blot immunoassay. However based on ELISA, the antibody titre was enhanced in both phage-infected and uninfected strain (Fig. 5.4). There were cross-reactions observed between phage-infected and non-infected strains 12 and 20. In several studies, the use of PAbs to detect antigens and to differentiate bacterial isolates had caused problems such as cross-reactivity (Kerr *et al.*, 2001), polyreactivity and lack of reproducibility (Stemke and Robertson, 1985), non-specific and false-positive reactions (Liu *et al.*, 2002) and weak reactivity (Sithigorngul *et al.*, 2002). Thus, the presence of cross-reacting antigenic determinants in the antigen, and the low affinity and weak sensitivity of the PAbs may explain the lack of significant difference in antibody titre between phage-infected and uninfected strains (Fig. 5.4).

Monoclonal antibodies (MAbs) production had been widely used for the detection and differentiation of different antigens from bacteria. For example, 3 MAbs raised against pathogenic yellowhead virus were used to detect and differentiate some viruses in the yellowhead virus complex (Soowannayan *et al.*, 2003). Likewise, MAbs specific to an envelope protein of 28kDa from white spot syndrome virus (WSSV) was used to differentiate WSSV-infected shrimp from uninfected shrimp (Liu *et al.*, 2002). In this particular study, six monoclonal antibodies were produced from two fusions: four from 55kDa specific toxin sub-unit (3A7-3, 3E9-3, 3E1-6 and 3A1-9) and two from 45kDa molecular weight toxic protein band (5A1-7 and 5A2-1). One of the four MAbs (3A1-9) reacted with the 55kDa and 45kDa protein bands whilst only one of the two MAbs (5A2-1) reacted with the 45kDa protein band and weakly with the 55kDa protein using western blot analysis. Both the reacting MAbs showed cross-reactivity against

CFSE from the naïve bacterial strains. Accordingly, these MAbs were isotyped and all belonged to the IgM sub-class. This may explain the low affinity and heterospecificity of the antigen. Poorly soluble antigens generally result in MAbs of the IgM isotype and often with low affinity (Zola, 1995).

The two MAbs characterised in this study reacted to a number of other protein bands in addition to the two major toxic protein bands. There are possible causes for this to occur. One is the production of polyproteins that is subsequently cleaved by proteases to produce precursor and mature polypeptides. These cleavages may result in the production of various proteins with different molecular weight from the same epitope (Cann, 1997). If the protein is repeatedly cleaved with the epitope attached, numerous protein bands will be recognised to that specific epitope (Cann, 1997). Another possible cause is the glycosylation of proteins. The monosaccharide units in oligosaccharides and polysaccharides can attach to one another at multiple points thus the carbohydrate can carry much more information per weight than either proteins or nucleic acids (Cann, 1997). Glycosylation can cause the production of numerous bands being recognised by the antibody such that some proteins in the solution are slightly heavier than the same protein with no sugar on it resulting to numerous bands with the same epitope with slightly different molecular weights (Cann, 1997).

An up-regulation of protein band as indicated by a heavy band intensity of approximately 45kDa molecular weight was observed in *V. harveyi* strain 30 infected with phage. This might be due to additional extracellular protein cleaved and containing this particular epitope. Zhang *et al.* (2001) described double copies of haemolysin genes in the most pathogenic strain, *V. harveyi* VIB645, whilst only one copy was in the majority of avirulent strains. These toxin genes may be responsible for the intense band in this strain. Cloning of these genes in *E. coli* lead to very high haemolytic activity, a virulence factor largely involved in the pathogenesis of salmonids (Zhang *et al.*, 2001).

A down-regulation of protein band was observed in *V. harveyi* strain 12 where bands having molecular weights of 30kDa and 10kDa, respectively were missing in the infected strain and were present in the uninfected strain. This is similar as

in the case of *V. harveyi* 643 where a band of 50kDa was lacking in the infected strain. This might be due to changes in biochemical activities as a result of phage transduction to the naïve bacteria e.g. this could be cleavage of these products not to be detectable or it may be disruption of the genes due to insertion of prophage into these genes.

It is interesting to note that the uninducible *V. harveyi* strain 20 as observed in mitomycin C assay (Chapter 4) produced pronounced changes in protein profile using western blot assay. A 20kDa protein band as observed in *V. harveyi* 47666-1 was also seen in the infected strain but not in uninfected strain. An extra-protein band of approximately 50kDa was observed in the uninfected strain and was absent in infected strain. These findings suggest that phage had entered the bacterial genome and may have altered structural characteristics as a result of phage transduction. These results further confirmed the results from Chapter 4 such as increased haemolysin production, increased chitinase production and increased mortality in a prawn bioassay.

In conclusion, monoclonal and polyclonal antibodies were able to detect at least one of the two major toxic protein subunits from CFSE of *V. harveyi* strain 47666-1. These antibodies were used to detect these subunits in previously naïve strains of *V. harveyi* challenged with phage VHPL. Western blot analysis using these MAbs identified additional protein bands which were missing in either phage-infected or uninfected strains. It is clear that phage infection had caused changes in biochemical activities and altered structural characteristics in strains of *V. harveyi* which may explain the increased virulence to the larvae of *P. monodon*. Other phages may either introduce new genes to previously naïve bacteria that induce the production of exotoxins or promote the production of an existing toxin (Ruangpan *et al.*, 1999). Further work is needed to determine what virulent genes are encoded for by the phage genome. Gene-based techniques that study virulence genes like polymerase chain reaction (PCR), colorimetric DNA-DNA-hybridisation and multiplex PCR can be valuable in determining these toxin genes.

CHAPTER 6

GENERAL DISCUSSION

Vibrio harveyi is an economically significant bacterial pathogen responsible for luminous vibriosis in prawns and other marine organisms. Virulence within strains of *V. harveyi* was uncommon (Pizzutto and Hirst, 1995). DNA profiling and M13 finger printing cannot differentiate virulent from avirulent strains (Pizzutto and Hirst, 1995). They suggested that the possession of a genetically mobile element such as a bacteriophage might be responsible for its virulence. A number of pathogenic bacteria had been found to possess bacteriophage. For example, β prophage of *Corynebacterium diptheriae* (Freeman, 1951), pathogenic strains of *Shigella* producing shigella toxin (Hale, 1991), *Staphylococcus* enterotoxin A (Betley and Mekalanos, 1985), *Clostridium botulinum* toxins (Johnson *et al.*, 1980), *Pseudomonas aueroginosa* cytotoxins (Hayashi *et al.*, 1990), *Escherichia coli* verotoxins and cytotoxins (Takeda and Murphy, 1979), *Vibrio cholerae* enterotoxin (Waldor and Mekalanos, 1996) and *Mycoplasma arthritidis* bacteriophage (MAV1 (Voelker *et al.*, 1995). Recently, Munro *et al.* (2003) studied a bacteriophage from *V. harveyi* known as *Vibrio harveyi* Myovirus like (VHML) and found that this phage converts non-virulent strains to virulence. They demonstrated it by infecting the bacteriophage to four known naïve strains of *V. harveyi* using various virulence assays such as increased haemolysin production, increased protein excretion by SDS-PAGE and increased mortality in a prawn bioassay.

This study was geared towards identifying if a more virulent strain, *V. harveyi* 47666-1 (Muir, 1991) which is infected by a bacteriophage tentatively identified as *Vibrio harveyi* Podovirus like (VHPL) (Oakey, 2000) can elicit the same changes as the previously studied *V. harveyi* strain 642 (Munro *et al.*, 2003).

To aid in the interpretation of results, a table that summarises all phenotypic changes was produced.

Table 6.1. A summary table showing all phenotypic changes in strains of *V. harveyi* experimentally infected with phage VHPL and uninfected strains.

	4766 6-1	643*	643	30*	30	12*	12	20*	20
Haemolysin assay	+	+	-	-	-	+	-	-	-
Chitinase assay	+	+	-	+	-	+	-	+	-
Siderophore production	-	-	-	-	-	-	-	+	+
Prawn bioassay	+	+	-	+	-	+	-	+	-
Protein excretion by SDS-PAGE	+	+	-	+	-	+	-	-	-
PAbs	55, 45	-	-	55	-	55	55	55	55
MAb3A1-9	55, 45	55,45	50	-	-	55	55,30, 10	-	-
MAb5A1-2	55, 45	55	-	55,45	55,45	45,30	45,30, 10	55,45, 43,20	55,50, 45

+ means increased mortality in a prawn bioassay and increased band intensity in SDS-PAGE.
- means down-regulation in haemolysin assay and chitinase assay, negative for siderophore production, decreased mortality in a prawn bioassay and down-regulation in SDS-PAGE.
* means phage-infected strains
numbered entries refer to protein bands stained and are expressed in kilodaltons (kDa)

From the above experiments, it is clear that phage infection into previously naïve strains 643,30,12 and 20 have resulted to up-regulation of haemolysin, an up-regulation of chitinase, changes in protein profile as revealed in SDS-PAGE, production of extra-proteins as recognised by MAbs and a greater mortality rate to larvae of *P. monodon*. Perhaps, there is a growing trend for bacteriophage in the

marine environment to copy their terrestrial brethren in conveying virulence to bacteria. Studies revealed that infection with bacteriophage had been associated with the virulence of *V. harveyi*. Pasharawipas *et al.* (1998) reported a bacteriophage in *V. harveyi* strain VH1039 from a black tiger prawn (*P. monodon*) showing Tea Brown Gill Syndrome (TBGS). Likewise, Ruangpan *et al.* (1999) described a bacteriophage-mediated toxicity in *V. harveyi* strain VH1039 and suggested that this phage may be responsible for the production of new genes encoding toxin or promotes the production of an existing toxin. Oakey and Owens (2000) isolated a bacteriophage from *V. harveyi* 642 termed as *Vibrio harveyi Myovirus-like* (VHML) and was further studied by Munro *et al.* (2003) by infecting VHML to naïve strains of *V. harveyi*. Their results confirmed that VHML conferred virulence to the four naïve strains of bacteria. Certainly the studies by Ruangpan *et al.* (1998), Munro *et al.* (2003) and this present study support this growing trend.

There are many directions for future research that would be followed from this study. Firstly, a study that is directed against definitive confirmation of phage transduction. This can be achieved by the use of transmission electron microscope (TEM) to identify the structures by which phage attached, the use of polymerase chain reaction (PCR) using appropriate probes to identify phage DNA, and dot blot hybridisation using DNA probes to confirm the integration of prophage DNA into the chromosome of the lysogen. Secondly, a study geared towards identification of putative toxin genes and the extracellular toxins encoded by the bacteriophage VHPL. It can be recalled that the related sequence SLSGDN from CFSE of strain 47666-1 has 83% homology to the sequence SLTGDN from virulent *Salmonella* and *Shigella* species (Harris and Owens, 1999). In a recent study with VHML phage, Oakey *et al.* (2002) found a complete N6-adenine methyltransferase (Dam) gene encoded by ORF 17 which reportedly associated to the regulation of pathogenicity island in *Salmonella typhimurium*. It could be hypothesised that some putative genes carried on by VHPL phage may have possible link to the putative Dam protein encoded by VHML phage. To determine the relationship between these phages and other known putative toxins, the same procedures developed for VHML phage by Oakey *et al.,* (2002) could be used in

VHPL phage. These include genome nucleotide sequencing, open reading frame analysis and putative gene identification.

This study had shown that the virulence in *V. harveyi* strain 47666-1 is mediated by a bacteriophage VHPL. With the advent of phage therapy as a biological control of aquatic diseases, understanding the nature of the phage-host interaction and of the pathogenesis of infection would be a prerequisite tool to develop techniques to control this disease in prawns and other marine animals.

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APPENDICES

APPENDIX 1

MEDIA FOR BACTERIAL CULTURE

1.1 PYSS broth

Bacteriological peptone	5g
Yeast extract	1g
Synthetic sea salt	33g

Make up with distilled water to 1 litre, and autoclave at 121 ^{0}C for 30 min.

1.2 PYSS agar

Bacteriological peptone	5g
Yeast extract	1g
Synthetic sea salt	33g
Agar No. 1 (Oxid L11)	15g

Make up with distilled water to 1 litre, and autoclave at 121 ^{0}C for 30 min.

1.3 CAMP agar plates (dual layer agar)

Bottom layer

Bacteriological peptone	5g
Yeast extract	1g
Synthetic sea salt	33g
Agar No. 1 (Oxid L11)	15g

Make up with distilled water to 1 litre, and autoclave at 121 ^{0}C for 30 min.

Top layer

Bacteriological peptone	4.75g
Yeast extract	0.95g
Synthetic sea salt	31.35g
Agar No. 1 (Oxid L11)	14.25g

Make up with distilled water to 1 litre, and autoclave at 121 ^{0}C for 30 min.
Then when cooled to 40 ^{0}C, add 50 ml of sheep blood

APPENDIX 2

PREPARATION OF CHROME AZUROL S (CAS) AGAR

1,4-piperazinediethanesulfonic acid	30.24g
50% (w/w) NaOH solution	12g
Agar No. 1 (Oxoid L11)	12g
Distilled water	750ml

Add the above reagents to an acid washed bottle. Adjust to pH 6.8 and autoclave at 121 ^{0}C for 15 min. Allow to cool.

Autoclave the following separately and add when the agar above has cooled to approximately 55 ^{0}C:

*Iron depleted 10% casamino acids solution	
(Appendix 3.1)	30ml
1 M MgCl$_2$	0.4ml
CAS Solution (Appendix 3.2)	100ml

Add CAS solution along the wall of the bottle. Mix the solution without frothing. Dispense agar into sterile petri dishes.
* Filter sterilise the iron depleted 10 % casamino acids solution before addition, using a 0.2 μm filter (Sartorius, Germany).

2.1 Preparation of iron depleted 10% casamino acids solution

2.1.1 The extraction of iron

One volume of a 10% (w/v) casamino acids (Difco Laboratories, Detroit, USA) solution in distilled water, was shaken overnight, on a rotary shaker with two volumes of chloroform containing 3% (w/v) 8-hydroxyquinoline (Sigma, St. Louis, USA). After the overnight extraction, the two immiscible layers were separated using a separating funnel. Two volumes of a fresh 3% (w/v) 8-hydroxyquinoline/chloroform solution were added to the aqueous phase and shaken on a rotary shaker for a further four hours. The phases were then separated using a separating funnel.

2.1.2 Removal of 8-hydroxyquinoline from the aqueous phase

Residual 8-hydroxyquinoline in the aqueous, casamino acids phase was extracted by repeated shaking with equal volumes of chloroform in a separating funnel. The progression of this extraction was monitored by the UV adsorption at 244 nm of the chloroform phase after each extraction. The extraction was deemed complete when the adsorption of the chloroform phase was either the same as that of pure chloroform or the adsorption at 244 nm remained constant for several chloroform volumes.

2.1.3 Removal of chloroform from the aqueous phase

Iron-depleted, 8-hydroxyquinoline-free, 10% casamino acids solution was transferred to an acid washed glass bottle and the remaining chloroform removed by heating at 60 ^{0}C in a water bath. Absence of chloroform was determined by smell.

2.1.4 Removal of particulate matter from the aqueous phase

Treatment of 10% casamino acids solution with chloroform led to the formation of a precipitate. This was removed by filtration through a 0.45 μm Minisart NML filter (Sartorius, Goettingen, Germany).

2.2 Chrome azurol S (CAS) solution

Chrome azurol S	60.5mg
Distilled water	50ml
FeCl$_3$ solution (Appendix 2.3.1)	10ml

Add slowly, with stirring, to a solution containing:

Hexadecyltrimethylammoniumbromide	72.9mg
Distilled water	40ml

Sterilise by autoclaving.

2.3 FeCl$_3$ solution, 1 mM

Anhydrous FeCl$_3$	0.1624g
10 M HCl	1ml

Make up to 1 litre with distilled water. Filter sterilise using a 0.2 μm filter (Sartorius, Goettingen, Germany).

APPENDIX 3

PREPARATION OF CHITIN OVERLAY AGAR

Chitin (Practical grade from crab shells) powder	50g
Chilled stirring acid solution	1050ml
Distilled water	400ml
Ice cold distilled water	2500ml

A 50 g of chitin powder was added to 400 ml of distilled water to form chitin slurry. Approximately 450 ml of the chitin slurry was added to a chilled stirring acid solution (Appendix 4.1). The mixture was mixed for approximately 30 to 60 minutes in an ice-water bath with constantly stirring until it looked like a brown syrup. The solution was precipitated by adding 2.5 litres of ice-cold distilled water. After a brief period, the chitin appeared as a white fluff of precipitate. The suspension was allowed to settle overnight at 4^0C. Then the clear supernatant was decanted from the chitin solution. The re-precipitate chitin was concentrated by centrifugation at 300 *g* for 5 minutes. The mixture was then neutralised by adding 10 N NaOH. When the chitin mixture has been concentrated to 4 bottles, at least two centrifuge bottle volumes of distilled water were used to remove the excess salt present in the mixture. The resulting chitin was refrigerated until use. For long storage, the chitin was frozen at -70^0C.

3.1 Chilled stirring acid solution

Sulfuric acid, concentrated	525ml
Distilled water	525ml

Concentrated sulfuric acid in 525 ml was diluted with 525 ml of distilled water. Acid was added to water. The solution was chilled in an ice-water bath.

3.2 Chitin Overlay Medium

Chitin (prepared from above)	30g
Agar	15g
Distilled water	1000ml
NaCl	2%

The above medium was autoclaved for 15 min at 121 ^{0}C. A PYSS agar was prepared which served as base layer medium. After the base layer has solidified, it was overlaid with molten chitin medium. Approximately 7 ml of overlay medium was added as a complete, but thin layer over the base medium. Chinolytic bacteria formed a clear zone around the colony.

APPENDIX 4

Buffers and SDS-PAGE reagents

4.1 10X Phosphate buffered saline (PBS)

NaCl	80g
KH_2PO_4	2g
Na_2HPO_4	11.5g

Make up to 1 litre with distilled water and adjust pH to 7.2 then autoclave.

4.2 SM buffer

NaCl	5.8g
$MgSO_4$	2g
Tris (50 ml)	1 $molL^{-1}$ (pH7.5)
Gelatin	100mg
Distilled water	945ml

4.3 10X TEN-Tween 20 buffer

Tris	0.50 M
EDTA	0.01 M
NaCl	1.50 M

Make up to 1 litre with distilled water and adjust pH to 8 then autoclave. To make 1x TEN-Tween 20 buffer, mix 100ml of stock solution to 900ml of distilled water and then tween 20 at 0.5ml.

4.4 SDS-PAGE Stock Solutions and Reagents

4.4.1 SDS reducing buffer

Distilled water	4.0ml
0.5 M Tris-HCL, pH 6.8	1.0ml
Glycerol	0.8ml
10% (w/v) sodium-dodecyl-sulphate	1.6ml
2-bete-mercaptoethanol	0.4ml
0.05% (w/v) bromophenol blue	0.2ml
	8.0ml

4.4.2 1.5 M Tris-HCL buffer, pH 8.8

Tris[hydroxymethyl]-aminomethane (Tris)	18.15g

Dissolve in distilled water, adjust pH to 8.8 with HCL and make up to a final volume of 100 ml. Store at 4^0C.

4.4.3   0.5 M Tris-HCL buffer, pH 6.8

Tris[hydroxymethyl]-aminomethane (Tris)   6.0g

Dissolve in distilled water, adjust pH to 6.8 with HCL and make up to a final volume of 60 ml. Store at 4^0C.

4.4.4   1 M Tris-HCL buffer, pH 6.8

Tris[hydroxymethyl]-aminomethane (Tris)   12.0g

Dissolve in distilled water, adjust pH to 6.8 with HCL and make up to a final volume of 60 ml. Store at 4^0C.

4.4.5   5X SDS running buffer, pH 8.3

Tris[hydroxymethyl]-aminomethane (Tris)	15g
Glycine	72g
Sodium-dodecyl-sulphate	5g

Make up to 1 litre with distilled water and store at 4^0C. Dilute to 1x prior to use.

4.4.6   12% SDS resolving gel

distilled water	4.9ml
30% Acrylamide mix	6.0ml
1.5 M Tris pH 8.8	3.8ml
10% SDS	0.15ml
10% Ammonium persulphate	0.15ml
TEMED	0.006ml

(Note: prepare the ammonium sulphate fresh prior to pouring the gel).
(Mix gently to aerate).

4.4.7   4% SDS Stacking gel

30% Acrylamide mix	0.83ml
distilled water	3.4ml
0.5 M Tris pH 6.8	0.63ml
10% SDS	0.05ml
10% ammonium persulphate	0.05ml
TEMED	0.005ml

(Note: prepare the ammonium sulphate fresh prior to pouring the gel).
(Mix gently to aerate).

4.4.8 Coomassie blue stain

Coomassie blue	2g
Methanol	800ml
Acetic acid	200ml
Distilled water	1000ml

4.4.9 Destain solution

Ethanol	200ml
Acetic acid	200ml
Distilled water	1600ml

4.4.10 10% Ammonium Persulphate (APS) Solution
Dissolve:

APS	0.05g
ddH$_2$O	0.50ml

Make fresh daily.

4.4.11 10% Sodium Dodecyl Sulphate (SDS) Solution
Dissolve:

SDS	10g
ddH$_2$O	100ml

APPENDIX 5

SOLUTIONS FOR WESTERN BLOT

5.1 Transfer buffer

Tris base	3.03g	25mM
Glycine	14.4g	192mM
Dissolve in 500ml ddH$_2$O add;		
Methanol	200ml	20%

Should be pH 8.3 without adjusting. Make up to 1L in dH$_2$O and chill before use.

5.2 DAB Substrate Solution

DAB solution:		
DAB	60mg	0.3mM
Dimethyl sulfoxide (DMSO)	3ml	
Dissolve DAB in DMSO		
PBS	200ml	

Can be frozen at this stage. Add 30% H$_2$O$_2$ to final concentration of 0.1%.