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**INVESTIGATION INTO THE MORTALITIES OF LARVAL  
MUD CRABS, *Scylla serrata* AND METHODS OF  
CONTROL.**

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**In partial fulfilment of the requirements for the degree of Master of  
Science in the Discipline of Microbiology and Immunology, James Cook  
University, Townsville, Australia October 2005**

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Laurence Andreas Liessmann

October 2005

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Laurence Andreas Liessmann

October 2005

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**ABSTRACT**

Pathogens and disease throughout the larval stage of the mud crab *Scylla serrata* are said to be responsible for preventing the establishment of a viable mud crab aquaculture industry within Australia.

Vibriosis has been demonstrated as the underlying cause for inconsistent survival of larvae of the mud crab *S. serrata* (Mann *et al.* 1998). Initial investigations of this study were directed at gaining an understanding of virulence factors of vibrios, namely siderophores, haemolysin, chitinases and bacteriophages. Haemolysin, chitinase and siderophore activity were assessed through observation on specifically prepared agars. Detection of a bacteriophage was conducted by mitomycin C induced growth curves. It was visibly evident that the LLD1 strain of *V. harveyi* had the greatest haemolysin production compared to the other strains. The *V. harveyi* strain 12 produced the least haemolytic activity. Colony and halo diameter of the strains were as a group, significantly different ( $F=70.78, df=4, 100, P<0.0001$ ) and ( $F=148.31, df=4, 100, P<0.0001$ ) respectively. When examining chitinase activity, strains 12 and LLB2 were the only isolates that did not produce an evident zone of clearance. Colony and halo diameter of chitinases of the above strains were as a group, significantly different ( $F=41.46, df=5, 60, P<0.0001$ ) and ( $F=118.03, df=5, 60, P<0.0001$ ) respectively. It was visibly evident that the selected probiotic isolate *V. harveyi* LLB1 had the greatest siderophore production compared to the other strains. Colony and halo diameter of siderophores of the strains were as a group significantly different ( $F=22.79, df=4, 50, P<0.0001$ ) and ( $F=172.97, df=4, 50, P<0.0001$ ) respectively. From the four isolates that were concentrated through ultracentrifugation and analysed by TEM, *V. harveyi* 642 was the only isolate to possess a bacteriophage.

The effectiveness of an environmental probiotic, referred to as the *V. alginolyticus* LLB2, was assessed on the survival of larvae of *S. serrata* when challenged with a virulent *V. harveyi* strain. The factors of the experimental trial, the bacterial isolate and concentration of bacteria, were significantly different as a group ( $P<0.0001$ ) indicating that survival of zoea were affected by these factors. It was evident that there was a definite trend between increase in concentrations of bacteria and an increase in mortalities. It was also evident that treatments with *V. alginolyticus* LLB2 led to the

highest survival, which also happened to be at a concentration of  $10^5$  CFU ml<sup>-1</sup>. Treatment consisting of *V. harveyi* LLD1 obtained the lowest survival at a concentration of  $10^5$  CFU ml<sup>-1</sup>. Treatments that were inoculated at a cell density of  $10^5$  CFU ml<sup>-1</sup> with the virulent *Vibrio harveyi* strains LLD1 and 642 and supplemented with the probiotic *V. alginolyticus* LLB2 had significantly higher survival rate ( $P>0.05$ ) than the treatment inoculated with *V.harveyi* LLD1 and 642 alone. These results suggest that the probiotic *V. alginolyticus* LLB2 demonstrated a protective effect towards the larvae of *S. serrata* when challenged with a virulent *V. harveyi* isolate.

A histological investigation was carried out on the possible diseases associated with the larvae of *S. serrata*, and adult mud crabs. Cells within the hepatopancreas in both the larvae and adults showed nuclear pathology. Amorphous basophilic intranuclear inclusions, which were markedly hypertrophied were observed. These observations were similar to the initial surveys of the intranuclear bacilliform virus *Scylla baculovirus virus* (SBV) in adult *S. serrata* (Anderson and Prior 1992). From 15 batches of larvae that were surveyed only two batches of individuals were found to carry SBV. The prevalences of infected larvae were 32.26% and 52.17 %, respectively, where as 8% of 25 adult crabs from the Townsville region were infected.

Following the detection of an intranuclear bacilliform virus within the larvae of *S. serrata*, presumably SBV, an attempt was made to amplify the nucleotide sequence of this virus. Novel primer sets were designed from the protein-binding gene from WSSV which is said to be homologous to that of the insect baculovirus. Only the primer set BP2, BP2 5' – AAAAATGGTTGCCCGAAGCTC and BP2 5' – TGAGGAACGGCGACGGACAG-3' were successful in producing amplicons. When the relatedness of the amplicon sequences were compared to available databases using Basic Local Alignment Search Tool (BLAST), there were no significant phylogenetic matches.

This has been the first time the intranuclear bacilliform virus SBV has been identified in larvae of *S. serrata*, as well as the first partial sequence from SBV. The investigation of virulence mechanisms possessed by *Vibrio* sp. as well as the use of environmental probiotics in attempt to control vibriosis, will provide a sound basis for future studies in diseases in the mud crab *S. serrata*.

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**LIST OF ABBREVIATIONS**

<b>ACMM</b>	Australian collection of marine microorganism
<b>BP</b>	Base pair
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>CCEAD</b>	Consultative Committee on Emergency Animal Diseases
<b>CFU</b>	Colony forming units
<b>DAB</b>	Diaminobenzidine Tetrahydrochloride
<b>DNA</b>	Deoxyribonucleic acid
<b>DPI&amp;F</b>	Department of Primary industries and Fisheries.
<b>ECP</b>	Extracellular products
<b>EDTA</b>	Ethyl diaminetetraacetic acid
<b>IBV</b>	Intranuclear bacilliform virus
<b>LA</b>	Luminous agar
<b>LBB</b>	Luria bertani broth
<b>LB</b>	Luminous broth
<b> LSD</b>	Least significant difference
<b>MARFU</b>	Marine and Aquaculture Research Facility
<b>OD</b>	Optical density
<b>PCR</b>	Polymerase chain reaction
<b>PYSS</b>	Peptone yeast and sea salt
<b>SBV</b>	<i>Scylla</i> baculovirus
<b>TEM</b>	Transmission electron microscopy
<b>UV</b>	Ultra-violet
<b>VHML</b>	<i>Vibrio Harveyi</i> Myovirus Like
<b>TBE</b>	Tris Borate - EDTA
<b>TE</b>	Tris, EDTA buffer
<b>TES</b>	Tris, EDTA, NaCl buffer
<b>TCBS</b>	Thiosulphate-citrate-bile salts-sucrose
<b>WSSV</b>	White spot syndrome virus

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## CHAPTER 1

### GENERAL INTRODUCTION

Crabs of the genus *Scylla* are associated with mangrove habitats throughout the tropical and subtropical Indo-West Pacific region and form a substantial component of tropical inshore fisheries (Keenan 1998). Mud crabs are considered a quality food item due to such attractive qualities as delicate flavour and high meat yield. The market for mud crabs has been reported as being promising, resulting in high market prices and high rates of crab consumption being experienced in many countries in Asia, America, Europe and Australia (Aldon and Dagoon 1997).

Within Australia, the culture of mud crabs is not extensive, with few operators producing marketable quantities (McCormack 1989). Pathogens and disease have been reported as the major constraint in the establishment of a successful hatchery production (Cholik 1999; Yamaguchi 1991). Boer *et al.* (1993) and Parenrengi *et al.* (1993) both reported that *Vibrio harveyi* was found to be the major contributor to disease. *V. harveyi*, as well as posing a serious threat to a number of economically important cultured marine organisms, has also been reported as a major pathogen of cultured penaeids in all phases of production throughout South East Asian countries (Lavilla-Pitogo *et al.* 1990; De la Pena *et al.* 2000; Karunasagar *et al.* 1994). Mann *et al.* (1998) reported that for the first few days of culture, bacterial communities within the culture system are relatively unstable. Total heterotrophic and presumptive vibrio levels rapidly increase to day 2 of culture, whereby at approximately day 3 and 4 bacterial levels rapidly decline to a lower concentration. Therefore, bacterial infection could be an instrumental factor in the heavy mortalities experienced around early zoeal stages throughout the industry (Dr C. Zeng James Cook University pers comm. 2002)

*V. harveyi* is often considered an opportunistic pathogen when the host animal is compromised (Karunasagar *et al.* 1998). However, primary disease caused by highly virulent strains has also been reported. Factors involved in the pathogenicity of bacteria are not well understood despite extensive studies. It is thought that two major characteristics are involved in virulence:

- 1) Ability of microorganisms to attach to and colonise specific sites in the host.
- 2) Formation of substances (toxins, enzymes and other molecules) that cause damage to the host (Brock *et al.* 1994).

There are numerous mechanisms that have been reported to confer virulence. Extracellular products (ECP) including proteases, haemolysins and cytotoxins have been reported to allow bacteria to survive and replicate within the hosts tissues (Montero and Austin 1999; Liu *et al.* 1996).

The expansion of the aquaculture industry has seen a corresponding increase in the incidences of microbial diseases, which is considered a major constraint in consistent larval production (Tanasomwang and Ruangpan 1995; Moriarty 1988). Presently the most significant method of controlling disease in cultured organisms has been through the prophylactic use of antibiotics and other chemotherapeutics (Gomez-Gil *et al.* 2000). This indiscriminate use of antibiotics has resulted in; the incidence of bacterial resistance to antibiotics of cultured organisms and possibly human pathogens; an increase in virulence of some bacteria; and an increase in traces of restricted chemicals identified in the tissues of cultured animals. Consequently to improve public perception of aquaculture and protect human health, alternatives to the regular use of antibiotics are essential. Such alternatives involve the use of probiotic microorganisms, "vaccination" and other forms of immunostimulation (Gomez-Gil *et al.* 2000). The use of probiotics to control and combat the presence of disease while at the same time creating a sustainable environmentally friendly industry, has attracted considerable interest.

Mortality rates are also high between molts of zoea<sub>5</sub> to megalopa, which is known as moulting death syndrome (MDS) (Fielder and Heasman 1998). The underlying reason for the high mortalities associated with this particular larval phase is unclear, however nutritional deficiency has been regarded as a leading factor. Zeng and Li (1998) reported that poor nutritional status during zoeal stages might play a crucial role in why larvae are unable to pass through this critical stage successfully. Yet to be reported as a major disease causing agent in larvae of *S. serrata*, are viruses pertaining to the *family Baculoviridae* which have been reported in many crustaceans of economic importance (Sindermann 1989), including cultured penaeids, which has resulted in disease accompanied by high mortality rates (Lightner 1985). Anderson and Prior (1992)

reported finding a nonoccluded rod shaped nuclear virus in *S. serrata* from Darwin. These infections were observed in juvenile, subadult and adult specimens. However this virus did not cause clinical disease. Viral prevalence and disease, in addition to a wide variety of other infectious agents in marine invertebrates have been shown to be exacerbated by stress. Stress may be a direct result of dietary inadequacies, pollution and adverse environmental conditions (Stewart 1991). Therefore under intensive rearing conditions which commonly give rise to such stress factors, this intranuclear bacilliform virus may prove to be lethal to the mud crab *S. serrata*. The fact that the commercial hatchery phase of *S. serrata* in Australia has not become fully established, could possibly account why viral infection has yet to be reported in larvae of *S. serrata*. Although vertical transmission of viruses from broodstock to larvae is possible, as demonstrated with prawn viruses, the most likely transmission of viruses is through the ingestion of diseased tissue and cohabitation with diseased or latent virus carriers (Fegan *et al.* 1991).

To support the development of a commercial industry in mud crab aquaculture, extensive research is warranted into the diseases and pathogens affecting the success in larviculture of *S. serrata*. This thesis will involve the investigation of possible diseases associated with mortalities experienced at specific stages during larval production of *S. serrata*. Environmental bacterial probiotics will be selected and trialled as a possible control method against pathogenic bacteria, which have been demonstrated to be virulent to mud crab larvae. Virulence mechanisms associated with *Vibrio* sp. will also be investigated to gain a greater understanding of how these mechanisms are employed by bacteria to increase their pathogenicity towards mud crab larvae

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Introduction

The mud crab, *Scylla serrata*, is widely distributed throughout the tropical and subtropical Indo-West Pacific and supports the basis of a substantial inshore fishery. Also called mangrove crabs because of their association with intertidal and subtidal mangrove habitats, the culture of *Scylla serrata* has received increasing attention in a number of countries (Rattanachote and Dangwatanakul 1992; Yamaguchi 1991). The mud crab has a number of attractive qualities that lends itself to the development of its culture. These include a high meat yield, high acceptability and market price in addition to their decreasing availability resulting from direct fishing pressure (Aldon and Dagoon 1997; Yamaguchi 1991).

Presently in Australia, the culture of mud crabs is not widespread with few operators having produced marketable quantities (McCormack 1989). A major constraint restricting further expansion of mud crab culture is the limited supply of crab larvae or 'seed' (Keenan 1999). Hatchery production of crab larvae has been attempted in several countries. However, a reliable hatchery protocol has not been fully established (Keenan 1999). Therefore the majority of mud crabs cultured have relied on wild caught stock. However, the quantities of crab larvae being caught by fishermen are not sufficient to meet demands even at the mud crab industry's current level (Cowan 1984). This insufficiency of larval supply is principally due to loss of mud crab habitat and over exploitation of wild stocks combined with the recent growth in crab culture operations (Le Vay, 2001; Keenan, 1999).

Although adults of *Scylla* sp. are considered fairly robust, easy to culture and said to have relatively few associated diseases, a major constraint to their successful hatchery production and their commercial viability is the mass mortality of larvae due to pathogens and disease (Cholik 1999; Yamaguchi 1991). To date, there has been very little investigation into diseases of cultured mud crabs, which is probably due to the industry still being in its infancy.

Traditionally, the major method of controlling disease in cultured organisms has been through the administration of chemical compounds such as antibiotics (Gomez-Gil *et al.* 2000). Controversy continues to gain momentum concerning the use of antibiotics for disease prevention and management practices in aquaculture. This is due to their potential role in the development of resistance in bacterial pathogens of cultured organisms and possibly human pathogens. Decreased efficacy has been documented in a wide range of antimicrobial drugs regardless of their mode of action (Dixon 2001; Lewin 1992). Resistance can be readily transferred to other strains by either altering the existing genome or by transferring genetic material between cells via plasmids or bacteriophages (Towner 1995). In addition to the threat of antibiotic resistance, the use of antibiotics in the hatchery culture of *S. serrata* appears to have other limitations. Brick (1974) reported that, although using a combination of antibiotics (penicillin-G + polymyxin-B) enhanced the premetamorphic survival of zoea, they had a detrimental effect on the survival of megalopae. Brick (1974) suggested that these antibiotics might have indirectly affected survival of the megalopa by acting on essential symbiotic bacteria. Symbiotic bacteria, which may possess certain favourable characteristics such as enzymes relevant for digestion, have been found in the digestive tracts of many invertebrates (Xianghong *et al.* 2000; Dempsey *et al.* 1989).

In order to improve public perception of aquaculture and protect human health, alternatives to the regular use of antibiotics are essential. Such alternatives involve the use of probiotic microorganisms, "vaccination" and other forms of immunostimulation (Gomez-Gil *et al.* 2000).

Therefore, to increase the production of mud crab larvae, which in turn will allow for the expansion of the industry, extensive research is warranted into the diseases and pathogens that may be affecting hatchery production of the larvae of *S. serrata*. The use of probiotics as a possible control method against harmful bacteria could prove to be a useful tool as an alternative to antibiotics in combating such disease.

## 2.2 General biology of *Scylla serrata*

### 2.2.1 Taxonomy and Distribution

The mud crab *Scylla serrata* belongs to the family Portunidae within the order Decapoda (Keenan *et al.* 1998). For some time there was considerable confusion on taxonomic nomenclature in the genus of *Scylla*. A recent taxonomic revision of the genus suggests that *Scylla* includes 4 species: *S. serrata*; *S. tranquebarica*; *S. parmamosain* and *S. olivacea* (Keenan *et al.* 1998). Two species, namely *S. serrata* and *S. olivacea* were identified as inhabiting northern Australia and Queensland waters with the predominant species in Queensland being *S. serrata* (QFMA 1991).

### 2.2.2 Lifecycle and Reproduction

Maturation and spawning in *Scylla* species occurs continuously throughout the year with some seasonal peaks (Le Vay 2001). In tropical regions peak in maturation coincide with high seasonal rainfall, probably resulting from an increase in productivity in coastal waters. Conversely, maturation in subtropical regions is related more to temperature and day length (Heasman *et al.* 1985). The offshore migration of gravid females to spawn has become a frequently recognised phenomenon (figure 2.1) (Yamaguchi 1991). This migration may represent a dispersal mechanism rather than a method of optimising conditions for larval survival because some inshore areas are suitable for larval development (Hill 1994). Depending on size, a single female may extrude clutches of up to 6 million eggs, and eggs can hatch in the range of 10 to 20 days depending on the environmental temperature (QFMA 1991). Larvae hatch as planktonic protozoae and pass through five zoeal stages and a megalopa larvae stage before settling to the sea floor and metamorphosing into juveniles. At this stage, juveniles adopt the benthic existence characteristic of adults. The duration of the larval phase takes approximately three weeks to complete during which they gradually move back inshore (QFMA 1991).



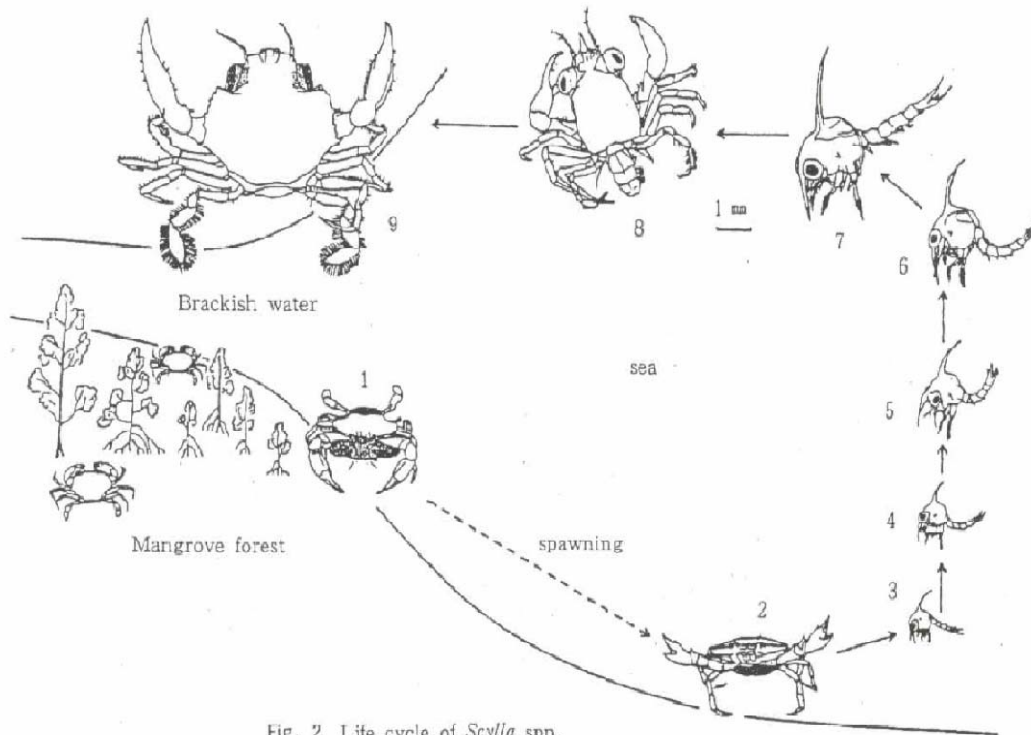
**Figure 2.1** Life cycle of the mud crab *Scylla serrata*.

Fig. 2 Life cycle of *Scylla* spp.  
 1, mating; 2, gravid female (for 2–4 weeks);  
 3, zoea I (3 days after hatching);  
 4, zoea II (2 days after molting);  
 5, zoea III (2 days); 6, zoea IV (4 days);  
 7, zoea V (4 days); 8, megalopa (7 days);  
 9, juvenile crab.

From Yamaguchi (1991)

### 2.3 Major diseases associated with *Scylla serrata*

The rearing of marine crustaceans under environmental conditions associated with aquaculture has led to serious disease problems. Diseases associated with the culture of *S. serrata* have not been extensively researched, however initial investigations indicate that bacterial infection may be largely responsible for high mortality rates observed in early larval stages (Boer *et al.* 1993; Cholik 1999). Understanding the diseases involved in culture of *S. serrata* is of primary importance when developing effective disease control strategies. The major diseases and pathogens that have been found associated with *S. serrata* are discussed below.

#### 2.3.1 Intranuclear bacilliform viruses

Viruses pertaining to the family *Baculoviridae* have been reported in many crustaceans of economic importance (Sindermann 1989), such as cultured penaeids. This disease accompanies high mortality rates (Lightner 1985). Baculoviruses pathogenic to crustaceans are classified as non-occluded (subgroup C) or occluded (subgroup A and B) genera (Johnson and Lightner 1988). However, because of recent research into the family of *Baculoviridae*, it is now recognised as being composed of two genera, *Nucleopolyhedrovirus* and *Granulovirus* and the unaligned intranuclear bacilliform viruses.

Baculoviruses are dsDNA; rod-shaped, enveloped nucleocapsids, which are identified on the basis of enveloped nucleocapsid morphology and an occlusion of proteinic crystal (Summers 1977). Best known from insects, they also occur in the marine crustaceans, penaeid prawns and brachyuran crabs (Johnson 1984). Baculovirus are said to form the largest group of viruses known in decapod crustaceans (Johnson and Lightner 1988).

An intranuclear bacilliform virus that first appeared in northeast Asia in 1992-93, with its associated mortality, has emerged as one of the most challenging problems for the global shrimp industry, especially in Asia (Rajendran *et al.* 1999; Shi *et al.* 2000). Known as white spot syndrome virus (WSSV), this virus was previously an unassigned member of the *Baculoviridae* because of its rod-shaped, enveloped morphology (Van Regenmortel *et al.* 2000). After completing phylogenetic analysis on the viral DNA polymerase of WSSV, Van Hulten *et al.* (2000) suggested that WSSV differs profoundly

from all reported viruses, proposing that WSSV belongs to the *genus Whispovirus* within a new family called *Nimaviridae* (ICTV). The causative agent is a dsDNA virus, which consists of an enveloped, rod-shaped nucleocapsid. It has been reported in a variety of crustaceans including wild and cultured penaeid shrimp, crabs and crayfish (Lo and Kou 1998). Because WSSV can develop in different crustacean species, it is considered to have a low degree of specificity, which is unusual for viruses. In addition to its pathogenicity, its low specificity makes this one of the most dangerous viruses so far observed in crustaceans (Shi *et al.* 2000). WSSV infection is characterised by rapid disease onset and high mortality in which mortality typically reaches 90 % within 2 to 7 days after the onset of disease (Lo and Kou 1998). Under stressful conditions WSSV can be triggered to replicate rapidly and subsequently resulting in mortalities of animals infected (Lo and Kou 1998).

As investigations into decapods continue, further intranuclear bacilliform viruses are being detected thereby widening the known host range of these viruses (Table 2.1).

**Table 2.1** Morphological comparison of crustacean intranuclear bacilliform viruses.

Name	Host(s)	Occlusion body	Average size of nucleocapsid (nm)	Key References
<b>Gut-Infecting</b>				
BP	<i>Penaeus</i> spp.	Yes	50 × 270	Couch 1981
MBV	<i>Penaeus</i> spp.	Yes	42 × 246	Lightner <i>et al.</i> 1983
BMNV	<i>Penaeus</i> spp.	No	36 × 250	Sano <i>et al.</i> 1981
Tau	<i>Carcinus mediteraneus</i>	No	68 × 310	Pappalardo & Bonami 1979
Baculo-A	<i>Callinectes sapidus</i>	No	43 × 247	Johnson 1976
Baculo-PP	<i>Paralithodes platypus</i>	No	38 × 200	Johnson & Lightner 1988
SBV	<i>Scylla serrata</i>	No	24 × 205	Anderson & Prior 1992
CBV	<i>Cherax quadricarinatus</i>	No	34 × 154	Anderson & Prior 1992
<b>Haemocyte-infecting</b>				
WSSV	<i>Penaeus</i> spp.	No	80 × 270	Chou <i>et al.</i> 1995
Baculo B	<i>Callinectes sapidus</i>	No	100 × 335	Johnson 1983
Haemocytic rod-shaped virus	<i>Penaeus</i> spp.	No	588 × 119	Owens <i>et al.</i> 1993

Abbreviations: BP, *Baculovirus penaei*; MBV *Monodon* baculovirus; BMNV, baculoviral midgut gland necrosis virus; SBV, *Scylla* bacilliform virus; CBV, *Cherax* bacilliform virus; WSSV, white spot syndrome virus.

### 2.3.1.1 Intranuclear bacilliform viruses of crabs

#### 2.3.1.1.1 *Scylla* bacilliform virus (SBV)

Anderson and Prior (1992) reported finding two non-occluded rod-shaped nuclear viruses, indicating they were clearly related to the known family *Baculoviridae*. Baculovirus related nuclear pathology in hepatopancreatic epithelial cells was identified from the crayfish *Cherax quadricarinatus* and from *S. serrata*. These infections were observed in juvenile, subadult and adult specimens, however neither of these viruses caused clinical disease. The baculovirus found in *S. serrata* termed *Scylla* baculovirus (SBV) was found to have a similar nuclear distribution to Baculo A (Johnson 1976, 1983) and Tau (Pappalardo and Bonami 1979) with regular arrays of virions tending to align along the inner nuclear membrane. Anderson and Prior (1992) suggested that a range of subtypes of a single branchyuran crab baculovirus may exist, based on similarities found among SBV, Tau and Baculo A, even though their dimensions differed. As these two viruses do not produce occlusion bodies, they clearly belong in the unassigned bacilliform virus group.

#### 2.3.1.1.2 Baculo-A

Baculo-A (Baculovirus A) is a non-occluded virus that infects the differentiating cells of the hepatopancreatic epithelium (Johnson 1984; Johnson and Lightner 1988). Juveniles and adults of the blue crab *Callinectes sapidus* from the Atlantic coast of Northern America were consistently infected with the non-occluded hepatopancreatic virus Baculo A (Baculovirus A). Although the infection is not known to cause disease in blue crabs, infected cells are undoubtedly unable to perform normal functions. However these cells are almost always found in isolated groups and few in number. Baculo-A did not attack stems cells and infection occurred only in those cell populations, undergoing constant replacement (Johnson and Lightner 1988).

### 2.3.1.1.3 Baculo-PP

Baculo-PP is a non-occluded virus that has occurred in at least two populations of Alaskan blue king crab, *Paralithodes platypus* (Johnson and Lightner 1988). Baculo-PP was isolated from the epithelial cells of the hepatopancreas of *P. platypus*. Similar to Baculo-B virus of *Callinectes sapidus*, Baculo-PP was shown to be focal and thus may not be pathogenic, however Johnson and Lightner (1998) reported that it probably infects stem cells as well as differentiating cells.

### 2.3.1.1.4 White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV), the causative agent of white spot syndrome, is known to possess a wide host range having not only been observed in shrimp but also crabs, copepods and other arthropods (Lo *et al.* 1996). Natural WSSV infections have been found in captured and cultured specimens of *S. serrata* in Taiwan coastal waters (Lo *et al.* 1996). In some species of prawns such as *P. monodon* and *P. japonicus*, preliminary diagnosis in acutely affected shrimp is made by the presence of white spots, while in the portunid crab *S. serrata* the infection can be detected by the observation of clouded areas in the last two segments of the fifth pereopod (Lo *et al.* 1996; Peng *et al.* 1998). Rajendran *et al.* (1999) was able to experimentally transmit WSSV to *S. serrata*. Despite presence of the infection being identified by histological and bioassay methods, the crabs survived without exhibiting clinical symptoms. Rajendran *et al.* (1999) suggested that these animals might act as an asymptomatic carrier rather than an indicator species of infection. Supamattaya *et al.* (1998) observed mortality in adult *S. serrata* after they were infected with WSSV by injection. Chen *et al.* (2000) confirmed larvae of *S. serrata* were susceptible to WSSV via waterborne pathway after observing an increase in mortalities of the experimental groups corresponding to the level of infection. Chen *et al.* (2000) also suggested that WSSV first infects the external organs before the internal organs. The virus was also able to replicate over time especially in the cuticular epithelial lining of the stomach (Chen *et al.* 2000). Tissue tropism observed in larvae of *S. serrata* was also similar to that found in many species of prawns (Chen *et al.* 2000). A high prevalence of the infection was observed in benthic larvae captured from coastal waters and those used throughout the experiment. This is further evidence

of WSSV rather severe natural infection rate and its stable prevalence in the mud crab population (Chen *et al.* 2000).

### 2.3.2 Vibrios

Pathogenic *Vibrio* spp. have been implicated as one of the major causes of disease in the culture of aquatic organisms (Ruangpan and Kitao 1993; Lightner 1993). Boer *et al.* (1993) reported that the mud crab zoea were very sensitive to luminous bacteria with *V. harveyi* said to be a major contributor to disease from a number of luminescent species. Parenrengi *et al.* (1993) isolated the species of vibrio namely *V.harveyi*, *V. alginolyticus* and *V. parahaemolyticus* and tested their pathogenicity to zoea of mud crabs. Results indicated that all isolates were found to be pathogenic to zoea, however their pathogenicity was considered moderate in comparison to *V. harveyi* (Parenrengi *et al.* 1993).

Vibrios have also been found to be involved in shell disease associated with captive broodstock of *S. serrata* (Yamaguchi 1991; Lavilla-pitogo *et al.* 2001). Lavilla-Pitogo *et al.* (2001) reported that shell disease was a major problem in captive broodstock due to a combination of fouling organisms and chitinoclastic bacteria. Bacterial populations that were isolated from the coloured and diseased areas of the shell were very high, with up to  $10^7$  total bacteria per 0.1 g of sample, consisting of 50 to 75 % of chitinoclastic bacteria. As well as possessing chitinase, these vibrios also possessed the enzymes lipase and gelatinase, which were considered to enhance shell disease (Lavilla-Pitogo *et al.* 2001). Fouling organisms such as filamentous blue green algae, sessile bacteria, ciliated protozoans, saprophytic ciliated protozoans and some flagellates developed providing a favourable substrate for the establishment of chitinoclastic bacteria on the dorsal regions of the body (Lavilla-Pitogo *et al.* 2001). After investigating lesion location patterns on the edible crab *Cancer Pagurus* as a result of their back burrowing behaviour, Vogan *et al.* (1999) suggested that sand abrasion injuries may also lead to the formation of shell disease. Although these conditions result in extensive shell erosion and perforation that may create portals of entry for secondary bacterial or parasitic infections, this condition is said to seldom lead to mortality (Lavilla-Pitogo *et al.* 2001). However, as McCormack (1989) suggests, even though the flesh remains

untainted as a result of infection, their market price may be affected due to their eroded appearance.

### 2.3.3 Fungi

A number of fungal diseases have been frequently observed infecting the larvae and ova of *S. serrata*, including, *Lagenidium scyllae* (Cholik 1999; Zafran 1993), *Haliphthoros philippinicus* (Hatai *et al.* 1980) and *Atkinsiella hamanaensis* sp. (Bian and Egusa 1980).

*Scylla serrata* suffers from devastating infections caused by the non-host specific pathogen *Lagenidium sp.* (Bian *et al.* 1979), resulting in high mortalities of reared larvae. Pathogenesis of *Lagenidium sp.* infections among crustacean hosts involves the sequential production and release of zoospores into the rearing medium (Lio-Po *et al.* 1982). *Lagenidium callinectes* has also resulted in the destruction of up to 25% of egg masses and occasional larvae of the crab *Callinectes sapidus* (Sindermann and Lightner 1988). Bian and Egusa (1980) demonstrated that *A. hamanaensis* is pathogenic to *S. serrata* eggs and that this marine mastigomycete has a strong destructive potential even greater than that of *Lagenidium scyllae*.

### 2.3.4 *Hematodinium sp.*

Following an intensive study of the parasites and symbionts of wild mud crabs and their potential significance in aquaculture, Hudson and Shields (1994) described the protozoan *Hematodinium sp.* as an important pathogen of cultured mud crabs. *Hematodinium sp.* is a parasitic dinoflagellate, which infects the haemolymph and tissues of crabs giving the haemolymph a cloudy appearance and reducing its ability to clot (Hudson and Shields 1994). Shields (1992) reported a similar infection in the commercially important sand crab *Portunus pelagicus* in Moreton Bay. In Alaska *Hematodinium sp.* has been identified as causing “bitter crab disease” (BCD) in the Tanner crab (*Chionoecetes bairdi*), which eventually leads to the crab’s death (Meyers *et al.* 1987). This disease causes the meat of processed crabs to have a bitter after taste leaving the product unmarketable. Meyers *et al.* (1990) reported that 5 % of the total 1988/89 season of Tanner crab catch was rejected due to this disease, which caused a direct economic losses to both fishermen and processors.



### 2.3.5 Peritrich ciliates

Protozoans such as the stalked peritrich ciliates *Epistylis* sp., *Zoothamnium* sp. and the suctorian ciliates *Acineta* sp. and *Lagenophrys* sp. have been observed attached to the shell and gills of *S. serrata* (Hudson and Lester 1994; Couch 1983). Cholik (1999) has also observed the ciliates *Zoothamnium* and *Lagenidium* attached to incubated eggs of berried females harvested from brackish water. Duc Dat (1999) reported that the ciliate *Zoothamnium* sp. infected a high number of larvae from the mud crab *Scylla paramamosian*. By parasitising the shell and gills, the ability of larvae to catch food was potentially reduced. Couch (1966) reported that heavy peritrich infestations were accountable for the death of blue crabs which were held in floating cages and shedding tanks. Hudson and Lester (1994) identified ciliates as potential problems in mud crab aquaculture where high densities can interfere with respiration.

Peritrichs are said to have similar effects on the gills of cultured prawns especially under crowded conditions or during periods of low oxygen availability (Overstreet 1973). Peritrich infestations have also been suggested to affect larvae and postlarvae of prawns in other ways, including interfering with normal feeding behaviour, locomotion and moulting which inevitably leads to stress, causing possible death (Lightner 1983). The impact of microbial gill infestations on the pathology and physiology of the freshwater crab *Potamonautes warreni* has also been investigated (Schuwerack *et al.* 2001). The attachment of stalks of *Zoothamnium* and *Epistylis* resulted in the dilation of lamellar tissues, the formation of vacuoles and an increase in subcuticular spaces in the epithelia. (Schuwerack *et al.* 2001). Physiological changes in infected crabs compared to uninfected included; significant differences in increments of wet body mass and a reduced growth rate over time compared with uninfected crabs, significant Increase in specific oxygen consumption in rested infected crabs and a significantly lower heart rate of infected rested crabs (Schuwerack *et al.* 2001).

A number of peritrichs such as *Lagenophrys* sp. have been shown to be responsible for causing gill damage at its attachment site (Couch 1983). However peritrichs including *Epistylis* sp. and *Zoothamnium* sp. have not been shown to cause gill damage in this way (Foster *et al.* 1978). Suctorians feed mainly on bacteria and smaller protozoans and in high numbers may interfere with respiration. Because of the piercing and sucking

action of their “tentacles”, some have been shown to cause stress by extracting cytoplasm from their host (Gucatan *et al.* 1979; Overstreet 1987).

Peritrichs are usually associated with water that is high in organic matter and other nutrients. Apart from suctorians that feed on other protozoans, most peritrichs feed primarily on bacteria. Bacteria which thrive in water that is high in organic matter probably suggests why peritrichs are also common in such conditions (Couch 1983).

### 2.3.6 Parasitic barnacles

#### 2.3.6.1 *Loxothylacus ihlei*

Boschma (1949) described *Loxothylacus ihlei* as a parasite of *S. serrata* in Indonesian waters. *Loxothylacus ihlei* is a rhizocephalan parasite that influences the size and morphometry of *S. serrata*, similar to other sacculinid infections (Hochberg *et al.* 1992; Knuckey *et al.* 1995). Once the parasite externa has developed, it inhibits moulting which results in a stunted size in infected crabs (O'Brien and Skinner 1990). The broader abdominal widths of infected mud crabs are an indication of feminisation (Knuckey *et al.* 1995). Weng (1987) suggested that this broadening might facilitate the survival and reproduction of the parasite. The degree of change is said to depend on the number of moults the host undergoes before the externa erupts and the age of host at the time of infection (Weng 1987). Knuckey *et al.* (1995) reported the presence of the parasite *L. ihlei* parasitising *Scylla serrata* for the first time in Australian waters. *S. serrata* were collected from commercial catches in the Northern Territory. The findings of Knuckey *et al.* (1995) were similar to other reports on crabs infected with *L. ihlei*, where infected *S. serrata* were significantly smaller than crabs uninfected. However, Knuckey *et al.* (1995) reported that the annual prevalence of infection was relatively low and therefore suggested that no alteration of the present management practices was necessary.

### 2.3.6.2 *Octolasmis* sp.

The crustacean symbiont *Octolasmis* sp. is often found attached to the gills or in the gill chambers of *S. serrata*. *Octolasmis* sp. may interfere with respiration by competing for oxygen and decreasing the amount of gill surface available for respiration. Debris may accumulate on colonized respiratory surfaces compounding the problem (Overstreet 1978). Hashmi and Zaidi (1964) reported that this barnacle was responsible for causing death of *S. serrata* by reducing their respiratory efficiency. Gannon and Wheatly (1992) and Gannon and Wheatly (1995) studied the physiological effects of the barnacle *Octolasmis muelleri* on the gas exchange of the blue crab *Callinectes sapidus*. Infested crabs maintained their oxygen uptake at the same rate as controls, however heart rate and scaphognathite rate increased. Although heavily infested crabs appeared to compensate for barnacle presence, extremely heavily infested crabs were not able to survive the experimental stress. Because natural infestation levels are low Gannon and Wheatly (1992) suggested the barnacle probably does not pose a serious threat to the blue crab population (Gannon and Wheatly 1992). Hudson and Lester (1994) noticed that the prevalence of *Octolasmis cor* increased with crab size and more were present on female crabs than male crabs. Shields (1992) found similar relationships with sand crabs suggesting that the longer moult interval of female crabs compared with that of males allowed more time for barnacles to accumulate. Crabs heavily infested with *Octolasmis cor* could also be disadvantaged due to the extra weight carried as well as being less marketable (Overstreet 1978; Hudson and Lester 1994).

### 2.3.7 Nicothoid copepod (*Choniosphaera indica*)

The nicothoid copepod *Choniosphaera indica* was reported by Shields (1992), as an egg predator of sand crabs (*Portunus pelagicus*) found in Moreton bay. However, Hudson and Lester (1994) were unable to catch any ovigerous mud crabs and did not detect the egg predator *Choniosphaera indica*. Therefore Hudson and Lester (1994) relied on investigations by Gnanamuthu (1954), which reported that the copepodite stage of *Choniosphaera indica* was found in the gills of the infected crab. As Hudson and Lester (1994) were unable to detect *C. indica* in the gills of mature female crabs, they suggested that the parasite might not infect *S. serrata*.

## 2.4 Probiotics as a possible control method

The expansion of the aquaculture industry has seen a corresponding increase in the incidence of microbial diseases, which is considered a major constraint in consistent larval production (Tanasomwang and Ruangpan 1995; Moriarty 1998). Intensive aquaculture systems are often characterised by high culture densities and high organic loads, which can prove stressful to the organism being cultured (Garriques and Arevalo 1995). Slight environmental changes can result in disruption of the natural dynamic balance of the bacterial flora and the organism's ability to tolerate them. The resultant imbalance in the microbial flora possessed by these organisms or of their aquatic environment often leads to pathogenesis (Garriques and Arevalo 1995; Rengpipat 1996).

Indiscriminate use of antibiotics in attempts to control pathogenic bacteria in aquacultural hatcheries has led to the development of resistant strains (Sorgeloos 1995; Brown 1989; Weston 1996; Tanasomwang *et al.* 1998). More recently, interest is increasing towards alternative methods of control including probiotic microorganisms, "vaccination" and other forms of immunostimulation (Gomez-Gil *et al.* 2000). The push towards environmentally friendly aquaculture has led to a demand for bacterial probiotics. Bacterial probiotics have displayed a proven record of success in hatcheries when they are added to clean water with no preexisting microbial community (Douillet 2000, Garriques and Arevalo 1995, Douillet and Langdon 1994).

Jory (1998) defined probiotics which are used in aquaculture as "a culture (single or mixed) of selected strains of microorganisms that are used in culture and production systems to modify or manipulate the microbial communities in water and sediment, reduce or eliminate selected pathogenic species of microorganism, and generally improve growth and survival of the target organism". Probiotics used in aquaculture systems may differ in their mechanism of action. Some of these may include the enhancement of digestion through the supply of essential enzymes, by moderating and promoting the direct uptake of dissolved organic materials (Ukeles and Bishop 1975; Hagiawara *et al.* 1994), production of pathogen-inhibitory reagents (Lemos *et al.* 1985; Vandenberghe *et al.* 1999), competitive exclusion of pathogens for nutrients or adhesion

sites (Phianphak *et al.* 1999; Ringo and Vadstein 1998), and immunity enhancement against pathogens (Vadstein 1997; Skjermo *et al.* 1995).

The successful use of bacterial probiotics in hatcheries has been achieved by the utilisation of appropriated strains in combination with effective management. Microorganisms selected for probiotic application should be determined by their effectiveness on the culture conditions and on target organisms (Douillet 2000). Rengpipat *et al.* (1998b) suggested using local isolates for biosafety reasons and to avoid the sudden changes in microbial flora of the ecosystem. Effective management involves creating favourable conditions for probiotic activity through the limitation or removal of microbial contamination, removal of bacterial predators and provision of optimal environmental conditions for activity of the probiotic (Douillet 2000).

As the information available on probiotics in crabs, especially *S. serrata* is limited the literature review has been expanded to include probiotics in prawn culture. The information obtained from other crustaceans may be analogous to the *S. serrata* thus providing a larger information base. Use of probiotics and their relationship with crab and prawn culture are dealt with below.

#### **2.4.1 Probiotics in crustacean culture**

The majority of studies involving probiotics in crustacean culture have been undertaken on prawns, probably as a direct result of the vast size of industry and its economic importance. Probiotics have been applied to prawn larviculture for a number of years now with promising results including a marked reduction in outbreak of bacterial disease (Griffith 1995). Following the introduction of probiotics in Ecuador in 1992, Griffith (1995) reported an increase in production by 35%, a reduction in hatchery down time between batches from 7 days per month to between 5 and 21 days annually, and total antimicrobial use decreased by 94%.

### 2.4.1.1 *Thalassobacter utilis*

Maeda and Liao (1992) attempted to elucidate the role of microorganisms in the culture of larvae of *P. monodon* after evidence that microorganisms were ingested throughout their larvae stages. Bacterial strains were isolated from the culture water of *P. monodon* and the effectiveness of each strain was tested against larvae. Larvae of *P. monodon* were also cultured in the presence of soil extract combined with the diatom *Navicula* sp. Soil extract was reportedly added to promote the growth of microorganisms by possibly acting as a source of nutrients (Maeda and Liao 1992). From seven strains of bacteria that were isolated and cultured with larvae of *P. monodon*, the strain PM-4 gave the greatest survival and moulting rates. The addition of soil extract together with the diatom also resulted in higher survival rate than that of the control groups. Interpretation of the results was made difficult due to the very limited data present (table 2.2).

Nogami and Maeda (1992) again used the isolate PM-4, which was later identified as *Thalassobacter utilis* (Nogami *et al.* 1997), as a possible bacterial probiotic in the culture of the blue crab, *Portunus trituberculatus*. An average survival of 27.2% was obtained from tanks that were inoculated with the strain *T. utilis* PM-4, compared with 6.8% in control tanks. The isolate *T. utilis* PM-4 was also found to inhibit the growth of *V. anguillarum*. Possible explanations of why *T. utilis* PM-4 was able to repress the growth of *Vibrio* sp. were either through competitive exclusion or through the production of vibriostatic reagents. In experiments that did not involve the addition of the probiotic *T. utilis* PM-4, survival rates from zoea 1 to zoea 4 were high, however, larvae in some experiments died when they reached megalopa 1. Nogami and Maeda (1992) suggested the probiotic *T. utilis* PM-4 may have also possessed another mechanism in which it served as a nutrient source during growth, thus improving the physiological state of the larvae. Despite the repeated inoculation of *T. utilis* PM-4 into tanks, Nogami and Maeda (1992) observed that bacterial levels did not exceed  $10^6$  cell ml<sup>-1</sup>. Nogami and Maeda (1992) suggested that protozoan populations grazing on bacteria were responsible for keeping bacterial levels within the tanks at  $10^6$  cell ml<sup>-1</sup>. Another possible explanation is that necessary nutrients within the system were inadequate to support higher bacterial densities (Gomez-Gil *et al.* 2000). Nogami and Maeda (1992) also reported that similar bacterial levels of  $10^6$  cell ml<sup>-1</sup> have been observed in contrasting locations such as the open Pacific Ocean and eutrophicated coastal areas. This could

possibly indicate that there is a stable maximum density that bacteria reach even in cultured conditions. Therefore, by ensuring bacterial densities are kept at  $10^6$  cell  $\text{ml}^{-1}$ , this may reduce the risk of other bacteria from becoming established, particularly those that are pathogenic in nature.

**Table 2.2** Environmental probiotics used in the culture of crustaceans.

Probiot(s)	Mechanism of action	Target Organisms	Pathogen	Reference
<i>Bacillus</i> sp. (S11)	Organic acid	Prawn ( <i>Penaeus monodon</i> )	<i>Vibrio harveyi</i>	Rengpipat <i>et al.</i> 1998a.
<i>Bacillus</i> sp. (S11)	Immunity enhancement	Prawn ( <i>Penaeus monodon</i> )	<i>Vibrio harveyi</i>	Rengpipat <i>et al.</i> 2000.
<i>Lactobacillus</i> sp.	Colonisation	Prawn ( <i>Penaeus monodon</i> )	<i>Vibrio harveyi</i>	Phianphak <i>et al.</i> 1999.
<i>Thalassobacter utilis</i> (PM-4)	Colonisation	Crab ( <i>Portunus trituberculatus</i> )	<i>Vibrio anguillarum</i>	Nogami and Maeda 1992.
<i>Thalassobacter utilis</i> (PM-4)	Colonisation	Prawn ( <i>Penaeus monodon</i> )	Non specific	Maeda and Liao 1992.
<i>Vibrio alginolyticus</i>	Competitive inhibition	Prawn ( <i>Penaeus vannamei</i> )	<i>Vibrio parahaemolyticus</i>	Garriques and Arevalo 1995.
<i>Pseudoalteromonas</i> sp.	Antibiotic	Prawn ( <i>Palaemon macrodactylis</i> )	<i>Lagenidium callinectes</i>	Gil-turnes <i>et al.</i> 1989.
<i>Bifidobacterium thermophilum</i>	Immunity enhancement	Prawn ( <i>Penaeus japonicus</i> )	<i>Vibrio penaeicida</i> and WSSV	Itami <i>et al.</i> 1998

#### 2.4.1.2 *Vibrio alginolyticus*

From literature concerning bacterial probiotics, *V. alginolyticus* is a frequently tested probiont that has shown promising results (Harris 1998; Gatesoupe 1999; Austin *et al.* 1995; Garriques and Arevalo 1995). Although *V. alginolyticus* has been reported to be a suitable probiotic candidate for shrimp larviculture, caution should be advocated since some strains are pathogenic (Lightner 1993). Vandenberghe *et al.* (1999) reported that *V. alginolyticus* is the dominant vibrio species in the larval stages of healthy and diseased *Penaeus vannamei*. Genetic fingerprinting showed a high genetic heterogeneity among *V. alginolyticus* strains suggesting that putative probiotic and pathogenic strains each have specific genotypes. Therefore to avoid the danger of using pathogenic *V. alginolyticus* as a probiont, Vandenberghe *et al.* (1999) suggested the use of only genotypically well-characterised strains.

Garriques and Arevalo (1995) isolated *V. alginolyticus* from seawater and selected it as a probiotic based on its apparent lack of pathogenicity and tested its affect on the larvae of *Penaeus vannamei*. Results showed that groups receiving the inoculation of the probiotic bacteria *V. alginolyticus* had the highest survival of 90.1% and wet weight of 7.8 mg. The other groups that received prophylactic doses of oxytetracycline and the control group receiving no antibiotics or bacterial supplements had lower survivals and wet weights of 83.8% and 6.0 mg, and 74.5% and 7.1 mg, respectively. Furthermore, the control group and the group treated with antibiotics had *V. parahaemolyticus* present in 10% of the samples at late postlarval stage, whereas larvae that received only bacterial inoculations had no *V. parahaemolyticus* detected in any of the samples. Garriques and Arevalo (1995) believed these results were due to *V. alginolyticus* successfully out-competing pathogenic strains of bacteria. Despite these findings, it should be noted the control treatment had a higher wet weight than that of the antibiotic treatments of 7.1 mg to 6.0 mg respectively. This may suggest that the prophylactic use of antibiotics may interfere or prevent symbiotic microorganisms that are necessary in digestion and nutrient enhancement from becoming established. These findings are similar to those of Brick (1974), where antibiotics were thought to affect survival of *S. serrata* megalopa indirectly by acting on essential symbiotic bacteria.



Harris (1998) also demonstrated that a strain of *V. alginolyticus* was able to protect larvae of *P. monodon* from the effects of pathogenic strains of *V. harveyi* in larval pathogenicity assay. An increase in larval survival was experienced when the density of *V. alginolyticus* was at least two-log steps greater than the density of the pathogen *V. harveyi* used to challenge the larvae. The *V. alginolyticus* strain used as a probiont by Harris (1998) failed to show any vibriostatic activity similar to the *V. alginolyticus* strain tested by Garriques and Arevalo (1995). Competitive inhibition was also believed to be responsible for the probioitic effect of *V. alginolyticus* towards larvae of *P. monodon*. However more complex interactions may have taken place between the probiont and pathogen. For example, the probiont may have interfered with the AHL-regulated expression of virulence factors possessed by *V. harveyi*, as some bacteria are said to produce compounds know as cyclic dipeptides which have been shown to interfere with AHL-mediated systems (A/Prof. R. de Nys, James Cook University pers comm., 1997). Future studies are necessary to investigate whether competitive inhibition was responsible for its probiotic effect or the involvement of other mechanisms. Competitive inhibitions have reportedly resulted from such interactions as competition of attachment sites (Ringo and Gatesoupe 1998) or through siderophore-mediated competition (Pybus *et al.* 1994), in which siderophore complexes have been identified in a strain of *V. alginolyticus* (Gatesoupe 1997).

#### **2.4.1.3 *Lactobacillus***

*Lactobacillus*, a group of lactic acid bacteria, has been a widely used probiotic for terrestrial animals in recent years (Ringo and Gatesoupe 1998). Phianphak *et al.* (1999) isolated a number of *Lactobacillus* species and strains from chicken gastrointestinal tracts and mixed them with a formulated shrimp diet. After 100 days, shrimp survival was 34 % in the treatment group compared to 16% in the control group. It appeared that *Lactobacillus* contributed to both increased yields and growth. However, from the five strains of *Lactobacillus* used in this experiment, the only strain to survive and predominate in the gastrointestinal tract of the shrimp was the strain *L. casei* subsp. *tolerans*. It is thought that *Lactobacillus* may have increased survival through colonising and adhering to the gastrointestinal tract of the shrimp resulting in the competitive exclusion of other microbes (Phianphak *et al.* 1999).

#### 2.4.1.4 *Bacillus* S11

Rengpipat *et al.* (1998a) isolated the bacterium *Bacillus* S11 from the habitat of *P. monodon* and added it to shrimp feed as a probiotic. Prawns in the probiotic treatment experienced better growth and survival than the control treatment. After feeding for 100 days, both groups were challenged with the luminous bacteria *V. harveyi* D331. The treatment group again obtained the best survival of 100 % compared to that of the control group of 26%. The treatment group also appeared to be healthy and normal whereas the control group had an unhealthy external appearance and deformed texture of the intestine and hepatopancreas (Rengpipat *et al.* 1998a). It was thought that *Bacillus* S11 had colonised the gastrointestinal tract of the *P. monodon*, thus out-competing the luminous strain of *V. harveyi* D331. However, *Bacillus* may have also acted as an antagonist as indicated by its previous ability to produce antimicrobial substances against *V. parahaemolyticus* and *V. harveyi* D311 (Rengpipat *et al.* 1998a).

Rengpipat *et al.* (1998b) again applied the *Bacillus* strain S11 as a probiotic through the pathway of *Artemia*, which were fed to *P. monodon*. The addition of *Bacillus* strain S11 resulted in significantly reduced development times of *P. monodon* with an increase in body length and weight and reduced disease problems compared to the control group. The treatment also showed a higher survival when challenged with *V. harveyi* (Rengpipat *et al.* 1998b).

In a recent study, Rengpipat *et al.* (2000) attempted to identify the effects of probiont *Bacillus* S11 on the immunological responses of *P. monodon*. As well as experiencing an increase survival and growth in the probiotic treated group, *Bacillus* S11 was also found to provide disease protection by activating both cellular and humoral immune defences.

#### 2.4.1.5 *Pseudoalteromonas* sp.

*Pseudoalteromonas* sp. and its ability to produce antimicrobial compounds responsible for antagonistic interactions against fungi and bacteria have been frequently documented (Ruiz *et al.* 1996; Gil-Turnes *et al.* 1989).

A strain of *Pseudoalteromonas* sp. that is frequently isolated from healthy *Palaemon macrodactylus* embryos, was shown to effectively inhibit the growth of the opportunistic pathogen fungus *Lagenidium callinectes in vitro* (Gil-Turnes *et al.* 1989). Investigations revealed that the antifungal compound was released into the culture medium in relatively large quantities, and was later identified as the compound 2,3-indolinedione, also known as isatin (Gil-Turnes *et al.* 1989).

An investigation by Ruiz *et al.* (1996) isolated a bacterial strain from both larval cultures and collectors of the great scallop (*Pecten maximus*), which was found to produce an antagonistic effect. Early indications suggested that this strain belonged to the genus *Altermonas* now *Pseudoalteromonas*. Ruiz *et al.* (1996) tested the antagonistic effect on a number of bacteria and other potential pathogens, and observed that some bacteria such as *V. anguillarum* were very sensitive to the action of the presumed *Pseudoalteromonas* strain. Interestingly enough when this strain was isolated no Vibrionaceae were found in a qualitative analysis of the natural microflora of larvae and collectors. Further investigations are necessary to accurately identify the strain of bacterium and test the isolates effectiveness as a biocontrol agent against pathogenic strains in larval scallop cultures.

The investigation of Riquelme *et al.* (1996) found that a strain of *Pseudoalteromonas haloplanktis* clearly suppressed the growth of *Vibrio alginolyticus* and *Vibrio anguillarum*, two pathogens that have shown to cause severe mortalities in larval cultures of the scallop *Argopecten purpuratus*. Riquelme *et al.* (1996) suggested that the inhibition of these bacterial strains were remarkable considering they showed resistance to several strains of antibiotics. Although the phenomenon of autoinhibition has been observed in isolates of *P. haloplanktis*, it was not detected in this particular species. Riquelme *et al.* (1996) also reported that this strain of *P. haloplanktis* was able to protect *Argopecten purpuratus* from infection of *V. anguillarum* more effectively at a shorter preincubation time compared to a longer preincubation time. Greater survival was also obtained when larvae were challenged with a lower concentration of pathogenic *V. anguillarum* of  $10^3$  cells/ml than those challenged at  $10^6$  cells/ml. Toxicity may have played a role in reducing *Argopecten purpuratus* protection due to long preincubation times.

#### **2.4.1.6 *Bifidobacterium thermophilum***

The potential of peptidoglycan (PG) derived from *Bifidobacterium thermophilum* to enhance disease resistance in the kuruma prawn (*Penaeus japonicus*) larvae was investigated by oral administration (Itami *et al.* 1998). PG-treated *P. japonicus* larvae displayed significant protection against *Vibrio penaeicida* and white spot syndrome virus, with 81.7% and 95% survival respectively compared to 20% and 15%, respectively in control groups (Itami *et al.* 1998). Cell activating factors in the blood of PG-fed prawns were examined with respect to phagocytosis to aid in the characterisation of the prawns response mechanisms to disease. Itami *et al.* (1998) suggested that oral administration of PG enhanced the production of cell activating factors in the haemocytes, thereby enhancing the phagocytic activity of the granulocytes.

### **2.5 Inhibition of virulence**

Manefield *et al.* (2000) documented on the direct inhibition of virulence factors possessed by some pathogenic bacteria. In their study Manefield *et al.* (2000) suggested that virulence together with luminescence is dependent on an intercellular signalling mechanism for expression. A halogenated furanone, which has been shown to interfere with N-acylated-L-homoserine lactone (AHL) regulated gene expression, was used to inhibit the quorum sensing-regulated luminescence phenotype of two virulent strains of *V. harveyi* (47666-1 & ACMM 642). This resulted in luminescence being inhibited without affecting growth of the bacteria and additionally the signal antagonist also inhibited toxin production. When the larvae of *P. monodon* were challenged *in vivo* with the prawn pathogen *V. harveyi*, toxicity was found to be noticeably lower in the furanone-treated *V. harveyi* than the control.

### **2.6 Bacteriophage Therapy**

Although there have been no reported studies on the therapeutic use of bacteriophages in crustacean disease, recent research on the efficacy of bacteriophages for both the treatment and prophylaxis of bacterial disease has shown successful beginnings in the use of fish culture and is worth mentioning. Bacteriophages and research into their therapeutic use has continued without interruption since 1926 in Eastern Europe,

however this concept was somewhat abandoned in the West largely due to the development of antibiotics. Since the 1980s there is renewed interest involving bacteriophages and their theoretical use in the treatment of bacterial infections. This renewed interest has emerged primarily due to the increase in antibiotic resistant bacteria and the lack of new antibiotics being developed (Cervený *et al.* 2002; Park *et al.* 2000).

The efficacy of phage therapy in the treatment of bacterial infections has been demonstrated by using numerous animal models including *Escherichia coli* in mice and farm animals (Smith and Huggins 1982; Smith and Huggins 1983), *Salmonella typhimurium* in poultry (Berchieri *et al.* 1991), and *Pseudomonas aeruginosa* in mice and guinea-pigs (Soothill 1992; Soothill 1994).

Only recently has the use of bacteriophages as a biological control agent, been trialed in aquatic organisms. Phage therapy has shown promising results in initial experiments used in the treatment of *Pseudomonas plecoglossicida*, the causative agent of bacterial haemorrhagic ascites disease in cultured ayu fish (*Plecoglossus altivelis*) (Park *et al.* 2000; Park and Nakai 2003) and *Lactococcus garvieae*, a pathogen of yellowtail (*Seriola lalandi*), suggesting that bacteriophages could be useful for controlling bacterial infections of fish. From their initial investigations, Park *et al.* (2000) isolated two phages namely PPpW-4 (Podoviridae) and PPpW-3 (Myoviridae) specific to the fish pathogen *Pseudomonas plecoglossicida*. The investigation reported that the oral administration of these phages was effective in treating experimentally infected with *P. plecoglossicida* (Park *et al.* 2000). Park and Nakai (2003) in recent experiments fed *P. plecoglossicida*-impregnated feed ( $10^7$  CFU fish<sup>-1</sup>) and then fed phage-impregnated feed ( $10^7$  PFU fish<sup>-1</sup>) to the fish ayu. Mortalities of fish receiving PPpW-3, PPpW-4, and the combination PPpW-3/W-4 and a control receiving no phage treatment were 53.3, 40.0, 20.0 and 93.3% respectively. Phage impregnated feed was also administered to ayu in a pond where disease occurred naturally. Mortalities of ayu within the pond decreased to around 30% after two weeks. Although this field trial lacked an untreated phage control, results gave notable evidence that the phage treatment was responsible for the successful treatment against *Pseudomonas plecoglossicida*.

Three hours after the administration of phage-impregnated feed, phages were isolated at rate of 90 % from ayu kidneys and were detected in live fish for a considerably long period of 15 days at a prevalence of 7.1%, while the pathogen *P. plecoglossicida* was undetectable in live fish by day 8. Evidence indicating increased survival was phage related was due to the constant decrease in daily mortalities from Days 3 to 15 was roughly parallel to estimated phage carrying rate of live fish of 7.1 %.

Previous research on phage therapy has reported a number of constraints such as the apparent low host specificity of phages which causes complexity in the preparation of therapeutic phage strains, quick appearance of phage-resistant organisms, similar as during chemotherapy, and production of phage-neutralizing antibodies, which inevitably results in ineffectiveness of repeated phage therapy (Park *et al.* 2000; Barrow and Soothill 1997; Smith and Huggins 1982). However, Park and Nakai (2003) suggested in their investigations that these factors did not act as constraints due to a number of findings. Park *et al.* (2000) reported that therapy against *P. plecoglossicida* is composed of only one phage type, which eliminates the complexity in preparation of phage strains. However the high specificity of bacteriophages could on the other hand be advantageous by preserving existing useful bacteria. From the same investigation Park *et al.* (2000) showed that phage resistant strains of *P. plecoglossicida* lacked virulence for ayu, and that no phage resistant bacteria were isolated from dead fish after phage treatment. The phage used, possibly recognised virulence factors as receptors, therefore phage-resistant mutants have lost their receptors limiting them to other undesirable bacteriophages. Another suggested obstacle is that fish may be able to produce phage-neutralizing antibodies leading to the ineffectiveness of repeated phage therapy. There were also no neutralizing antibodies present within fish that repeatedly received phage treatment (Park and Nakai 2003).

Phages have the capability to transfer genetic information between bacterial cells either by generalised or specialised transduction. This transfer of genetic information allows for the possible dispersal of antibacterial resistance or virulence genes among the bacterial population. Consequently phages isolated for therapeutic purposes should not possess the ability of transduction (Cerveny *et al.* 2002). Phages have also been known to confer virulence in bacteria upon infection, in a process known as lysogenic conversion. Waldor and Melakalanos (1996) reported that bacterial virulence factors such as toxins were

often encoded by accessory genetic elements such as bacteriophages. Therefore sequencing the genomes of potentially therapeutic phages may be required to avoid phages that code for such undesirable features (Cervený *et al.* 2002).

From initial investigations the use of bacteriophages for the prophylaxis and treatment of bacterial disease seems attractive. However in order to optimise the beneficial attributes while limiting potential constraints will require further research. With obvious advantages over antibiotics, even if phage therapy is unable to fully replace antibiotics it may have a substantial role in conjunction with antibiotics or where they are no longer effective.

## 2.7 Conclusion

The expansion of penaeid prawn culture into a worldwide industry has resulted in increasing awareness of the importance of disease, especially those caused by infectious agents. The intensive nature of aquaculture has inevitably enhanced the development and transmission of disease in cultured crustaceans (Dobrovsky *et al.* 1988). The most important diseases of cultured penaeid prawns have viral or bacterial aetiologies, with viral disease becoming an important limiting factor for prawn production in recent years (Lightner and Redman 1998; Lo and Kou 1998). Fungal and protozoan agents have been the cause of few important diseases (Lightner and Redman 1998). Viral prevalence and disease, in addition to a wide variety of other infectious agents in marine invertebrates, is said to be exacerbated by stress. Stress may be a direct result of dietary inadequacies, pollution and adverse environmental conditions (Stewart 1991). An example of how stress can affect the outcome of a disease can be illustrated by the infectious nature of WSSV. The principle clinical sign of WSSV is identifiable by white spots in the exoskeleton and epidermis of the diseased prawn. However the presence of these white spots does not mean the condition is terminal and the infected prawn may survive indefinitely, under non-stressful conditions (Lo and Kou 1998).

The penaeid culture industry and research undertaken in associated diseases could prove an important resource when developing culture methods for the mud crab *Scylla serrata*. Although *S. serrata* are said to have relatively few diseases, the aquaculture industry has barely reached a viable commercial status due to the non-existence of a reliable hatchery phase. As well as the obvious advantages of having a consistent

supply of larvae for culture, developing a reliable hatchery phase for *S. serrata* could also prevent the entry of infectious agents associated with wild caught larvae. For example natural WSSV infections have already been identified in natural specimens of *S. serrata* in Taiwan's coastal waters (Lo *et al.* 1996).

A major factor affecting the success of larval production in commercial hatcheries, including that of *S. serrata*, has been the ability to control the occurrence of pathogenic bacteria in intensive larval rearing. Most management practices have directed their attention towards controlling opportunistic pathogens by water disinfection, using clean algal stocks, washing *Artemia* and maintaining hygienic practices (Garriques and Arevalo 1995). The prophylactic use of antibiotics and chemotherapeutics have also been used extensively throughout the industry in an effort to reduce total bacterial numbers and to control pathogenic bacteria. This indiscriminate use of antibiotics has resulted in the increase in bacterial resistance to antibiotics, increase in virulence of some bacteria as well as traces of restricted chemicals identified in the tissues of cultured animals. Consequently, the use of probiotics and their substantial potential for improving hatchery efficiency and creating a sustainable, environmentally friendly industry has attracted considerable interest. Microbes have critical roles in culture conditions due to disease control and water quality being directly related and closely affected by their activity (Jory 1998). The principle probiotic bacteria that have been investigated have been *Vibrio*, *Pseudomonas*, *Bacillus* and several *Lactobacillus* which all vary in their mechanism of action (Gomez-Gil *et al.* 2000).

In order to increase the survival of mud crab larvae, which is said to be responsible for preventing the further expansion of the industry, research is warranted into investigating diseases concerned with mud crab larviculture and methods of control. The potential application and use of probiotics in the hatchery phase of *S. serrata* may play an integral part in establishing a reliable and commercially viable production of mud crab larvae



## CHAPTER 3

### GENERAL METHODS AND MATERIALS

#### 3.1 Source of bacterial strains

The majority of bacterial strains used in this study were obtained from the Microbiology and Immunology collection at James Cook University. Strain numbers and the source of isolates are listed below (table 3.1).

**Table 3.1** The strain numbers and the source of isolation of *V. harveyi* isolates used in this investigation.

Strain Number	Source
ACMM #642	<i>P. monodon</i> larvae
#12	Environmental: seawater
#645	Environmental: seawater
#ISO7	Environmental: seawater
#LLD1	Environmental: seawater

Avirulent and virulent strains of bacterium were isolated from the rearing water of *S. serrata* larvae from tanks kept at the Marine and Aquaculture Research Facility Unit (MARFU). Potential probiotic strains were isolated from tanks that showed a better than average survival and only from sucrose-positive colonies on Thiosulphate-citrate-bile salts-sucrose (TCBS). Virulent strains were isolated from those tanks that showed poor survivals and from colonies that displayed such properties as being haemolytic, sucrose negative on TCBS and luminescent.

#### 3.2 Storage and propagation of stock isolates of bacterial strains

Throughout this study bacterial strains were cultured on luminous (LM) (Reichelt and Baumann 1973) and peptone yeast and sea salt (PYSS) media (appendix 1). The luminous agar (LA) was streaked from a thawed frozen bacterial suspension from a

cryotube. After incubation overnight at 28°C, the culture was examined for purity based on colony morphology before being used for experimental purposes. Liquid cultures were grown in luminous and PYSS broth. Several isolated colonies from an agar plate were suspended in broth before being placed in an orbital incubator at 110 rpm, at a temperature of 28°C until the appropriate cell density was achieved.

All Isolates were prepared for storage 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). Stocks were then stored at -70°C.

### **3.3 Cell density determination of bacterial strains**

Cell density of bacterial strains was estimated by spectrophotometric analysis. Overnight cultures of bacterial isolates were used to inoculate 2 ml of seawater liquid with vitamins (SWLV) to the equivalent turbidity of a McFarland's Column 0.5. The suspension was further diluted to  $10^2$  colony forming units (CFU ml<sup>-1</sup>) in 20 ml aliquots of luminous broth (LB) (appendix 1) and was incubated as stated previously overnight. Two ml disposable cuvettes (Sarstedt, Adelaide, Australia) were used to measure absorbance in an UV mini – 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Optical density readings were taken every two hours to a maximum of 30 hours post induction. Absorbance at 600 nm was correlated quantitatively with a ten-fold series dilution of the LB onto LM for the respective bacterial strain to plot a growth curve. The appropriate cell density of each bacterium was obtained from their respective growth curve.

### **3.4 Bacterial identification**

Environmental bacterial isolates were identified using the keys based from Alsina and Blanch (1994a, b) and further modified by Department of Primary Industries and Fisheries (DPI&F).

### **3.5 Broodstock**

#### **3.5.1 Capture Site**

Female mud crabs were caught from Railway Estate Bridge, Townsville Queensland. Female mud crabs that had a carapace length of at least 150 mm were kept for broodstock (Permit No. PRM02078H). Female crabs obtaining an average of 150 mm have mated and can spawn two to three times without the need for re-mating as spermatophores are stored in the female and can last for long periods (Robertson and Kruger 1994). For this reason, male crabs were not required.

#### **3.5.2 Maintenance**

*S. serrata* broodstock were kept at MARFU at James Cook University. The broodstock were held in 2 x 1000 L circular tanks in which the water was re-circulated. Water was exchanged within the broodstock tanks at a rate of 400 to 500 percent daily. Salinity ranged from 28 to 36 ppt while temperature ranged from 26 to 29°C.

Crabs were fed at a rate 5 to 8 % of their body weight, this amount was adjusted according to their consumption the previous day. The crabs were fed once daily in the evening with mussel, shrimp, squid or fish meat. Uneaten food was removed the following morning.

#### **3.5.3 Spawning**

Once crabs spawned they were disinfected using a 50-80 ppm formalin bath for 6 hours and transferred into a 300-litre indoor tank where they stayed for the remainder of the egg incubation period. Water which was used for the indoor tank was placed on a recirculation system, constantly being mechanically filtered and UV sterilised (254 nm). Water in the tanks was exchanged at a rate of 600-800 % daily. Salinity ranged from 32 to 36 ppt while the temperature was kept at 26 to 29°C. Egg incubation lasted for approximately 12 to 14 days. During this period the berried crabs were not fed in an attempt to reduce the particulate and dissolved organic matter in the tank. Eggs were sampled regularly to monitor embryonic development and to estimate time of hatching. One or two days prior to hatching the crabs were again disinfected in 50 ppm of formalin

for 6 hours. During this procedure the incubation tanks were cleaned in readiness for hatching.

### 3.6 Larval rearing conditions

Larvae were stocked at a density of 25 per 500ml beaker of water. Water prior to being used in the experiment was firstly filtered to 1µm pore size before being disinfected using UV sterilisation. The salinity was adjusted to 25 ppt. The beakers were placed in a water bath with a constant temperature of 28°C with each beaker being gently aerated. If a water exchange was required, then water was exchanged at 20 % per day. Old rotifers were removed by using a 53 µm filter and a wide mouth pipette was used to transfer the larvae from beaker to beaker.

### 3.7 Live feed

During the first two stages of zoea, the larvae were fed on rotifers (*Brachionus rotundiformis*), before being weaned onto *Artemia* nauplii when larvae developed into zoea 3. Rotifers were kept in an 80 L container with seawater and fed algae twice daily. Rotifers were harvested by siphoning (~20 L) them into firstly a 100 µm sieve to collect clumped algae and other floating debris before being filtered through a 53 µm to collect rotifers. After harvesting, the rotifers were thoroughly washed with fresh water. A sample of one millimetre was taken from the collected rotifers and was fixed with formalin before being counted on a Sedgewick-Rafter chamber. Larvae were fed at a rate of 60 rotifers per mL of water.

*Artemia* cysts were hydrated in water for one hour before being placed into a tank and vigorously aerated for 24 hours. Before harvesting, aeration was turned off and the tank was covered with black plastic. After 30 to 60 minutes giving sufficient time for hatched *Artemia* nauplii to settle to the bottom and unhatched cysts to float to the top, *Artemia* nauplii were then siphoned from the bottom of the tank. *Artemia* nauplii were then washed in fresh water before being fed to larvae at a rate of 20 per mL of water.

### 3.8 Larval pathogenicity assays

A short term, larval pathogenicity assay was used to determine effects of bacterial strains on larval survival. Modification of the short-term larval pathogenicity assay developed by Muir (1991) and used by Harris (1998) was used. Two-day-old larvae were used for the experiment. Twenty-five larvae were placed in each of five replicate beakers containing 500 ml of sterile seawater. Larvae of penaeids possess their own internal nutrition when first hatched; therefore, it is not necessary to provide any feed for the first two days of its life cycle. However larvae of mud crabs do not possess their own source of nutrition when hatched; therefore, they rely on an external food source immediately. After hatching, larvae were cultured under normal rearing conditions for two days. Animals were then used for challenge experiments. For this reason, rotifers were also stocked in to beakers at a density of 60/ml.

### 3.9 Quantification of DNA

Purity and concentration of nucleic acid present in DNA extractions was determined by the use of an Ultrospec III Spectrophotometer (Pharmacia, Sydney) with a 1 cm light path length. An aliquot of 60  $\mu$ l was pipetted into a sterile Eppendorf cuvette where wavelengths of 260 nm ( $A_{260}$ ) and 280nm ( $A_{280}$ ) were used to determine absorbance levels. The concentration of nucleic acid was calculated from the optical density (OD) at an absorbance of 260 nm where an OD of 1.0 is equivalent to 50 mg / ml DNA. Purity of the nucleic acid is determined by the ratio between  $A_{260}$  and  $A_{280}$ , where the values 1.8 – 2.0 are an indication of pure preparations.

### 3.10 PCR Amplification

Products were quantified and analysed by electrophoresis. A 1.0% agarose gel was prepared using working stock of Tris Borate – EDTA (TBE) buffer (appendix 6). Ethidium bromide solution at a concentration of 10  $\mu$ m / 100 ml was added to molten gel. A total of 10  $\mu$ l of samples and appropriate markers were mixed on parafilm with 2  $\mu$ l of loading dye before being pipetted into gel wells. Visualisation of products was undertaken through the use of an ultraviolet illuminator.

## CHAPTER 4

### INVESTIGATION OF POSSIBLE VIRULENCE FACTORS ASSOCIATED WITH *Vibrio* sp.

#### 4.1 Introduction

Factors involved in the pathogenicity of bacteria are not well understood despite extensive studies. It is thought that two major characteristics are involved in virulence:

- 1) Ability of microorganisms to attach to and colonise specific sites in the host.
- 2) Formation of substances (toxins, enzymes and other molecules) that cause damage to the host (Brock *et al.* 1994).

There are numerous mechanisms that have been reported to confer virulence. Extracellular products (ECP) including proteases, haemolysins and cytotoxins are just a few of the mechanisms that have been reported to allow bacteria to survive and replicate within the tissues of the host (Montero and Austin 1999; Liu *et al.* 1996).

*Vibrio harveyi*, a luminous Gram-negative bacterium that can be found in a free-living state or as a commensal organism in the enteric contents of marine organisms (Ruby and Neilson 1973; Ruby and Morin 1979), has been reported as being responsible for the disease phenomenon luminescent vibriosis and has been implicated as a major pathogen in an expanding list of cultured organisms. This includes penaeids particularly *P. monodon* in Australia (Pizzuto and Hirst 1995), Thailand (Jiravanichpalsal *et al.* 1994), Philippines (Lavilla-Pitogo *et al.* 1990), and India (Karunasagar *et al.* 1994), as well as posing a serious threat to a number of other economically important cultured marine fish and shellfish. *V. harveyi* has also been implicated as the major cause for inconsistent survival in mud crab larvae (Mann *et al.* 1998).

There is great variation in terms of virulence in strains of *V. harveyi*. *V. harveyi* is generally found to be non-pathogenic and may be found associated with apparently healthy larvae of penaeids (Xianghong *et al.* 2000; Ruangpan *et al.* 1994). *V. harveyi* is often considered to be an opportunistic pathogen when the host animal is compromised (Karunasagar *et al.* 1998); however primary disease caused by highly virulent strains has also been reported. Lavilla-Pitogo *et al.* (1990) reported that virulent strains from the Philippines resulted in 100 % mortality rates in larvae of *P. monodon* at densities as low

as  $10^2$  CFU ml<sup>-1</sup>. Pizzutto and Hirst (1995) on the other hand reported avirulent strains, which produced no mortality at  $10^6$  CFU ml<sup>-1</sup>. As a result of examining 17 virulent and avirulent isolates of *V. harveyi* by protein analysis and M13 DNA fingerprinting Pizzutto and Hirst (1995) found *V. harveyi* to be genetically diverse. Pizzutto and Hirst (1995) suggested that *Vibrio harveyi* acquired virulence factors as a result of the transfer of possible genetically mobile elements.

Bacteriophages have been known for some time to confer virulence to bacteria upon infection, a process known as lysogenic conversion. Bacterial virulence factors such as toxins are often encoded by accessory genetic elements (bacteriophages, plasmids, chromosomal islands and transposons) (Waldor and Melakalanos 1996). Examples include diphtherial toxin of *Corynebacterium diphtheriae*, known to be mediated by a bacteriophage (Rajadhyaksha and Rao 1965), as well as a potent enterotoxin produced by *Vibrio cholerae*, which is encoded by a filamentous bacteriophage (Waldor and Mekalanos 1996).

Haemolysin and siderophore production are also known to enhance the invasive capability of the bacteria, and therefore, are significant virulence factors for some pathogens. Iron is said to be an essential nutrient for growth in virtually all bacteria (Neilands 1981). Reasons limiting bacteria's iron acquisition is the extremely poor solubility of iron at physiological pH and the resulting form of the element being strongly bound to various iron-binding proteins of animal hosts (Nishina *et al.* 1992). Some common mechanisms of iron uptake are by the secretion of a low molecular weight iron chelator known as a siderophore or by directly utilising iron of transferrin or lactoferrin by cell surface receptors (Mckenna 1988). Siderophores are the most common iron uptake mechanism and are reported as being responsible as the major cause of disease in the following pathogens; *Vibrio anguillarum* (Pybus *et al.* 1994; Crosa *et al.* 1980), *Vibrio cholerae* non-01 (Amaro *et al.* 1990), *Shigella flexneri* (Crosa 1989) and *Aeromonas salmonicida* (Hirst *et al.* 1994). Haemolytic activity is yet another strategy for obtaining iron. Lysed blood cells of the host result in an increase in iron availability and subsequent increase in the growth of the bacteria (Black 1996). Haemolysin has been correlated with virulence (Fiore *et al.* 1997; Zhang and Austin 2000). Haemolysin production has been found associated with a number of *Vibrio* species including *V.*

*harveyi* (Zhang and Austin 2000; Munro 2003), *V. parahaemolyticus* (Rodrigues *et al.* 1993), *V. cholerae* and *V. mimicus* (Chakraborty 1997).

In order for bacteria to optimise the utilization of biogenic particles as nutrient sources, they need to attach. When attached they may optimise contact between cell surface enzymes and the substrate allowing for efficient uptake of the soluble by-products of hydrolysis. This particular mechanism may describe the degradation of chitin (Montgomery and Kirchman 1993). It is thought that bacteria, which can specifically attach to chitin particles may gain a nutritional advantage over those that attach to surfaces non-specifically (Montgomery and Kirchman 1994). More importantly chitinases may assist in the invasion of the host ultimately leading to luminous vibriosis.

The primary aim of this work was firstly to isolate a virulent and probiotic bacterial strain from the rearing water of *S. serrata*. An attempt was also made to detect and investigate possible virulence factors that are said to promote virulence, namely; haemolysin, siderophores, chitinases and bacteriophages.



## **4.2 Material and Methods**

### **4.2.1 Sources of bacterial isolates**

Bacterial isolates used for the investigation were the potential virulent and probiotic *Vibrio* sp. referred to as *V. harveyi* LLD1 and *V. alginolyticus* LLB2 respectively. Other *V. harveyi* strains used were 642, 12, ISO 7 and 645 obtained from the Microbiology and Immunology collection at James Cook University.

### **4.2.2 Isolation of probiotic and virulent strains**

A proposed environmental probiotic that was isolated by Nguyen (2002) was used to inoculate beakers at a rate of  $10^5$  CFU ml<sup>-1</sup> as the initial bacterial strain. Guidelines for selecting potential environmental probiotics involved isolating bacteria from beakers that displayed the best larval survival and colonies that were sucrose positive on thiosulphate-citrate-bile salts-sucrose (TCBS). Bacteria were plated using Luminous agar LA agar and TCBS agar. Four percent LA agar was used to inhibit swarming, a characteristic of some marine bacterial isolates.

The virulent strain was isolated from the water in beakers displaying the poorest survival of crab larvae and those colonies that were sucrose negative on TCBS.

### **4.2.3 Growth curve and selection of the probiotic isolate**

A growth curve was done on bacteria isolated for these studies. Growth was measured using a spectrophotometer using the procedure described in section 3.3. The growth curve was used to determine the fastest growing isolate. Data was visualised as linear graphs using the program Excel, and standard errors were also calculated. Once the fastest growing bacterium was identified, this bacterial strain was then passaged by repeating the protocol a number of times until the final isolate was selected as the probiotic strain.

#### 4.2.4 Bacteria identification

The potential probiotic bacteria and virulent strain were identified using the key from Alsina and Blanch (1994a,b) modified by DPI&F. The identification of the virulent bacterial strain was later confirmed by PCR (section 4.3).

#### 4.2.5 Extraction and purification of *Vibrio harveyi* DNA

DNA was extracted from a *Vibrio harveyi* isolate cultured overnight in a broth of seawater with vitamins (appendix 1), using the High Pure PCR Template Preparation Kit (Roche Applied Science) (Roche product no. 1796828) according to the manufacturer's instructions.

#### 4.2.6 PCR amplification using *Vibrio harveyi* specific primers

*V. harveyi* strain was confirmed by the PCR developed by Oakey *et al.* (2003). Primers used in this reaction are specific to a 413-base pair (bp) segment of the 16S rDNA gene. Although this product is specific to *Vibrio harveyi* species, *V. alginolyticus* strains have been known to possess a product of similar size (Oakey *et al.* 2003). Therefore, this PCR method was recommended for the confirmation of suspect *V. harveyi* isolates that were identified using other techniques. Primer sequences used in this procedure were VH1b (5' – aac gag tta tct gaa cct tc – 3') and VH2b (5' – gca gct att aac tac act acc – 3'). Controls used for this PCR were *V. harveyi* Australian Collection of Marine Microorganism (ACCM 642) and *Vibrio harveyi* University of Queensland Microorganism (UQM) for positive controls and *V. parahaemolyticus* for a negative control. The master mix prepared for the PCR is presented in table 4.1.

**Table 4.1** Master mix prepared for the PCR used for the confirmation of *V. harveyi* isolates according to Oakey *et al.* (2003).

	Single Reaction	Final concentration
10× reaction buffer (MBI Fermentas)	5 µl	1×
MgCl <sub>2</sub> (25mM)(MBI Fermentas)	6 µl	3 mM
DNTP (10mM each)	1 µl	200 µM each
Primer VH1b, working strength, 10 pmol / µl)	1 µl	0.2 µM
Primer VH2b, working strength, 10 pmol / µl)	1 µl	0.2 µM
Taq polymerase 1 U / µl (MBI Fermentas)	1 µl	1.0 U / 50 µl
Nuclease-free water	31 µl	
TOTAL	46 µl	

A total of 46 µl of master mix was aliquoted into each test. A negative control was prepared before and after sample preparation by aliquoting 4 µl nuclease-free water. A further 4 µl of each template was added to the remaining PCR tubes.

The amplification was performed in an Eppendorf Mastercycler gradient PCR machine. Amplification reactions were subject to an initial denaturation of 94°C for 2 minutes, 40 cycles of 94°C for 1 minute and 65°C for 1 minute, with a final extension of 72°C for 2 minutes. The expected amplicon size of the 16S rDNA gene is 413 bp. PCR products were visualised by electrophoresis (section 3.10).

#### 4.2.7 Detection of haemolysin activity

Observation of haemolysin production was achieved on dual layered agar plates prepared as stated in appendix 4. Bacteria were streaked onto LM agar plates using isolation streaks and incubated overnight. Single colonies were transferred from LM agar plates onto dual layered plates using sterile toothpicks. Each bacterial strain was inoculated in five well separated locations on the plate. Plates were sealed with parafilm

and incubated at 30°C for 48 hours before both colony diameter and the area of clearance was measured at both 24 and 48 hours.

#### **4.2.8 Detection of chitinase activity**

Chitinase plates used for the observation of chitinase production were prepared by a method described in appendix 3. Streaked plates were sealed with parafilm and incubated at 30°C for 7 days before both colony diameter and the area of clearance was measured.

#### **4.2.9 Detection of siderophore activity**

Chrome azurol S (CAS) agar plates used in this study were prepared by the method of Drew (1992), being a modification of the method described by Schwyn and Neilands (1981) (appendix 2). Streaked plates were sealed with parafilm and incubated at 30°C for 48 hours before both colony diameter and the area of clearance was measured.

#### **4.2.10 Detection of a phage**

The cultures were grown in 100ml aliquots of Peptone yeast and sea salt (PYSS) and incubated at 28°C at 110rpm overnight. The cultures were diluted until O.D. 600 nm was approximately 0.2 ABS. The cultures were then aseptically divided into two equal aliquots. All tubes were induced with 50 ng ml<sup>-1</sup> mitomycin C (Sigma-Aldrich, Castle Hill, NSW, Australia) and further incubated. Optical density readings were then taken periodically through the experiment to a maximum of 24 hours post-induction. A decrease in the O.D. 600 nm relative to the control cultures was taken as a presumptive infection of the bacteriophage

#### **4.2.11 Bacteriophage extraction and concentration**

Strains 642, 12, 645 and LLB2 were cultured in PYSS broth. The broth was inoculated from frozen cultures and incubated for 10 -12 hr on an orbital shaker at 28 °C. Concentration of 50 ng ml<sup>-1</sup> of mitomycin C was added to all strains to induce the isolates into a lytic cycle and were further incubated for 10 -12 hr on an orbital shaker at 28 °C. Cultures were centrifuged at 5000 × g for 10 min to pellet bacterial cells and the supernatant fluids were then filtered through 0.45 µm membranes. Filtered extracts were ultracentrifuged at 200 000 × g for 4 h. Pellets were resuspended in 250 µl of sterile SM buffer (appendix 6). Aliquots of 25 µl were removed for transmission electron microscopy (TEM) and the remaining concentrates were stored at 4 °C.

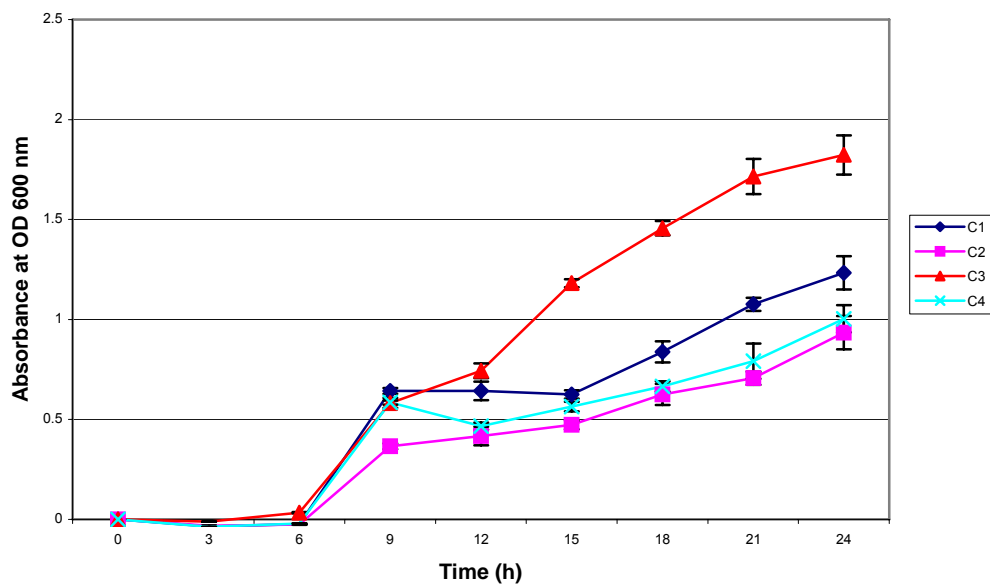
#### **4.2.12 Statistical analysis**

Statistical analysis was performed using SPSS version 10. All data were examined for normality and homogeneity using Q-Q plots and logarithmic transformation was necessary due to high variances. All data was compared using univariant analysis of variance (ANOVA) and comparisons of individual means were performed using least significant differences (LSD) multiple comparisons. All P-values less than or equal to 0.05 were considered as statistically significant.

## 4.3 Results

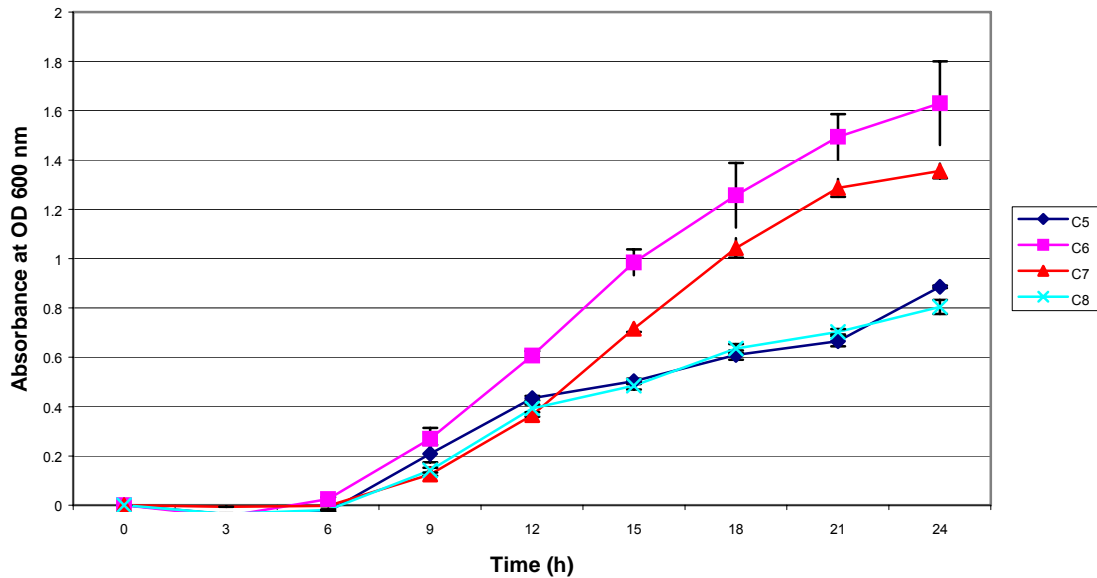
### 4.3.1 Comparative bacterial growth curve of environmental isolates

Isolates C1, C3 and C4 had similar growth rates until hour 9 (figure 4.1) whereafter C3 exhibited a faster growth rate than that of the other three isolates. Isolate C2 had the lowest growth rate for the entire 24 hour period.



**Figure 4.1** Growth curves of isolated presumptive *Vibrios* from the first passage as measured by optical density, at a wavelength of 600 nm. Colonies were isolated from different cultures of mud crab larvae.

In passage 2, isolate C6 had a faster growth rate than the other isolates throughout the 24 hour period (figure 4.2). Isolates C5 and C8 initially had similar growth rates until 12 hours where their growth rates were slower for the remainder of the time.



**Figure 4.2** Growth curves of isolated presumptive *Vibrios* from the second passage as measured by optical density, at a wavelength of 600 nm. Colonies were isolated from different cultures of mud crab larvae.

#### 4.3.2 Identification of suspect virulent and probiotic isolate

A suspected virulent bacterial strain isolated from the larval rearing tanks of *S. serrata*, which had experienced poor survivals was presumptively identified as a *Vibrio harveyi* LLD1 (table 4.2). The isolated potential probiotic bacteria was presumptively identified as *V. alginolyticus* designated LLB2 (table 4.2).

**Table 4.2** Identification of the virulent strain *Vibrio harveyi* and a probiotic strain *Vibrio alginolyticus* using the key devised by Alsina and Blanch's (1994a,b) keys and modified by DPI&F.

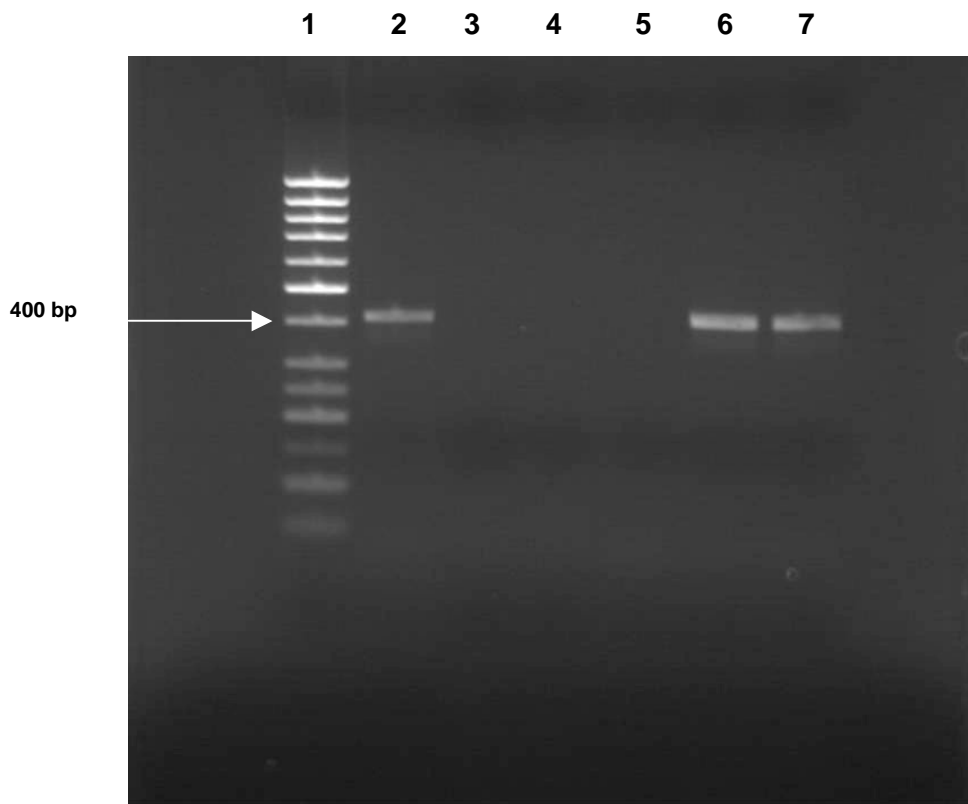
Biochemical test	<i>Vibrio alginolyticus</i> (LLB2)	<i>Vibrio harveyi</i> (LLD1)
Arginine decarboxylase	-	-
Lysine decarboxylase	+	+
Ornithine decarboxylase	+	+
0 % NaCl	-	-
Gelatin	+	+
ONPG	-	-
VP	+	-
Swarm blood agar	+	-
Indole	+	
Sucrose acid		+

+. Positive; -. Negative; |. Inconclusive



### 4.3.3 PCR confirmation of suspected *V. harveyi* using *V. harveyi* specific primers

PCR was successfully performed on DNA extracted from a suspected *Vibrio harveyi* strain, using primers specific to the 16S rDNA gene (Oakey *et al.* 2003). A 413bp amplicon was observed in lane 2, lane 6 and lane 7 (figure 4.3) representing *Vibrio harveyi* LLD1, *Vibrio harveyi* Australian Collection of Marine Microorganism (ACCM 642) (positive control) and *Vibrio harveyi* University of Queensland Microorganism UQM (positive control) respectively.

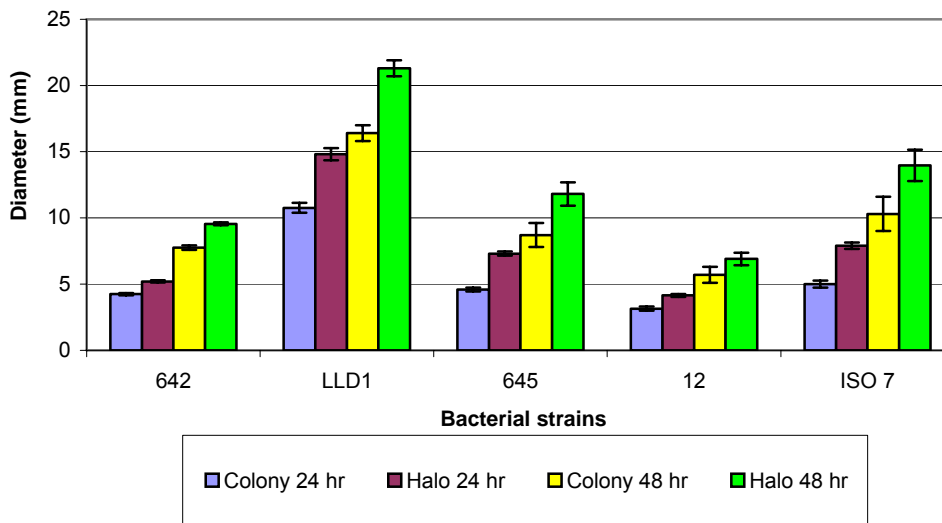


Lane 1: Generuler DNA ladder plus 50bp marker (MBI Fermentas); Lane 2: *V. harveyi* LLD1; Lane 3: (negative control); Lane 4: (negative control); Lane 5: *Vibrio parahaemolyticus* (negative control); Lane 6: *Vibrio harveyi* ACCM (positive control); Lane 7: *Vibrio harveyi* UQM (positive control).

**Figure 4.3** Amplification of the 16SrDNA sequence coding for *Vibrio harveyi* by PCR in a presumptive *Vibrio harveyi* LLD1 isolate.

#### 4.3.4 Haemolysin production on blood agar

*V. harveyi* strain LLD1 had the greatest haemolysin production compared to the other strains, where as *V. harveyi* strain 12 produced the least haemolytic activity (figure 4.4). Colony and halo diameter as a group were significantly different between strains ( $F=70.8, df=4, 100, P<0.0001$  and  $F=148.3, df=4, 100, P<0.0001$ ) respectively (table 4.3). *V. harveyi* strain 645 colony diameter was not significantly different ( $P>0.05$ ), to strains 642 and ISO7, however the halo diameter of the strain 645 was significantly different to strains 642 and ISO 7 ( $P<0.0001, P<0.016$ ) respectively (table 4.3). It should be noted that isolate LLB2 was not screened for haemolysis because of its swarming characteristics. However visually LLB2 appeared to produce the greatest area of haemolysis.



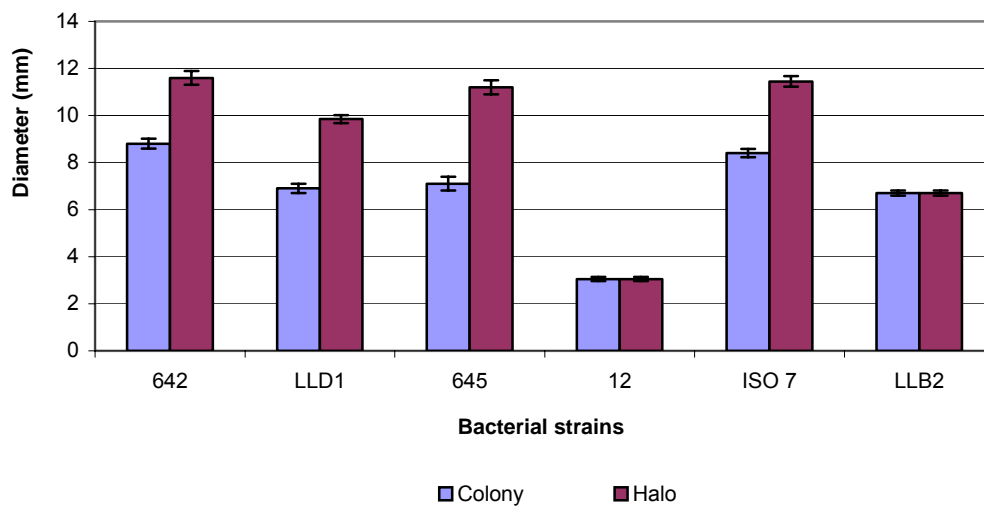
**Figure 4.4** Mean colony diameter and haemolysin diameter at 24 and 48 hours of *Vibrio* strains 642, LLD1, 645, 12 and ISO 7  $\pm$  S.E.

**Table 4.3** A comparison of significance differences (P value) of haemolysin halo diameter and colony diameter (shaded area) of *V. harveyi* strains grown on dual layered blood plates. Haemolysin production at 48 hours was compared using univariate analysis of variance (ANOVA) and comparisons of individual means were performed using LSD multiple comparisons.

	<b>642</b>	<b>LLD1</b>	<b>645</b>	<b>12</b>	<b>ISO 7</b>
<b>642</b>		0.000	0.000	0.001	0.000
<b>LLD1</b>	0.000		0.000	0.000	0.000
<b>645</b>	0.273	0.000		0.000	0.016
<b>12</b>	0.009	0.000	0.000		0.000
<b>ISO 7</b>	0.006	0.000	0.093	0.000	

#### 4.3.5 Chitinase production on chitin agar

On chitin agar, strains 12 and LLB2 were the only isolates that did not produce an evident zone of clearance (figure 4.5). Colony and halo diameter of the above strains were significantly different ( $F=41.5, df=5, 60, P<0.0001$  and  $F=118.0, df=5, 60, P<0.0001$ ) respectively (table 4.4). *V. alginolyticus* strain LLB2 colony diameter was not significantly different ( $P>0.05$ ) to *V. harveyi* strains LLD1 and 645. Strain 642 colony diameter was also not significantly different ( $P>0.05$ ) to that of strain ISO 7. However halo diameter of strain LLB2 was significantly smaller than strains LLD1 and 645 ( $P<0.0001$ ). Strain 642 did not have a significant difference ( $P>0.05$ ) in halo size compared to strains 645 and ISO 7. Strain 645 also did not have a significant difference ( $P>0.05$ ) in halo size to strain ISO 7 ( $P<0.016$ ) (table 4.4).



**Figure 4.5** Mean colony diameter and zone of clearance at seven days resulting from chitinase production of *Vibrio* strains 642, LLD1, 645, 12, ISO 7 and LLB2  $\pm$  S.E.

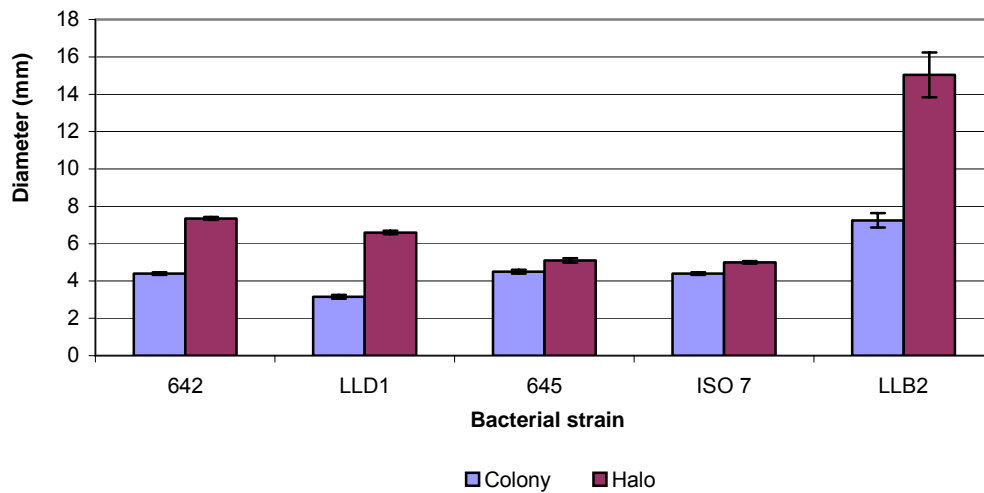
**Table 4.4** A comparison of significance differences (P value) of halo diameter and colony diameter (shaded area) of strains grown on chitin agar. Chitinase production at seven days was compared using univariate analysis of variance (ANOVA) and comparisons of individual means were performed using LSD multiple comparisons.

	642	LLD1	645	12	ISO 7	LLB2
642		0.000	0.190	0.000	0.621	0.000
LLD1	0.000		0.000	0.000	0.000	0.000
645	0.000	0.000		0.000	0.411	0.000
12	0.000	0.000	0.000		0.000	0.000
ISO 7	0.149	0.000	0.000	0.000		0.000
LLB2	0.000	0.467	0.149	0.000	0.000	

#### 4.3.6 Siderophore production on CAS agar

Strain LLB2 had the greatest siderophore production compared to all other strains. Colony and halo diameter of the above strains were significantly different ( $F=22.8, df=4, 50, P<0.0001$  and  $F=173.0, df=4, 50, P<0.0001$ ) respectively (table 4.5). Strain 642 colony diameter was not significantly different ( $P>0.05$ ) to strains 645 and ISO 7. Colony diameter of strain 645 was also not significantly different ( $P>0.05$ ) to strain LLB2. Strain LLD1 halo size was not significantly different ( $P>0.05$ ) to that of strains 642 and 645.

Strain 645 was also halo size was also not significantly different ( $P>0.05$ ) than that of ISO 7 (table 4.5).



**Figure 4.6** Mean colony diameter and siderophore diameter at 48 hours of *Vibrio* strains 642, LLD1, 645, 12, ISO 7 and LLB2  $\pm$  S.E.

**Table 4.5** A comparison of significance differences (P value) of halo diameter and colony diameter (shaded area) of strains grown on CAS agar. Siderophore production at 48 hours was compared using univariate analysis of variance (ANOVA) and comparisons of individual means were performed using LSD multiple comparisons.

	642	LLD1	645	ISO 7	LLB2
642		0.334	0.005	0.004	0.000
LLD1	0.000		0.057	0.043	0.000
645	0.713	0.000		0.897	0.000
ISO 7	1.000	0.000	0.000		0.000
LLB2	0.000	0.000	0.713	0.000	

#### 4.3.7 Detection of bacteriophage infection in bacterial strains

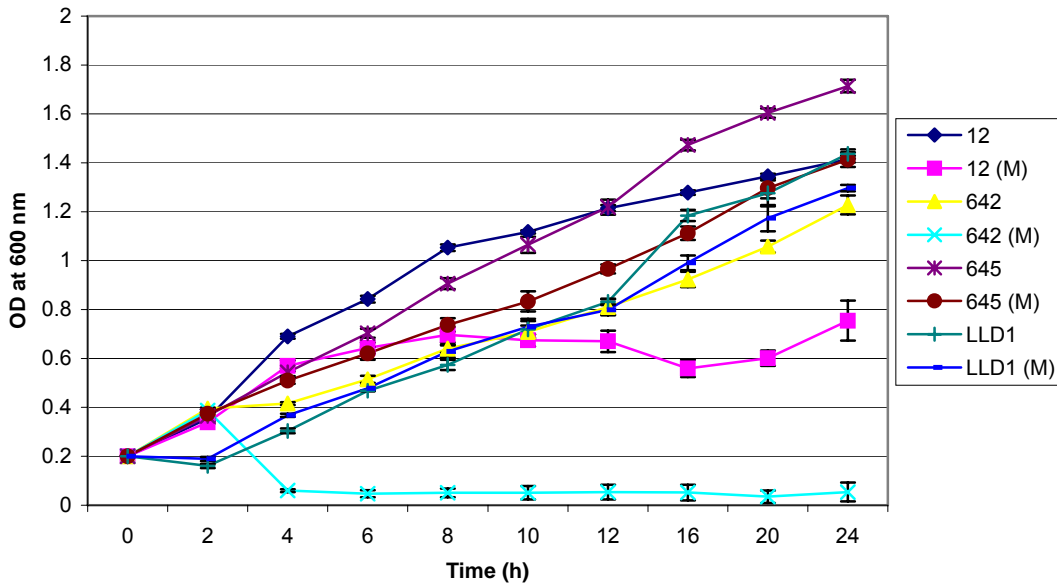
Isolate LLD1 did not show signs of cell lysis (figure 4.7), remaining relatively unaffected by the addition of the mutagen. In figure 4.7a it was apparent that the mitomycin C concentration of 50 ng was too toxic for the isolate 642, resulting in death of the bacterial strain. In figure 4.7b isolate 642 again showed that its growth was severely affected with the addition of the mutagen before slowly increasing in later stages. Supposedly isolate 12 which was uninfected with bacteriophage, displayed dramatic changes in growth as if the strain was infected with a prophage (figure 4.7a and 4.7b). Similar growth curves were obtained from isolate LLB2, which showed a dramatic change in growth as if it was infected with a prophage (figure 4.7b).

#### 4.3.8 Bacteriophage extraction and concentration

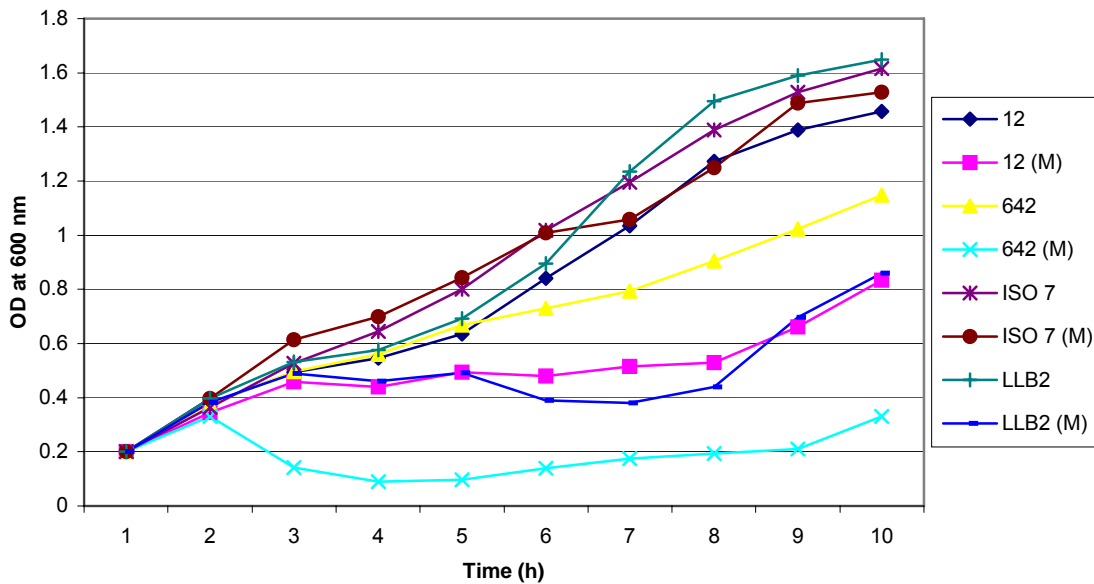
From four isolates, namely: *V. harveyi* 642, 12, ISO7 and *V. alginolyticus* LLB2 that were concentrated through ultracentrifugation and analysed by TEM, 642 was the only isolate to possess bacteriophage (figure 4.8). The phage-like particles from the isolate 642 had an icosahedral-shaped head of 40-50 nm in diameter and a sheathed rigid tail of 15 x 200 nm in length. These phage particles also had collar/neck zone between the capsid and tail. The described bacteriophage was similar to the *Vibrio harveyi* Myovirus Like (VHML) phage characterised by Oakey and Owens (2000). It should be noted that the isolate 642 was the only one found to have visible clearing over 12 – 14 hours after the addition of the mutagen mitomycin C, indicating that prophages present had converted to a lytic cycle. After ultracentrifugation of the isolates, 642 was again the only isolate with a visible pellet.

**Figure 4.7** Growth curve of bacterial strains using an absorbance of 600nm with and without 50 ng of the stressor mitomycin C. In all figures (M) indicates that sample has been stressed with mitomycin C.

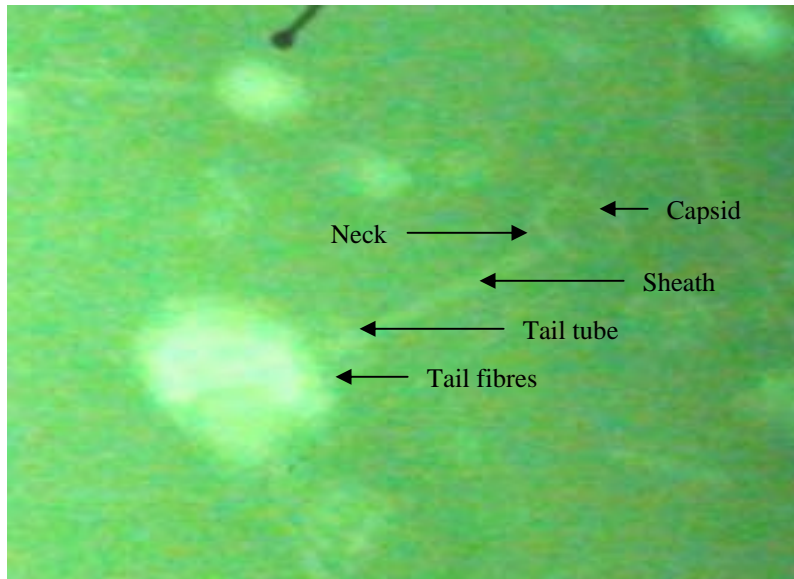
**Figure 4.7a**



**Figure 4.7b**



**Figure 4.8** Electron microscopy of concentrated bacteriophage particles of *V. harveyi* 642



#### 4.4 Discussion

The isolated probiotic and virulent bacterial strains were identified as *V. alginolyticus* referred to as LLB2 and *V. harveyi* referred to as LLD1 respectively, by conventional biochemical tests using the key developed by Alsina and Blanch (1994a,b) and modified by the DPI&F. The identification of environmental *Vibrio* species using biochemical keys has certain difficulties, such as the immense diversity of the taxonomy of Vibrionaceae (Alsina and Blanch 1994a,b), which is under constant revision, and the close phylogenetic relationship that is shared between some of these organisms (Conejero and Hedreyda 2003).

Although biochemical keys can provide the presumptive identification of *Vibrio* sp., in this investigation the identification of the virulent isolate was confirmed using PCR as *Vibrio harveyi*. PCR was performed with the primers specific to the 16S rDNA gene designed by Oakey *et al.* (2003). Despite the 16S rDNA gene being heterogenous in the *Vibrio* genus, this region is said to be conserved in bacteria closely related to *V. harveyi*



(Gauger and Gomez-Chiarri 2002). Conejero and Hedreyda (2003) have also reported the development of a PCR protocol to discriminate *V. harveyi* from other phylogenetically related *Vibrio* species. Primers were designed from a conserved region of the *toxR* gene of *Vibrio harveyi* targeting a 390 bp amplicon. The development of a PCR for the rapid and specific detection of aquatic pathogens such as *V. harveyi*, allows for rapid diagnostics and the implementation of control strategies.

*V. harveyi* strain 642, which has previously shown to possess the bacteriophage VHML by researchers Oakey and Owens (2000), we presented similar results in this investigation. A dramatic change in growth curves (figure 4.7 a,b) was experienced with the addition of mitomycin C indicating the lyses of the bacterial strain, possibly resulting from the abundance of prophage present within the strain. There was strong evidence that isolates LLB2 and 12 possessed a prophage, because initial growth curves (figure 4.7 a, b) displayed signs of cell lysis after the mutagen mitomycin C was introduced. Even though the presumptive phage was absent when viewed by TEM, it is possible that the phage had reattached to remnants of the bacterial cell wall. Therefore, the phage may have either been incorporated into the pellet after ultracentrifugation or filtered from supernatant fluids, therefore resulting in the phage being absent from the TEM sample. When mitomycin C is introduced to cultures which possess a phage, they were expected to lyse due to the bacteriophage changing from its lysogenic phase into a lytic phase thereby rupturing the cells. The stressed strains in which the phage was absent should not show signs of cell death. The lysogeny can be greatly enhanced by a process known as induction, or by the exposure of cells to UV light, or in this instance, the addition of the DNA synthesis inhibitor, mitomycin C (Levy *et al.* 1993; Pennington and Ritchie 1975). However not all prophages can be induced to lyse and as a result some temperate prophage expression occurs only when triggered by natural events (Brock *et al.* 1994). From the mitomycin C induced growth curves, it is likely that strains 12 and LLB2 possessed a prophage. Therefore remnants of the bacterial cell wall may also need to be examined by TEM in an attempt to observe phage presence. Another possibility to consider is that contamination of *V. harveyi* 12 may have occurred. However contamination was assessed previous to any experimentation by visual observation of colony morphology. Single colony type was observed on general marine media (LM) and TCBS agar. TCBS agar facilitates the growth of sucrose positive, alkaline and thiosulphate tolerant *Vibrio* while inhibiting other faecal and environmental bacteria

under these conditions (Kobayashi *et al.* 1963). Although the isolate *V. harveyi* 12 appeared not to be contaminated with other bacterium, there may be a possibility that the naive isolate of *V. harveyi* 12 was contaminated with a *V. harveyi* 12 that had previously been experimentally infected with the phage VHML by Munro *et al.* 2003, from whom the isolate was initially obtained. Indeed the later hypothesis is favoured as Vidgen (2005) had similar problems with these *Vibrio* isolates and had to clone purify before use in the author's experiments.

Various bacteria are able to produce proteins (cytotoxins) that react on the animal cytoplasmic membrane causing cell lysis. The action as a result of these toxins is most easily detected with red blood cells, often called haemolysins (Brock *et al.* 1994). The production of haemolysins can also be a strategy for sequestering iron, by lysing the blood cells of the host, there is an increase in iron availability and increase in growth of the bacteria (Black 1996).

When screening for haemolysin production, strain LLD1 had the greatest haemolysin production (figure 4.4). However isolate LLB2 was unable to be screened for haemolysis because of its swarming characteristics. However, visually, LLB2 produced the greatest area of haemolysis. Haemolysin may importantly enhance the bacterium's invasive capability. It therefore may be a significant virulence factor for some pathogens. Wolk (1975) demonstrated that fish infected with *V. angillarum* exhibited anaemia, which led them to suggest that haemolytic toxin played a role in the pathogenesis of vibrosis. Austin and Austin (1951) also believed the level of production of haemolysin could be used as an indicator of virulence. Munro *et al.* (2003) demonstrated that an increase in halo and colony size of *V. harveyi* correlated with a significant increase in mortality to larvae of *P. monodon*. A total of 100% of *V. harveyi* strains infected with the phage VHML showed an increase in haemolysin activity along with up-regulation and excretion of protein bands, which were very similar to identified toxic subunit bands of haemolysin from strain 642. These strains showed limited production of haemolysin activity prior to the infection with the phage VHML. Munro *et al.* (2003) suggested that the increase in haemolytic activity could result from the up-regulation of the haemolysin gene from VHML, which had been inserted into the genome of the bacterium.

Pelczar *et al.* (1977) suggested that haemolytic strains of pathogenic bacteria were found to be more virulent than non-haemolytic strains of the same species. In some reports both non-pathogenic and pathogenic vibrios produced haemolysin including *V. anguillarum* (Toranzo *et al.* 1983; Inamura 1984) and *V. harveyi* (Zhang and Austin 2000). Zhang and Austin (2000) demonstrated that both pathogenic and non-pathogenic strains of *V. harveyi* produced extracellular products (ECPs) containing haemolysin. Strain VIB645 was found to be the most pathogenic isolate, also contained the highest titre of haemolytic activity. Toranzo *et al.* (1983) showed similar results with all of the pathogenic *V. anguillarum* isolates that produced haemolysin. Although most non-pathogenic *V. anguillarum* displayed haemolytic activity, some of these strains produced a very weak haemolysin or no haemolysin at all. It appears to have a varying effect in virulence. It may be a cytotoxin that is possibly one of several factors that determine pathogenicity. Haemolysin may be one factor that appears to increase pathogenicity, however the ability of the bacterium to attach to and colonise specific sites in the host may ultimately determine if haemolysin becomes a virulence factor. When established within the host, the properties of haemolysin production such as the formation of toxins or enzymes and its ability to lyse cells may than be able to harm the host.

Although sheep blood agar is currently used by many researchers in the study of bacterial diseases, because it shows a general cytolytic response, due to the fundamental difference between mammalian non-nucleated blood cell with haemoglobin compared to that of the non-pigmented, nucleated crustacean haemocyte it may be wrong to suggest that haemolytic production observed using sheep blood agar would indicate haemolytic activity for crustacean haemocytes. Chang *et al.* (2000) were able to determine the haemolytic activity of bacterial isolates by using a newly developed prawn blood agar consisting of tiger prawn haemolymph in medium containing Rose Bengal. Prawn blood agar showed an accurate correlation between haemolytic activity and mortality of injected tiger prawns (*P. monodon*) in comparison to sheep blood agar, which showed a poor correlation. Therefore, agar containing crustacean haemocytes may be an accurate way of determining virulence between potential bacterial pathogens isolated from crustacean culture systems.

Another strategy to overcome these iron limitations within the aquatic environment is by the production of iron-chelating agents known as siderophores. The majority of bacterial

pathogens possess some form of iron uptake system. Siderophores are the most commonly found mechanism. Siderophore production likely enhances the bacterium's invasive capability, therefore it is a significant virulence factor for some pathogens (Neilands 1981). *Vibrio alginolyticus* LLB2 isolate had the greatest siderophore production compared to all other isolates. The *V. harveyi* isolates 642 and LLD1 also produced high levels of siderophore. It was interesting to note that the *V. alginolyticus* strain LLB2 which was isolated for use as an environmental probiotic, had the highest siderophore production considering siderophore complexes were initially investigated as a virulence factor. Siderophore complexes have been previously identified in *V. alginolyticus* strains (Gatesoupe 1997). Although the siderophores possessed by some virulent isolates are thought to aid in invasion of an organism, it may on the other hand prove beneficial by preventing or limiting the establishment of pathogenic bacteria through competitive inhibition for iron.

Most reported incidents of siderophore-mediated iron acquisition systems in pathogens associated with virulence have occurred in vertebrate pathogens. From 92 isolates of *V. harveyi*, Owens *et al.* (1996) showed siderophore production was greater in environmental and fish isolates than in strains isolated from invertebrates. Invertebrates tend to lack the extensive iron-binding compounds such as lactoferrin, transferrin and haemoglobin that vertebrates possess. Therefore the conflict for iron between bacteria and invertebrates does not seem to be as intense as that between bacteria and vertebrates. Because invertebrates lack methods of sequestering iron away from bacteria and the lack of competition between pathogens and invertebrates results in a decreased necessity to maximise siderophore expression (Harris 1993; Owens *et al.* 1996). Harris (1993) working with 16 strains of *V. harveyi*, including two strains that were proven highly virulent to larvae of *P. monodon*, failed to show that enhanced siderophore production was associated with virulence in larvae of *P. monodon*. As a consequence of the low availability of certain elements present in seawater it is not surprising that siderophore activity is high in environmental isolates. Siderophores production might be an important mechanism for *V. harveyi* isolates living in an iron restricted environment like seawater or in association with vertebrates, but not for those pathogenic to invertebrates (Owens *et al.* 1996).

In order for bacteria to optimise the utilization of biogenic particles as nutrient sources they need to attach. When attached they may optimise contact between cell surface enzymes and the substrate allowing for efficient uptake of the soluble by-products of hydrolysis. This particular mechanism has been used to describe the degradation of chitin (Montgomery and Kirchman 1993). Isolates 12 and LLB2 were the only isolates that failed to produce any evident zone of clearance as a result of chitinase production. All other strains including 642, LLD1, 645 and ISO7 produced a distinct zone of clearance where colony to halo ratio was comparably similar amongst isolates (figure 4.5).

Although the roles of chitinolytic mechanisms in *V. harveyi* have not been thoroughly studied, it is thought that bacteria that can specifically attach to chitin particles may gain a nutritional advantage over those that attach to surfaces non-specifically (Montgomery and Kirchman 1994). Presumably chitinases may more importantly aid in the invasion of the host ultimately leading to luminous vibriosis. Lavilla-Pitogo *et al.* (1990) observed colonisation by bacteria occurred specifically on the feeding apparatus and oral cavity of larvae suggesting an oral route of entry for the initiation of infection. This may indicate that this is the preferred location of attachment of *V. harveyi*. Baumann and Schubert (1984) suggested that only chitinase-positive *Vibrio* sp. were found to be pathogenic to animals. In contrast Jirarvanichpacsal *et al.* (1994) demonstrated that a chitinase-negative isolate of *V. harveyi* was more pathogenic towards juvenile *P. monodon* than the chitinase positive isolate. However this experiment is highly suspect, as the methodology may have prevented the advantage of the chitinolytic mechanism by injecting the isolates intramuscularly by-passing the chitinous cuticle. Liu *et al.* (1996) demonstrated that *V. harveyi* isolates had similar chitinase activities, and that chitinase was not crucial for development of infections. However again isolates were injected intramuscularly therefore preventing the chitinolytic mechanism from being any advantage. The experiment carried out in this study indicate that chitinase activity may be associated with virulence, isolates 642 which has previously been shown to be virulent (Munro *et al.* 2003) and the potential virulent bacterium isolated from *S. serrata* rearing tank LLD1, produced the greatest chitinase activity. This is further supported by the fact that *V. harveyi* 12 is relatively non virulent to *P. monodon* (Munro *et al.* 2003) and the potential probiotic *V. alginolyticus* LLB2, which failed to produce any chitinase activity (figure 4.5).

Pizzutto and Hirst (1995) concluded that virulence is associated with mobile genetic elements after demonstrating that virulence in *V. harveyi* was not related phenotypically. Their work was supported by Munro *et al.* (2003), who demonstrated that the bacteriophage VHML was responsible for mediating virulence in 642 by infecting other avirulent strains with the same bacteriophage. A total of 100 % of the infected strains had an up regulation of proteins, resembling identified toxins of 642. Strains infected with the bacteriophage VHML also had significantly higher mortality rates in larvae of *P. monodon* prawns than strains uninfected with the bacteriophage. Another example of how genetically mobile elements determine virulence is given by Crosa *et al.* (1980). They demonstrated that avirulent strains of *Vibrio anguillarum* harboured a specific plasmid class designated pJM1, which was absent in avirulent strains. This particular plasmid was found to encode for an efficient iron-sequestering system. It was linked to the ability of *V. anguillarum* ability to establish infections in marine fish (Tolmasky and Crosa 1984). The fact that the relatively non-pathogenic *V. harveyi* 12 and the proposed environmental probiotic *V. alginolyticus* LLB2 appeared to possess a bacteriophage (figure 5.7 a, b), their non-pathogenicity may simply suggest that the bacteriophage present does not encode for any virulent characteristics.

*V. harveyi* strain 642 which was shown previously to be virulent to larvae of *P. monodon*, consistently displayed high activity in all of the detection assays for virulence. Alternatively *V. harveyi* 12, which has shown to be relatively nonvirulent, consistently displayed less reactive results in the virulent detection assays. However *Vibrio alginolyticus* LLB2, which was isolated as a proposed probiotic, displayed high activity in pathogenicity assays, producing the highest siderophore activity and possibly the highest activity of haemolysis. High haemolysin activity displayed by the potential probiotic LLB2 may not be a favourable trait for a probiotic bacterium to possess. This is due to the ability of the bacteria to lyse cells of the host resulting from haemolysin production. On the other hand, high siderophore activity displayed by LLB2 may prove to be a favourable characteristic for the potential probiotic to possess. The acquisition of free iron by means of siderophore complexes may contribute to the ability of the probiotic bacteria to out-compete pathogenic bacteria.

Although genetic mobile elements appear to be largely responsible for mediating virulence throughout the *Vibrio* genus, and ultimately enhance other virulence factors such as haemolysin activity, generally speaking the possession of specific virulence mechanisms may aid the bacteria to attach and colonise to specific sites, or form toxins that can cause damage for the host. However the absence of one of these traits may lead to the bacterial isolate being non-virulent. As a consequence of the extensive research that has been conducted on virulence factors amongst *Vibrio* sp., we are slowly gaining a better understanding why there is such a large variation in virulence amongst bacteria isolates. The identification of virulence mechanisms in the bacterium *Vibrio harveyi* is of primary importance when developing effective disease control strategies.

## CHAPTER 5

### THE EFFICACY OF A ENVIRONMENTAL PROBIOTIC ON THE LARVAL *Scylla serrata* WHEN CHALLENGED WITH VIRULENT *Vibrio harveyi*.

#### 5.1 Introduction

The intensive nature of aquaculture is conducive to the spread of opportunistic and pathogenistic bacteria, namely species of *Vibrio* and *Pseudomonas* which can cause high mortalities in cultured organisms (Murchelano and Bishop 1975). *Vibrio* spp. frequently appear in high numbers in the natural flora of aquatic systems. Generally found to be non-pathogenic, they may be found associated with apparently healthy larvae of penaeids (Xianghong *et al.* 2000; Ruangpan *et al.* 1994) as well as a commensal organism in the enteric contents of other marine organisms (Ruby and Neilson 1973; O'Brien and Sizemore 1979). Boer *et al.* (1993) and Parenrengi *et al.* (1993) both reported that from a number of *Vibrio* species that *Vibrio harveyi* was considered to be the major contributor to disease of the larvae of the mud crab *S. serrata*. *V. harveyi* has also been implicated as a major pathogen of cultured penaeids in all phases of production throughout south east Asian countries (Lavilla-Pitogo *et al.* 1990).

Antibiotics and other chemical treatments have been extensively used in an attempt to control or suppress the growth of *V. harveyi* in culture systems. Although antibiotics have been widely adapted for the control of diseases, its potential benefits are clearly outweighed by the apparent risks. Risks that have been implicated in the prophylactic use of antimicrobials is the development of resistance in bacterial pathogens, transfer of resistance to other pathogenic bacteria and the wavering efficacy of antibiotic treatment due to resistant bacteria (Moriarty 1999; Spannggard *et al.* 1993). Furthermore, an additional hazard associated with the application of antibiotics in aquaculture is that bacteria pathogenic to humans occur naturally in aquatic systems, which could ultimately pose a serious health risk to humans (Brown 1989).



The use of beneficial bacteria as a control strategy against pathogenic bacteria in intensive aquatic systems while creating an environmentally sustainable industry has attracted increasing attention. Microbes are said to have critical roles in culture conditions, due to disease control and water quality being directly related and closely affected by their activity (Jory 1998). A number of studies have reported on the successful reduction of luminous vibriosis while enhancing growth and production of culture organisms by changing the existing microbial diversity of hatchery and larval rearing tanks by the addition of selected probiotic strains (Harris 1998; Gatesoupe 1999; Garriques and Arevalo 1995).

Environmental probiotics that have been applied to aquatic systems have exhibited a number of diverse mechanisms. Competitive inhibition and/or exclusion is one mechanism that has been manipulated to successfully modify the composition of the bacterial community. The ability of beneficial bacteria to out compete pathogens for a limited resource namely nutrients and adhesion sites, may be considered an ideal mechanism for reducing the direct impact it has on the host organism. *Vibrio alginolyticus* has been a frequently tested as an environmental probiotic. It has been reported as possessing characteristics capable of conferring some degree of protection against disease (Garriques and Arevalo 1995).

The aim of this study was primarily to assess the effectiveness of an environment probiotic isolated as described in chapter 4 on the survival of larvae of *S. serrata* when challenged with virulent *V. harveyi* strains.

## 5.2 Materials and Methods

### 5.2.1 Challenge of larvae of *S. serrata*.

Larval pathogenicity assays were conducted as described in section 3.8.

The experiment was terminated after 48h where larval survival was then calculated.

Bacterial strains used in the experiment were *V. harveyi* ACMM 642, 12 and LLD1, and also the potential probiotic *V. alginolyticus* LLB2. Initial assays were performed to test the suitability of virulent bacteria strains for future challenge experiments against the environmental probiotic *V. alginolyticus* LLB2. The presumptive virulent bacterial strains were inoculated at a cell density of  $10^2$  CFU mL<sup>-1</sup> to  $10^5$  CFU mL<sup>-1</sup>. Experiments in which the environmental probiotic was challenged against the virulent strains, both the environmental and virulent strains were inoculated into beakers at a cell density of  $10^5$  CFU mL<sup>-1</sup>. The equivalent volume of LM broth used in the treatment with the highest concentration, was used as a broth control.

As there was a lack of viable broodstock, a total of 6 experiments were conducted over a ten-month period. Due to physical limitations of housing the crab larvae as well as the possibility of compromising the accuracy of results, only 8 treatments were conducted at any one time with a total of five replicates of 25 larvae per beaker for each treatment. This lead to an experimental matrix that was not balanced in terms of the strains of bacteria tested and the concentrations tested (table 5.1).

**Table 5.1** Number of replicates of *S. serrata* larval pathogenicity assays performed for various bacterial isolates at different concentrations.

Bacterial isolate	Number of replicates			
	Concentration			
	$10^2$	$10^4$	$10^5$	
642	10	20	25	
LLD1	10	30	10	
12	10	10	15	
LLB2			20	
642 + LLB2		10	15	
LLD1 + LLB2		15	15	
Untreated control				30
Broth control				15

### 5.2.2 Screening of vibriostatic activity

Using the method described by Nogami and Maeda (1992) the potential probiotic strain was examined for vibriostatic activity against a pathogenic strain of *V. harveyi* ACMM 642. Both luminous and TCBS agars were used as media for this assay.

The cross-streaking method was used to determine vibriostatic activity of the environmental probiotic LLB2 towards the virulent *V. harveyi* strain ACMM 642. *V. harveyi* strain ACMM 642 was streaked three times on an LM plate. The environmental probiotic LLB2 was then streaked perpendicularly across two of the pre-existing *V. harveyi* ACMM 642 streaks. After 24 hours, vibriostatic activity was determined by the inhibition of growth of *V. harveyi* ACMM 642.

### 5.2.3 Statistical analysis

The normality of all data was determined by using a Q-Q plot and the data met the requirements of the four conditions for parametric tests, those being random selection, normal distribution, homogeneity of variances and an interval level of measurements.

All data from larval pathogenicity assays were accepted as normally distributed so no data was transformed. Analysis between groups was performed using Univariate Analysis of Variance (ANOVA) and a comparison of individual means was performed using LSD multiple comparisons.

### 5.3 Results

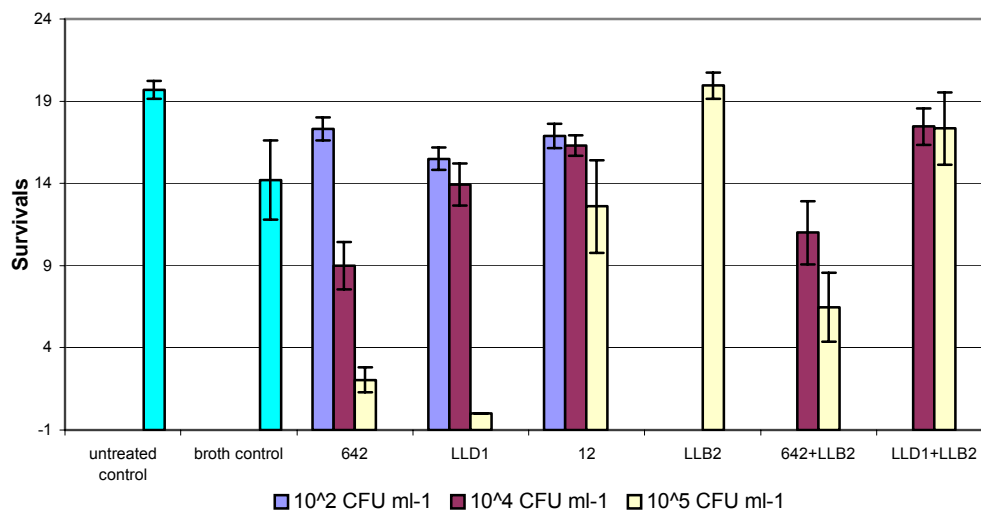
#### 5.3.1 The effectiveness of environmental probiotic on larval of *S. serrata*.

The design of the analysis had the factors of replicate, experiment number, bacterial isolate and concentration versus survival. The replicate effect was removed from the analysis as it was not found to be significant ( $P > 0.05$ ). The experiment, the bacterial isolate and concentration of bacteria were significantly different as a group ( $P < 0.0001$ ) indicating that survival of mud crab larvae varied and was affected by these factors (table 5.2). There was a definite trend between increase in concentrations of bacterial isolates and increase in mortalities across all treatments except *V. harveyi* LLD1 when supplemented with *V. alginolyticus* LLB2. Treatments with *V. alginolyticus* LLB2 had the greatest survival of all tested strains and it was significantly different ( $P < 0.0001$ ) from all strains at a concentration of  $10^5$  CFU ml<sup>-1</sup> except for the untreated control and *V. harveyi* LLD1 supplemented with the environmental probiotic *V. alginolyticus* LLB2 ( $P > 0.05$ ) (table 5.3, figure 5.1). At a concentration of  $10^5$  CFU ml<sup>-1</sup> *V. harveyi* strain LLD1 had the lowest survival of larvae than all other tested strains. Survival in larvae exposed to LLD1 was significantly different ( $P < 0.0001$ ) than all other strains except for *V. harveyi* 642 ( $P > 0.05$ ) (table 5.3). Survivals of treatments that were inoculated with the virulent *V. harveyi* strains LLD2 and 642 at a cell density of  $10^5$  CFU ml<sup>-1</sup> and supplemented with the probiotic *V. alginolyticus* LLB2 had significantly higher survival ( $P > 0.05$ ) than treatments inoculated with single strains of *V. harveyi* LLD2 and 642 respectively.

**Table 5.2** Univariate ANOVA results of the number of the experiment of trial (EXP), the bacterial isolate (ISOLATE) and concentration of bacteria (CONC).

Source	df	F	Sig.
Corrected model	50	698.469	0.000
Intercept	1	8770.666	0.000
EXP	5	65.749	0.000
ISOLATE	7	100.548	0.000
CONC	2	339.453	0.000
EXP*ISOLATE	10	15.029	0.000
EXP*CONC	3	5.905	0.001
ISOLATE*CONC	6	12.155	0.000
EXP*ISOLATE*CONC	4	9.209	0.000
Error	209		
Total	260		
Corrected Total	259		

**Figure 5.1** Mean survival of 25 larvae of *S. serrata* at different concentrations of bacterial treatments  $\pm$  S.E.



**Table 5.3** The significant values and mean differences from the LSD analysis of the survival rates of the larvae between bacterial treatments at a concentration of  $10^5$  CFU ml<sup>-1</sup>.

	Untreated Control	Broth Control	642	LLD1	12	LLB2	642 + LLB2
Broth control	P = 0.008 MD = -0.308*						
642	P = 0.000 MD = -1.052	P = 0.000 MD = -7.44*					
LLD1	P = 0.000 MD = -1.311*	P = 0.000 MD = -1.003*	P = 0.059 MD = -0.259				
12	P = 0.000 MD = -0.465*	P = 0.240 MD = -0.157	P = 0.000 MD = 0.588	P = 0.000 MD = 0.846*			
LLB2	P = 0.974 MD = 0.003	P = 0.013 MD = 0.311*	P = 0.000 MD = 1.056*	P = 0.000 MD = 1.315*	P = 0.000 MD = 0.468*		
642 + LLB2	P = 0.000 MD = -0.723*	P = 0.002 MD = -0.415*	P = 0.006 MD = 0.329*	P = 0.000 MD = 0.588*	P = 0.053 MD = -0.259	P = 0.000 MD = -0.727*	
LLD1 + LLB2	P = 0.317 MD = -0.115	P = 0.149 MD = 0.192	P = 0.000 MD = 0.937*	P = 0.000 MD = 1.196*	P = 0.009 MD = 0.349*	P = 0.341 MD = -0.119	P = 0.000 MD = 0.608*

P. Significance value; MD Mean Difference (I-J); I. Vertical column; J. Horizontal column.

\*. The mean difference is significant at the 0.05 level.

### 5.3.2 Screening of vibriostatic activity

*V. alginolyticus* strain LLB2 did not produce any vibriostatic substances as the growth of ACMM 642 remained uninhibited.

## 5.4 Discussion

The isolated probiotic produced a degree of protection to the larvae of *S. serrata* when challenged with a virulent strain of *V. harveyi*. Larval survivals in treatments that were inoculated with the virulent *V. harveyi* strains LLD2 and 642 at a cell density of  $10^5$  CFU  $\text{ml}^{-1}$  and challenged with the probiotic *V. alginolyticus* LLB2 were significantly higher ( $P > 0.05$ ) than treatments inoculated with single strains of *V. harveyi* LLD2 and 642 respectively (table 5.3). The containers of larvae of *S. serrata* which were inoculated with *V. alginolyticus* LLB2 resulted in the ( $P < 0.0001$ ) highest survival from all other tested strains except for the untreated control ( $P > 0.05$ ) (figure 5.1, table 5.3).

The environmental probiotic identified as *Vibrio alginolyticus* was selected on the basis of having the fastest growth rate (section 4.3.2). The ability of LLB2 to confer a degree of protection may be associated with the strain's fast growing characteristics, allowing the isolate to out-compete coexisting opportunistic and virulent bacteria for a limited resource, such as nutrients and adhesion sites. Another mechanism, which would allow the environmental probiotic to out-compete existing bacteria attributing to the higher survival of *S. serrata* larvae, is through the up take of free iron by means of siderophore complexes. Pybus *et al.* (1994) suggested that iron uptake seemed to be involved in competition among *Vibrio* spp., such siderophore complexes have been identified in *V. alginolyticus* strains (Gatesoupe 1997). Other investigations support this hypothesis, for example are Smith and Davey (1993) suggested *Pseudomonas fluorescens* was able to inhibit the growth of *Aeromonas salmonicida* through the competition for free iron. Gram (1993) and Gram *et al.* (1999) also reported that the inhibitory activity of many *Pseudomonas* strains seemed to be mediated by siderophores. Previous experiments in this study have demonstrated that the environmental probiotic identified as *V. alginolyticus* LLB2, displayed the greatest siderophore production compared to all other tested strains (chapter 4). This could indicate that siderophore expression may have contributed to *V. alginolyticus* LLB2 probiotic effect.

Another mechanism by which beneficial bacteria can displace pathogens by competitive inhibition is through the production of antibacterial substances. Ruangpan *et al.* (1998) reported evidence indicating that *V. alginolyticus* produced antibacterial substances that inhibited the growth of *V. harveyi*. I concluded that *V. alginolyticus* strain LLB2 did not

produce any vibriostatic substances. This was supported by the growth of ACMM 642 in mixed cultures of LLB2. These results are similar to those obtained by Harris (1998) and Garriques and Arevalo (1995) with *V. alginolyticus*, which also failed to show any vibriostatic activity. Although environmental probiotics have been frequently proposed as a friendly alternative to the prophylactic use of synthetic antibiotics, caution is still warranted. Antibacterial substances capable of displacing virulent bacteria also have the ability to promote resistance genes in pathogenic bacteria (Gibson *et al.* 1998). Furthermore Rosser *et al.* (1999) proposed that resistance to naturally occurring antibiotics produced by bacteria, has greater longevity than that of synthetic antibiotics.

An additional mechanism which is also worth mentioning is the use of beneficial bacteria to enhance digestion through the supply of essential enzymes, thereby moderating and promoting the direct uptake of dissolved organic materials (Ukeles and Bishop 1975; Hagiawara *et al.* 1994). Garriques and Arevalo (1995) isolated a strain of *V. alginolyticus* and selected it as a probiotic based on its apparent lack of pathogenicity. Using this probiotic they tested its affect on the larvae of *Penaeus vannamei*. Results showed that groups receiving the probiotic bacteria *V. alginolyticus* had the highest survival and wet weight compared to the antibiotic and control treatments. *V. alginolyticus* was believed to be able to successfully out-compete pathogenic strains of bacteria. The higher wet weight of animals inoculated with the probiotic *V. alginolyticus* suggests that the prophylactic use of antibiotics may interfere or prevent symbiotic microorganisms that are necessary in digestion and nutrient enhancement from establishing themselves (Garriques and Arevalo 1995). These findings are similar to those of Brick (1974), where antibiotics were thought to affect survival of *S. serrata* megalope indirectly by acting on essential symbiotic bacteria.

Although there was no significant difference ( $P>0.05$ ) between the survival of larvae in containers inoculated with the *V. alginolyticus* strain LLB2 and the non-treated control, it may be more advantageous to inoculate larval tanks with a known avirulent bacterium to prevent the establishment of pathogenic bacteria (table 5.3).

The experiment trial was also found to be significantly different as a group ( $P<0.0001$ ). The significant difference obtained may have been due to a number of variables, such



as the varying quality of broodstock and larvae which may have influenced the experiment.

The concentration of bacteria was also significantly different as a group ( $P < 0.0001$ ), indicating that survival of mud crab larvae was affected by this factor (table 5.2).

It was evident that there was a definite trend between increasing concentrations of bacterial isolates and increasing mortalities (figure 5.1). The increase in mortalities could have been related to the increase in volume of broth used to achieve higher bacterial concentrations or directly related to increased bacterial concentrations.

It was apparent that there was a significant difference ( $P < 0.0001$ ) between the untreated control and broth control, where the untreated control had higher survivals than the broth control treatments (table 5.3). The detrimental effect that the broth appeared to have on the larvae of *S. serrata* may be directly related to the broth, or the broth may have simply provided a nutritional source for opportunistic pathogens already present within the system to utilise. Similar results were obtained by Harris and Owens (1999) and Leibovitz (1978) where broth controls appeared to have a detrimental effect on experimental organisms. In both studies it was concluded that the broth acted as a nutritional source allowing pathogenic bacteria to reach lethal concentrations. Leibovitz (1978) stated that in an commercial hatchery environment it would merely mimic the presence of food concentrations, dead or decaying algal foods or larvae, which may intensify the pathogenic effect of both extrinsic and intrinsic microbes.

It was interesting to note that the environmental probiotic and virulent bacterium isolated from larval rearing tank of *S. serrata* were identified as *V. alginolyticus* and *V. harveyi*, respectively. This supports an ongoing inclination to describe *V. alginolyticus* as a probiont, possessing characteristics capable of conferring some degree of protection against disease (Gatesoupe 1999; Austin *et al.* 1995; Garriques and Arevalo 1995). It also supports *V. harveyi* as the major contributor of disease to a number of economically important cultured marine organisms (Lavilla-Pitogo *et al.* 1990; Pass *et al.* 1987; Zhang and Austin 2000).

Although there may be an underlying trend it may be inappropriate to typecast these bacteria. *V. alginolyticus* has been found predominantly associated with healthy larvae stages (Vandenbergh *et al.* 1999); however pathogenic strains of *V. alginolyticus* have

also been reported (Riquelme *et al.* 1996; Vandenberghe *et al.* 1999). Genetic fingerprinting showed a high genetic heterogeneity among *V. alginolyticus* strains suggesting that putative probiotic and pathogenic strains each have specific genotypes. Therefore to avoid the danger of using pathogenic *V. alginolyticus* as a probiont, Vandenberghe *et al.* (1999) suggested the use of only genotypically well-characterised strains. This is similar to *V. harveyi*, which is frequently correlated with disease; they are generally found to be non-pathogenic and may be found associated with apparently healthy larvae of penaeids (Xianghong *et al.* 2000; Ruangpan *et al.* 1994). Within this experiment *V. harveyi* 12 when challenged with larvae of *S. serrata* also exhibited above average survivals (figure 5.1).

The probiotic *V. alginolyticus* and the virulent *V. harveyi* strains were inoculated at the same times when performing the pathogenicity assays. The fact that the probiotic *V. alginolyticus* LLB2 was not given sufficient time to establish itself within the beaker and possibly the digestive tract of the larvae of *S. serrata* before the addition of the virulent *V.harveyi* strains, may have ultimately compromised the effectiveness of the probiotic.

A major factor affecting the success of *S. serrata* larval production in hatcheries has been the ability to control the occurrence of pathogenic bacteria in intensive larval rearing. From this investigation there is evidence that environmental probiotics namely *V. alginolyticus*, may have a beneficial role when applied to larvae of *S. serrata*. This protective effect acts through various modes, such as competitive exclusion. With further investigations, the potential application and use of probiotics in the hatchery phase of *S. serrata* may help in establishing a more sustainable and commercially viable production of mud crab larvae.

**CHAPTER 6****INVESTIGATION INTO AN INTRANUCLEAR BACILLIFORM VIRUS OBSERVED IN  
THE LARVAE OF THE MUD CRAB SCYLLA SERRATA.****6.1 Introduction**

A major constraint preventing the establishment of a reliable and commercially viable hatchery phase of the mud crab *S. serrata*, has been the mass mortality of larvae due to pathogens and disease (Cholik 1999; Yamaguchi 1991). From a number of potential diseases, vibriosis has been implicated as the major cause for inconsistent survival in mud crab larvae (Mann *et al.* 1998). It is believed that these bacteria proliferate in the host digestive tract where the establishment of pathogenic strains enviably leads to mass mortalities (Colorni 1985). Although a number of *Vibrio* species have been shown to be pathogenic to the larvae of *S. serrata*, most have moderate pathogenicity in comparison to that of *V. harveyi* (Boer *et al.* 1993; Parenrengi *et al.* 1993). Mann *et al.* (1998) reported that for the first few days of culture, bacterial communities within the culture system are relatively unstable. Total heterotrophic and presumptive vibrio levels rapidly increase to day 2 of culture, whereby day 3 and 4 approximately, bacterial levels rapidly decline to a lower base level. Therefore, high mortality rates experienced by *S. serrata* larvae at early zoeal stages, are possibly caused by bacterial infection (Dr C. Zeng, James Cook University pers comm., 2002).

Other critical larvae stages where high mortalities rates occur, have been reported as being zoea5 to megalopa, known as the moulting death syndrome (MDS) (Fielder and Heasman 1998; Zeng and Li 1998). The underlying reason for the high mortalities associated with this particular larval phase is unclear; however, nutritional deficiency has been regarded as a leading factor. Zeng and Li (1998) reported that poor nutritional status during zoeal development might play a crucial role in why larvae are unable to pass through this critical stage successfully.

One possibility that has had little mention is that of viral infection. There have been few reports concerning viral diseases within the mud crab *S. serrata*. Additionally investigations into viral infections associated with the larval phase of *S. serrata* have yet to be reported. Anderson and Prior (1992) reported finding a nonoccluded baculovirus in adult mudcrabs

captured from Darwin in the Northern Territory, described it as *Scylla baculovirus* (SBV), now termed *Scylla bacilliform virus*. Intranuclear inclusions were observed in the hepatopancreatic epithelial cells and were not found to be associated with disease or mortality. A prevalence of 27 % was observed from a total of 11 mud crabs sampled. Nguyen (2002) also reported finding a nonoccluded virus, presumptively SBV, from adult *S. serrata* sampled from the Railway Estate, in Townsville. Prevalence was 10% from a total of 20 mud crabs.

Although the mud crab aquaculture industry is very much in its infancy, it may be appropriate to draw analogies between the disease status of mud crabs and that of the well established prawn industry. The rapid expansion and intensive nature of prawn farming inevitably enhanced the development and transmission of disease in cultured crustaceans (Dobrovsky *et al.* 1988). Viral diseases in recent times have become the single most important limiting factor in the production of prawns worldwide (Lightner and Redman 1998; Lo and Kou 1998). Although Anderson and Prior (1992) reported that SBV did not cause clinical disease in adult *S. serrata*, a wide variety of other infectious agents in marine invertebrates, are exacerbated by stress.

The objective of this study was to investigate possible bacterial or viral infections associated with the larval stages of *S. serrata* by conventional histological techniques.

## **6.2 Material and Methods**

### **6.2.1 Sampling procedure**

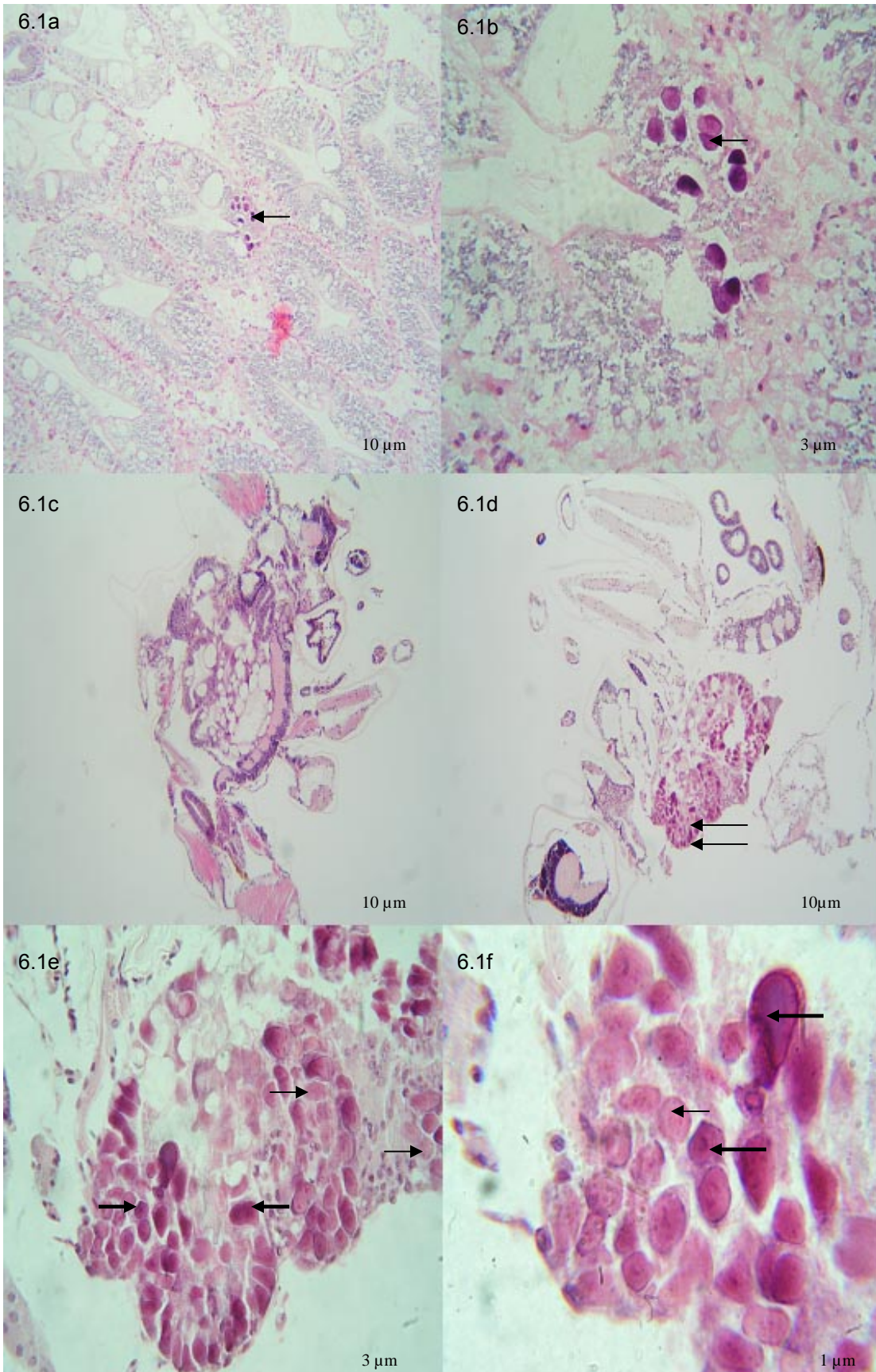
The majority of batches sampled were larvae reared at MAUFU at James Cook University. Staff from the QDPI&F at Bribie Island and Darwin also supplied samples and sent them for histological examination. Sampling took place every second day from the larval stages of zoea<sub>1</sub> to crablet. Two samples were acquired throughout sampling. One lot of specimens were fixed immediately following removal from the water in 10% formalin or Davidson's fixative (Humason 1972) for histology purposes and the other was stored in 70% ethanol for PCR. Volume to tissue ratio when applying the fixative to the specimens was approximately 10 x volume of fixative to tissue. A total of 25 adult mud crabs were also sampled from Railway Estate, Townsville. Their hepatopancrei were removed before being fixed using the same procedure as above.

### **6.2.2 Histological procedures**

Crab larvae were fixed in Davidson's fixative for 24 hours before being transferred to 70 % ethanol or 10% formalin. A few drops of eosin were added to the ethanol in order to give the larvae a pink appearance, assisting in later processing by allowing easy visualisation of larvae. The larvae were then placed into the wells of a 24 well culture plate (Corning, New York, USA) where 2 % agar was used to overlay the larvae. After setting, the agar moulds were removed from the 24 well culture plate and transferred to 10% (v/v) phosphate buffered formalin, where they remained for 24 hours. Agar moulds, which contained the larvae, were then processed by conventional histological methods (Bell and Lightner 1988). Tissues were sectioned at 4 - 5 µm in thickness and stained with haematoxylin and eosin. Examination of the processed samples was performed using standard light microscopy.

### 6.3 Results

One adult *S. serrata* possessed 12 cells in one hepatopancreatic tubule that showed nuclear pathology. Adjacent cells appeared normal (figure 6.1a). Infected nuclear were significantly hypertrophied, either irregular or round in shape (figure 6.1b). The nucleus was completely occupied by basophilic inclusions. Viral inclusions were normally distributed in a dense cluster (figure 6.1e). Nuclear pathology of infected cells was contained to the hepatopancreas of larvae of *S. serrata* (figure 6.1c,d). Early infected cells displayed slight hypertrophy of the nucleus, a pale nucleolus, a band of strongly basophilic chromatin with sporadic fine eosinophilic granules scattered throughout the nucleus. Nuclear pathology of advanced infected cells consisted of hypertrophied nucleus with inclusion becoming a darker purple-red colour (figure 6.1f). The nucleolus was central with the band of basophilic chromatin becoming more distinctive (figure 6.1f).



**Figure 6.1** Light micrograph of amorphous, basophilic intranuclear inclusions in adult *S. serrata* hepatopancreatic tubule epithelial cells (a, b). Infected nuclei (arrows) were markedly hypertrophic with a thin marginal band of basophilic chromatin. Light micrograph of uninfected larvae of *S. serrata* (c). Light micrograph of basophilic intranuclear inclusions in larvae of *S. serrata* (d,e,f). Infected nuclei are markedly hypertrophic. Advanced (large arrow) and early (small arrow) nuclear changes are present. All H&E stain.

From 15 batches of larvae sampled from MARFU at James Cook University and DPI&F laboratories, only two were found to carry IBV presumptively SBV. Both batches that tested positive were reared at James Cook University MARFU (table 6.1). In the two infected batches, the percentage of larvae infected was 32.26% and 52.7%, respectively (table 6.2). From twenty-five crabs sampled from Railway Estate, only two crabs possess IBV (table 6.3), indicating a prevalence of 8% in wild populations of mud crab from the area. The intensity of tubules infected with a IBV infection within the adult crabs was 12.95% (table 6.4).

**Table 6.1** Prevalence of SBV in batches of *S. serrata* larvae

No. of batches sampled	No. of infected batches	Prevalence of batches infected (%)	95% Confidence limits (%)
15	2	13.33	2 - 40

**Table 6.2** Prevalence of SBV in cells within infected batches of *S. serrata* larvae

Batches	No. of infected larvae	No. of larvae examined	Prevalence of larvae infected (%)	95% Confidence limits (%)
1	10	31	32.26	17 – 51
2	12	23	52.17	31 – 73

**Table 6.3** Number of adult crabs sampled that were infected with SBV.

No. of crabs sampled	No. of infected Crabs	Prevalence of crabs infected (%)	95% Confidence limits (%)
25	2	8	0.1 - 26



**Table 6.4** Intensity of SBV infection within an infected adult crab.

No. of positive tubules	No. of tubules examined	Prevalence of tubules infected (%)	95% Confidence limits (%)
50	386	12.95	9 - 16

## 6.4 Discussion

Visual observations by light microscopy were similar to the initial surveys of the intranuclear bacilliform SBV in *S. serrata* (Anderson and Prior 1992). Nuclear pathology of infected cells was limited to the hepatopancreatic region of crabs. The characteristic signs of the IBV was nuclear hypertrophy (figure 6.1e,f). This description is also similar to that of the intranuclear bacilliform viruses Baculo-A and Baculo-PP in blue crabs (Johnson 1977; Jonhson and Lightner 1988).

The prevalence of SBV in wild populations of *S. serrata* was 8% from the Railway Estate region within Townsville (table 6.1). This was similar to that documented by Nguyen (2002) who observed a prevalence of 10 % in 20 crabs. From larvae of *S. serrata*, 2 of 12 batches (16%) of larvae were infected with the SBV. Prevalence rates of SBV infected batches were within the 95 % confidence limits of infection rates of adult crabs. Cultured mud crabs could possibly have higher infection rates than that of the wild populations due to the intensive nature of the culture system, inevitably enhancing the development and transmission of the disease. Stewart (1991) determined that infections occurring frequently in larval stages are enhanced by stress induced by overcrowding, abnormal temperatures and pollutants. For a more accurate indication of prevalence of crabs infected with SBV, the sampling of a greater number of crabs may be required.

The detection of SBV in *S. serrata* in Darwin by researchers Anderson and Prior (1992) and now recently by its observations of the SBV in mud crabs located in the Townsville region, suggests this intranuclear bacilliform virus is probably present along Australia's vast coastline, at least from Darwin to Townsville.

It was difficult to determine the effects of SBV on larvae. Even though there appeared to be a reduction in survival within some infected batches, many uninfected batches displayed similar unexplained declines in survivals. Histological findings revealed the absence of cell necrosis

within tissues infected with SBV, suggesting the virus does not cause widespread necrosis. Anderson and Prior (1992) also reported infections of SBV were not associated directly with disease or mortality in the mud crab. The occurrence of a virus does not necessarily mean that disease will result. Many viruses are suppressed by the host's defences and some have never been associated with any pathological condition (Stewart 1991). Although it appears that SBV does not appear to cause disease, infected cells are undoubtedly unable to perform normal functions. Couch (1983) documented that heavy infections of Baculo-A presence within the blue crab *Callinectes sapidus* did not appear to affect the crabs. He associated this absence of disease due to the constant renewal of epithelial cells of hepatopancreas. Unlike Baculo A, which infects *Callinectes sapidus*, a bacilliiform virus was fatal to *Carcinus mediterraneus* (Pappalardo and Bonami 1979).

It is difficult to suggest what the commercial importance SBV would have on mud crab aquaculture if its culture was to become established within Australia. Extensive techniques such as those currently employed by overseas countries such as rearing mud crabs in earthen ponds and applying only supplement as food are likely to be adopted for growout purposes within Australia. However in suggesting this, a much more intensive approach would be applied for rearing of mud crab larvae. Until mud crab aquaculture has become established within Australia, where the rapid expansion and the intensive culturing may enhance the development and transmission of this disease, it would be premature to suggest that SBV would not be of any economic importance to mud crab culture.

## CHAPTER 7

### THE USE OF POLYMERASE CHAIN REACTION FOR THE DETECTION OF *Scylla* BACILLIFORM VIRUS IN *Scylla serrata*

#### 7.1 Introduction

The intranuclear bacilliform virus WSSV, which first appeared in northeast Asia in 1992-93, has emerged as one of the prawn industries greatest threats where infection can reach a cumulative mortality of up to 100 % within 3 to 10 days (Cai *et al.* 1995; Lightner 1996). As well as being a major economic threat to cultured prawns, WSSV has also been found to infect other marine and freshwater crustaceans including crayfish and crabs, thus becoming a major threat to marine ecology (Flegel *et al.* 1997).

Previously, WSSV was an unassigned member of the *family Baculoviridae*, due its morphological similarities, such as its rod shape and enveloped capsid (Takahashi *et al.* 1994; Durand *et al.* 1997). Phylogenetic analysis of WSSV genes has indicated that it shares no close relationship with any of the baculoviruses. In fact it is said to be distinct from previously identified DNA viruses. WSSV is now a representative of a new virus genus known as *Whispovirus* confirmed by the International Committee for the Taxonomy of Viruses (ICTV) as belonging to the *family Nimaviridae* (Van Hulten *et al.* 2001).

However, there may be determinants that suggest some relatedness between WSSV with other bacilliform viruses. These suggestions are supported by a number of observations, such as SBV sharing similar morphological traits with that of WSSV. Similarly unconfirmed reports in Ecuador and Spain suggested WSSV was detected by PCR in *Cherax quadricarinatus* (A/Prof. L. Owens, James Cook University pers comm., 2003). It was suggested WSSV primers were amplifying another nonoccluded IBV known as Cherax bacilliform virus (CBV), which has been reported infecting *C. quadricarinatus* in Australia (Anderson and Prior 1992). It was from this conjecture that Claydon *et al.* (2004) attempted using PCR primers developed by Lo *et al.* (1996) to amplify CBV. Although positive PCR results were attained, the results were questionable because DNA from putative virus-free crayfish also amplified. Chapman *et al.* (2004) after conducting an extensive survey to assess the presence of

WSSV in stocks of commercially significant penaeid species, also produced false positive results while using primers developed by Lo *et al.* (1996). These findings support an incident which occurred in Australia in late 2000. PCR findings indicated a possible infection of WSSV in prawns within aquaculture facilities at the Northern Territory University and crabs within the Darwin Aquaculture Centre. After the slaughter of all crustaceans in the two facilities, the Consultative Committee on Emergency Animal Diseases (CCEAD) agreed to conduct a survey of wild crustaceans to determine if in fact WSSV was present in Australia. Similarly positive reactions were obtained through PCR but when retested and investigated by bioassay, histology and *in situ* hybridisation, all retested negative (Bernoth 2002). It is possible that the native IBV of crustaceans had enough similarity with WSSV to cause false positives from these crustaceans. This is the logic that lead to this “shotgun” approach to obtaining genetic sequence of SBV.

This investigation will involve trying to amplify SBV using primers designed from the protein binding gene of WSSV and not WSSV primers developed from Lo *et al.* (1996). The amino acid profile of the protein binding region has been reported to be analogous to that of insect baculoviruses (Yang *et al.* 2001). Not only will the use of these primers allow us the investigation of the relationship between WSSV and SBV, but it will also allow us to examine the relationship between SBV and insect baculoviruses. If PCR products can be amplified, an attempt will be made to sequence the DNA through plasmid cloning. If successful this will also allow for the nucleotide comparison between SBV and other IBVs.

## 7.2 Material and Methods

### 7.2.1 Viral extraction and DNA purification

DNA extraction was performed on crab larvae that were identified as being heavily infected with the *Scylla* bacilliform virus via conventional histological techniques (chapter 6). Infected larvae were from the first batch, which were reared at James Cook University. DNA was extracted from the tissues using the High Pure PCR Template Preparation Kit (Roche Applied Science) according to the manufacturer's instructions. Purified DNA was quantified by spectrophotometry (section 3.9).

### 7.2.2 Testing DNA template integrity using decapod specific primers

Decapod specific primers 143F/143R were used to verify that DNA was successfully extracted from the infected crab larvae tissue (Lo *et al.* 1996). The decapod primer sequences were 143F, 5' –TGC-CTT-ATC-AGC-TAC-GTT-CGA-TTG-TAG-3' and 145R, 5'-TTC-AGA-CGT-TTT-GCA-ACC-ATA-CTT-CCC-3'. A total of 49  $\mu$ l of master mix was aliquoted for each test together with 1  $\mu$ l of DNA template (table 7.1).

**Table 7.1** Master mix prepared for PCR.

	Single Reaction	Final concentration
10 $\times$ reaction buffer with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (MBI Fermentas)	5 $\mu$ l	1 $\times$
MgCl <sub>2</sub> (25mM)(MBI Fermentas)	3 $\mu$ l	1.5 mM
DNTP (2mM each)	5 $\mu$ l	200 $\mu$ M each
Primer 143F, (100 $\mu$ m stock)	1 $\mu$ l	0.2 $\mu$ M
Primer 143R, (100 $\mu$ m stock)	1 $\mu$ l	0.2 $\mu$ M
Taq polymerase 1 U / $\mu$ l (MBI Fermentas)	1 $\mu$ l	1.0 U / 50 $\mu$ l
Nuclease-free water	34 $\mu$ l	
TOTAL	49 $\mu$ l	

The amplification was performed in an Eppendorf Mastercycler gradient with PCR profile consisting of one cycle of 94°C for 4 minutes followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. The expected amplicon size is 848 bp.

A tissue extract from *Penaeus monodon* was used as a positive control and two negative controls were used both containing no DNA template.

### 7.2.3 PCR amplification using primers designed from the White Spot Specific Genome

Two primers were designed from the protein binding gene from the White Spot Syndrome virus genome. The sequences for the first binding protein (BP) primer were BP1F 5' - ATCAATGTTCTGCGTGCAATTT-3' and BP1R, 5'-GAACGGCGACGGACAGT-3' and the second was BP2F 5' – AAAAATGGTTGCCCGAAGCTC and BPR2 5' – TGAGGAACGGCGACGGACAG-3'. A total of 49 µl of master mix was aliquoted for each test together with 1 µl of DNA template (Table 7.2).

**Table 7.2** Master mix prepared for PCR.

	Single Reaction	Final concentration
10× reaction buffer with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (MBI Fermentas)	5 µl	1×
MgCl <sub>2</sub> (25mM)(MBI Fermentas)	4 µl	1.5 mM
DNTP (2mM each)	5 µl	200 µM each
Primer BPF, (100 µm stock)	0.25 µl	0.2 µM
Primer BPR, (100µm stock)	0.25 µl	0.2 µM
Taq polymerase 1 U / µl (MBI Fermentas)	1 µl	1.0 U / 50 µl
Nuclease-free water	34 µl	
TOTAL	49 µl	

The amplification was performed in an Eppendorf Mastercycler gradient with PCR profile consisting of one cycle of 94°C for 4 minutes followed by 30 cycles of 94°C for 1 minute, a

gradient of 56°C ± 5°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. The amplicon size of 196 bp for BP1 and 150 bp for BP2 was expected.

#### **7.2.4 Analysis of products**

PCR products were visualised by electrophoresis (section 3.10).

#### **7.2.5 PCR product purification**

Products that were chosen for purification were run on a gel using 30µl of each reaction mixture. The gel was visualised under shortwave ultraviolet light before excising DNA fragments of the appropriate size using a scalpel blade. DNA was purified from the PCR gel using Promega's Wizard SV Gel and PCR clean-up system according to the manufacturer's instructions.

#### **7.2.6 Cloning of PCR products**

PCR products were cloned into bacterial plasmids, using pGEM-T Easy Vector System (Promega, Madison, USA) according to the manufacturer's instructions. From the blue/white screening process relevant bacterial colonies that appeared to possess plasmids with DNA inserts were subcultured into Luria bertani broth (LBB) (appendix 1) with ampicillin before being frozen at -80°C.

#### **7.2.7 Isolation and confirmation of plasmid DNA**

Plasmid DNA was then purified from cultures of *E. coli* according to the protocol of Wizard plus SV minipreps (Promega). To ensure plasmids contained the appropriate DNA insert a PCR was conducted on purified plasmid DNA using M13 primers. Primer sequences were M13 5'-TGAAAACGACGGCCAGT-3', and M13 5'-AGCGGATAACAATTTTCACAC-3'. A total of 49 µl of master mix was aliquoted for each test together with 1 µl of DNA template (table 7.3).

**Table 7.3** Master mix prepared for PCR.

	Single Reaction	Final concentration
10× reaction buffer with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (MBI Fermentas)	5 µl	1×
MgCl <sub>2</sub> (25mM)(MBI Fermentas)	4 µl	1.5 mM
DNTP (2mM each)	5 µl	200 µM each
Primer M13F, (100 µM stock)	0.25 µl	0.2 µM
Primer M13R, (100µM stock)	0.25 µl	0.2 µM
Taq polymerase 1 U / µl (MBI Fermentas)	1 µl	1.0 U / 50 µl
Nuclease-free water	32.5 µl	
TOTAL	49 µl	

The amplification was performed in an Eppendorf Mastercycler gradient with PCR profile consisting of one cycle of 94°C for 4 minutes followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. The amplicon size of insert length plus 254 bp is expected.

### 7.2.8 DNA sequencing

DNA sequencing was carried out using the Beckman Coulter CEQ DTCS Quick Start Kit, according to the manufacturer's instructions. After vacuum drying, samples were taken on ice to the Advanced Analytical Center at James Cook University in Townsville for subsequent sequencing. Two forward and two reverse sequence reactions from two 150 bp clones (eight sequences) and two forward and two reverse sequence reactions from three 350 bp clones were aligned using Sequencher software (Genecodes Corporation), to give an overall consensus sequence. Consensus sequences obtained were compared to available databases using Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

### 7.2.9 *In situ* hybridisation of histology slides

The *in situ* hybridisation of histology slides used was based on that described by Rolighed & Lindeberg (1996) with some modifications.



### 7.2.9.1 Construction of labelled probes

A probe was prepared using PCR products that were observed at the region of the expected amplicon of approximately 150 bp when using BP2 specific primers. Products were cloned into bacterial plasmids using pGEM-T Easy Vector before purifying cultures of *E. coli* according to protocol of Wizard plus SV minipreps (Promega, Madison, USA).

Dig labelled probe<sub>150</sub> was constructed by direct incorporation of digoxigenin-11-dUTP (Boehringer Mannheim, Australia) and primers into the product during PCR amplification. Primers BP2 5' – AAAAATGGTTGCCCGAAGCTC and BP2 5' – TGAGGAACGGCGACGGACAG-3' were incorporated with complementary sequences into the vector.

Direct labelling of the probe was done using a dNTP mix that consisted of 10mM dATP, 10mM dCTP, 10mM dGTP, 9.5mM dTTP and 0.5mM dig-11-dUTP, giving a final ratio of 19:1 for dig-11-dUTP:dTTP. This was then used as a stock dNTP mix for PCR as previously described.

A total of 24  $\mu$ l of master mix was aliquot for each test together with 1 ng of purified DNA template (table 7.4).

**Table 7.4** Master mix prepared for PCR.

	Single Reaction	Final concentration
10 $\times$ reaction buffer with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (MBI Fermentas)	2.5 $\mu$ l	1 $\times$
MgCl <sub>2</sub> (25mM)(MBI Fermentas)	2.5 $\mu$ l	2.5 mM
DNTP's mix (10 $\mu$ M each)	0.5 $\mu$ l	200 $\mu$ M each
Primer BPF, (100 $\mu$ M stock)	0.25 $\mu$ l	0.2 $\mu$ M
Primer BPR, (100 $\mu$ M stock)	0.25 $\mu$ l	0.2 $\mu$ M
Taq polymerase 5 U / $\mu$ l (MBI Fermentas)	0.3 $\mu$ l	0.3 U / 50 $\mu$ l
Nuclease-free water	34 $\mu$ l	
TOTAL	24 $\mu$ l	

The amplification was performed in an Eppendorf Mastercycler gradient with PCR profile consisting of one cycle of 94°C for 4 minutes followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes.

Following hybridisation treatments were applied to the sections

1. Full protocol – probe<sub>150</sub> and anti-dig both applied to the sections
2. No probe – probe was not applied to the sections

With treatments in which probe was absent the sections were incubated as per normal in the relevant buffer.

### 7.2.9.2 Preparation of tissue sections

Larvae of *S. serrata* were fixed in 70% ethanol before being embedded in paraffin. Sections were cut from histology blocks from larvae of *Scylla serrata*, which were determined to be infected with SBV by histology. Tissues were sectioned at 5 µm in thickness before being placed on positively charged slides (Menzel-Glaser, Braunschweig, Germany). Tissue sections were deparaffinized through xylene, washed several times in ethanol and rehydrated in distilled water.

Slides were then treated with a working solution of 100µg/ml proteinase K in TES buffer (appendix 7). Each section was treated with 20-30µl of the working solution of proteinase K. During the proteolytic digestion, coverslips were placed on sections and placed in a humid chamber at 37°C for 5 minutes.

### 7.2.9.3 Hybridisation

After treatment with proteinase K, coverslips were removed and the digestion was blocked by the addition of glycine to a final concentration of 0.2% w/v. Sections were then fixed with 0.4% formaldehyde for 5 minutes at 4°C followed by immersing of the sections in distilled water for 5 minutes. The sections were then allowed to drip and air dry for 5 minutes. The probe<sub>150</sub> was applied at a concentration of 1 ng µl<sup>-1</sup> in hybridisation buffer (appendix 6). A negative control was prepared by covering one section from each block with a blind probe cocktail. Gene Frames (ABGene, Brighton, United Kingdom) were

placed over each section and DNA was denatured by placing the slides in an Eppendorf Master Cycler at 95°C for 8 minutes. Slides were then quenched on ice for 1 minute before being placed in a humid chamber and incubated overnight at 37°C. Coverslips were removed after incubation and sections were washed as follows; 2 × 5 minutes in 2 × sodium citrate (SSC) at 20°C and 1 × 10 minutes in 0.1 × SSC at 37°C (appendix 7).

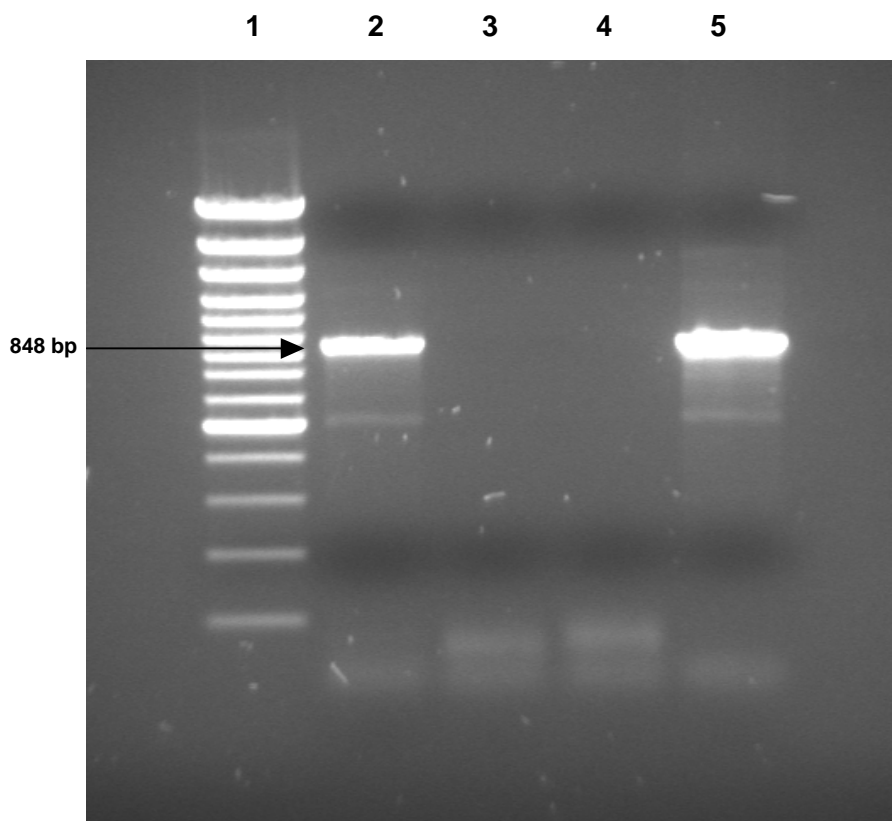
#### **7.2.9.4 Detection of probe hybridisation**

After being washed, each section was dipped into buffer 1 (appendix 5). Following this procedure each section was treated with 20-40µl of buffer 2 (appendix 5), which was then overlaid by a coverslip before incubating the sections at 20°C for 15 minutes. After incubation, coverslips were removed before dipping the sections into buffer 1. Antibody conjugate anti-digoxigenin – peroxidase (POD) (Roche Diagnostics GmbH Mannheim, Germany) was diluted 1:1000 in buffer 2. The diluted conjugate was then distributed at 10-20µl over each section including the negative control. The sections were overlaid with a coverslip before being placed in a humid chamber at room temperature for 1 hour. The coverslips were then removed before the sections were washed in 2 × 10 minutes in buffer 1 then equilibrated for 5 minutes in buffer 3 (appendix 5). Approximately 20 µl of colour solution DAB (3,3' – diaminobenzidine tetrahydrochloride with metal enhancer tablet sets DAB peroxidase substrate, Sigma-Aldrich CO. St Louis USA) was distributed onto each section. Sections were then overlaid with a coverslip and left in the dark overnight. After incubation coverslips were removed and sections were gently washed in TE buffer (appendix 5) for 5 minutes. Sections were finally counterstained with Mayer's haematoxylin before being mounted.

### 7.3 Results

#### 7.3.1 DNA quality from infected *S. serrata* tissue using decapod specific primers

A PCR using decapod specific primers was performed to ensure that DNA was successful extracted from *S. serrata* larvae. DNA was extracted from crab larvae and the *Penaeus monodon*, both displayed a 848bp amplicon, indicative of intact extracted DNA (figure 7.1).

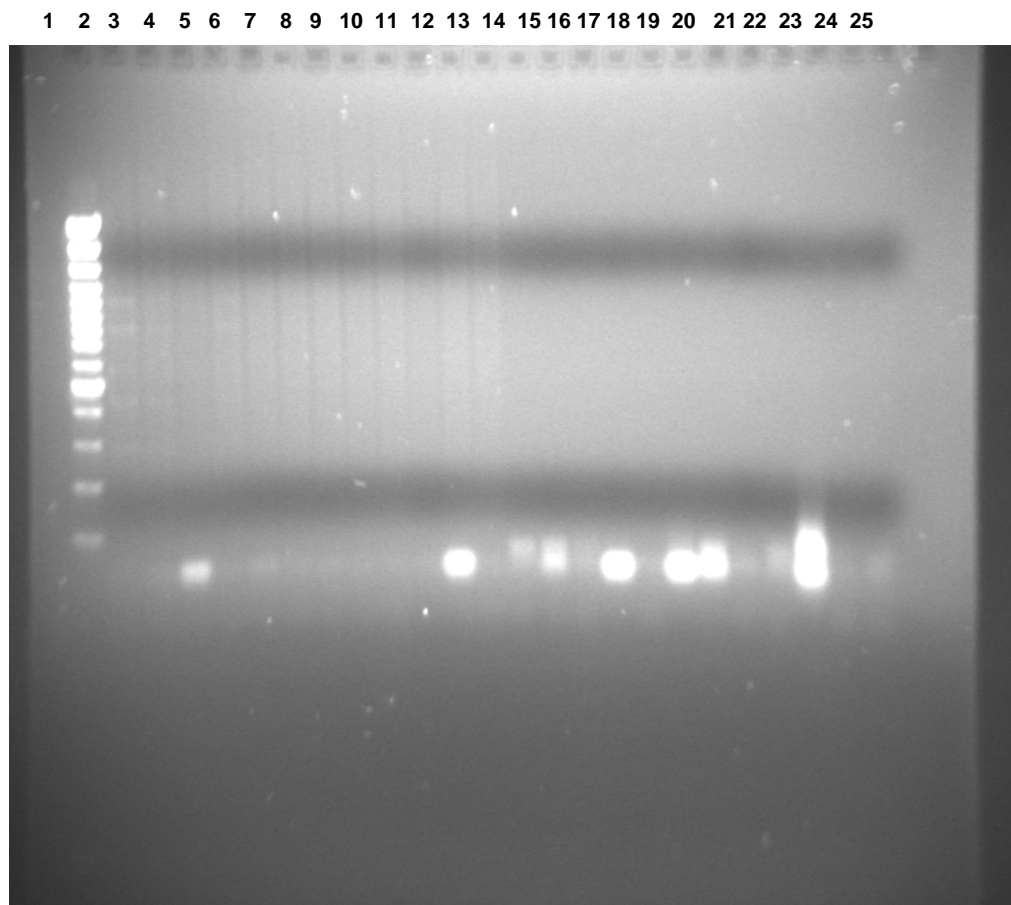


**Figure 7.1** Electrophoretic gel of a PCR using decapod specific primers.

Lane 1: Generuler DNA ladder plus 100bp marker (MBI Fermentas); Lane 2: *Scylla serrata*; Lane 3: blank extract (negative control); Lane 4: no template (negative control); Lane 5: *Penaeus monodon* (positive control).

### 7.3.2 PCR amplification of infected *S. serrata* tissue using WSSV – specific primer BP1.

No visualisation of products was obtained from the PCR utilising BP1 specific primers.

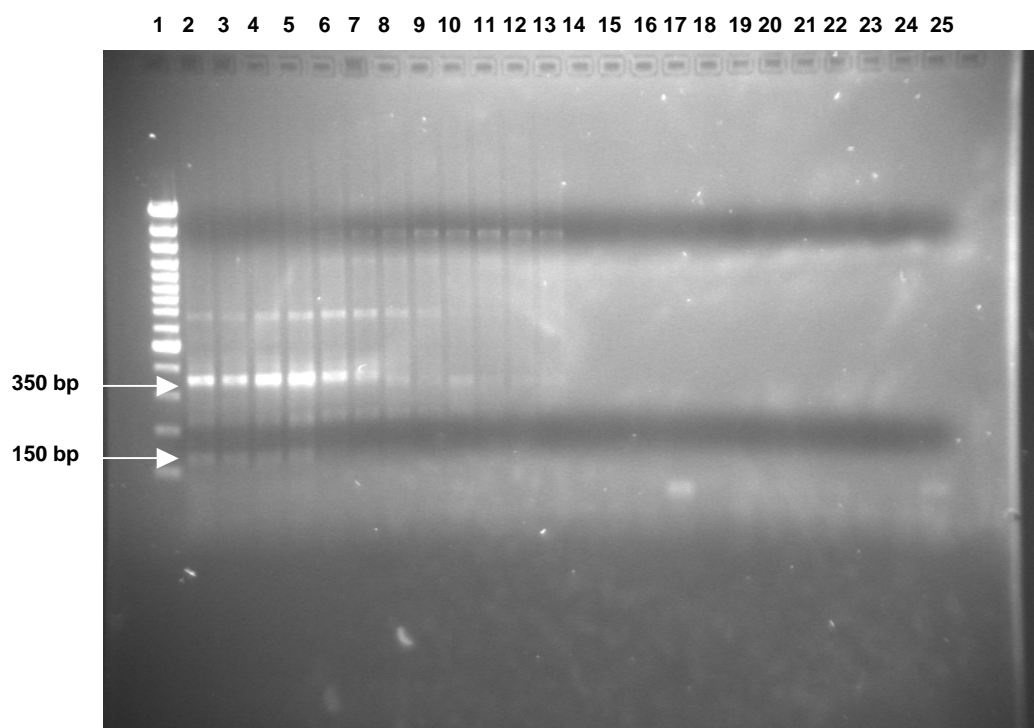


Lane 1: Generuler DNA ladder plus 100bp marker (MBI Fermentas); Lane 2: Crab A, gradient 1 (51°C); Lane 3: Crab A, gradient 2 (51.2°C); Lane 4: Crab A, gradient 3 (51.7°C); Lane 5: Crab A, gradient 4 (52.6°C); Lane 6: Crab A, gradient 5 (53.7°C); Lane 7: Crab A, gradient 6 (55°C); Lane 8: Crab A, gradient 7 (56.4°C); Lane 9: Crab A, gradient 8 (57.8°C); Lane 10: Crab A, gradient 9 (59.1°C); Lane 11: Crab A, gradient 10 (60.2°C); Lane 12: Crab A, gradient 11 (61°C); Lane 13: Crab A, gradient 12 (61.4°C); Lane 14: blank reaction, gradient 2 (51.2°C), (negative control); Lane 15: blank reaction, gradient 4 (52.6°C), (negative control); Lane 16: blank reaction, gradient 6 (55°C), (negative control); Lane 17: blank reaction, gradient 8 (57.8°C), (negative control); Lane 18: blank reaction, gradient 10 (60.2°C) (negative control); Lane 19: blank reaction, gradient 12 (61.4°C) (negative control); Lane 20: no template, gradient 2 (51.2°C), (negative control); Lane 21: no template, gradient 4 (52.6°C), (negative control); Lane 22: no template, gradient 6 (55°C), (negative control); Lane 23: no template, gradient 8 (57.8°C), (negative control); Lane 24: no template, gradient 10 (60.2°C), (negative control); Lane 25: no template, gradient 12 (61.4°C), (negative control).

**Figure 7.2** Amplification of the DNA coding for the WSSV binding protein by PCR from crab *Scylla serrata*.

### 7.3.3 PCR amplification of infected *S. serrata* tissue using WSSV – specific primers BP2.

Faint bands were observed at the region of the expected amplicon of 150bp when using BP2 specific primers. Bands were only visualised in the lower end of the gradient PCR. Highly illuminated bands were also observed at an approximate amplicon of 350bp, which were also observed in the lower end (figure 7.3).



Lane 1: Generuler DNA ladder plus 100bp marker (MBI Fermentas); Lane 2: Crab A, gradient 1 (51°C); Lane 3: Crab A, gradient 2 (51.2°C); Lane 4: Crab A, gradient 3 (51.7°C); Lane 5: Crab A, gradient 4 (52.6°C); Lane 6: Crab A, gradient 5 (53.7°C); Lane 7: Crab A, gradient 6 (55°C); Lane 8: Crab A, gradient 7 (56.4°C); Lane 9: Crab A, gradient 8 (57.8°C); Lane 10: Crab A, gradient 9 (59.1°C); Lane 11: Crab A, gradient 10 (60.2°C); Lane 12: Crab A, gradient 11 (61°C); Lane 13: Crab A, gradient 12 (61.4°C); Lane 14: blank reaction, gradient 2 (51.2°C), (negative control); Lane 15: blank reaction, gradient 4 (52.6°C), (negative control); Lane 16: blank reaction, gradient 6 (55°C), negative control; Lane 17: blank reaction, gradient 8 (57.8°C), (negative control); Lane 18: blank reaction, gradient 10 (60.2°C) (negative control); Lane 19: blank reaction, gradient 12 (61.4°C), (negative control); Lane 20: no template, gradient 2 (51.2°C), (negative control); Lane 21: no template, gradient 4 (52.6°C), (negative control); Lane 22: no template, gradient 6 (55°C), (negative control); Lane 23: no template, gradient 8 (57.8°C), (negative control); Lane 24: no template, gradient 10 (60.2°C), (negative control); Lane 25: no template, gradient 12 (61.4°C), (negative control).

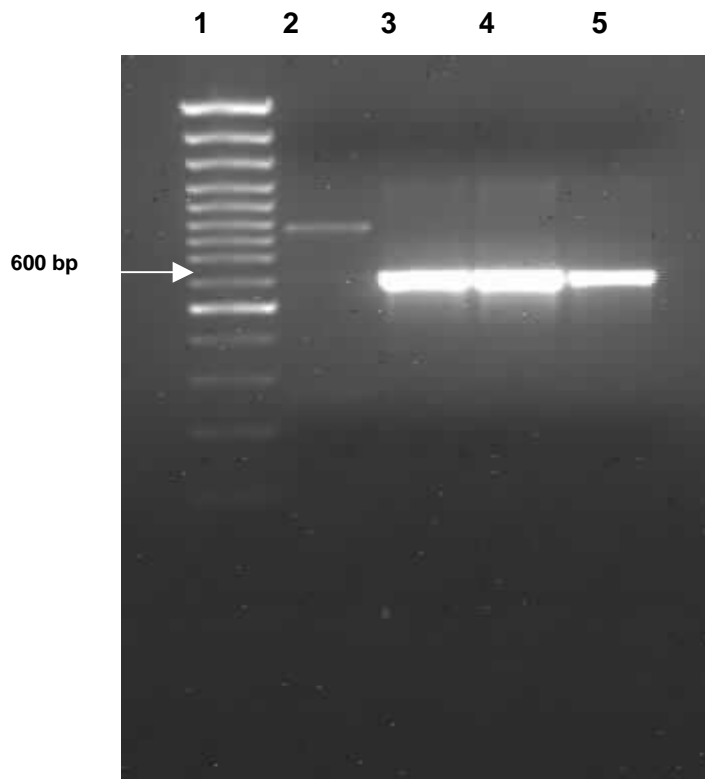
**Figure 7.3** Amplification of the DNA coding for the WSSV binding protein by PCR from the crab *Scylla serrata*.

#### 7.3.4 PCR Product Purification

Following the analysis of electrophoresis gels, two bands were selected for sequencing, a faint band at the region of the expected amplicon of 150 bp and a brighter band at an approximate amplicon size of 350 bp (figure 7.3).

#### 7.3.5 Cloning of PCR products

When attempting to clone PCR products into bacterial plasmids, white colonies were isolated representing *E. coli* containing an insert into the DNA plasmid. Plates containing the expected 350 bp amplicon yielded approximately 15 white colonies in which 10 were selected for further analysis. Plates containing the expected 150 bp amplicon only yielded 1 white colony. Attempted verification by PCR using M13 primers failed in that the appropriate sized DNA insert was absent (figure 7.4). Therefore, cloning was repeated using the 150 bp product. The repeated cloning yielded 3 white colonies. Relevant bacterial colonies that appeared to possess plasmids with DNA inserts were subcultured into LB broth with ampicillin before aliquots were frozen at -80°C.

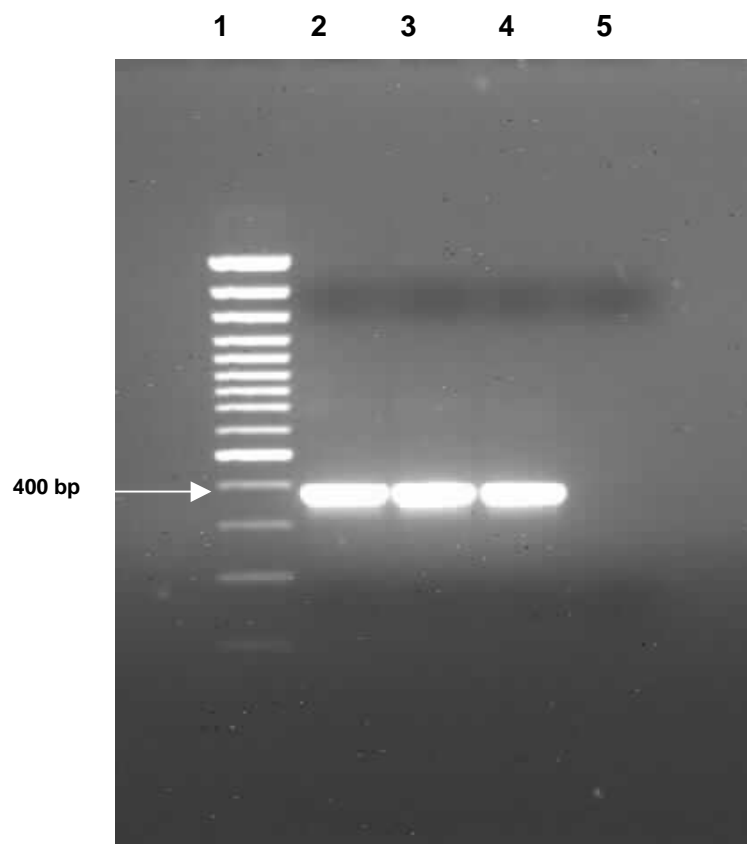


**Figure 7.4** Electrophoretic gel of a PCR product using M13 primer.

Lane 1: Generuler DNA ladder plus 100bp marker (MBI Fermentas); Lane 2: 150 bp amplicon (minor amplicon); Lane 3: 350 bp amplicon (major amplicon); Lane 4: 350 bp amplicon (major amplicon); Lane 5: 350 bp amplicon (major amplicon).

Size of the product is derived from the 251 bp of the plasmid plus the DNA insert of 350 bp.





**Figure 7.5** Electrophoretic gel of a PCR using M13 primers.

Lane 1: Generuler DNA ladder plus 100bp marker (MBI Fermentas); Lane 2: 150 bp; Lane 3: 150 bp; Lane 4: 150 bp; Lane 5: no template (negative control). Size of the product is derived from the 251 bp of the plasmid plus the DNA insert of 150 bp.

### 7.3.6 Isolation and confirmation of plasmid DNA

To ensure plasmids that contained the appropriate DNA insert, a PCR was conducted on purified plasmid DNA using M13 primers (figure 7.4, 7.5). Untransformed plasmids have an amplicon of 251 bp. Samples displaying greater than this amount, were assumed to contain DNA insert. The expected amplicon from this reaction was 251 bp of plasmid, plus the DNA insert of either 350 bp (major bands) or 150 bp (minor bands), giving a total length of approximately 600 bp and 400 bp respectively. Three plasmids of both of the expected 150 bp and 350 bp amplicon were used in the M13 PCR for future

sequencing (figure 7.4,7.5). As mentioned previously the first attempt at cloning the 150 bp product proved to be unsuccessful and a product of approximately 900 bp was obtained (figure 7.4). Lanes 3, 4 and 5 (figure 7.4) showed a product of approximately 600 bp indicating the successful cloning of the 350 bp product. Lanes 2,3,4 (figure 7.5) all produced products of approximately 400 bp indicating the successful cloning of the 150 bp product.

### 7.3.7 DNA Sequencing

The following sequences (figure 7.6 and 7.7) were obtained using the BP2 specific primers. No matches over 15 bp were obtained in the sample by BLAST searches.

```

1  AAAAATGGTT  GCCCGAAGCT  CCTACACTGG  ACAATACTAC  TGCTGCCAGA
51 CACCCACCA  GGCCGCCGTC  TCCAGCCCAC  ACCACCACTT  CCCGTACTAC
101 TAAATATGTG  TCTGTCCGTC  GCCGTCCTA  A

```

**Figure 7.6** Sequence of *S. serrata* 150bp into a plasmid of *E. coli* DNA insert of 131bp. Shaded areas represent primer sites.

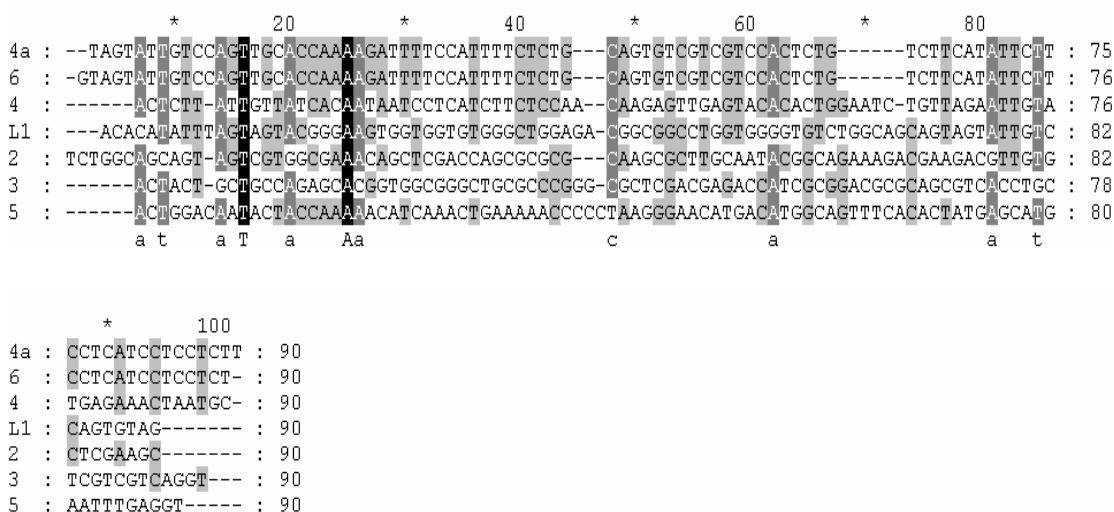
```

1  TGAGGAACG  CGACGGACAG  CGATGTATGG  GGGCTGGCAA  GGGCGGGGTC  51
   GGCGTCTCTT  AATGGAAAGG  GGCGCCACAG  AGGTGACTCC  ATAACTATTA
101 ACCCGCCCAT  TGTTGTCCTC  CTGTAGGCCG  CCACCCATTC  ACCAAGTTCC  151
   GCTGTGAGTG  CTGCGAGAGA  CTGTATGGCT  GACAGCCACT  TCCGTGATTA
201 TTTTTTCCTT  CTTTTCTTTT  CCTTCGCGAG  ATTTTTTTGGT  ATTTCTCCC
251 TCTCCCTCTT  CTTATTCCT  TTGCTATCGT  TCATTCTGTCG  TTTCCGCATC
301 ATTTGTTT  CTATCTGTCA  GGAATGTCTG  TCTCCTGTCC  GTCGCCGTTG
351 CTCA

```

**Figure 7.7** Sequence of *S. serrata* 350 bp *E. coli* plasmid DNA insert of 354 bp. Shaded areas represent primer sites.

The relatedness of SBV 131 bp product sequence to corresponding sequences using GeneDoc software is shown below (figure 7.8). The degree of nucleotide identity was as follows: 2 - 29%, 3 - 28%, 4 - 28%, 4a - 33%, 5 - 25%, 6 - 33%. Whilst the matches were poor, all the matches were with the bacilliform/ baculoviral group viruses.

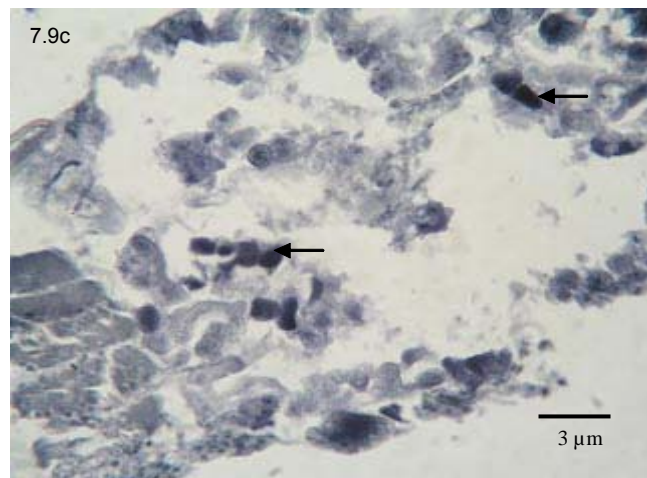
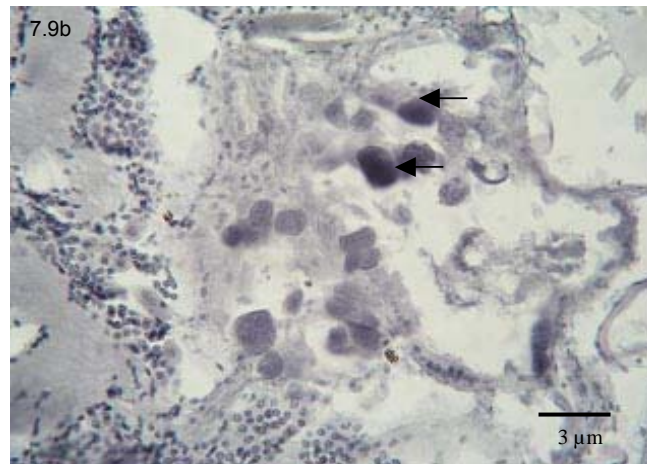
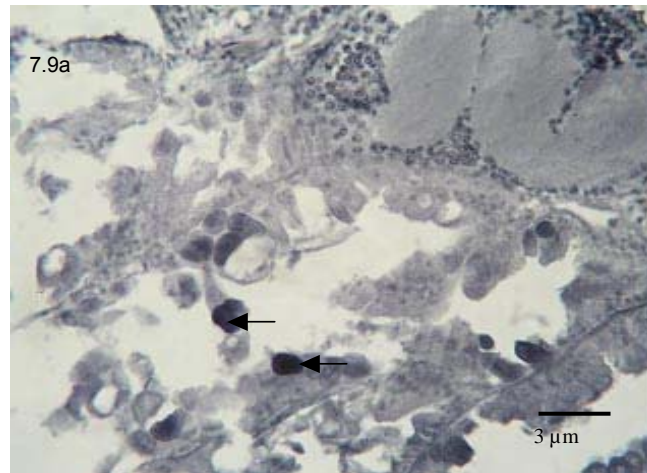


**Figure 7.8** The DNA nucleotide sequence of the SBV product 131 bp in comparison to its approximate phylogenetic affiliations with other intranuclear bacilliform viruses obtained using BLAST alignment. Sequence data was aligned using GeneDoc software. Shading indicates identical nucleotides. Dashes indicate simulated spaces introduced to achieve maximal alignment.

L1: sequence data, 2: *Lymantria dispar* nucleopolyhedrovirus, complete genome (20879 - 20893), (Kuzio *et al.* 1999) 3: *Lymantria dispar* nucleopolyhedrovirus immediate early) protein (ie- 0) and immediate early 1 protein (ie-1) genes (860 – 846), (Pearson and Rohrmann 1997) 4a: Shrimp white spot syndrome, complete genome (10327 – 10341), (Yang *et al.* 2001) 4b: Shrimp white spot syndrome, complete genome (283005 – 283019), (Yang *et al.* 2001) 5: Shrimp white spot syndrome virus isolate Korea 01 VP35 gene, (356 – 342), (Baek *et al.* 2003) 6: Shrimp white spot syndrome virus nucleocapsid protein (vp35) gene, (2149 – 2163) (Kou *et al.* 2004).

### 7.3.8 Hybridisation

Both sections, those treated with the DIG-labelled DNA probe and the untreated control had a dark, blue/purple precipitate (figure 7.9). The dig-labelled probe had not specifically bound to the inclusion body.



**Figure 7.9** (a). Light micrograph of hepatopancreas of larvae of *S. serrata* infected with SBV. SBV inclusions are visible (arrows) within infected cells. The section was a negative control where the probe<sub>150</sub> was not applied. Dark precipitate was present within a number of the infected cells. (b,c). Light micrograph of hepatopancreas of larvae of *S. serrata* infected with SBV. The probe<sub>150</sub> and anti-dig were applied to both sections. SBV inclusions are visible (arrows) within infected cells. Dark precipitate was also present within a number of infected cells.

#### 7.4 Discussion

The primary aim of this investigation was to attempt to amplify DNA sequence from SBV using a crossreaction from the designed primer sets. Only the primer PB2 successfully amplified SBV producing bands similar in size to that of the protein binding gene of WSSV of approximately 150 bp (figure 7.3). However when sequences were compared to available databases using BLAST to determine approximate affiliations, there was only slight homology with WSSV and a nucleopolyhedrovirus, which infects the insect *Lymantria dispar* (figure 7.8). The alignment of the WSSV and insect baculovirus genomes with the 131 bp sequence of SBV was not in the region of the targeted binding protein gene, which further supports that the amplification was a non-specific reaction. The PCR using PB2 primers on DNA template which amplified bands of 150 bp and 350 bp (figure 7.3), was repeated several times yielding equivalent bands. Future primers designed for the attempted amplification of SBV, may need to be specific as this “shotgun” method was unsuccessful.

The study also involved *in situ* hybridisation techniques to allow specific nucleic acid sequences to be detected in preserved tissue sections. The principle behind *in situ* hybridisation is specific annealing of labelled nucleic acid probe to complementary sequences in fixed tissue, followed by visualisation of location of the probe to locate viral DNA (Wilkinson 1992). DNA inserts obtained from a cloning of the amplified band at approximately 150 bp (figure 7.3), were non-radioactively labelled with digoxigenin for use as gene probes in a further attempt to confirm that DNA sequence obtained was specific to that of SBV in *S. serrata*. Results demonstrated that the probe was unable to detect the SBV in *S. serrata*, as it displayed no reaction to tissue infected with the virus. The dark precipitate identified in inclusion bodies was a blue/purple in colour most probably resulting from the haematoxylin counterstain. If the probe was to react with the

intranuclear bodies containing SBV, then a blue/brown colour as a resultant of DAB would have been visualised (figure 7.9). *In situ* hybridisation procedures represent a compromise between the detection of the genomic target, non-specific binding of probe and the loss of specific signal due to decreased diffusion and contaminating nucleases (Brahic and Haase 1989). The absence of a positive control brings with it the difficulty of optimising the procedure.

Further research is necessary to enhance diagnostic capabilities for the detection of SBV to facilitate future epidemiological, pathogenicity and carrier host studies. Traditional methods such as histopathology may be somewhat subjective and insensitive. Detection by PCR and labelled probes would allow for a more rapid and sensitive diagnostic capabilities. Future research on SBV is necessary to study the taxonomic position in relation to other IBVs and to allow a detailed understanding of the pathology of the virus in crabs. It is important to broaden our general knowledge of naturally occurring viral diseases of not only IBVs that infect crustaceans but also those affecting insects. Federici and Hazard (1975) remarked on the affinities of the viruses in insects. Insect viruses are being used as biological control agents and runoff might expose estuarine and coastal crustaceans in particular, to these viruses.

## CHAPTER 8

### GENERAL DISCUSSION

The culture of *Scylla serrata* in recent years has received increasing attention attributed to a number of attractive qualities including high meat yield, high acceptability and market price in addition to their decreasing availability resulting from direct fishing pressure (Aldon and Dagoon 1997; Yamaguchi 1991). Presently in Australia, the culture of mud crabs is not widespread with only few operators having produced marketable quantities (McCormack 1989). A limited supply of crab larvae is restricting further expansion of mud crab aquaculture (Keenan 1999). One major constraint to the hatchery production of mud crab larvae and their commercial viability is the mass mortality of larvae due to pathogens and disease (Cholik 1999; Yamaguchi 1991). To date, investigations into diseases of cultured mud crabs have been limited, possibly due to the industry still being in its infancy.

From a number of potential diseases, vibriosis has been implicated as the major cause for inconsistent survival in mud crab larvae (Mann *et al.* 1998). It is believed that these bacteria proliferate in the host digestive tract where the establishment of pathogenic strains invariably leads to mass mortalities (Colorni 1985). Although a number of vibrios species have shown to be pathogenic to the larvae of *S. serrata*, their pathogenicity has been considered moderate in comparison to that of *V. harveyi* (Boer *et al.* 1993; Parenrengi *et al.* 1993). Researchers Mann *et al.* (1998) reported that for the first few days of culture bacterial communities within the culture system are relatively unstable. Total heterotrophic and presumptive vibrio levels rapidly increase to day 2 of culture, where at approximately day 3 and 4 bacterial levels rapidly decline obtaining a lower concentration. Therefore bacterial infection could be considered to be an instrumental factor why high mortalities are experienced around early zoea stages throughout the industry (Dr C. Zeng, James Cook University pers comm. 2001).

Vibriosis has been previously identified as a major disease within the larvae phase of *S. serrata*. After an investigation into their virulence mechanisms there is evidence that the possession of such mechanisms may not necessarily determine the ability of an isolate to become virulent. Pathogenicity of bacteria is dependant on the particular virulence

mechanisms present and their ability to firstly to attach to and colonise specific sites in the host and the formation of substances that cause damage to the host. For example *V. alginolyticus* LLB2, which was isolated from the rearing environment of larvae of *S. serrata* as a proposed probiotic displayed possibly the highest haemolysin activity, however the isolate was shown to be non-virulent to *S. serrata* perhaps because of its low chitinase production. The ability of the bacterium to attach to and colonise specific sites in the host may ultimately determine if haemolysin becomes a virulence factor.

There are a number of other extracellular products which have not been investigated in this study but have been shown to cause damage to the host. Muir (1991) through histopathological evidence showed exotoxins produced by luminous bacteria were responsible for mediating disease in infected larvae of *P. monodon*. *V. harveyi* strain 642 has previously shown to be virulent to *P. monodon* larvae where the isolate was demonstrated to produce proteinaceous exotoxins with a molecular weight of 100kDa (Harris and Owens 1999). Toxins produced by the strain 642 had an LD<sub>50</sub> of 2.2µg g<sup>-1</sup>. Harris and Owens (1999) suggested the major effect of toxins probably occurs in the intestinal tract of infected *P. monodon* larvae. However, like most virulence factors, exotoxins rely on other bacterial components such as regulatory mechanisms and adherence factors to fully potentiate their expression, delivery and effects on the host (Finlay and Falkow 1997). Furthermore, toxin production in *V. harveyi* strain 642 was found to be mediated by the bacteriophage termed VHML. The mediation of toxin by a mobile genetic element such as a bacteriophage may suggest why virulence is not widely dispersed throughout members of the *Vibrio* genus.

Mobile genetic elements play a significant role in determining virulence. Accessory genetic elements such as bacteriophages have been shown on numerous accounts to encode for bacterial virulence factors such as toxins (Rajadhyaksha and Rao 1965; Johnson *et al.* 1980). These genetic elements have also shown to be responsible for the amplification of other virulence mechanisms such as haemolysin production (Munro *et al.* 2003). Crosa *et al.* (1980) demonstrated that virulent strains of *Vibrio anguillarum* harboured a specific plasmid class designated pJM1, which was absent in avirulent strains. This particular plasmid was found to encode for an efficient iron-sequestering system and linked to *V. anguillarum* ability to establish infections (Tolmasky and Crosa



1984). This research again demonstrated the involvement of genetically mobile elements that ultimately determined the isolate's virulence.

The fact that the isolate *V. alginolyticus* LLB2 was non-pathogenic and conferred a degree of protectiveness may be associated with the strains fast growing characteristics, allowing the isolate to out-compete coexisting opportunistic and possibly virulent bacteria for a limited resource such as nutrients and adhesion sites. Siderophore complexes possessed by the environmental probiotic could have also contributed to the out-competition of existing bacteria through the acquisition of free iron, attributing to the higher survival of *S. serrata* larvae. Pybus *et al.* (1994) suggested that iron uptake seemed to be involved in the competition among *Vibrio* sp. in which siderophore complexes have been identified in *V. alginolyticus* strains (Gatesoupe 1997).

Other critical larvae stages where high mortalities rates occur have been reported as being zoea<sub>5</sub> to megalopa known as moulting death syndrome (MDS) (Fielder and Heasman 1998; Zeng and Li 1998). It's uncertain why significant mortality rates are experienced in this particular region of the larvae phase, however poor nutritional status during early larval development has been suggested to be responsible, preventing zoea<sub>5</sub> from successfully moulting into megalopa (Zeng and Li 1998). One possible causative agent that has little mention is viral aetiologies. There have been few reports concerning viral diseases within the mud crab *S. serrata*.

Histological examination of larvae of *S. serrata* revealed nuclear pathology where amorphous, basophilic intranuclear inclusions were observed in location of the hepatopancreas region. Visual observations by light microscopy were similar to the initial surveys of the intranuclear bacilliform virus SBV in *S. serrata* described by Anderson and Prior (1992). Histological findings revealed the absence of cell necrosis within tissues infected with SBV suggesting the virus appear to lack pathogenicity. Anderson and Prior (1992) also reported infections of SBV were not associated directly with disease or mortality observed in the mud crab. The occurrence of a virus does not necessarily mean that disease will result. Many viruses are suppressed for large parts of crustacean life cycle and some have never been associated with any pathological condition (Stewart 1991). Couch (1983) documented that heavy infections of Baculo-A within the blue crab

*C. sapidus* did not appear to affect the crabs. They associated this absence of disease due to epithelial cells of hepatopancreas constantly renewing the cell population.

Following the detection of an intranuclear bacilliform virus within the larvae of *S. serrata* presumably SBV, an attempt was made to amplify sequence from this virus. As there was no available sequence data and SBV's relationships with other intranuclear bacilliform viruses remains uncertain, it was necessary to design novel primer sets. The IBV WSSV maybe related to SBV in some form due to a number of determinants, such as SBV sharing similar morphological traits with that of WSSV. However probably the major underlying factor correlating the two IBVs is unconfirmed reports in Ecuador and Spain suggesting WSSV was detected by PCR in *C. quadricarinatus* (A/Prof. L. Owens, James Cook University pers. comm 2004). Its suggested WSSV primers were amplifying the nonoccluded IBV known as Cherax baculovirus (CBV), which has been reported as infecting *C. quadricarinatus* in Australia (Anderson and Prior 1992). It was from this conjecture that Claydon (2004) attempted using PCR primers developed by Lo *et al.* (1996) to amplify the IBV CBV. Although positive PCR results were attained, results should be handled with scepticism as putative virus-free crayfish also displayed a positive cross reaction with crayfish genome. Therefore to try and eliminated the possibility of primers cross reacting with the crustacean DNA, this experiment was performed using primers which targeted a specific feature of the genome. This investigation involved trying to amplify SBV using primers designed from the protein binding gene of WSSV, whereby the amino acid profile of this particular gene has been reported to be homologous to that of insect baculoviruses (Yang *et al.* 2001). BP2 primers were the only primer sets to amplify product of desired size. When sequence was compared to available databases using BLAST there no significant phylogenetic relatedness to any sequences.

The primary difficulty that has affected this investigation has been the low availability of infected tissue. The use of crustacean cell lines to propagate IBVs *in vitro* has continued to elude researchers (Kim *et al.* 1998). Further methods may be devised in an attempt to amplify SBV in *S. serrata*. Viral prevalence and disease, in addition to a wide variety of other infectious agents in marine invertebrates, is said to be exacerbated by stress. Stress may be a direct result of dietary inadequacies, pollution and adverse environmental conditions (Stewart 1991). Environmental factors such as physiochemical parameters may

be altered in *S. serrata* culture conditions in order to enhance viral loads within animals that can be used for further characterisation.

It is the first reported time that SBV has been observed in larvae of *S. serrata*. It remains unclear on the pathogenicity of SBV has in larvae however pathologically the infection does not appear to be associated with disease. The virulence of this virus will not be fully recognised until the crab industry has become fully established. With the intensive nature of aquaculture, inevitably the development and transmission of this disease will be enhanced. Presently WSSV is one of the four crustacean viral genomes that have been completely sequenced. Future research on SBV may provide us with genetic information necessary to investigate its taxonomic position and to allow a detailed understanding of the pathology of SBV in *S. serrata*.

Future research should be directed at assessing a larger group set of environmental probiotic bacteria and their relationship between mechanisms which may aid in the bacteria's ability to exist and their protective effect towards mud crab larvae when challenged with virulent bacteria. We may then be able to obtain a greater understanding of what mechanisms attribute to the environmental probiotics ability to confer a degree of protectiveness.

Although results demonstrate that the digoxigenin – labelled PCR generated probe was unable to detect SBV in *S. serrata*, from the comparison of the DNA nucleotide sequence of the SBV product 131 bp, using BLAST, it was interesting to note that the majority of phylogenetic affiliations share a close relatedness mainly with bacilliform/baculoviral group viruses. In the future, a procedure for increasing the sensitivity of the probe may involve tailing the probe. Terminal transferase may be used to add a mixture of labelled dUTP and dATP to the 3' ends of an oligonucleotide in a template independent reaction . Within this tailing reaction, concentrations of labelled dUTP and unlabelled dATP are adjusted to produce the highest hapten incorporation, optimal spacing of the hapten, and ultimately the highest sensitivity (Schmitz *et al.* 1991).

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**APPENDIX 1****GENERAL BACTERIA CULTURE MEDIA****Luminous Agar (LA)** (Reichelt and Buamann, 1973)

Glycerol	3 ml
Tryptone	5 g
Yeast extract	5 g
Artificial sea salt	30 g
Agar	20 g
Distilled water	1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**4 % Luminous Agar (LA 4%)** (Reichelt and Buamann, 1973)

Glycerol	3 ml
Tryptone	5 g
Yeast extract	5 g
Artificial sea salt	30 g
Agar	40 g
Distilled water	1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**Luminous Broth (LB)** (Reichelt and Baumann, 1973)

Glycerol	3 ml
Tryptone	5 g
Yeast extract	5 g
Artificial sea salt	30 g
Distilled water	1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**Luria Bertani Broth (LBB)**

Tryptone	2.5 g
Yeast extract	1.25 g
NaCl	5 g

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**PYSS Agar**

Bacteriological peptone	5 g
Yeast extract	1 g
Artificial sea salt	33 g
Agar	15 g
Distilled water	1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**PYSS Broth**

Bacteriological peptone	5 g
Yeast extract	1 g
Artificial sea salt	33 g
Distilled water	1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**Thiosulphate Citrate Bile Salts Media (TCBS)**

TCBS media (Oxoid CM 333)	88 g
Distilled water	1000 ml

Dissolve media in water bath.

**Seawater liquid with vitamins (SWLV)**

Peptone	1 g
Yeast extract	1 g
Vitamin solution	5 ml
Filtered seawater	1000 ml

Autoclave at 121°C for 15 minutes.

**Vitamin solution**

Thiamin HCl (aneurine hydrochloride)	0.02 g
Biotin (0.1 mg/ml)	0.1 ml
Distilled water	100 ml

Autoclave at 121°C for 15 minutes.

## APPENDIX 2

## PREPARATION OF CHROME AZUROL S (CAS) AGAR

1,4-piperazinediethanesulfonic acid (Sigma P-3896)	40.24 g
50 % (w/w) NaOH solution	12 g
Agar No. 3 (Oxoid L11)	12 g
Distilled water	750 ml

Add the above reagents to an acid washed bottle. Adjust pH to 6.8 and at 121°C for 15 minutes. Allow to cool.

Autoclave the following separately and add when the agar above has cooled to approximately 55°C.

*Iron depleted 10% casamino acids solution (Appendix 2.2)	30 ml
1 M MgCl <sub>2</sub>	0.4 ml
CAS solution (Appendix 2.1)	100 ml

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, Goettingen, Germany).

## 2.1 Chrome azurol S (CAS) solution

Chrome azurol S (Sigma C-1018)	60.5 mg
Distilled water	50 ml
FeCl <sub>3</sub> solution (Appendix 3.1.1)	10 ml

Add slowly, with stirring, to a solution containing:

Hexadecyltrimethylammoniumbromide (Sigma H-5882)	72.9 mg
Distilled water	40 ml

Sterilise by autoclaving at 121°C for 15 minutes.

2.1.1 FeCl<sub>3</sub> solution, 1 mM

Anhydrous FeCl <sub>3</sub>	0.1624 g
10 M HCl	1 ml

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

## **2.2 Preparation of iron depleted 10% casamino acids solution**

### **2.2.1 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.

### **2.2.2 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 244nm 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.

### **2.2.3 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°C in a water bath. Absence of chloroform was determined by smell.

### **2.2.4 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).



**APPENDIX 3****PREPARATION OF CHITIN AGAR****Top layer**

Marine agar 2216 (Difco)  
Distilled water 1000 ml

Adjust pH 7.5, Autoclave at 121°C for 15 minutes.

**Bottom layer**

Purified chitin 30 g  
Agar No. 3 15 g  
NaCl 20 g  
Distilled water 1000 ml

Autoclave at 121°C for 15 minutes.

**3.1 Purification of crude chitin****3.2.1 Precipitation of crude chitin**

50 g of chitin powder was added to 400 ml of distilled water. Concentrated sulphuric acid was diluted into 525 ml of distilled water, making sure the acid is added to the water. This process was undertaken while chilling the solution in an ice-water bath. 450 ml of the chitin slurry was added to the chilled stirring solution and constantly stirred for approximately 30 to 60 minutes in an ice-water bath. In this the solution the crude chitin is hydrolysed. If the mixture is exposed to acidic conditions for too long, the chitin will not precipitate. The chitin was then precipitated from the solution by adding 2.5 litres of ice-cold distilled water. After a short period chitin will appear as a white fluff of precipitate. This suspension was then left in the refrigerator overnight to allow for it to settle.

**3.2.2 Centrifugation of chitin**

The following morning the clear supernatant from the chitin solution was decanted. The remaining of the solution was then centrifuged to concentrate reprecipitated chitin. Throughout centrifugation process the extremely acidic suspension was neutralized several times using 10 N NaOH. The mixture was centrifuged a number of times using distilled water to remove the excess salt present in the mixture. The chitin was then refrigerated ready for use.

**APPENDIX 4****PREPARATION OF DUAL LAYER BLOOD PLATES****Bottom Layer**

Bacteriological peptone	5 g
Yeast extract	1 g
Synthetic sea salt	33 g
Agar No.3	15 g
Distilled water	1000 ml

Autoclave at 121°C for 15 minutes.

**Top Layer**

Bacteriological peptone	4.75 g
Yeast extract	0.95 g
Synthetic sea salt	31.35 g
Agar No. 3	15 g
Distilled water	950 ml

Autoclave at 121°C for 15 minutes. When cooled to 55°C, add 50 ml of sheep blood.

## APPENDIX 5

## DIFFERENTIAL AND SELECTIVE BACTERIA CULTURE MEDIA

**Falkow's Decarboxylase Media** (Atlas and Parks, 1993)

Peptone	5 g
Yeast extract	3 g
Glucose	1 g
Artificial sea salt (Aquasonic)	30 g
0.2% (w/v) Bromocresol purple	10 ml
Distilled water	950 ml

**Decarboxylases:**

L – arginine	10 g / L
L – lysine	10 g / L
L – orthithine	10 g / L

Dissolve ingredients (except decarboxylases) and adjust pH 6.7, autoclave at 121°C for 15 minutes and cool to 55°C.

Dissolve 10g of decarboxylase and glucose into 50mL distilled water. Filter sterilise 50 mL solution into sterile decarboxylase media into sterile bijoux bottles.

**Gelatinase (GA)**

Basal medium	1000 g
Yeast extract	5 g

Adjust pH to 7.5

Agar	40 g
Gelatin	10 g

Note: Boil gently to dissolve agar and gelatin, autoclave at 121°C for 15 minutes.

**Indole media** (Atlas and Parks, 1993)

K <sub>2</sub> HPO <sub>4</sub>	15.65 g
L – Tryptophan	5 g
NaCl	5 g
Distilled water	1000 ml

Adjust to pH 7.5, Autoclave at 121°C for 15 minutes.

**Oxidation – Fermentation Media (OF)**

Oxidation – Fermentation media (Oxoid CM 883)	9.8 g
Glucose	1 g
Artificial sea salt (Aquasonic)	30 g
Distilled water	1000 ml

Adjust to pH, autoclave at 115°C for 10 minutes.

**Simmon's Citrate Media (Atlas and Parks, 1993)**

NaCl	10 g
Sodium citrate	2 g
K <sub>2</sub> HPO <sub>4</sub>	1 g
(NH <sub>4</sub> )H <sub>2</sub> PO <sub>4</sub>	1 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g
Bromothymol blue	0.08 g
Agar	20 g
Distilled water	1000 ml

Adjust to pH 6.8, autoclave at 121°C for 15 minutes.

**Salt Agar (Requirement/Tolerance)**

Peptone	5 g
Yeast extract	1 g
NaCl	0 – 8 % (w/v)
Distilled water	1000 ml
Agar	20 g

Adjust to pH 7.5, autoclave at 121°C for 15 minutes.

**APPENDIX 6****BUFFERS AND REAGENTS****Buffer 1**

100mM Tris-HCl  
150mM NaCl

Adjust to pH 7.5, autoclave at 115°C for 10 minutes.

**Buffer 2**

0.5% (w/v) blocking reagent in buffer 1

**Buffer 3**

100mM Tris-HCl  
100mM NaCl  
50 mM MgCl<sub>2</sub>

Adjust to pH 9.5, autoclave at 115°C for 10 minutes.

**Catalase Reagent**

Hydrogen peroxide	10 ml
Distilled water	90 ml

Store in the dark at 4°C.

**Colour developer**

Dissolve one tablet of DAB (3,3' – diaminobenzidine tetrahydrochloride with metal enhancer tablet sets DAB peroxidase substrate, Sigma-Aldrich CO. St Louis USA) in 5 ml deionized water.

**20 × Denhardt's**

BSA (fraction v)	0.4 g
Ficoll 400	0.4 g
PVP 360 (polyvinyl pyrrolidone)	0.4 g
Distilled water	100 ml

**Hybridization buffer**

(50ml final volume)  
 50% formamide (25ml 100% formamide)  
 5% dextran sulfate (10ml 25% dextran sulfate)  
 0.5 mg ml<sup>-1</sup> sperm DNA (2.5ml of 0.5mg/ml<sup>-1</sup> salmon sperm)  
 1 × Denhardt's solution (2.5 ml 20 × Denhardt's)  
 4 × saline sodium citrate (SSC) (10 ml 20 × SSC)  
 Store at 4°C.

**Kovac's Reagent**

Paradimethylaminobenzaldehyde	5 g
Amyl alcohol	75 ml
HCl	25 ml

In the fume hood add amy alcohol to HCl in a dark reagent bottle, then add paradimethylaminobenzaldehyde.

**Oxidase Reagent**

Tetramethyl – p – phenylenediamine	5 g
Ascorbic acid	0.02 g
Distilled water	20 g

Stored in the dark at 4°C.

**20 × SSC**

3M NaCl  
 0.3M sodium citrate

Adjust to pH 9.5, autoclave at 115°C for 10 minutes  
 Store at 4°C.

**10 x TBE buffer**

Tris base	108 gm
Boric acid	55 gm
Na <sub>4</sub> EDTA	9.3 gm

Add ddH<sub>2</sub>O to 1 litre, adjust pH to 8.3.

**TES buffer**

5mM Tris-HCl  
10 mM EDTA  
10 mM NaCl

Adjust to pH 7.4, autoclave at 115°C for 10 minutes.

**TNE buffer**

50mM Tris  
10mM NaCl  
1 mM EDTA

Adjust to pH 7.4, autoclave at 115°C for 10 minutes  
Store at 4°C.

## APPENDIX 7

## WALNES MEDIA FOR ALGAE CULTURE (Walnes, 1974)

<b>Stocks (1)</b>	<b>Trace metal solution (TMS)</b>	per 100 ml
	ZnCl <sub>2</sub>	2.1 g
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	2.0 g
	(Na <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub>	0.9 g
	CuSO <sub>4</sub> ·5 H <sub>2</sub> O	2.0 g

Acidify with a few drops of HCl to give a clear solution.

<b>Stocks (2)</b>	<b>Vitamin solution</b>	
	B12	50 mg / L
	D Biotin	50 mg / L
	B 1	1 g / L
	Distilled water	1000 ml

<b>Stocks (3)</b>	<b>Nutrient solution</b>	
	FeCl <sub>3</sub> ·6H <sub>2</sub> O	1.3 g / L
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.36 g / L
	H <sub>3</sub> BO <sub>3</sub>	33.6 g / L
	EDTA (disodium salt)	45 g / L
	NaNO <sub>3</sub>	100 g / L
	TMS (see above)	1 g
	Distilled water	1000 ml

Dissolve boric acid, EDTA and sodium nitrate using boiling distilled water.  
Add remainder of ingredients.

<b>Stocks (4)</b>	<b>Phosphate solution</b>	
	NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	20 g / L
	Distilled water	1000 mL

Add 300 ml of stock solutions 2, 3, and 4 to make up 900 mL of working solution.  
Working solution to be used at 3mL / L.



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**APPENDIX 8****FIXATIVE****Davidson's Fixative**

Ethanol	1.5 L
Formaldehyde	1 L
Acetic acid	500 mL
Distilled water	2 L

Pour distilled water in first before pouring the acid.  
Place tissue in sealed container overnight and then take to histology.