1 Introduction

1.1 Challenges for successful spiny lobster larviculture

One of the primary challenges facing the successful commercialisation of larviculture systems for economically important spiny lobsters (family Palinuridae) is bacterial disease leading to mass attrition rates during the extended larval phase (Kittaka 1997; Diggles et al. 2000; Bourne et al. 2004; Ritar et al. 2006). There is a general lack of knowledge on cultured Palinurid phyllosoma (larvae) disease aetiologies and pathologies, and efficient disease management strategies. Due to the global antibiotic resistance crisis (Davies, 2007), there is now a shift from conventional chemotherapeutic treatment of infectious disease in aquaculture systems towards developing sustainable biocontrol methods such as probiotics (Defoirdt et al. 2007). Herein resides a research niche that demands further investigation.

The objectives of this project were to identify and study pathogens responsible for epizootics in the larviculture of an important Palinurid aquaculture candidate, ornate spiny lobster (*Panulirus ornatus*), and to identify potential probiotic bacteria antagonistic to the pathogen capable of protecting cultured *P. ornatus* phyllosomas from disease.

1.2 Importance of aquaculture

Wild capture fisheries and aquaculture had a combined global seafood harvest of 142 million tonnes in 2008, with resources used for human consumption reaching an all-time high per capita supply of 17 kg in a world population of 7 billion (FAO, 2010). Nevertheless, supply is still primarily sourced from wild capture fisheries, which has stabilised over the last decade (Figure 1-1) and continues a disconcerting trend indicating many important fisheries have reached their maximum sustainable limits or are being overfished with declining yield. It is estimated more than half of the world's fishery stocks are fully exploited, and the proportion of overexploited, depleted or recovering stocks has increased from 10% in the 1970s to 32% in 2008 (FAO, 2010). A number of the most voluminously-produced marine species are either fully- or overexploited including anchoveta (*Engraulis ringens*), Alaska pollock (*Theragra chalcogramma*), Atlantic herring (*Clupea harengus*), and up to 95% of important tuna stocks.

Nevertheless, consumer demand for high quality seafood continues to increase against a limited, to declining, supply (FAO, 2010).

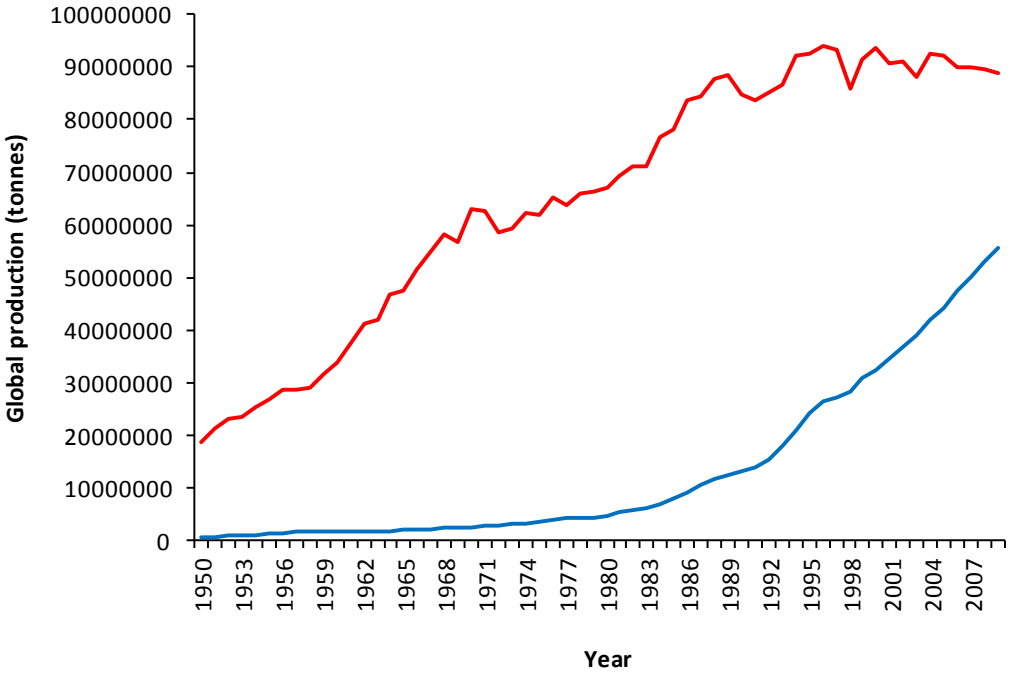

Figure 1-1. Global production of (—) wild capture fisheries (excluding plants, mammals, reptiles and amphibians) and (—) aquaculture (excluding plants) since 1950 (FAO, 2011).

Marine lobsters belonging to the spiny lobster family Palinuridae are some of the most coveted and economically important seafood groups, but harvest has stagnated or declined on a global scale in recent years (Figure 1-2) indicating yield has reached maximum limits (Jeffs, 2010). The decline in Palinurid fisheries in most cases can be attributed to a lack of sufficient fisheries management and overfishing (Guzman and Tewfik, 2004; Radhakrishnan et al. 2005; Guzman et al. 2008). However, erratic annual harvest is also facilitated by natural environmental fluctuations (Cruz et al. 2001; Arteaga-Ríos et al. 2007; Caputi, 2008; Linnane et al. 2010).

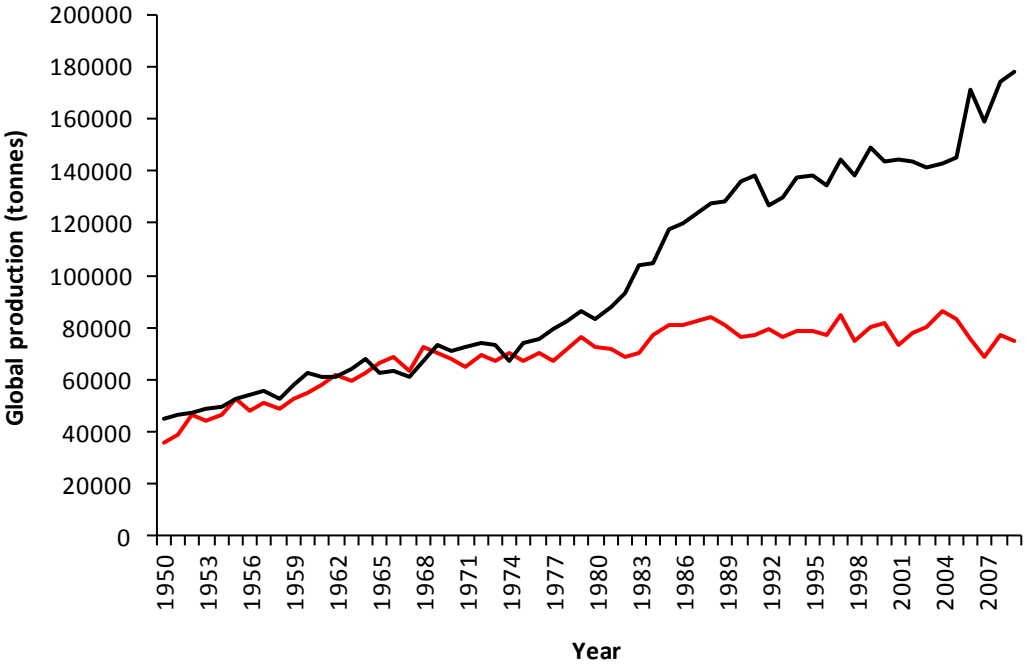

Figure 1-2. Annual global fisheries production of marine lobster families (—) Nephropidae (clawed lobsters) and Palinuridae (—) since 1950 (FAO, 2011).

With a projected population of >9 billion by 2050 (UN, 2010), and a significant portion being within the Asian region, the demand for quality protein sources such as seafood will skyrocket, placing food security alongside climate change as some of the foremost challenges facing humanity (Cribb, 2010). As researchers begin to unravel the effects of climate change on oceanic conditions, species diversity and productivity (MacNeil et al. 2010; Lough and Hobday, 2011), the valuable Australian Palinurid lobster fisheries emerges as prime examples of how changing oceanic conditions affect population recruitment. In these cases, the effects are driven by the weakening of the Leeuwin Current and the southward penetration of the East Australian Current on the west and east sides of the Australian continent, respectively (Pecl et al. 2009; Caputi et al. 2010). Given these climatic, economic and demographic challenges, there appears to be limited potential to enhance global productivity of wild fisheries and it is unlikely they can supply the forecasted 27 million tonnes of additional seafood per year to maintain current per capita levels by 2030 (FAO, 2010). It is clear the additional supply will need to be sourced from aquaculture.

Aquaculture is the fastest growing food production sector in the world, increasing at a rate of 8.3% since 1970 and valued close to $US100 billion (FAO, 2010). Global annual production has grown from less than 1 million tonnes in 1950 to 52.5 million tonnes in 2008 (Figure 1-1), increasing at three times the rate of combined poultry and livestock production. Aquaculture accounts for around 46% of total fish products destined for human consumption, translating to an annual per capita supply of 7.8 kg in 2008 and representing a 10 fold increase since 1970 (FAO, 2010). Aquaculture is a also a major source of income and livelihood particularly for coastal communities in developing nations, and conservative estimates places 11 million people in fish farming worldwide (FAO, 2010). In particular, the number of aquaculturists is growing significantly in Asia (increasing by 189% in the period 1990-2008) which contributes to the region's dominant position in world aquaculture production. With the pending rise of the middle classes in Asia, where seafood consumption on a per capita basis is three times that in the west, demand for high value seafood, such as marine lobsters, will dramatically increase offering wealth creation opportunities in aquaculture to produce such products (Chun, 2010).

1.3 Australian aquaculture

Similar to global trends, Australian wild capture production has decreased from 236 300 tonnes in 2004–05 to 171 000 tonnes in 2008–09 (ABARES, 2011). In contrast, the contribution of aquaculture production has risen 12% since 2004–05 and now represents 29% (70 000 tonnes) of gross fishery production. The expansion has been driven primarily by increases in salmonid production, which has tripled in volume (30 000 tonnes), corresponding to 37% ($AU326 million) of total aquaculture production. The southern bluefin tuna (*Thunnus maccoyii*) sector is the second most valuable aquaculture species, producing 8800 tonnes for a value of $AU158 million. Clearly, there is untapped potential in the cultivation of other high-value species to maximise economic returns, to alleviate pressures on wild stocks and protect marine ecosystems, as well as supplying rapidly increasing demand from Asia. .

Currently, the most valuable Australian export products are spiny rock lobsters of the family Palinuridae, all harvested from wild stocks, representing 32% ($AU400 million) of the total fisheries export value ($AU1.2 billion). Almost two-thirds of production is sourced from the western rock lobster (*Panulirus cygnus*) fishery in Western Australia, with southern rock

lobster (*Jasus edwardsii*) fisheries in South Australia and Tasmania accounting for 15% and 13%, respectively, of total catch (ABARES, 2011). In recent years, Hong Kong and China have emerged has strong export markets of rock lobsters, accounting for 61% of total fisheries export value to these regions. However, due to concerns of changing oceanic cycles on recruitment and fishable biomass (Pecl et al. 2009; Caputi et al. 2010) and ecological consequences of removing certain size-distributions of predatory lobsters (Ling et al. 2009), the main lobster producing jurisdictions in Australia have introduced Total Allowable Catch (TAC) and Individual Transferable Quotas (ITQs) to cap production (ABARES, 2011). Yet even with stringent management plans to maintain sustainable wild stocks for harvesting, demand for spiny lobsters is likely to supersede natural production limits, especially with China's rapid economic development and expanding prosperity in middle class consumers (Kharas, 2010). It is clear that the only solution to produce sustainable supplies of spiny lobsters is through aquaculture.

1.4 Spiny lobster aquaculture

Palinurid spiny lobsters have been subject to considerable aquaculture research and commercial interest for decades. At present the only significant aquaculture sector for Palinurid lobsters is in Vietnam, operating open-life cycle production of selected tropical *Panulirus* species with primary focus on the ornate spiny lobster *P. ornatus* (Jones, 2010). This type of culture relies on capturing wild 'seedstock' comprising pueruli and early stage juveniles and grow-out to market size in sea cages. Since the early 1990s, sea cage production had flourished and by 2006 the industry peaked at almost 2000 tonnes with a value of approximately $US90 million (Hung and Tuan, 2009). However, productivity has since declined dramatically largely due an epizootic known as milky 'disease' (Hung and Tuan, 2009) and production currently sits at 1500 tonnes worth $US60 million (Jones, 2010).

Clearly, the long-term sustainability of these farming methods is uncertain and is of major concern. To remain viable, the industry will depend on locating reliable and abundant supplies of seedstock. Biological neutrality has been suggested as such a strategy for spiny lobsters, which involves harvesting pueruli to juveniles for aquaculture, and additionally, reseeding sufficient amounts into wild fisheries to ensure fishing effort and catch are not displaced

(Gardner et al., 2006). Such methods appear to have promise, providing contiguous reef habitats with abundant shelter can be located for the release of naïve juveniles (Mills et al. 2006). However, these methods may have little relevance in jurisdictions where wild stocks are unmanaged and largely understood (Jones, 2010). In any case, the expected demand will far exceed any supply that wild populations can possibly meet. It becomes obvious that the establishment of closed-life cycle larviculture systems is necessary to supply grow-out facilities with sustainable volumes of quality seedstock (Kenway et al. 2009).

The ornate spiny lobster (*Panulirus ornatus*) represents an ideal spiny lobster candidate in which to pursue commercial larviculture technology. The species is highly fecund, with females capable of producing over 10^6 eggs and spawn on average 2-3 times per year (Rogers et al. 2010). *P. ornatus* also possess one of the shortest larval phases of the Palinuridae (Williams, 2007) with scope for reducing larval duration in culture (Smith et al. 2009b). Growth of juveniles is also rapid (Dennis et al. 1997) and under culture conditions it is possible to achieve market size (1 kg) from a 3 g juvenile within 18 months (Jones et al. 2001). Lastly, juveniles are tolerant of a wide range of temperatures, salinities, and high density living, and there is even scope for culture under turbid and eutrophic conditions in shrimp ponds (Jones and Shanks, 2009). From an economic viewpoint, *P. ornatus* is highly coveted by Chinese consumers, which because of its large size, firm textured flesh, and vibrant colourations, is served as a sashimi centrepiece (Hart, 2009).

In Australia, the current wild capture industry is operated by two jurisdictions in northern Queensland (Australia) and Torres Strait yielding annually 1000 tonnes and valued at $AU17 million (Ye et al. 2008), mainly supplying frozen tail and live markets, with the latter fetching prices exceeding AU$100 per kg depending on season (Rogers et al. 2010). Demand for this particular species is predicted to explode in the future especially from China (Hart, 2009). The limited capacity for increased production in wild capture fisheries fortifies the need to expand production through sustainable aquaculture (Ye et al. 2008).

1.4.1 Larviculture of spiny lobsters

Since the first recorded hatching experiments of spiny lobsters in 1898 (Hattori and Oishi 1899), advancements in larviculture technology have only been incremental because of the zootechnical challenges faced in maintaining high survival during the complex and protracted planktonic larval stage. Palinurid larval development is unique among crustaceans as it is one of the longest cycles of marine invertebrates, ranging from 4-7 months for the tropical *P. ornatus* to 12-22 months for cold water *Jasus edwardsii* (Figure 1-3). In contrast, larviculture of successful crustacean aquaculture sectors are epitomised by species possessing short larval phases such as penaeid prawns (Rothlisberg, 1998).

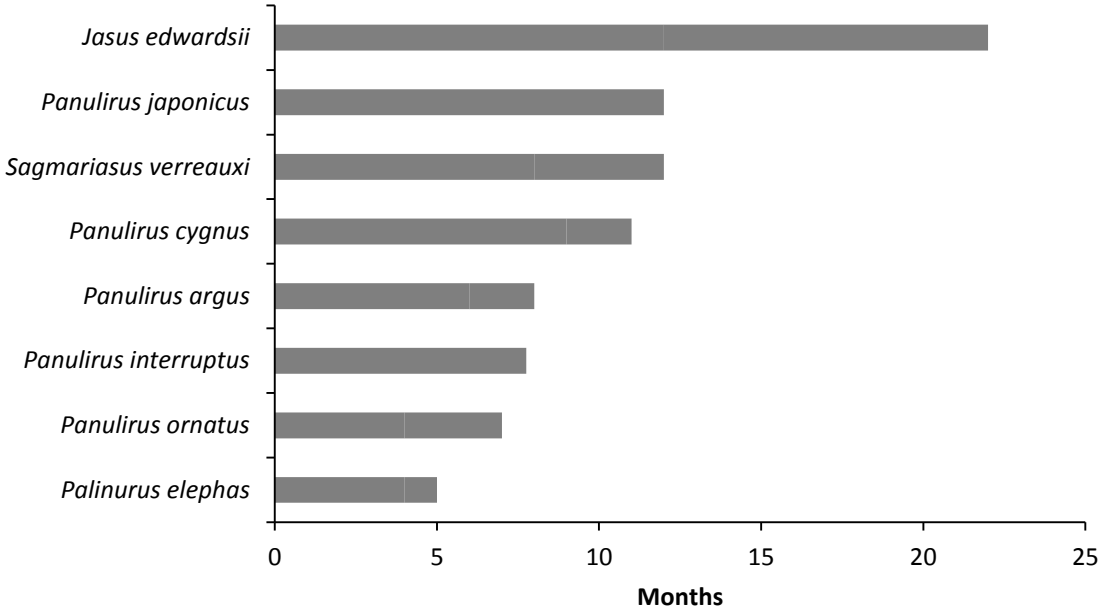

Figure 1-3. Estimated maximum larval periods in natural environments of selected Palinurid spiny lobsters. Graphed from a table in George (2005).

Following hatching, Palinurid larvae enter a planktonic zoeal larval phase known as phyllosomas (from Greek, “leaf-like” body). In natural settings, phyllosomas are released into the pelagic zooplankton and dispersal is influenced by numerous advective processes, including gyres, currents, eddies, upwelling, and wind-driven surface currents (Cobb, 1997). Phyllosomas are characterised by a transparent, dorso-ventrally flattened bodyplan and undergo a series of diecdysic moults to different morphological stages. For *P. ornatus*, there are 11 distinct morphological stages (Figure 1-4a) defined by progressive formation of pereiopods, antennule/antenna, mouthparts, pleon and gills, and within each stage are additional supernumerary growth moults known as instars (Smith et al. 2009b). Phyllosomas exhibit diel vertical migration, using exopodal natatory setae on maxillipeds and pereiopods for propulsion (Figure 1-4b). The final phyllosoma stage metamorphoses into a lecithotrophic (non-feeding) puerulus, which is non-pigmented and propelled by abdominal pleopods and capable of directional movement. In nature, navigation of pueruli from oceanic to coastal waters is thought to be mediated by a suite of cues detected by sensory receptors (Jeffs et al. 2005). The best evidence for this so far was reported by Goldsetin and Butler (2009) who showed *P. argus* pueruli were chemotactic to macroalgal (*Laurencia* spp.) metabolites and demonstrated barokinesis. Following benthic settlement, pueruli metamorphose into early stage juveniles and associate with macroalgal and seagrass environments (Dennis et al. 1997; Goldstein and Butler 2009) where feeding recommences and pigmentation develops with the deposition of calcium carbonate.

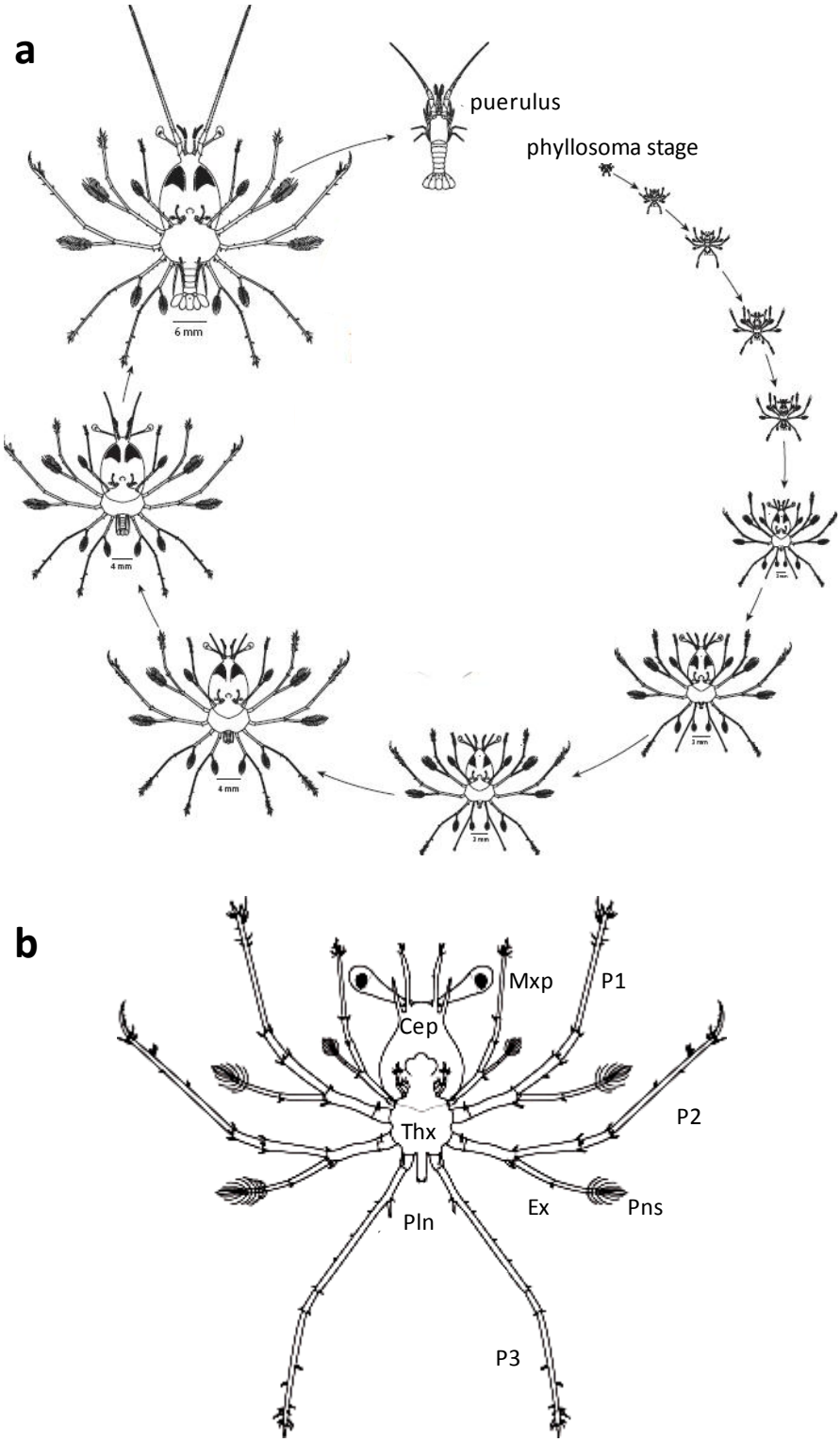

Figure 1-4. (a) Larval cycle of *Panulirus ornatus* and (b) ventral view of stage 1 *P. ornatus* phyllosoma illustrating major morphological features: cephalic shield (Cep); exopods (ex); maxilliped (Mxp); pereiopods (P1-3); pleon (Pln) plumose natatory setae (pns); thorax (Thx) (Images: AIMS).

Completed larval cycles, from phyllosoma through to puerulus, have so far only been described for 9 of the 20+ species in the Palinuridae ((Table 1-1; George, 1997). *P. japonicus* hatchery facilities in Japan regularly yield hundreds of juveniles from eggs annually (Murakami et al. 2007), yet production still lags behind commercially viable levels. In Australia, *J. edwardsii* and *P. ornatus* have been targeted for further research (Phillips and Matsuda, 2011), and hatchery technology for the latter is poised to become a commercial reality within the next 3 years (Rogers et al. 2010). However, some areas of Palinurid hatchery technology still need refinement, including reducing phyllosoma attrition rates by improving nutrition (Smith et al. 2009b) and managing bacterial epizootics (Bourne et al. 2004; 2007).

Table 1-1. Species of Palinuridae in which larval cycles have been completed in culture

Species	**Duration of phyllosoma phase (days)**	**Duration of puerulus phase (days)**	**References**
Palinurus sp.			
P. elephas	65-72	13.5	Kittaka et al. (2001)
Panulirus spp.			
P. argus	140-198	15-26	Goldstein et al. (2008)
P. japonicus	231-417	9-26	Sekine et al. (2000)
P. longipes	281-294		Matsuda and Yamakawa (2000)
P. ornatus	131-225	21-25	Smith et al. (2009b)
P. penicillatus	256-294	16-18	Matsuda et al. (2006)
Jasus spp.			
J. edwardsii	212-435	19	Kittaka et al. (2005)
J. lalandii	306	11	Kittaka (1988)
Sagmariasus sp.			
S. verreauxi	189-359	25.5	Kittaka et al. (1997)

Phyllosomas are opportunistic carnivores and recent observational and morphological studies, and molecular analysis on gut contents indicate they mainly prey upon gelatinous zooplankton such as chaetognaths and salps, but also more robust prey including icthyoplankton as development progresses (McWilliam and Phillips, 2007; Johnston et al. 2008; Suzuki et al. 2008; Smith et al. 2009a; Chow et al. 2010; Ikeda et al. 2011). It is likely that the biochemical composition of such organisms differs significantly from live *Artemia* provided in the

hatchery and may be suboptimal for larval development and growth (Smith et al. 2009b). Other factors impacting phyllosoma bioenergetic reserves in the culture system are nutrient absorption from the ambient environment (Rodriguez Souza et al. 1999; Söylemez et al. 2010), the metabolic expense of their photokinetic behaviours (Bermudes et al. 2008) and the requirement of fatty acids, amino acids and sterols for endocrine processes during metamorphosis (McWilliam and Phillips, 2007). Optimising nutrition could reduce the number of instars and override the plasticity of the phyllosoma larval cycle, and examples of this have been reported in the literature previously. Through dietary supplementation of larval sailfin sandfish (*Arctoscopus japonicus*) rich in lipid content, Kittaka et al. (2001) reduced the time taken for phyllosomas of *Palinurus elephas* to metamorphose by half, from 132 days to 65 days. Ironically, the phyllosoma survival rates were low due to a hepatopancreatic infection presumably caused by *Vibrio* spp. (Kittaka et al. 2001). Similarly, bacterial infections are major causes of mass attrition rates in cultured phyllosomas of *P. ornatus* (Bourne et al. 2004), *P. japonicus* (Kittaka, 1997), *S. verreauxi* (Diggles et al. 2000) and *J. edwardsii* (Ritar et al. 2006), and this warrants greater insight into the microbiology of the Palinurid hatchery ecosystem.

1.4.2 Microbiology of *P. ornatus* larviculture

Hatcheries can be highly eutrophic ecosystems due to the flux of organic matter from high larval densities and exogenous feeding (Verschuere et al. 1997; Olafsen, 2001; Bachère, 2003). These conditions are conducive to the proliferation of a range of heterotrophic microorganisms in the larviculture ecosystem, including pathogens, and differ markedly from the oligotrophic oceanic waters which wild conspecifics inhabit. The emergence of pathogenicity in aquaculture settings is likely an evolutionary response to high host availability and the selection of traits which enhance infectivity and transmission (Mennerat et al. 2010; Pulkkinen et al. 2010). Further, the susceptibility to disease is exacerbated in decapod larval stages which may not possess fully functional immune effectors (Jiravanichpaisal et al. 2007). These threats outline the necessity to identify sources of pathogens in the hatchery in order to implement appropriate disease management strategies.

Bourne et al. (2004) and co-workers initiated a series of fundamental microbiological studies on the *P. ornatus* larviculture ecosystem, focussing on four main interlinking compartments: the biofilm, water column, live prey and phyllosoma. Biofilm development on tank surfaces during early stage *P. ornatus* rearing is highly dynamic (Figure 1-5), but invariably dominated by members of Alpha-, Beta- and Gammaproteobacteria groups (Bourne et al. 2006). Importantly, though, the biofilm was shown to be a reservoir of a pathogenic *Vibrio harveyi* strain (Bourne et al. 2006). Some important attributes of bacterial biofilms compared to planktonic conspecifics are increased resistance to antimicrobial agents, host immune responses, and grazing by protozoans (Karunasager et al. 1996; Mah and O'Toole, 2001; Jefferson, 2004; Matz and Kjelleberg, 2005), which highlights a significant source of recurrent infection in hatcheries (Karunasager et al. 1996).

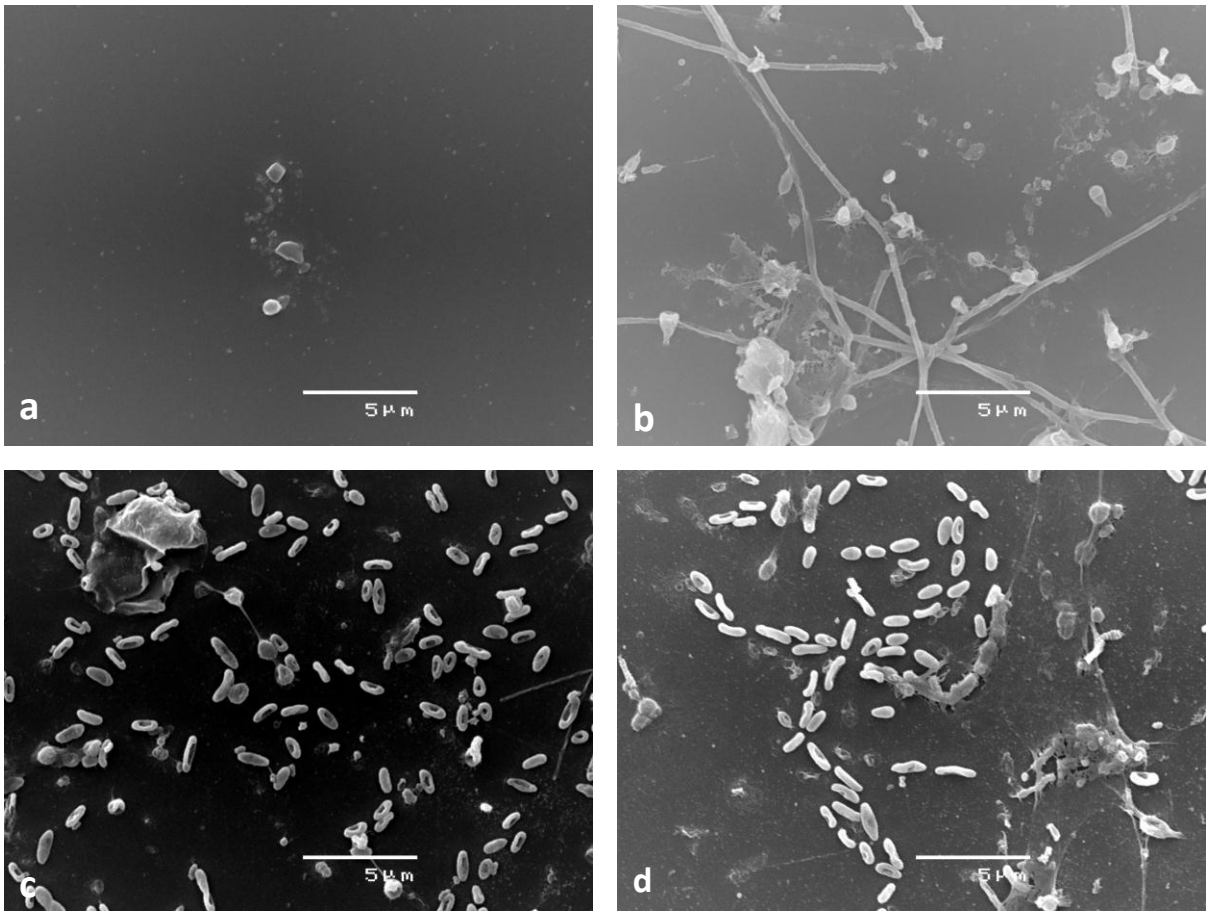

Figure 1-5. Scanning electron micrographs showing dynamic biofilm development during larval rearing of *P. ornatus*. (a) day 0; (b) day 9; (c) day 19; and (d) day 29 (Images: M. Hall).

The dispersal of planktonic bacteria from biofilms means the water column is constantly refuelled by transient free-living cells that forge associations with other microorganisms, microparticulates, and higher organisms (Grossart, 2010; Tang et al. 2010). The majority of free-living bacteria present in early stage *P. ornatus* larval rearing systems affiliate with microorganisms associated with biogeochemical cycling, including *Sulfitobacter* sp. and *Thiomicrospira* sp. (Bourne et al. 2004; Payne et al. 2006). However, a number of potential pathogens including *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* have also been recovered (Bourne et al. 2004; 2007; Payne et al. 2006). Mechanisms by which free-living pathogens affect *P. ornatus* phyllosomas is currently unknown, however Diggles et al. (2000) have shown experimentally injured *S. verreauxi* phyllosomas were more susceptible to challenge by immersion with *V. harveyi*, suggesting waterborne pathogens can invade hosts with compromised exocuticles. Another important consequence of free-living forms is that pathogenic microorganisms are subject to bioaccumulation by live prey organisms such as *Artemia* (Chair et al. 1994; López-Torres and Lizárraga-Partida, 2001; Vaseeharan and Ramasamy, 2003a) and subsequently transferred to aquatic larvae. Vectored transmission of pathogens is a potential hazard in Palinurid larviculture systems (Diggles et al. 2000; Ritar et al. 2006; Bourne et al. 2007) where animate prey (*Artemia*) is required to stimulate feeding (Johnston et al. 2008; Smith et al. 2009a). Furthermore, the *Artemia* gut can act as a safeguard from disinfectants and antimicrobials (Høj et al. 2009) which complicates microbial control strategies.

Bacterial communities of cultured *P. ornatus* phyllosomas differ significantly from their wild conspecifics (Payne et al. 2008). Cultured phyllosomas harbour a more diverse community which is reflective of the numerous environmental niches that exist within larval rearing systems, and include species belonging to the genera *Desulfobulbus*, *Methylobacter*, *Photobacterium*, *Pirulella*, *Pseudoalteromonas*, *Saprospira*, *Thiothrix*, and *Vibrio* (Bourne et al. 2004; Payne et al. 2007; 2008). By striking contrast, communities of wild phyllosomas are highly conserved and dominated by *Roseobacter* clade bacteria (*Sulfitobacter* sp. and *Roseobacter* sp.), Epsilonproteobacteria and *Erythrobacter* (Payne et al. 2008). The potential symbioses between these naturally occurring bacteria and wild phyllosoma may provide insightful clues into improving microbial health in *P. ornatus* larviculture.

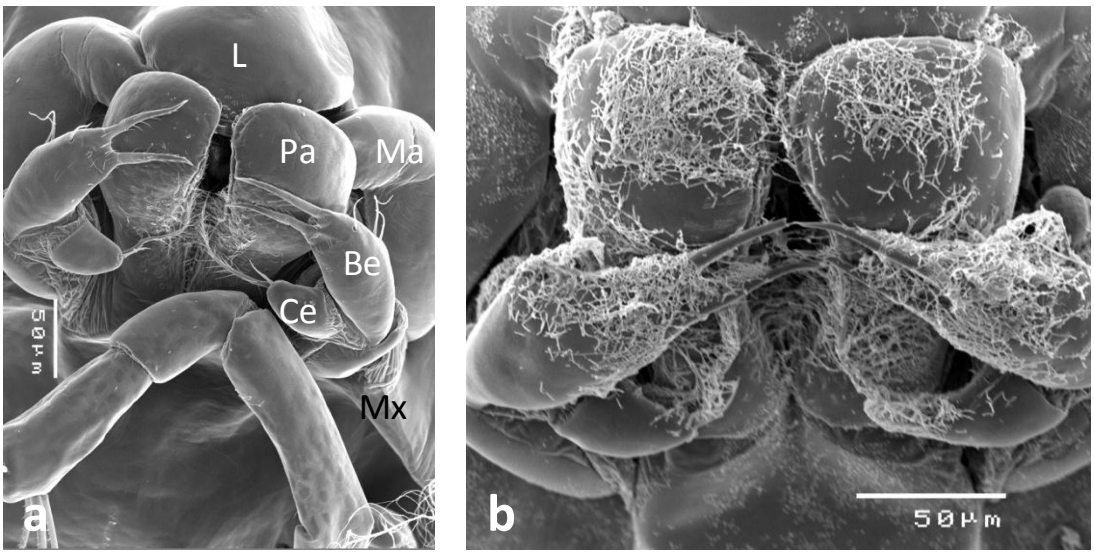

Figure 1-6. Scanning electron micrographs of mouthparts of (a) day 1 cultured phyllosoma and (b) higher magnification of mouthparts showing severe fouling in cultured larvae. Be = basal endites; Ce = coxal endites; L = labrum; Ma = mandible; Mx = 1st maxilla; P = paragnaths (Images: M. Hall).

P. ornatus phyllosomas are subject to both ectobiotic and endobiotic colonisation. In culture, severe biofouling of phyllosoma mouthparts (Figure 1-6) by filamentous bacteria (predominately *Thiothrix* spp.) is believed to hinder feeding and ecdysis (moulting), resulting in physiological decline (Bourne et al. 2007; Payne et al. 2007). Epistylis-like protozoans, and coccoid and rod-shaped bacteria including *Vibrionaceae*-affiliated microorganisms also form part of an overall diverse ectobiotic community (Bourne et al. 2004; Webster et al. 2006). By contrast, wild-caught conspecifics are devoid of such biofouling agents which exemplify how prevailing environmental conditions can influence bacterial community structure (Payne et al. 2008).

Using fluorescent *in situ* hybridisation (FISH), Payne et al. (2008) showed autochthonous endobionts of wild *P. ornatus* phyllosomas include distinct numbers of the groups *Bacteriodetes*, and Alpha-, Beta-, and Gammaproteobacteria. Small numbers of *Vibrionaceae* were also indigenous to phyllosomas, associated with gut tissues. In contrast, proliferation of *Vibrionaceae* in the hepatopancreas of cultured phyllosomas is associated with tissue necrosis and mass mortality events (Webster et al. 2006; Bourne et al. 2007). The symptoms present macroscopically as ‘white gut’ resulting from mass populations in the hepatopancreas and

epithelial cell sloughing, and similar pathologies have also been described in diseased phyllosomas of *S. verreauxi* (Diggles et al. 2000) and *J. edwardsii* (Handlinger et al. 1999). It is likely pathogenesis is mediated by enterotoxins given homologues of a *V. cholera* virulence regulatory gene (*toxR*) and a *V. parahaemolyticus* thermostable haemolysin gene (*tl*) have been found in a *V. harveyi* strain pathogenic to *P. ornatus* phyllosomas (Bourne et al. 2006).

It is clear from the extensive microbial analysis of *P. ornatus* phyllosomas that *Vibrio* spp. are significant disease causing organisms. In particular, the Harveyi-clade vibrios (Sawabe et al. 2007b) are economically important pathogens in aquaculture sectors around the world (Austin and Zhang, 2006; Cano-Gómez et al. 2009) and species within the Harveyi clade such as *V. alginolyticus* (Esteve and Herrera, 2000; Liu et al. 2004), *V. campbelli* (Hameed et al. 1996; Soto-Rodriguez et al. 2006), *V. harveyi* (Prayitno and Latchford, 1995; Robertson et al. 1998; Diggles et al. 2000; Aguirre-Guzmán et al. 2001), and *V. parahaemolyticus* (Aguirre-Guzmán et al. 2001; Cai et al. 2006; Martin et al. 2004; Khuntia et al. 2008) harbour strains which are virulent to aquatic invertebrates. A *V. harveyi* strain has shown to increase mortality of *P. ornatus* phyllosomas in preliminary infection experiments (Bourne et al. 2006), however further information on epizootic strains, pathology, and infection routes is lacking. It is paramount to the focus and timing of appropriate disease management in *P. ornatus* larviculture that pathogens are identified using experimental infection models that provide information on infection routes and infection dynamics (Saulnier et al. 2000). Further, fluorescently labelled bacteria is increasingly utilised in aquaculture as a powerful tool for elucidating pathogen-host interactions and identifying pathogen ecological niches (Ling et al. 2001; O'Toole et al. 2004; Chu and Lu, 2008; Sawabe et al. 2007a; Travers et al. 2008).

1.5 Disease management

As in other food production systems, antibiotics are also used in aquaculture to control infectious disease. In aquatic systems, there are significant environmental consequences relating to indiscriminate usage of antibiotics which may exert selection pressures on bacterial communities within and outside the aquaculture area (Cabello, 2006; Heuer et al. 2009). Increased circulation of antibiotic residues in the environment drives the selection of spontaneous mutants expressing antimicrobial resistance which may promote intra- and

intergenus dissemination of resistant determinants in bacterial populations through horizontal gene transfer (Cabello, 2006; Heuer et al. 2009; Burridge et al. 2010). One of the main risks to human health is the spread of antimicrobial resistance from aquatic bacteria to potential human pathogens including *Escherichia coli, Salmonella* spp. and *V. cholera* (Weber et al. 1994; Sørum and L'Abée-Lund, 2002; Molina-Aja et al. 2002; Heuer et al. 2009). Indeed, an epidemiological consequence of clinical antibiotic resistance is exemplified by multi-resistant *Staphylococcus aureus* (MRSA), which is a major public health problem implicated in the deaths of more than 18000 people in the US alone in 2005 (Klevens et al. 2007). Moreover, human consumption of aquatic products with antibiotic residues could alter the composition of intestinal microbiota, increasing susceptibility to disease and generating allergic hypersensitivity and antibiotic toxicity (Cabello, 2006; Willing et al. 2011).

Antibiotic usage is becoming increasingly obsolete in aquaculture as many economically important pathogens evolve resistance, including those belonging to the genera *Aeromonas* and *Vibrio* (Akinbowale et al. 2006; Sørum, 2006; Heuer et al. 2009). In addition, there is evidence for enhanced toxin production among bacteria exhibiting antibiotic resistance (Nakayama et al. 2006), and antibiotics are also implicated in the downregulation of some crustacean immune-related genes, which could have profound consequences on survival of the host (Aoki at al. 2010). With the risk of antibiotic resistance, many governing bodies especially in Europe, North America and Japan, have enforced bans or strict regulations on the use of antibiotics in animal production systems. Unfortunately, a large proportion of global aquaculture production takes place in countries which have less stringent governance on antibiotic usage. As the reforms and social attitudes against unregulated antibiotic use continue to develop, international cooperation is needed so regulatory frameworks can be implemented to control and monitor the use of antibiotics and antibiotic resistance (Heuer et al. 2009).

The Norwegian salmonid industry is a commendable example of an aquaculture sector which has significantly reduced antibiotic usage (by 99%) and simultaneously increasing productivity due to effective vaccines and improved husbandry (Heuer et al. 2009). However, as invertebrates do not possess immunological memory it is unlikely true vaccination

techniques can be applied to cultured crustaceans (Bachère, 2003; Smith et al. 2003), which instead rely on the upregulation of innate immune effectors including the phenoloxidase system, antimicrobial peptides, phagocytosis, and other haemocyte-mediated responses to combat invading microorganisms (Lee and Söderhäll, 2002; Aoki et al. 2010; Nayak et al. 2010; Somboonwiwat et al. 2010; Soonthornchai et al. 2010; Antony et al. 2011). While some of these immune genes such as crustins have been described in early stage crustaceans including Palinurid phyllosomas (Pisuttharachai et al. 2009) it is likely they have limited functionality (Jiravanichpaisal et al. 2007) meaning immunostimulation may not be a realistic option in invertebrate larviculture.

Other attempts to eliminate disease-causing bacteria and improve survival in Palinurid larviculture include seawater ozonation, which have yielded promising results (Ritar et al. 2006). However excessive ozonation can induce morphological abnormalities that incapacitate the ability of phyllosomas to undergo ecdysis and normal feeding, and other effects such as hepatopancreas degeneration (Jensen et al. 2011). Recent research suggests that bacteria associated with the body of zooplankton can be protected from ozonation (Tang et al. 2011), and this provides impetus to pursue alternative disease management regimes.

There is growing interest in sustainable biocontrol disease management strategies and the emergence of *P. ornatus* larviculture represents a unique opportunity to explore biocontrol options. The most successful strategies are likely to benefit from a microbial ecology approach, including defining and targeting microbial niches which harbour potential pathogenic microorganisms. It is not realistic to expect total eradication of pathogens from larviculture ecosystems, however with the appropriate biocontrol methods, targeted pathogen populations should be restricted to levels which do not cause disease.

1.6 Probiotics as a biocontrol strategy

The word probiotic derives from the Greek terminologies ‘pro’ and ‘bios’ and refers to ‘for life’. The functionality and therefore definition of a probiotic is largely dependent on the animal production system in which it will be used. Fuller’s widely cited “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”

(Fuller, 1989) does not strictly apply to aquaculture because aquatic animals are intimately associated with their ambient environment, thus microbial-induced improvements to the host ectobiota or host external environments may also be considered probiotic effects (Verschuere et al. 2000b). In their review, Irianto and Austin (2002) defined an aquaculture probiont as "an entire or component(s) of a microorganism that is beneficial to the health of the host". This all-encapsulating definition includes probiotic action on both internal and external environments, and introduces the prospect of microbial components, such as peptidoglycan and lipopolysaccharides, acting as immunostimulants (Smith et al. 2003). The Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) currently define human probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2001). Although this concept primarily relates to the beneficial effects of food with added *living* probionts on human health, it also infers that the benefits elicited by probiotics are not solely confined to the gastrointestinal region and may promote immunological and respiratory function and alleviate infectious disease.

It should be acknowledged that there are many emerging probiotic biocontrol methods in aquaculture, yet not all are relevant to the present research topic. Of particular interest are bacteriophage therapies (reviewed by Defoirdt et al. 2007; 2011a), which have shown promise in enhancing the survival of larval *Penaeus monodon* under hatchery conditions (Vinod et al. 2006; Shivu et al. 2007). However, in the following sections, the focus is more towards bacterial modes of action implicated in probiotic activity in aquaculture systems.

1.7 Probiotic modes of action

One of the main channels of a probiotic biocontrol approach is to preemptively colonise the hatchery environment with beneficial microorganisms to reduce the incidence of primary colonisation by unfavourable microorganisms, including pathogens (Verschuere et al. 2000b). Direct activity against pathogens may be facilitated through a number of possible mechanisms of action (MOAs) which include but are not limited to: antagonism (Verschuere et al. 2000b; Balcázar et al. 2006; Kesarcodi-Watson et al. 2008) competition for host adhesion sites and nutrients (Gatesoupe, 1999; Verschuere et al. 2000b; Vine et al. 2004b; 2006; Chabrillón et al.

2005a); quorum-sensing inhibition (Natrah et al. 2011); and predation (Qi et al. 2009). However, most of the perceived MOAs are based on *in vitro* observations and there is still a need for *in vivo* approaches, such as gnotobiotic models, to elucidate probiotic-host interactions and the effect of introduced probiotics on autochthonous microbiota (Tinh et al. 2008a).

1.7.1 Antagonism

Antagonistic microorganisms are commonly considered in probiotic screening processes and exert their MOA by producing bioactive compounds including antibiotics, bacteriocins, hydrogen peroxide, and organic acids which are inhibitory towards other microorganisms (Verschuere et al. 2000b; Vine et al. 2006). Inhibitory activity is commonly identified using *in vitro* antagonism assays in which known pathogens are exposed to putative probiotics or their extracellular products in solid or liquid medium (Figure 1-7). Electron microscopy reveals the action of bioactive substances can induce morphological changes to pathogen cells which disrupt physiology directly or make them susceptible to attack by other active compounds (Ringø, 2008; Ma et al. 2009; Rattanachauy et al. 2010). Further, bacteriocins produced by lactic acid bacteria are involved in depleting cell proton motive forces which are central to energetic processes of cytoplasmic membranes in bacteria (Bruno and Montville, 1993).

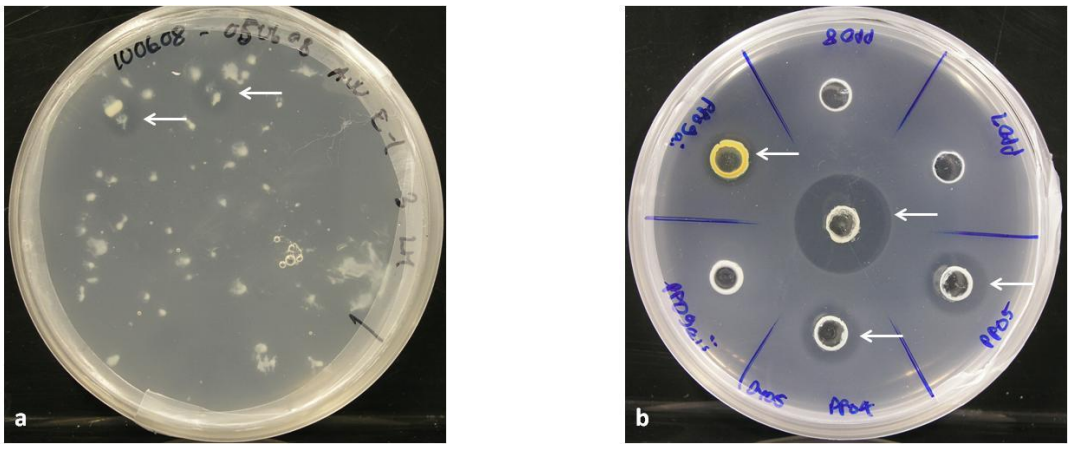

Figure 1-7. Common agar-based *in vitro* antagonism assays. A target pathogen is embedded in agar and test bacteria are (a) overlayed from environmental samples using the replica plate method or (b) inoculated from broth cultures into wells using the well diffusion method. Isolates positive for antagonism are represented by inhibition zones designated by white arrows (Images: E. Goulden).

Antagonism is a widespread trait among marine bacterial genera and is implicated in competitive interactions and ecological success of microorganisms (Kalinovskaya et al. 2003; Makridis et al. 2005; Martens et al. 2007; Fjellheim et al. 2007; Weitz et al. 2010; Gram et al. 2010). There are many listings in the literature where antagonistic bacterial isolates or their products have conferred a protective benefit or enhanced survival of larval and postlarval crustaceans (Nogami et al. 1997; Li et al. 2006; Ravi et al. 2007; Guo et al. 2009; Swain et al. 2009; Pai et al. 2010) and molluscs (Ruiz-Ponte et al. 1999; Riquelme et al. 2001; Longeon et al. 2004; Kesarcodi-Watson et al. 2010). These studies indicate a diversity of bacterial genera, including *Alteromonas, Arthrobacter, Bacillus, Enterococcus, Neptunomonas, Pseudomonas, Pseudoalteromonas, Roseobacter, Streptococcus, Thalassobacter,* and *Vibrio* harbour strains with probiotic potential.

The microbiota associated with larval hosts are good reservoirs of antagonistic bacteria (Hjelm et al. 2004; Fjellheim et al. 2007) and represent logical microenvironments in which to conduct initial searches for probionts. It is expected such microorganisms are intrinsically adapted to the dynamics of the culture system which thereby increases the chance of a desired probiotic effect (Kesarcodi-Watson et al. 2008). Preliminary probiotic screening work for *P. ornatus* larval rearing indicated wild Palinurid spp. and Scyllarid spp. phyllosomas harbour strains of *Pseudoalteromonas* spp., *Cytophaga* sp., *Nocardiodes* sp., *Tenacibaculum* sp., and *Winogradskella* sp. which are antagonistic to pathogenic *V. harveyi* CO71 (Wietz, 2007).

However, *in vitro* antagonistic activity is not always concomitant with an *in vivo* effect. For example, Gram et al. (2001) demonstrated that *Pseudomonas fluorescens* strain AH2, antagonistic to icthyopathogenic *Aeromonas salmonicida in vitro*, could not protect salmon (*Salmo salar*) from the pathogen *in vivo*. Similarly, Ruiz-Ponte et al. (1999) found an antagonistic *Roseobacter* strain could not protect scallop (*Pecten maximus*) larvae from *V. anguillarum*. In both these examples, the authors postulated that one of the reasons for the lack of an effect was due to differential niche specialisation exhibited by the pathogen and probiont. This illustrates that selected probionts must share and exert their perceived MOA in the ecological niche where the pathogen has its impact.

1.7.2 Competition for attachment sites and nutrients

Generally, a large pool of candidates will be generated from initial *in vitro* antagonism screening processes and it is highly impractical to validate the *in vivo* effects of all candidates due to the restricted accessibility and processing of large numbers of experimental animals (Kesarcodi-Watson et al. 2008). To supplement *in vitro* antagonism screens, probiotic candidates can be assessed for adhesive and competitive potential on mucous or intestinal epithelial cells (Vine et al. 2006), given the microbiota of the gastrointestinal tract serve as an important barrier to invading pathogens in aquatic larvae (Olafsen et al. 2001; Tinh et al. 2008a). Mechanisms by which microorganisms adhere to intestinal epithelial cells include van der Waal's forces, electrostatic and hydrophobic interactions, and specific receptor-ligand binding (Ringø et al. 2007). However, the temporal attachment of microbes can be influenced by flushing rates and transforming physicochemical characteristics during larval development (Olafsen 2001; Tinh et al. 2008a). As such, administered probionts may be evacuated from the gut before they can secrete antimicrobials, which for most aquaculture bacterial genera, occurs maximally in the stationary phase (Chythanya et al. 2003; Campos et al. 2006; Vijayan et al. 2006; Ringø, 2008; Rattanachuay et al. 2010).

Vine et al. (2004b) described an *in vitro* screening method using differently labelled radioactive isotopes to monitor the ability of candidate probionts to outcompete icthyopathogenic *V. alginolyticus* and *A. hydrophila* on mucous extracted from spotted grunter (*Pomadasys commersonnii*). The authors reported that only one strain, AP5, could reduce and displace *V. alginolyticus* attachment in the mucous, indicating a good potential to beneficially influence the intestinal microbiota. Similarly, Chabrillón et al. (2005a) found the antagonistic probiont Pdp11 could inhibit pathogenic *V. harveyi* attachment *in vitro* by exclusion and displacement on skin and intestinal mucous of sole (*Solea senegalensis*), and further could significantly enhance survival of *S. senegalensis* during *V. harveyi* challenge *in vivo*. Using intestinal epithelial cell lines of Chinese drums (*Miichthys miiuy*) as an attachment surface, Pan et al. (2008) showed the probiotic candidate *Clostridium butyricum* could significantly reduce attachment of icthyopathogenic strains of *V. anguillarum* and *A. hydrophila* regardless of whether *C. butyricum* was incubated prior to, simultaneously, or after the pathogens. In the case of phyllosoma larvae, epithelial mucous cannot be extracted without exigent

microdissection techniques and immortalised cell lines are difficult to generate for invertebrate tissues (Rinkevich, 2005), making *in vitro* screens for attachment and competition difficult for larval crustaceans. Further, as the physicochemical characteristics of the gut changes throughout larval development and metamorphosis, it is difficult to extrapolate observations on tissues from adult origin (Tinh et al. 2008a).

Probiotic microorganisms may also compete with pathogens for nutrients in the host or ambient environment. Siderophore-producing bacteria are prime examples of microorganisms which deprive competitors of iron by chelation. Gram et al. (1999) showed siderophore-containing supernatants produced by *Pseudomonas fluorescens* (AH2) inhibited the growth of icthyopathogenic *V. anguillarum* in medium deficient in iron, but not in iron replete medium. AH2 also reduced mortalities in rainbow trout (*Oncorhynchus mykiss*) experimentally challenged with *V. anguillarum*. A heat labile siderophore was also among the compounds produced by a *Bacillus* sp. strain isolated from dragonets (*Callionymus* sp.) with inhibitory activity against a range of intestinal-derived isolates including *Acinetobacter* spp., *Bacillus* spp., coryneforms, Enterobacteriaceae, *Flavobacterium* spp., *Moraxella* spp., *Pseudomonas* spp., and *Vibrionaceae* (Sugita et al. 1998). In fact, recent research indicates siderophore-producing bacteria, including *Enterovibrio* spp., *Vibrio* spp. and *Photobacterium* spp., are widespread intestinal microorganisms among fish species, which could be used to suppress pathogens in culture environments (Sugita et al. 2012).

1.7.3 Quorum-sensing disruption

Quorum sensing (QS) is a type of bacterial cell-cell communication system used to coordinate the expression of genes in response to small signal molecules known as autoinducers (Hentzer et al. 2002). There are two common types of QS systems, mediated respectively by the signal molecules *N*-acylated homoserine lactones (AHLs) and autoinducer 2 (AI-2; a furanosyl borate diester), and a third pathway exclusive to *Vibrio* spp. facilitated by CAI-I, the cholerae autoinducer 1. The widespread appearance of analogous QS systems among bacterial genera reflects possible roles in interspecies communication (Waters and Bassler, 2005; Boyer and Wisniewski-Dyé, 2009). Importantly, the AI-2 multichannel system has been found in some aquaculture pathogens such as *V. anguillarum*, *V. harveyi*, and *V. vulnificus* (Milton, 2006).

A number of *Vibrio* virulence factors are regulated by QS, including a type III secretion system (Henke and Bassler, 2004), siderophores (Lilley and Bassler, 2000), extracellular toxins (Manefield et al. 2000) and chitinases (Defoirdt et al. 2010). Experimental exposure to mixed AHLs caused mortality in giant freshwater prawn (*Macrobrachium rosenbergii*) larvae, putatively due to the induction of virulence factors in associated microbial communities (Baruah et al. 2009). Further, elevated levels of AHL molecules were detected in conjunction with mass mortalities of *P. ornatus* phyllosomas (Bourne et al. 2007), indicating quorum sensing likely regulates virulence mechanisms of some phyllosoma pathogens.

Recently, Defoirdt and co-workers demonstrated that virulence of *V. harveyi* towards gnotobiotic brine shrimp *Artemia franciscana* could be attenuated using AI-2 defective mutants (Defoirdt et al. 2005), paving the way for anti-virulence therapies targeting disruption of QS-regulated virulence factors. Aquatic bacteria are capable of QS signal antagonism and degradation, most notably the Gram positive genera *Bacillus* and *Halobacillus* (Defoirdt et al. 2011b; Teasdale et al. 2011). The sea-grass associated bacterium, *H. saliu*, produce metabolites containing phenetylamide which act as signal antagonists that inhibit a number of QS-regulated phenotypes of distantly related bacteria, including *V. harveyi* and *Chromobacterium violaceum* (Teasedale et al. 2009). *Bacillus* spp. are reputed producers of lactonases, encoded by the *aiia* gene, which hydrolyse the lactone ring of AHLs (Lee et al. 2002; Dong et al. 2002; 2007). Screening for AHL-degrading bacteria is usually achieved by enrichment cultures (bacteria selectively enriched from environmental samples that utilise AHLs as a nitrogen source) and have been produced from gastrointestinal tracts of shrimp *Peneaus monodon* (Tinh et al. 2007) and fish (Cam et al. 2009a,b). Strains derived from enrichment cultures have neutralised the growth retarding effect of *V. harveyi* on rotifers (Tinh et al. 2007) and improved survival in turbot (*Schopthalmus maximus*) larvae (Tinh et al. 2008b) and *M. rosenbergii* larvae (Cam et al. 2009b) when exposed to AHLs. Such observations support bacterial-mediated QS-disruption as a probiotic mode of action. Furthermore, AHL-acylases, encoded by the *aac* gene, were found in a fish-derived *Shewanella* strain which interrupted QS-regulated biofilm formation in icthyopathogenic *V. anguillarum* (Morohoshi et al. 2008) and interfered with AHL-mediated production of exoproteases in an *Aeromonas* strain (Morohoshi et al. 2005).

Unlike antibiotics, one of the main attractions of QS disruption is that pathogen growth is unaffected and selective pressures and risk of resistance is confined only to environmental situations where QS-regulated metabolism is essential (Defoirdt et al. 2011a). In this respect, knowledge of genetic and phenotypic factors regulated by QS in relevant pathogens is crucial prior to implementing such anti-virulence strategies.

1.7.4 Predation

The use of the bacterivorous bacterial genus *Bdellovibrio* as biocontrol agents in aquaculture is another promising avenue (Qi et al. 2009). These obligate predators of Gram-negative bacteria are highly motile, seeking prey cells by propulsion mediated by polar flagellum. Once attached to the outer membrane of prey cells, *Bdellovibrio* cells penetrate the periplasm and grow using the cytoplasm as a substrate before replicating and releasing themselves from the host (Jurkevitch, 2007). Very recently, Cao et al. (2012) isolated a strain from sturgeon (*Acipenser baerii*) gut with broad spectrum lytic activity against pathogenic *Aeromonas* strains, supporting these bacteria as promising probiotic agents against pathogen infections.

1.8 Research aims: Pathogen identification and probiotic biocontrol for *P. ornatus* larviculture

As the infective processes of *P. ornatus* phyllosoma pathogens remain to be elucidated, it comes to no surprise that very little research has been invested in probiotic biocontrol strategies in the larviculture of this species. Earlier work by Igarashi et al. (1990) have indicated the addition of beneficial bacteria to phyllosoma rearing systems of *Jasus edwardsii* can promote survival and metamorphosis to puerulus, extending the notion that bacteria can be employed as biocontrol methods in Palinurid larviculture. However, a detailed understanding of probiont-pathogen-host interactions is required to successfully implement biocontrol strategies which target specific pathogens.

The main objectives of this thesis were to study pathogens responsible for epizootics and their infective processes in *Panulirus ornatus* larviculture, and to identify bacteria antagonistic to the pathogen and capable of protecting *P. ornatus* phyllosomas from disease. Knowledge and

tools generated in this thesis can be used in the development of disease management strategies using biocontrol agents in Palinurid larviculture systems.

This chapter (Chapter 1) of the thesis provides an introduction to the importance of aquaculture against a backdrop of dwindling global wild-capture fisheries, with focus on Palinurid spiny lobsters. This is followed by a review of strategies and limitations of prospective Palinurid aquaculture and larviculture, with reference to *P. ornatus*. A synthesis of *P. ornatus* larviculture microbiology with disease management options through probiotic biocontrol is also provided.

Chapter 2 describes elements of the serious pathogen (*Vibrio owensii* DY05) responsible for epizootics during early stage *P. ornatus* larviculture. Experimental infection models were used to validate Koch's postulates, and determine the route of infection and pathologies concomitant with infection. Modes of pathogenesis were also discussed based on *in vitro* production of enzymes associated with virulence.

Chapter 3 provides a conceptualisation of the infective cycle of *V. owensii* DY05 based on *in situ* spatiotemporal localisation of a green fluorescent protein expressing transconjugant of DY05 during infection of *P. ornatus* phyllosomas. This research tool was used to examine and identify ecological niches in the hatchery environment to guide probiotic biocontrol options.

Chapter 4 describes an extensive probiotic screening strategy for bacteria antagonistic towards *V. owensii* DY05. This part of the investigation was developed using conventional and novel *in vitro* antagonism assays. From this effort, the combination of two probionts, *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 (prospected from natural prey items of phyllosomas), were shown to significantly enhance survival of *P. ornatus* phyllosomas during challenge with *V. owensii* DY05.

Chapter 5 describes and identifies the respective niches of the *P. ornatus* phyllosoma probionts *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107. This study examined the pathogen-probiont-phyllosoma interactions *in situ* using fluorescent protein expressing

transconjugants. It was hypothesised that niche specialisation demonstrated by the probionts contributed to the additive and enhanced survival benefit of PP05 and PP107 during pathogen challenge. A biocontrol strategy for early stage larviculture is proposed.

Chapter 6 draws conclusions from the previous chapters and prospective research is proposed.

2 *Vibrio owensii* DY05: a significant pathogen of cultured ornate spiny lobster (*Panulirus ornatus*) phyllosoma

2.1 Abstract

The type strain of *V. owensii* (DY05) was isolated during an epizootic of aquaculture-reared ornate spiny lobster (*Panulirus ornatus*). The present study investigated the pathogenicity of *V. owensii* DY05 in early stage *P. ornatus* phyllosomas using an experimental infection model. *V. owensii* DY05 was formally demonstrated as the aetiological agent of disease causing rapid and reproducible larval mortalities with similar pathologies as seen during disease epizootics. Vectored challenge via the aquaculture live feed organism *Artemia* caused consistent cumulative mortality rates of 84-89% after 72 h in contrast to variable mortality rates seen after immersion challenge. This result highlights live feed vectors as crucial determinants in the infection process. Histopathological examination of vector-challenged phyllosomas revealed bacterial proliferation in the hepatopancreatic lumen concomitant with epithelial cell necrosis. *In vitro* enzyme assays identified proteases, phospholipases, and haemolysins as potential virulence factors. This study provides the first detailed description of an emerging bacterium, *V. owensii*, as a virulent pathogen responsible for epizootics in the larviculture of *P. ornatus*.

2.2 Introduction

Major steps towards the commercialisation of closed life-cycle aquaculture production of the ornate spiny lobster (*Panulirus ornatus*) have been reported recently (Rogers et al. 2010), yet nutritional deficits (Smith et al. 2009b) and mortalities caused by bacterial disease (Bourne et al. 2004) remain key constraints to hatchery productivity. Mass mortalities of larvae (phyllosomas) are often associated with enteric vibriosis, an infection of the midgut gland (hepatopancreas) caused by *Vibrio* species (Webster et al. 2006; Bourne et al. 2007). Furthermore, additional afflictions such as biofouling by filamentous bacteria including *Thiothrix* spp. can restrict the capacity of phyllosomas to capture and masticate prey and

interfere with ecdysis, resulting in physiological decline and vulnerability to opportunistic infection (Bourne et al. 2007; Payne et al. 2007).

Vibrio harveyi is one of the most significant and economically important pathogens of cultured aquatic organisms (Austin and Zhang 2006). These microorganisms belong to the Harveyi clade, which includes close relatives *V. alginolyticus*, *V. campbelli*, *V. mytili*, *V. natriegens*, *V. parahaemolyticus*, *V. rotiferianus* (Sawabe et al. 2007b) and the recently described *V. owensii* (Cano-Gómez et al. 2010). Many virulence factors have been identified in Harveyi clade vibrios, including the production of proteases, haemolysins, phospholipases and chitinases (Liu et al. 1996; Zhang and Austin, 2000; Aguirre-Guzmán et al. 2004; Austin et al. 2005; Defoirdt et al. 2010). Certain virulence factors are controlled by gene regulatory systems, including quorum sensing and the transmembrane transcriptional regulator, ToxR (Milton, 2006; Ruwandeepika et al. 2011). In crustaceans, vibrio virulence is commonly associated with infection and degeneration of hepatopancreatic tissues (Lavilla-Pitogo et al. 1998; Robertson et al. 1998; Esteve and Herrara, 2000; Khuntia et al. 2008).

It is paramount to the development of efficient disease management strategies that pathogens are identified using experimental infection models that provide information on infection routes (Saulnier et al. 2000). This study describes the development of a robust experimental infection model to evaluate the pathogenicity of bacteria associated with mass mortality events in the larviculture of *P. ornatus*. The study included (1) a comparison between immersion and vector-challenge as natural routes of infection; (2) verification of Koch's postulates; (3) investigation of bacterial toxins produced *in vitro* as potential virulence factors; and (4) pathological assessment of experimentally infected animals.

2.3 Materials and Methods

2.3.1 Larviculture

P. ornatus phyllosomas were sourced from broodstock held at the Tropical Aquaculture Facility of the Australian Institute of Marine Science (AIMS), Townsville, Australia. Maintenance of broodstock, production of phyllosomas, and larviculture were performed

according to Smith et al. (2009b). Only apparently healthy individuals as assessed by positive phototaxis were used for experiments.

2.3.2 Isolation of pathogen

Vibrio spp. were isolated from moribund stage 3 phyllosomas during an epizootic in the AIMS larval rearing system. Briefly, phyllosomas were washed in sterile artificial seawater (ASW; Instant Ocean®) to remove loosely attached epibionts and excess detritus, homogenised in ASW and plated on thiosulphate citrate bile sucrose agar (TCBS; Becton, Dickinson and Company). Dominant and unique colony morphotypes were cultured to purity on TCBS and cryopreserved (-80^{o}C). Preliminary infection experiments (described below) indicated that 1 of 3 putative disease causing isolates, DY05, caused significant mortalities (85%) of stage 3 phyllosomas 72 h after vectored challenge (data not shown). DY05 was subsequently characterised by exhaustive phenotypical, biochemical and phylogenetic analyses and determined to belong to the novel species *Vibrio owensii*, of which DY05 is the type strain (Cano-Gómez et al. 2010).

2.3.3 Bacterial culture and inoculum preparation

Cryopreserved stocks of *V. owensii* DY05 were revived and cultured in 5 mL MB (28^{o}C, 170 rpm) for 18-24 h. Inoculums were prepared by washing cells 3 times by centrifugation (10 min at 4560 rpm; 10^{o}C) and resuspension in 0.22 µm filtered seawater (FSW). The final cell suspension was adjusted to absorbance OD_{600nm} 0.1 (Nanodrop ND1000). Corresponding total viable counts were determined in triplicate by spiral plating (Eddy Jet; IUL) on marine agar (MA; Becton, Dickinson and Company) and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL). This information was used to calculate the volume of the OD_{600nm} 0.1 suspensions needed to achieve the desired starting concentrations in the phyllosoma *in vivo* infection experiments described in sections 2.3.4 – 2.3.5.

2.3.4 Experimental infection by immersion

P. ornatus phyllosomas (stage 1) were washed twice in FSW and distributed into Nunc^{TM} 12-well cell culture plates (NUN150628) at 1 larva well^{-1} with 3 mL FSW per well. Animals were acclimated in darkness (28^{o}C, 45 rpm) for 2 h before adding *V. owensii* DY05 inoculum directly into wells at low (1 x 10^3 CFU mL^{-1}), moderate (1 x 10^5 CFU mL^{-1}) or high (1 x 10^7 CFU mL^{-1}) doses. Experimental controls were not exposed to *V. owensii* DY05. Each treatment was performed in separate plates and run in quadruplicate (n = 48). Larval mortality was assessed every 24 h for 5 days with phyllosomas not displaying any active movement after prolonged inspection being recorded as dead. No feeding or water changes were performed during the experiment. The immersion challenge experiment was repeated three times using progeny produced by different broodstocks and genetic lineages.

2.3.5 Experimental infection by vectored challenge

P. ornatus phyllosomas (stage 1 and stage 3) were exposed to *V. owensii* DY05 using live *Artemia* stage II (nauplii) as vectors. Formalin-disinfected nauplii (200 nauplii mL^{-1}) were enriched through filter feeding with the pathogen *V. owensii* DY05 (1 x 10^6 CFU mL^{-1}) in tissue culture flasks (Sarstedt) for 2 h (28^{o}C, 45 rpm). Control nauplii were treated similarly except no bacteria were added. Both control and enriched nauplii were homogenised and plated on TCBS.

Phyllosomas were distributed to cell culture plates and acclimated for 2 h (stage 1) or 24 h (stage 3) prior to experimental infection. On day 0, phyllosomas were fed with live *V. owensii* DY05-enriched or non-enriched *Artemia* nauplii (control) at approximately 3 nauplii mL^{-1}. Each treatment was performed in separate plates and run in quadruplicate (n = 48). One stage 3 experiment included a control with phyllosomas treated with an antibiotic cocktail (25 mg L^{-1} erythromycin, 25 mg L^{-1} oxytetracycline, 10 mg L^{-1} streptomycin, 40 mg L^{-1} ciprofloxacin) for 24 h prior to collection, then rinsed, acclimated, and challenged with control *Artemia* nauplii as outlined above. This experiment also included additional replicate plates for sacrificial sampling for analysis of culturable vibrios (section 2.3.6). Larval mortality was assessed every 24 h for 5 days as described above. Water changes and additional feeds were

not performed during the experiment. For each stage, three replicate experiments were performed using progeny from different broodstocks and genetic lineages.

2.3.6 Re-isolation of *Vibrio*

Vibrios were recovered from *Artemia* cultures and experimentally infected phyllosomas (stage 3) in sacrificial plates. Briefly, six live and six dead (within 4 h *post mortem*) phyllosomas were sampled within the first three days of the experiment, homogenised in ASW and plated on TCBS. Dominant colony morphotypes were cultured to purity and cryopreserved.

For each isolate, colony material from TCBS plates (28^{o}C, overnight) was used as template in PCR reactions with universal bacterial 16S rRNA gene primers 27F and 1492R (Lane, 1991) under standard amplification conditions. The PCR products were purified and sequenced in both directions by Macrogen (Seoul, Korea). Sequences were edited with 4Peaks (Mekentosj) software and analysed using the BLAST algorithm on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/) to determine nucleotide-nucleotide similarity with sequences in the nr/nt database. Isolates with high 16S rRNA gene sequence identity (>99%) to *V. owensii* were analysed further by sequencing *topA* (topoisomerase I, DNA replication and repair) and *mreB* (rod shaping protein gene, B subunit) using the primers VtopA400f/VtopA1200R or VmreB12F/VmreB999R, respectively (Sawabe et al. 2007b). Reaction conditions for each primer set and PCR reagents were as described by Thompson et al. (2005). Sequences were generated and analysed as described above.

2.3.7 Histopathological analysis

Stage 3 phyllosomas were sampled prior to and 18, 24 and 42 h after vector-challenge. Following removal of the legs (pereiopods), phyllosomas were fixed in Bouin's Fixative (75 mL saturated picric acid, 25 mL formaldehyde [40%], 5 mL glacial acetic acid) for 20 h at room temperature with gentle shaking. Fixed samples were washed and stored in 70% ethanol at 4^{o}C until further processing. Samples were dehydrated in an ethanol series (70%, 96% and 100%; 30 min) followed by pre-infiltration and embedding in 2-hydroxyethyl-methacrylate resin (Technovit 7100; Heraeus-Kulzer) according to the manufacturer's protocol.

Phyllosomas were sagittally sectioned (2 µm) using a carbide tungsten blade (Delaware Diamond Knives Inc.) on a rotary microtome (HM360, Microm). Sections were progressively stained with Gill's No. 2 haematoxylin and aqueous eosin (1%) and examined using light microscopy (AxioSkop 2 mot plus; Carl Zeiss). Histological images were captured using an AxioCam MRc5 camera (Carl Zeiss) and processed using AxioVision Rel. 4.8 software (Carl Zeiss). Special interest was given to the hepatopancreas tubules.

2.3.8 Enzymatic activities

Amylase, protease (caseinase and gelatinase), chitinase, lipase and phospholipase activities were performed as previously described for broth cultures (Liu et al. 1996; Austin et al. 2005) and extracellular products (Liu, 1957; Sudheesh and Xu, 2001; Austin et al. 2005) with some minor modifications. Briefly, *V. owensii* DY05 cultured in tryptic soya broth supplemented with 2% NaCl (TSB2) for 24 h was spot inoculated onto tryptone soya agar supplemented with 2% NaCl (TSA2) containing 1% soluble starch, 0.5% casein, 0.5% gelatin, 0.3% technical grade shrimp chitin (Sigma), 1% (v/v) Tween 80 (Sigma), or 0.2% egg yolk emulsion, respectively. All plates were incubated at 28^{o}C for 48 h. Substrate lysis was confirmed by the appearance of clearance zones around colonies. Lytic zones of the amylase and protease (caseinase and gelatinase) assays were visualised by flooding with Lugol's iodine solution (Sigma) or saturated ammonium sulphate, respectively. Lipase and phospholipase activity was indicated by appearance of opalescent halos resulting from the formation of a calcium oleate precipitate. Production of haemolysins was examined using Columbia agar base (Oxoid) supplemented with 5% (v/v) sheep erythrocytes.

To extract extracellular products, 24 h TSA2 colonies were suspended in 5 mL PBS (8 g L^{-1} NaCl; 0.2 g L^{-1} KCl; 1.44 g L^{-1} Na_2HPO_4; 0.24 g L^{-1} KH_2PO_4; pH 7.2) and 200 µL spread inoculated in triplicate onto sterile, non-plasticised cellophane (NatureFlex™ 23NP) overlayed on TSA2. Following incubation, colony material from each cellophane sheet was scraped into 2 mL ice cold PBS. Suspensions were centrifuged at 14000 g for 15 min (4^{o}C) and supernatants pooled before successive filtration (0.45 µm and 0.22 µm). Freshly prepared exoenzyme stocks were spot inoculated onto 1% agar in PBS amended with the respective

enzyme substrate, and substrate lysis confirmed by the appearance of clearance zones as outlined above.

2.3.9 Statistical analysis

Differences between survival curves were determined using the product-limit (Kaplan-Meier) estimator, employing log rank and Wilcoxon Chi-squared statistics, and confirmation with ANOVA. A *post hoc* Dunnett's test was used to compare treatments to defined control groups. Statistical significance was standardised at $\alpha = 0.05$. Analyses were performed using the statistical software package JMP®7 (SAS).

2.4 Results

2.4.1 Pathogenicity of *V. owensii* DY05 towards *P. ornatus* phyllosoma

Immersion challenge experiments were performed with non-feeding phyllosomas and low, moderate and high concentrations of *V. owensii* DY05 (Figure 2-1a). Significant inter-experimental differences were seen for groups exposed to the same pathogen dosage (ANOVA $p < 0.05$), but not for control phyllosomas (ANOVA $p > 0.05$) so data for the controls were pooled before further analyses. In two of the three replicate experiments mortalities were significantly increased relative to the control for moderate (Dunnett's test $p < 0.05$) and high (Dunnett's test $p < 0.0005$) pathogen concentrations.

Vectored challenge (via *Artemia*) of stage 1 phyllosomas resulted in reproducible mortality rates, with no significant inter-experimental differences for *V. owensii* DY05-challenged or control phyllosomas (ANOVA $p > 0.05$); hence data from the three experiments were pooled (Figure 2-1b). Vector-mediated exposure to *V. owensii* DY05 had a significant (ANOVA $p < 0.0001$) detrimental effect on phyllosoma survival. The highest number of phyllosoma deaths occurred within the first 24 h (49% of the total) and cumulative mortality after 72 h was 89% (Figure 2-1b).

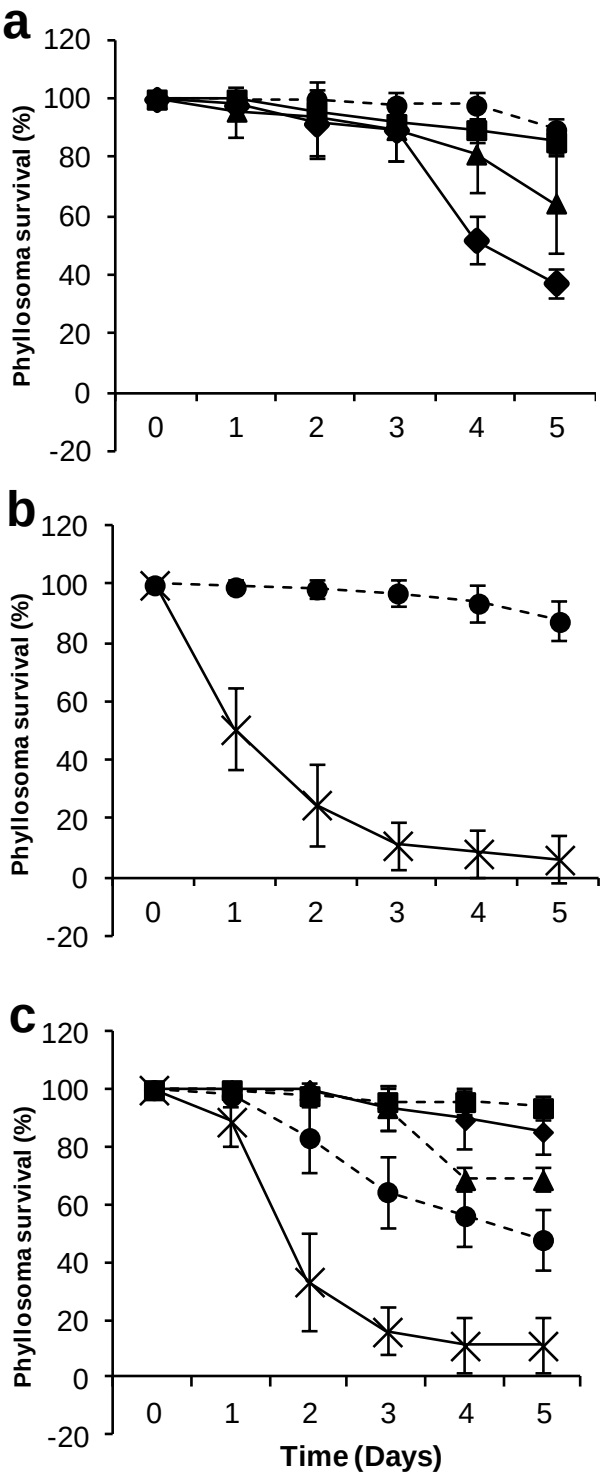

Figure 2-1. Survival of *P. ornatus* phyllosoma experimentally infected with *V. owensii* DY05 by immersion (a) or vectored challenge via *Artemia* nauplii (b-c). (a) Stage 1 phyllosoma survival after immersion with *V. owensii* DY05 at concentrations of 0 (control; ●), 1 x 10^3 (■), 1 x 10^5 (▲), and 1 x 10^7 (♦) CFU mL^{-1}. Data from one of three independent experiments are presented as high inter-experimental variability prevented pooling of data. (b) Stage 1 phyllosoma survival after vectored challenge with non-enriched nauplii (control; ●) or nauplii enriched with *V. owensii* DY05 (×). Pooled data from three independent experiments are presented. (c) Stage 3 phyllosoma survival after vectored challenge with non-enriched nauplii (controls; ●, ■, and ▲) or nauplii enriched in *V. owensii* DY05 (×). Data from three independent experiments are shown; data for controls were not pooled due to high inter-experimental variability, while data for *V. owensii* DY05 challenged phyllosomas were pooled. The first experiment (control; ●) included a treatment with phyllosomas exposed to an antibiotic cocktail (♦) prior to feeding with nauplii. Shown are Means ± SD.

Vectored challenge experiments of stage 3 phyllosomas showed significant inter-experimental differences for control phyllosomas (ANOVA $p < 0.0001$) but not for *V. owensii* DY05-challenged phyllosomas (ANOVA $p > 0.05$) so the latter data were pooled (Figure 2-1c). Vector-challenged phyllosomas showed decreased survival relative to each control group (Dunnett's test $p < 0.0001$). Similar to vector-challenged stage 1 phyllosomas, cumulative mortality of stage 3 phyllosomas was 84% after 72 h, however in contrast, deaths were most prolific (56% of the total) between 24 and 48 h (Figure 2-1c). A control group treated with antibiotics was included in one experiment to evaluate the contribution of opportunistic infections by autochthonous bacteria to stage 3 phyllosoma survival. Antibiotic treatment increased survival significantly relative to the corresponding control (Dunnett's test $p < 0.0001$) suggestive of a protective benefit.

2.4.2 Confirmation of Koch's postulates and identification of phyllosoma-associated vibrios

Isolates recovered from enriched *Artemia* and vector-challenged stage 3 phyllosomas are listed in Table 2-1. No vibrios were recovered on TCBS from control *Artemia* cultures. In contrast, a strain showing 16S rRNA, *mreB* and *topA* gene sequence identity to *V. owensii* DY05 was recovered from enriched *Artemia*. For five out of six dead vector-challenged phyllosomas, the dominant bacterial morphotypes were identical to *V. owensii* DY05 as determined by sequencing of the 16S rRNA, *mreB* and *topA* genes thereby satisfying Koch's postulates. The two-locus (*mreB* and *topA*) approach was previously found to be the most discriminative for *V. harveyi*-related species identification (Cano-Gómez et al. 2011) and in this study, sequencing of these loci allowed identification to the strain level as the 16S rRNA sequences were also identical to the penaeid pathogen *V. owensii* 47666-1 (Cano-Gómez et al. 2010). It should be noted that for the sixth vector-challenged individual, a *V. neptunis*-like strain (A37) was the dominant member in the culturable community. Importantly, *V. owensii* DY05 was not recovered from apparently healthy live phyllosomas or dead control individuals, for which recovered strains affiliated with *V. harveyi*, *V. neptunis*, and *V. parahaemolyticus*.

Table 2-1. Phylogenetic identity of isolates recovered from stage 3 *P. ornatus* phyllosomas and *Artemia* in an infection experiment

Isolate	Treatment and time (h) of sampling	Source	Gene	Nearest taxonomic relative (accession number)	Sequence identity (%)
A01	DY05 t = 2	Live *Artemia*	16S rRNA	*V. owensii* DY05 (GU018180)	1420/1421 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259)	860/860 (100%)
A06	Control t=0	Live phyllosoma	16S rRNA	*V. parahaemolyticus* (GU726844)	1400/1402 (99%)
A07	Control t=0	Live phyllosoma	16S rRNA	*V. harveyi* (DQ146937)	1414/1414 (100%)
A08	Control t=0	Live phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1408/1422 (99%)
A09	Control t=0	Live phyllosoma	16S rRNA	*V. parahaemolyticus* (EU660326)	1427/1430 (99%)
A10	Control t=0	Live phyllosoma	16S rRNA	*V. harveyi* (DQ146937)	1422/1423 (99%)
A39	Control t =48	Dead phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1414/1427 (99%)
A40	Control t =48	Dead phyllosoma	16S rRNA	*V. harveyi* (AM422800)	1414/1418 (99%)
A41	Control t =48	Dead phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1406/1420 (99%)
A42	Control t =48	Dead phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1404/1418 (99%)
A43	Control t =48	Dead phyllosoma	16S rRNA	*V. parahaemolyticus* (EU660326)	1422/1424 (99%)
A44	Control t =48	Dead phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1409/1424 (99%)
A37	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. neptunis* (NR025476)	1395/1408 (99%)
A45	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. owensii* DY05 (GU018180)	1420/1421 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259	860/860 (100%)
A46	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. owensii* DY05 (GU018180)	1420/1421 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259)	860/860 (100%)
A47	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. owensii* DY05 (GU018180)	1420/1421 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259)	860/860 (100%)
A49	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. owensii* DY05 (GU018180)	1420/1421 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259)	860/860 (100%)
A50	DY05 t = 48	Dead phyllosoma	16S rRNA	*V. owensii* DY05 (GU018180)	1390/1391 (99%)[a]
			topA	*V. owensii* DY05(GU111255)	625/625 (100%)
			mreB	*V. owensii* DY05 (GU111259)	860/860 (100%)

[a] One bp mismatch due to an uncertain nucleotide position ‘n’ in the BLAST database sequence.

2.4.3 Histology

Prior to experimental infection with *V. owensii* DY05, stage 3 phyllosomas demonstrated structural integrity of the bifurcating lateral hepatopancreas tubules, characterised by large basophilic and cuboidal epithelial cells anchored to an intact basement membrane (Figure 2-2a). Most tubule lumen appeared dilated and harboured eosinophilic particulates and B-cell vacuolisations were more numerous in the distal ends of the tubules (Figure 2-2a), while the mid and proximal regions of the tubules occupied more space in the cephalic shield and possessed a larger lumen. Eighteen hours after infection, most tubule sections showed pathologies similar to those of control phyllosomas; however some exhibited dissociation of intercellular junctions (Figure 2-2b). Proliferation of rod-shaped bacteria throughout the distal, mid and proximal regions of the tubule lumen was observed 24 h post exposure to *V. owensii* DY05, and was concomitant with epithelia cell rounding, detachment of epithelial cells from the basement membrane, and sloughing of necrotic cells into the lumen (Figure 2-2c-d). Forty-two hours after experimental infection, the hepatopancreas tubules of dead phyllosomas were necrotic and in some instances the tubules had completely disintegrated with the tissue vestiges and intertubular spaces inhabited by masses of bacterial cells (Figure 2-2e-f). Examination of eyes and thoracic musculature also indicated bacterial infiltration, suggesting progression to systemic infection in late stages of moribundity or *post mortem*.

2.4.4 Potential virulence factors

Broth cultures of *V. owensii* DY05 produced strong amylolytic, proteolytic (caseinase and gelatinase), alpha-haemolytic, lipolytic and phospholipolytic activity, but not chitinase activity. ECPs were deficient in haemolytic and lipolytic activity.

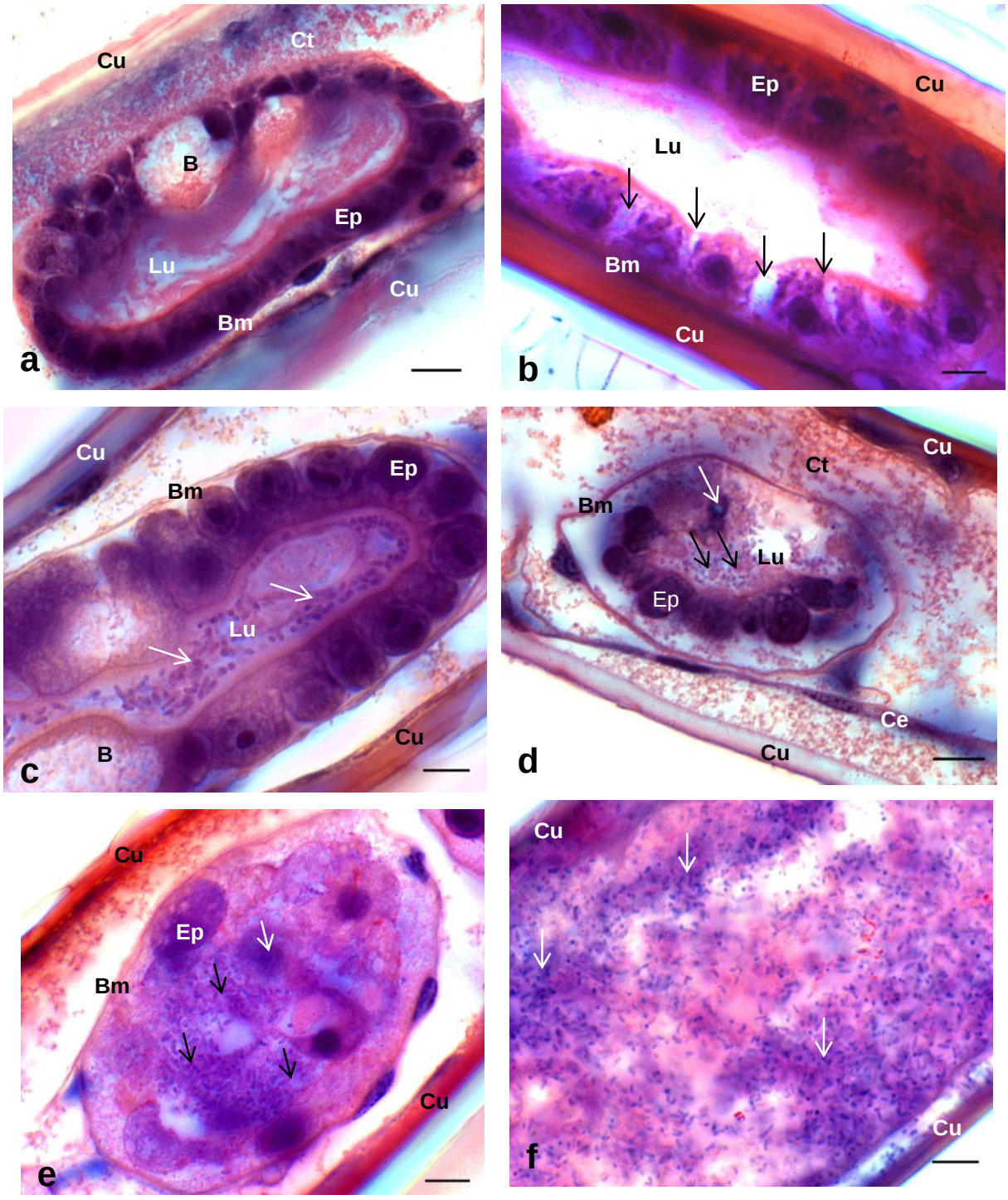
Cu
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Figure 2-2. Histopathological analysis of stage 3 *P. ornatus* phyllosoma hepatopancreas tubules during vectored challenge with *V. owensii* DY05. (a) Distal end of tubule before exposure to *V. owensii* DY05 showing no visible bacterial cells, cuboidal epithelial cells, large lumen and B-cell vacuoles. (b) Tubule mid-region 18 h after exposure, indicating dissociation of intercellular junctions (black arrows). (c) Tubules after 24 h exposure demonstrating bacterial proliferation (white arrows) within the lumen and rounding of epithelial cells. (d) Degenerative tubule after 24 h exposure showing detachment of epithelial cells from basement membrane, sloughing of necrotic cells into the lumen (white arrow), bacterial proliferation within the lumen (black arrows) and detachment of cuticular epithelia from cuticle. (e) Necrotic tubule 42 h after exposure characterised by disintegration of epithelial cells (white arrow), cell sloughing, absence of defined lumen and proliferation of bacteria (black arrows). (f) Total disintegration of hepatopancreas tubule showing distended space between ventral and dorsal cuticles and massive bacterial proliferation (white arrows) in remaining tissue. B = B-cell vacuole; Bm = basement membrane; Ce = cuticular epithelia; Ct = connective tissue; Cu = cuticle; Ep = epithelial cells; Lu = tubular lumen. All scale bars = 10 µm.

2.5 Discussion

This study provides the first description of the pathogenicity and pathology of the *V. owensii* type strain (DY05), which causes epizootics in the larviculture of the ornate spiny lobster *P. ornatus*. The causative effect of *V. owensii* DY05 infection on phyllosoma mortality was demonstrated by reisolating the agent from moribund experimentally infected phyllosomas, thereby fulfilling Koch's postulates. Identification of the reisolated strains was performed by sequencing multiple loci (16S rRNA, *topA*, and *mreB* genes) in order to enable discrimination between *V. harveyi*-like species at the strain level, which cannot reliably be achieved by sequencing the 16S rRNA gene alone (Cano-Gómez et al. 2010; 2011).

Members of the *Vibrio* genus form major constituents of the microbiota of healthy and diseased cultured *P. ornatus* phyllosomas (Webster et al. 2006; Payne et al. 2007). We isolated strains affiliated with *V. harveyi*, *V. neptunis*, and *V. parahaemolyticus* from both apparently healthy stage 3 phyllosomas and from dead control individuals. These species include strains pathogenic to cultured aquatic invertebrates and fish (Liu et al. 1996; Robertson et al. 1998; Sudheesh and Xu, 2001; Austin et al. 2005; Austin and Zhang, 2006; Cai et al. 2006; Khuntia et al. 2008) and it is possible they, together with other autochthonous microbiota, contributed to the interexperimental variability for control stage 3 phyllosomas.

This is supported by the enhanced survival of the control group exposed to antibiotic treatment. Another factor likely contributing to the differences in survival between stage 3 control cohorts is larval batch variability arising from underlying differences in genotype and physiological condition. It should also be noted that for one of the six experimentally-infected individuals, a *V. neptunis* strain was dominant in the cultured community. This introduces the possibility of synergistic interaction between other vibrios and *V. owensii* and the involvement of multiple aetiological agents in disease epizootics (Lightner and Redman, 1998; Gay et al. 2004). Despite the use of xenic hosts in our infection models, the consistent and reproducible mortality patterns of vector-challenged phyllosomas is suggestive of a singular effect imposed by *V. owensii* DY05.

Clear pathological changes were visualised in experimentally-infected phyllosomas, including bacterial proliferation in the hepatopancreas tubule lumen, rounding of epithelia cells, dissociation of intercellular junctions, detachment and necrosis of epithelial cells from the basement membrane, and eventually, complete disintegration of tubules associated with systemic infection. Similar pathologies have been reported previously in cultured *P. ornatus* phyllosomas during disease epizootics (Bourne et al. 2004; 2007), reinforcing the epizootiological relevance of our experiments. Detachment and rounding of epithelial cells was also reported by Martin et al. (2004) in the gut of ridgeback rock shrimp (*Sicyonia ingentis*) exposed to *V. harveyi* and *V. parahaemolyticus*. Comparable pathologies concomitant with *Vibrio* infections have also been described in cultured phyllosomas of packhorse rock lobster (*Sagmariasus* (*Jasus*) *verreauxi*) (Diggles et al. 2000), southern rock lobster (*Jasus edwardsii*) (Handlinger et al. 1999) and various life stages of penaeid shrimp (Lavilla-Pitogo et al. 1998; Soonthornchai et al. 2010).

Mortality was more rapid within the first 24 h in stage 1 compared to stage 3 phyllosomas, suggesting that newly hatched phyllosomas are more susceptible to invasion by *V. owensii* DY05. Increased sensitivity to pathogens in early developmental stages of crustaceans has been reported previously (Prayitno and Latchford et al. 1995; Aguirre-Guzmán et al. 2001). Factors that could contribute to delayed onset of disease in more developed phyllosoma

include a more complex hepatopancreas structure (Smith et al. 2009a), potential antagonistic activity from resident gut microbiota (Fjellheim et al. 2007) and differential expression of immune related genes (Jiravanichpaisal et al. 2007). Putative genes corresponding to antimicrobial peptides and the prophenoloxidase system have been reported in *P. japonicus* phyllosomas (Pisuttharachai et al. 2009), however the expression of such genes during phyllosoma development or in response to pathogenic invasion is currently unknown.

A key determinant of a reliable infection model is the delivery of putative pathogens to the host using natural infection routes (Saulnier et al. 2000), which can significantly influence the onset and pathology of disease (Magnadóttir et al. 2002; Magi et al. 2009). The aquaculture live feed organism *Artemia* have long been recognised as important disease vectors in marine larviculture (Chair et al. 1994; López-Torres and Lizárraga-Partida, 2001; Vaseeharan and Ramasamy, 2003a) and in the present study, vectored challenge via *Artemia* was tested as a possible infection route as we have previously shown them as carriers of *Vibrio* spp. in our system (Høj et al. 2009). Vectored challenge with *Vibrio owensii* DY05 via *Artemia* consistently caused 84-89% cumulative mortality in stage 1 and 3 *P. ornatus* phyllosomas 72 h post exposure. Mass mortalities of comparable magnitude and across similar temporal scales have been reported previously in larval rearing runs of *P. ornatus*, usually coinciding with moulting (Bourne et al. 2004). In contrast, mortality patterns of stage 1 phyllosomas challenged by immersion were more erratic and variable between experiments, suggestive of a less reliable model.

Incidental ingestion of *V. owensii* DY05 by non-feeding phyllosomas likely occurred during the immersion challenge at moderate and high concentrations ($\geq 1 \times 10^5$ CFU mL^{-1}) when mortality was significantly increased relative to controls. Entry through breakages in the exocuticle is also a possible infection route. Diggles et al. (2000) have demonstrated that experimentally-injured *Sagmariasus verreauxi* phyllosomas were more susceptible to *V. jasicida* (formerly *V. harveyi*; Yoshizawa et al. 2011) in similar immersion experiments and suggested inadvertent injuries acquired during experimental processing could have contributed to some inconsistent mortality patterns (Diggles et al. 2000).

While the mechanism by which *V. owensii* DY05 caused hepatopancreas epithelial cell detachment and necrosis is unknown, it can be hypothesised it was related to production of toxins. A number of virulence factors previously identified in Harveyi clade vibrios were produced by *V. owensii* DY05 including haemolysins, proteases (gelatinases and caseinases), and phospholipases (Liu et al. 1996; Zhang and Austin, 2000; Sudheesh and Xu, 2001; Soto-Rodriquez et al. 2003a; Austin et al. 2005; Manilal et al. 2009). Interestingly, haemolysins were absent from ECPs extracted from *V. owensii* DY05 despite the detection of haemolytic activity in the bacterial cultures. Using the same ECP extraction technique, Zhang and Austin (2000) observed a similar effect for some but not all *V. harveyi* strains, particularly when sheep erythrocytes were used as a substrate. The authors suggested the effect was due to a requisite for substrate internalisation by the bacterial cells or the hemolytic activity being associated with the bacterial cell envelope (Zhang and Austin, 2000). Haemolysins are considered one of the major virulence factors in the Harveyi clade (Ruwandeepika et al. 2011) and the gene encoding *V. harveyi* haemolysins (*vhh*) was present in all 48 Harveyi clade isolates tested by Ruwandeepika and co-workers (Ruwandeepika et al. 2010). Proteases and phospholipases were also detected in enzyme assays and have possible roles in the disruption of ion flux through cell membranes, inhibition of protein synthesis and cell lysis (Aguirre-Guzmán et al. 2004).

Chitin degradation metabolism appears to be an ancestral feature of vibrios (Hunt et al. 2008b) and recent research indicates that the chitinase gene *chiA* is widespread in the Harveyi clade (Ruwandeepika et al. 2010). In the present study, it is probable that chitinases produced by *V. owensii* DY05 were unable to lyse the substrate presented, and further studies using alternative forms of chitinous substrates are required to identify the bacterium's chitin degradation pathways. It is likely that the pathogenicity of *V. owensii* DY05 is a multifactorial process given some virulence genes, including those encoding haemolysins and proteases, may be linked or controlled by the same regulatory mechanism (Ruwandeepika et al. 2011).

2.6 Conclusions

V. owensii DY05 is implicated in epizootics of early stage *P. ornatus* phyllosomas and poses a serious threat to the development of viable hatchery technologies. It is anticipated the robust infection model described here will be used as an important diagnostic tool to identify pathogens in *Panulirus* and *Jasus* spp. hatcheries.

2.7 Acknowledgments

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3 Infection cycle and *in situ* visualisation of pathogenic *Vibrio owensii* in ornate spiny lobster (*Panulirus ornatus*) phyllosoma using a fluorescent protein expressing transconjugant

3.1 Abstract

Vibrio owensii DY05 is an emerging pathogen causing disease epizootics in the larviculture of ornate spiny lobster (*Panulirus ornatus*), however further understanding of the infective process is required to develop targeted biocontrol strategies. A fluorescent protein labelled transconjugant of *V. owensii* DY05 was used to investigate the infection cycle and localisations of the pathogen during vectored challenge via the live feed organism *Artemia*. Single fluorescent cells were dispersed in the phyllosoma foregut and hepatopancreas (mid gut gland) 6 h post exposure, which preceded mass proliferation in the distal ends of the hepatopancreas and evacuation of planktonic cells into the ambient environment 12 h post exposure. Colonisation of the entire hepatopancreas was evident 18 h post exposure, eventually leading to systemic infection and continued bacterial proliferation in host tissue *post mortem*. *V. owensii* DY05 is a specialised enteropathogen of *P. ornatus* phyllosomas that uses vector-mediated transmission and release from host-associations to a planktonic existence to perpetuate transfer. This understanding of the infection process will improve targeted biocontrol strategies and enhance the prospects of commercially viable larviculture for this valuable spiny lobster species.

3.2 Introduction

Members of the genus *Vibrio* are natural marine inhabitants, playing important roles in nutrient cycling and forming associations with zooplankton (Thompson et al. 2004). Microhabitat preferences and ecological selection may be key factors in the speciation of vibrios (Hunt et al. 2008a) and the intensive aquaculture environment is thought to select for bacterial virulence, including traits which enhance infectivity and transmission (Mennerat et al. 2010; Pulkkinen et al. 2010). Accordingly, many *Vibrio* species are pathogenic to cultured crustacean zooplanktonic larval forms including the three closely related species *V. campbelli*

(Hameed et al. 1996; Soto-Rodriguez et al. 2006), *V. harveyi* (Prayitno and Latchford, 1995; Robertson et al. 1998), and the recently described *V. owensii* (Cano-Gómez et al. 2010).

V. owensii DY05 is an emerging pathogen causing disease epizootics in the larviculture of ornate spiny lobsters (*Panulirus ornatus*) and poses a serious threat to the development of viable hatchery technologies (Goulden et al. 2012; Chapter 2). *V. owensii* DY05 infection is facilitated by vectored transmission, eventually causing extensive hepatopancreas tubule necrosis in early stage *P. ornatus* phyllosomas and acute mortality 72 h after challenge (Goulden et al. 2012; Chapter 2). A further understanding of the niche specialisation exhibited by the bacterium associated with the phyllosoma host is required to aid development of targeted biocontrol strategies for *P. ornatus* larviculture.

Fluorescent proteins (FP), derived from *Aequorea victoria* and other marine organisms, are intrinsically fluorescent when expressed in heterologous hosts (Chalfie et al. 1994; Matz et al. 1999) providing a convenient biomarker to analyse the expression, localisation and interaction of genes and proteins, and bacteria-host associations (Bloemberg et al. 1997; Southward and Surette, 2002; Grall and Manceau, 2003; Hautefort et al. 2003; Werner et al. 2004; Cabello et al. 2005). Recently, microorganisms engineered to express fluorescent proteins have significantly increased the understanding of invasive pathways and infection dynamics of pathogens including *V. anguillarum, Aeromonas hydrophila* and *Edwardsiella tarda* in fish models (Ling et al. 2001; O'Toole et al. 2004; Chu and Lu, 2008) and *V. harveyi* in abalone (Sawabe et al. 2007a; Travers et al. 2008). *Panulirus* spp. phyllosomas (from Greek; leaf-like body) are pelagic zooplanktoners that exhibit transparency as a cryptic predator-evasion strategy (Johnsen, 2001) and display a dorsal-ventrally flattened morphology. Both traits make phyllosomas excellent candidates for live, non-destructive direct microscopic observations of bacteria-host symbioses *in situ*. The objective of this study was to visualise the spatiotemporal localisations and infection cycle of *V. owensii* DY05 *in situ* using an FP-tagged strain.

3.3 Materials and Methods

3.3.1 Bacterial strains, culture conditions and preparation of inoculums

Cryopreserved stocks of *V. owensii* DY05 were revived and cultured in 5 mL MB (28^{o}C, 170 rpm) for 18-24 h. Strains used in transconjugations were the helper strain *E. coli* CC118λpir harbouring pEVS104 (Stabb and Ruby, 2002) and the green fluorescent protein (GFP) donor strain *E. coli* DH5αλpir carrying the *V. fischeri* pES213-derived plasmid pVSV201 (Dunn et al. 2006), both encoding kanamycin resistance. Helper and donor strains were revived and cultured in LB broth (5 g L^{-1} yeast extract, 10 g L^{-1} neutralised peptone) supplemented with 40 µg mL^{-1} kanamycin (30^{o}C, 170 rpm).

Inoculums were prepared by washing cells 3 times by centrifugation (10 min at 4560 rpm; 10^{o}C) and resuspension in 0.22 µm filtered seawater (FSW). The final cell suspension was adjusted to absorbance OD_{600nm} 0.1 (Nanodrop ND1000). Corresponding total viable counts were determined by spiral plating (Eddy Jet; IUL) on MA and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL). This information was used to calculate the volume of the OD_{600nm} 0.1 suspensions needed to achieve the desired starting concentrations in the phyllosoma *in vivo* infection experiments described in section 3.3.5.

3.3.2 Transconjugation

Donor, helper and recipient strains were cultured in LB broth with 40 µg mL^{-1} kanamycin (for helper and GFP-donor) and LB20 broth (5 g L^{-1} yeast extract; 10 g L^{-1} neutralised peptone; 20 g L^{-1} NaCl; for *V. owensii* DY05) for 24 h at 30^{o}C before subculture in the respective media for a further 16 h. Two hundred and fifty microlitres of each helper, donor and recipient subculture were combined in a microfuge tube, centrifuged (4650 rpm for 5 min) and the pellet resuspended in 200 µL 30^{o}C LB20 without antibiotics. Fifty microlitres of the mixed culture was spot inoculated onto LB20 agar (5 g L^{-1} yeast extract, 10 g L^{-1} neutralised peptone; 20 g L^{-1} NaCl; 1.5% agar) and incubated for 24 h at 30^{o}C. The mixed bacterial colonies were streak inoculated onto selective LB20 agar plates (supplemented with 40 µg mL^{-1} kanamycin and 50 µg mL^{-1} colistin) and incubated for 24 h at 30^{o}C. Fluorescent transconjugant colonies were detected using a blue light transilluminator and re-streaked on

Vibrionaceae-selective TCBS. TCBS-viable colonies were re-checked under blue light to substantiate GFP expression. Isolates were cryopreserved at -80^{o}C in 30% glycerol with LB20 supplemented with 40 µg mL^{-1} kanamycin.

3.3.3 Expression of GFP

V. owensii DY05[GFP] was assessed for stable expression of GFP by continuous subculture in media without antibiotic selection pressure. DY05[GFP] was pre-cultured on selective LB20 agar for 24 h at 28^{o}C, and in triplicate, a single fluorescent colony was inoculated with 5 mL MB without antibiotics and cultured for 24 h (28^{o}C, 170 rpm). The cultures were continuously subcultured in MB by transferring a 100 µL aliquot to 5 mL of MB every 24 h. Subcultures were sampled in triplicate by spiral plating (Eddy Jet; IUL) on MA, incubation overnight (28^{o}C) and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL).

3.3.4 Growth of transconjugants

The growth curve of DY05[GFP] was compared to wild type *V. owensii* DY05 using a modified microgrowth assay (Brewster, 2003). *V. owensii* DY05 and DY05[GFP] were revived on agar media as described previously, colony material was suspended in 2 mL PBS (8 g L^{-1} NaCl; 0.2 g L^{-1} KCl; 1.44 g L^{-1} Na_2HPO_4; 0.24 g L^{-1} KH_2PO_4; pH 7.2) and suspensions were adjusted to absorbance OD_{600nm} 0.1 (Nanodrop ND1000). Total viable counts (expressed as CFU mL^{-1}) were determined for each bacterial strain by spiral plating as described above. MB was inoculated with PBS suspensions of *V. owensii* DY05 or DY05[GFP] at an initial density of 1 x 10^{3} CFU mL^{-1} in a Nunc™ (NUN167008) 96-well microtitre plate (final well volume 200 µL). Treatments were performed in hextuplicate well sets. Perimeter rows and columns were loaded with 200 µL sterile milli-Q water to minimise evaporative loss and plate covers were made hydrophilic by treatment with 0.1% Triton X-100 in 20% ethanol to smooth condensation. Plates were sealed with parafilm and incubated at 28^{o}C with agitation (170 rpm) and growth of the strains was monitored by absorbance (OD_{595nm}) with a Wallac Victor^{2} 1420 multilabel counter. Measurements were adjusted by subtracting the average background noise generated from a MB control.

3.3.5 Confirmation of virulence and *in situ* visualisation of infection process

Vectored challenge via *Artemia* nauplii of *P. ornatus* phyllosomas (stage 1) with DY05[GFP] was performed as outlined by Goulden et al. (2012; Chapter 2). Enriched *Artemia* nauplii cultures and four vector-challenged *P. ornatus* phyllosomas were live mounted in FSW and viewed using differential inference contrast (DIC) and fluorescence microscopy (AxioSkop 2 mot plus; Carl Zeiss). Fluorescence was detected using a dual band filter set (59004; Chroma Technology Corp.) and images were captured by an AxioCam MRc5 camera (Carl Zeiss) directed by the multidimensional acquisition module of the AxioVision Rel. 4.8 software (Carl Zeiss).

3.3.6 Statistical analysis

Differences between survival curves were determined using the product-limit (Kaplan-Meier) estimator, employing log rank and Wilcoxon Chi-squared statistics, and confirmation with ANOVA. A *post hoc* Dunnett's test was used to compare treatments to defined control groups. Statistical significance was standardised at $\alpha = 0.05$. Analyses were performed using the statistical software package JMP®7 (SAS).

3.4 Results

3.4.1 GFP expression, effect on growth and confirmation of virulence

The transconjugant DY05[GFP] stably expressed GFP after continuous subculture (Figure 3-1a) and showed no differences in growth profile to the wild type *V. owensii* DY05 (Figure 3-1b). The survival curves of stage 1 *P. ornatus* phyllosomas after vectored challenge with DY05[GFP] (Figure 3-1c) did not significantly differ from those of the wild type (ANOVA $p > 0.05$). These observations strongly indicate expression of the FP had a low bioenergetic cost and no detrimental effect on virulence, making DY05[GFP] a suitable biomarker to visualise the infection process of *V. owensii* DY05.

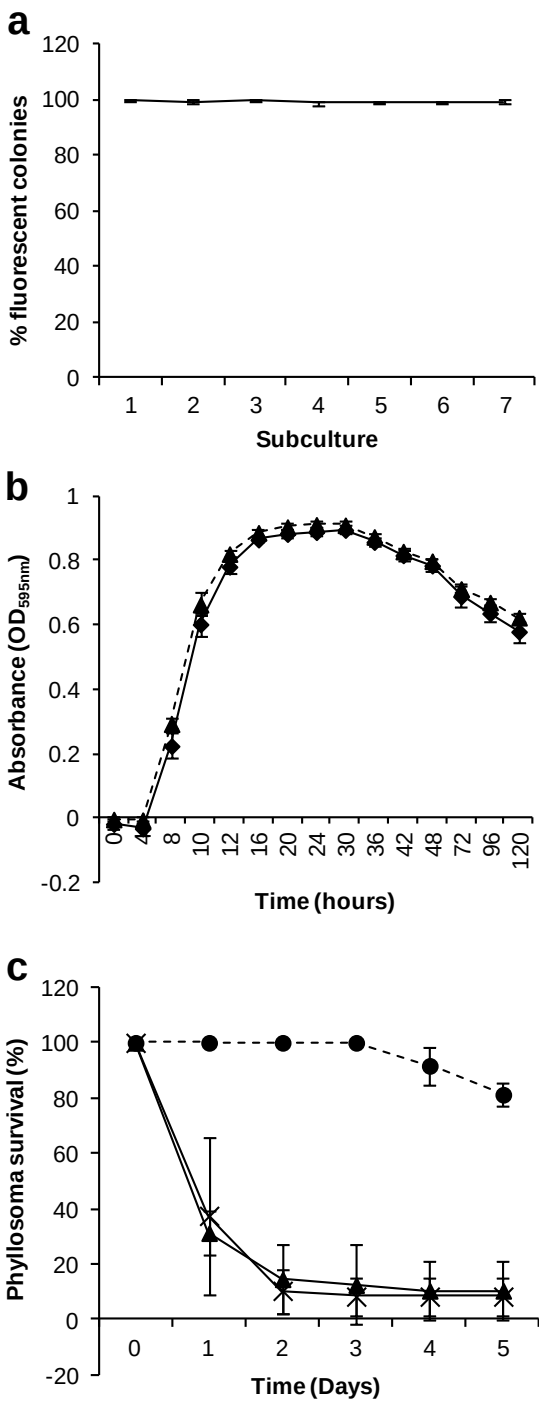


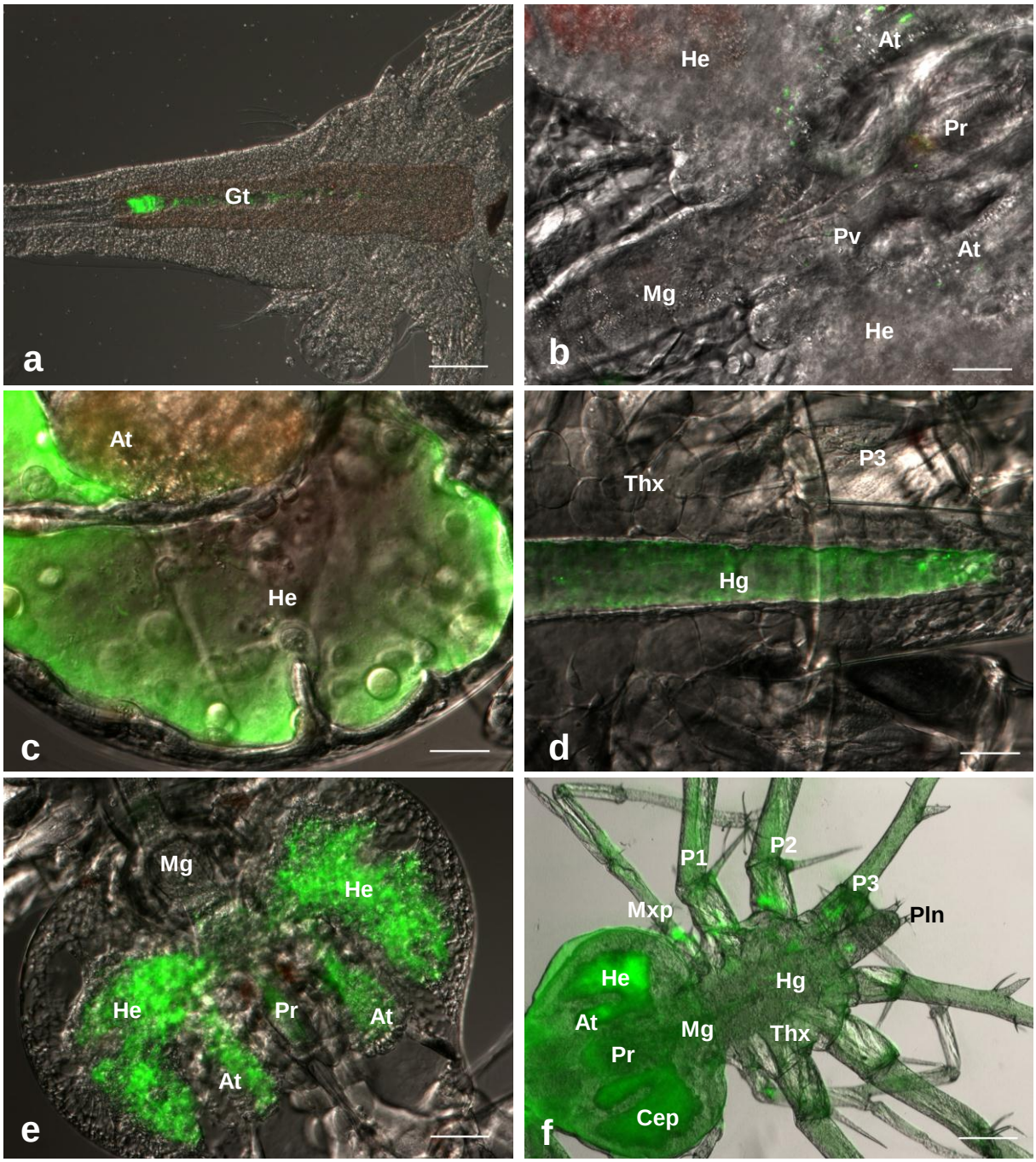
Figure 3-1. Stability of GFP expression and effects of transconjugation on growth and virulence of *V. owensii* DY05. (a) Expression of GFP in *V. owensii* DY05[GFP] following continuous subculture for 7 days in nonselective medium (MB). (b) Microgrowth profiles of wild type *V. owensii* DY05 (▲) and transconjugant DY05[GFP] (♦) over 120 h. (c) Stage 1 phyllosoma survival after vectored challenge with non-enriched *Artemia* nauplii (control; ●) or nauplii enriched with *V. owensii* DY05 (×) or DY05[GFP] (▲). Shown are Means ± SD.

3.4.2 *In situ* visualisation of pathogen infection process

The spatiotemporal dynamics of DY05[GFP] through the infection cycle was monitored during vectored (*Artemia* nauplii) challenge of stage 1 *P. ornatus* phyllosomas. Following the initial 2 h enrichment of *Artemia* culture, fluorescent cells were detected along the length of the *Artemia* nauplius gastrointestinal tract with colonisation typically more concentrated towards the posterior (Figure 3-2a). The degree of colonisation varied between individual nauplii suggesting bioaccumulation was not uniform. Small numbers of fluorescent cells were observed on appendages, indicating incidental entanglement.

After vectored challenge with *Artemia*, the *in situ* localisation of DY05[GFP] in stage 1 *P. ornatus* phyllosomas was monitored at 6 h intervals over a 24 h period. Six hours after vectored challenge, monodispersed and small aggregates of fluorescent cells were visualised within the proventriculum (foregut), hepatopancreas, and cells were seen transiting through the midgut (Figure 3-2b). At this stage, phyllosomas were still active, haemocytes were circulating and the structural integrity of tissues and organs was retained.

Figure 3-2. *In situ* spatiotemporal localisation of *V. owensii* DY05[GFP] in *Artemia* nauplius after 2 h enrichment (a) and during the infection process of vector challenged *P. ornatus* phyllosomas (b-f). (a) *Artemia* nauplius showing colonisation of DY05[GFP] in the gastrointestinal tract. Scale bar = 100 μm. (b) Hepatopancreas and mid gut region of phyllosoma after 6 h exposure, demonstrating presence of single cells or small aggregations of DY05[GFP] in the proventriculum, midgut, and anterior and lateral lobes of the hepatopancreas. Scale bar = 50 μm. (c) Proliferation of DY05[GFP] in the distal ends of lateral and anterior hepatopancreas lobes 12 h after exposure. Scale bar = 50 μm. (d) Hindgut 12 h after exposure showing trafficking of DY05[GFP] cells followed by evacuation. Scale bar = 50 μm. (e) Hepatopancreas 18 h after exposure, showing illumination of the entire organ with fluorescent DY05[GFP] cells concomitant with tissue granulation and loss of architecture. Scale bar = 100 μm. (f) Dead phyllosoma 24 h after exposure, showing colonisation of the entire body (systemic infection) by DY05[GFP] cells associated with loss of internal organ structural integrity. Scale bar = 200 μm. At = hepatopancreas anterior lobe; Cep = cephalic shield; Gt = gastrointestinal tract; He = hepatopancreas lateral lobe; Hg = hindgut; Mg = midgut; Mxp = maxilliped; P = pereiopod (1-3); Pln = pleon; Pr = proventriculum; Pv = pyloric valve; Thx = Thorax.


a
Gt
b
He
At
Pr
Pv
At
Mg
He
c
At
He
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He
Pr
At
At
f
P1
P2
P3
Mxp
Pln
He
Hg
At
Mg
Thx
Pr
Cep

After 12 h exposure, mass proliferation of fluorescent bacterial cells was visualised in the distal ends of the hepatopancreas lobes (Figure 3-2c). Most bacterial cells in the lobes appeared to be pulsating, indicating motility. At this phase of the infection, many bacterial cells were actively expelled from the hepatopancreas through the pyloric valve into the midgut and through a series of rhythmic convulsions, the cells were pushed through the hindgut and evacuated from the anus (Figure 3-2d). Eighteen hours post exposure, the entire hepatopancreas was illuminated by fluorescent cells, and this was associated with tissue granulation, loss of architecture, and detachment of the hepatopancreas gland from the cuticle epithelia (Figure 3-2e). At this stage, phyllosomas showed symptoms of lethargy and hepatopancreas opaqueness could be discerned macroscopically. After 24 h, when >63% of the population had died (Figure 3-1c), the entire dead phyllosoma was fluorescent, suggestive of systemic infection (Figure 3-2f). The vast majority of fluorescent cells were still motile while inhabiting internal tissues which appeared to lose structural integrity, with exception of the posterior hindgut. Bacterial cells transited through the vascular systems of the pereiopods, antennae, antennules and eyes, indicating translocation pathways.

In general, external colonisation was minimal during the infection process. Individual fluorescent cells, rather than clusters, were observed infrequently on the cephalic shield, thorax and pereiopods. However, incidental entanglement of fluorescent cells in the plumose natatory setae, a feathery appendage used for propulsion, was noted and seemed to increase over time in some individuals. Interestingly, in one individual, a severed pereiopod had extensive colonisation of cells around the lesion (data not shown), which could indicate an alternative route of infection.

3.5 Discussion

The present study provides the first *in situ* description of the infection dynamics of *V. owensii* DY05 associated with cultured *P. ornatus* phyllosomas. Infection of early stage phyllosomas appears to be a multifaceted process, involving 1) vector-facilitated transmission; 2) targeted colonisation and proliferation in the hepatopancreas; 3) evacuation of cells into the ambient environment; and 4) systemic infection and acute mortality. In addition, occasional cannibalism of moribund and dead phyllosomas by vigorous individuals (pers. obs. the author)

may serve as an additional infection route. A conceptualised model to describe and summarise the cycle of infectivity in the larviculture ecosystem is proposed (Figure 3-3).

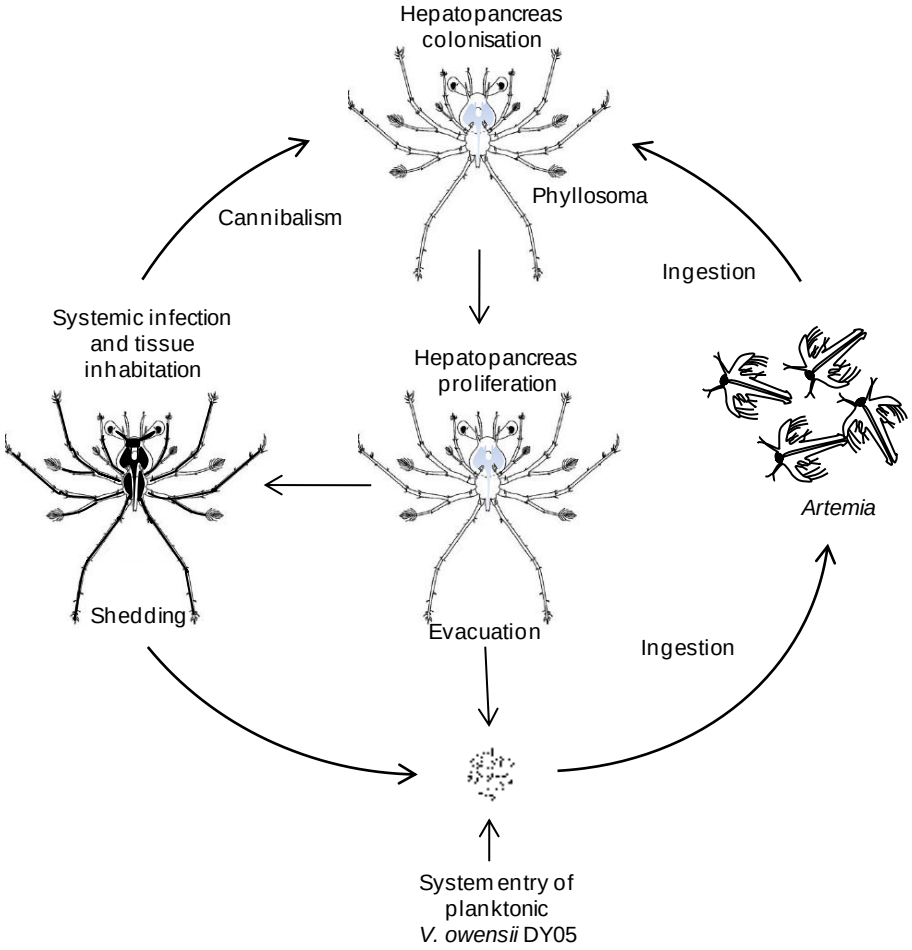


Figure 3-3. Infection cycle of *V. owensii* DY05 in the larviculture ecosystem of early stage *P. ornatus* phyllosomas. The pathogen enters the larviculture environment through an unascertained source as a free living planktonic form. Live prey *Artemia* nauplii actively ingest the bacteria, resulting in bioaccumulation in the gut. Early stage phyllosomas (1-3) capture and masticate the *Artemia* nauplii, triturating food particulates along with the pathogen through the proventriculum into the hepatopancreas. The pathogen proliferates in the distal ends of the hepatopancreas eventually colonising the entire organ, with planktonic cells dispelled from the midgut and evacuated back into the ambient environment. These planktonic cells are predisposed to ingestion by *Artemia* nauplii which perpetuates invasion of the phyllosomas through the oral route. Eventually, the condition progresses to systemic infection in association with acute mortality. By utilising and inhabiting the tissues of moribund and dead hosts, the pathogen can also be transmitted to new hosts through occasional cannibalism. Continuous shedding of bacteria from dead hosts likely contributes to the ambient bacterioplankton, again reactivating the oral infection pathway.

Visualisation of fluorescently labelled *V. owensii* DY05 in the *Artemia* gut affirmed previous observations that vectored transmission is a crucial determinant in the infection process of this pathogen (Goulden et al. 2012; Chapter 2). *Artemia* can ingest and bioaccumulate bacterioplankton at 10^2-10^4 CFU nauplii $^{-1}$ (Soto-Rodriguez et al. 2003a) thereby increasing the chance of delivery into the phyllosoma digestive tract. The rudimentary proventriculum of stage 1 phyllosomas has a limited ability to triturate finer particulates, and as a consequence large *Artemia* fragments (20 µm) can pass into the hepatopancreas (Smith et al. 2009a). Entry of these larger fragments may protect the pathogen from a potentially unfavourable gastrointestinal microenvironment, as previously suggested for *V. anguillarum* in turbot (*Scophthalmus maximus*) larvae (Grisez et al. 1996).

Bacterial proliferation in the hepatopancreas has been reported for pathogenic vibrios in spiny lobster phyllosomas (Diggles et al. 2000; Handlinger et al. 1999; Webster et al. 2006; Bourne et al. 2007), penaeid shrimp (Lavilla-Pitogo et al. 1998; Robertson et al. 1998; Aguirre-Guzmán et al. 2010), and molluscs (Yue et al. 2011). In the present study, proliferation of the pathogen occurred in the distal ends of the phyllosoma hepatopancreas 12 h after exposure. The distal region harbours a high proportion of digestive cells (Smith et al. 2009a) which could indicate a potential nutrient source for bacterial proliferation or the initial site for establishing contact with host epithelial cells. Evacuation of pathogen cells from the phyllosoma hepatopancreas (12 h after exposure) and reversion to planktonic forms may reflect a behavioural mechanism evolved to enhance the prospects of colonising other hosts (Tang et al. 2010). Reversion to a planktonic existence may be facilitated by quorum sensing, whereby cell-density dependent downregulation of virulence factors constitutes a switch from epithelia-attached forms to free-living lifestyles (Zhu and Mekalanos, 2003; Defoirdt et al. 2010). Shedding of large numbers of planktonic forms into the aquaculture environment increases the probability of uptake by *Artemia*, thus possibly perpetuating vector-mediated transmission through a feedback loop (Figure 3-3). This phase in the infection process may represent the survival strategy of a pathogen exquisitely adapted to an ecosystem where the availability of hosts is high (Pulkkinen et al. 2010; Mennerat et al. 2011).

The progression from hepatopancreas colonisation to systemic infection was likely facilitated by epithelial cell detachment and necrosis (Goulden et al. 2012; Chapter 2). Destruction of gut epithelial cells could enable bacteria unrestricted access to the basal lamina, ultimately leading to translocation to other tissues and organs (Ringø et al. 2007). For example, *V. proteolyticus* is known to interfere with gut epithelial cellular junctions of *Artemia*, enabling penetration through the intercellular spaces and eventual invasion of the body cavity (Verschuere et al. 2000a).

Colonisation of a severed pereiopod by FP-tagged *V. owensii* DY05 indicates surreptitious entry through breakages in the exocuticle is possible. However, ectobiotic colonisation was generally lacking, suggesting that the exocuticle is not a preferred microenvironment for *V. owensii* DY05 and that unaided invasion through the cuticle is a less likely portal of entry.

3.6 Conclusions

The present study provides a conceptualised snapshot of the adaptive strategy used by *V. owensii* DY05 to enhance infectivity in the *P. ornatus* larviculture ecosystem, including vector-mediated transmission and release from host-associations to a planktonic existence to perpetuate transfer. Importantly, identification of the bacterium's host-associated ecological niches will facilitate the development of targeted biocontrol strategies for larviculture of *P. ornatus.* As new pathogens emerge in larviculture settings, researchers are encouraged to take advantage of transparent zooplanktonic forms to elucidate bacterial-host symbioses *in situ*.

3.7 Acknowledgements

I wish to thank Eric Stabb (University of Georgia, USA) for kindly donating the helper and donor strains used to construct the fluorescent protein expressing transconjugants of *V. owensii*. I also acknowledge the zootechnical expertise of Greg Smith, Matt Kenway, Matt Salmon, Grant Milton, Justin Hochen, and Katie Holroyd (AIMS). I would also like to thank Rochelle Soo and Rose Cobb for their technical assistance (all AIMS). Financial support for the project was provided by AIMS and UNE.

4 Identification of an antagonistic probiotic combination protecting ornate spiny lobster (*Panulirus ornatus*) larvae against *Vibrio owensii* infection

4.1 Abstract

In the present study a multi-tiered probiotic screening strategy was used to identify antagonistic bacteria capable of protecting cultured *Panulirus ornatus* phyllosomas from the serious hatchery pathogen *Vibrio owensii* DY05. From a pool of more than 500 marine isolates, 91 showed definitive *in vitro* antagonism towards the pathogen with most belonging to the genera *Pseudoalteromonas* (66 strains) and *Vibrio* (16 strains). Antagonistic candidates were shortlisted based on phylogeny, strength of antagonistic activity, and isolate origin. Miniaturised assays used a green fluorescent protein labelled transconjugant of *V. owensii* DY05 to assess pathogen growth and biofilm formation in the presence of shortlisted candidates. This approach enabled rapid processing and selection of candidates to be tested in a phyllosoma infection model. When used in combination, strains *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 significantly and reproducibly protected *P. ornatus* phyllosomas during vectored challenge with *V. owensii* DY05, with survival not differing significantly from unchallenged controls. The present study has shown the value of multispecies probiotic treatment and demonstrated that natural microbial communities associated with wild phyllosomas and zooplankton prey harbour antagonistic bacteria capable of *in vivo* suppression of a pathogen causing epizootics in phyllosoma culture systems.

4.2 Introduction

The ornate spiny lobster (*Panulirus ornatus*) is considered a prospective aquaculture species based on encouraging grow-out potential (Jones and Shanks, 2009) and lucrative market value (Williams et al. 2009). However, commercialisation of a closed-life cycle sector for *P. ornatus* is currently unviable due to restricted production of pueruli and juveniles, resulting from nutritional deficits (Smith et al. 2009b) and bacterial disease (Bourne et al. 2004; 2007; Webster et al. 2006) during the 4-6 month long phyllosoma larval phase (Smith et al. 2009b).

Vibrio owensii DY05 is an emerging enteropathogen and the aetiological agent of a disease causing mass mortality in the larviculture of *P. ornatus* (Goulden, et al. 2012; Chapter 2). The pathogen can be transmitted through live feed vectors (*Artemia*) and proliferates in the phyllosoma hepatopancreas (midgut gland), causing extensive tissue necrosis and eventually major systemic infection (Goulden et al. 2012; Chapters 2 and 3).

In view of the global antibiotic resistance crisis (Davies, 2007) there is considerable interest in developing sustainable biocontrol methods such as probiotics for disease management in aquaculture (Defoirdt et al. 2007). Probiotics may briefly be defined as an entire microorganism (or components thereof) that confer a health benefit on the host (Irianto and Austin, 2002). A number of commercial probiotics have proven to increase disease resistance, growth or survival in invertebrates and fish (Moriarty, 1998; Decamp et al. 2006; Ziaei-Nejad et al. 2006; Saad et al. 2009; Aly et al. 2008; Faramarzi et al. 2011; Granados-Amores et al. 2011). However benefits may differ in trans-species applications (Decamp and Moriarty, 2007) dictating a need to develop host-specific probiotics (Gram et al. 2001; Hai et al. 2009).

The search for probionts is based on screening for beneficial microbial attributes such as antagonism, predation, anti-virulence, competition, attachment to host surfaces, and immunostimulation (Verschuere et al. 2000b; Vine et al. 2006; Kesarcodi-Watson et al. 2008; Defoirdt et al. 2007; 2011a). Antagonistic bacteria are frequently pursued as probionts due to their *in vitro* ability to inhibit pathogenic microorganisms by producing bioactive substances including antibiotics, bacteriocins, organic acids, and hydrogen peroxide (Verschuere et al. 2000b). However *in vitro* antagonism does not always correlate with beneficial *in vivo* effects (Riquelme et al. 1997; Gram et al. 2001; Spanggaard et al. 2001), warranting alternative probiotic *in vitro* screens for properties such as dominance, adherence to host surfaces and competition for attachment sites (Vine et al. 2004a,b; Chabrillón et al., 2005a,b; Balcázar et al. 2008; Pan et al. 2008; Fjellheim et al. 2010) to increase the potential for probiont-host interaction. Importantly, putative probionts must also be nonpathogenic to the host and demonstrate a protective benefit following challenge with specific pathogens *in vivo* (Verschuere et al. 2000b). It is logical to prospect for probionts in microbial communities

which share the same environment or are autochthonous to the cultured host (Kesarcodi-Watson et al. 2008).

It was shown previously that the planktonic form is central to vectored transmission of *V. owensii* DY05 (Goulden et al. 2012; Chapter 3), which warrants an investigation into the ability of probiotic candidates to inhibit planktonic growth. Moreover, since biofilms are refuges for pathogens in aquaculture systems (Karunasagar et al. 1996; Bourne et al. 2006) and pathogen biofilms on natural tissues are inherently tolerant to conventional antimicrobial therapies (Lynch and Robertson, 2008), this justifies an investigation into the ability of probiotic candidates to inhibit biofilm formation under conditions of exclusion, competition and displacement.

In this study, a multitiered probiotic screening process was used to identify probiotic bacteria capable of protecting *P. ornatus* phyllosomas from experimental *V. owensii* DY05 infection. Initially, a shortlist of probiotic candidates was generated from a large pool of bacteria showing *in vitro* antagonism towards *V. owensii* DY05. Two additional *in vitro* screens were developed using a green fluorescent protein (GFP)-transconjugant of the pathogen to assess its planktonic growth and biofilm formation in the presence of shortlisted candidates. Subsequently, promising candidates were assessed for inherent virulence and protective benefit *in vivo* using a *P. ornatus* phyllosoma experimental infection model.

4.3 Materials and Methods

4.3.1 Replica plate assay

Wild *Panulirus* spp. phyllosomas and putative zooplankton prey items were collected at Osprey Reef (13° 56′S, to 14° 03′ S and 144° 26′ E to 146° 48′ E) between 24 May-9 June 2008. Capture was achieved using a modified Isaac-Kidd mid-water trawl net according to Smith et al. (2009b). Briefly, specimens were washed 3x in 0.22 µm filtered artificial sea water (ASW; Instant Ocean®) to remove debris and loosely attached epibionts, homogenised in ASW, and serial dilutions were spread plated in triplicate on minimal marine agar (MMA; 0.3% casamino acids; 0.4% glucose; 1% bacteriological agar in 1 L ASW), modified from

Hjelm et al. (2004). After, incubation at ambient temperature (~24^{o}C) for 24-48 h, MMA plates with <300 colonies were replica plated (Hjelm et al. 2004) onto MMA seeded with 10 µL mL^{-1} of *V. owensii* DY05 grown overnight (24^{o}C, 170 rpm) in marine broth 2216 (MB; Becton, Dickinson and Company). Replica plates were incubated at ambient temperature for 72 h (24^{o}C) and inspected for clearing zones signifying antagonistic activity against *V. owensii* DY05. Antagonistic colonies were picked and cultured to purity on MMA, re-cultured in MB overnight (28^{o}C, 170 rpm), and cryopreserved in 30% (v/v) glycerol (-80^{o}C).

4.3.2 Well-diffusion agar assay (WDAA)

Antagonistic isolates recovered from replica plates and the Australian Institute of Marine Science (AIMS) culture collection were tested for growth-inhibitory activity against *V. owensii* DY05 in a well diffusion agar assay (WDAA). In brief, the pathogen was seeded into molten MMA as outlined above. Following solidification, wells (diameter 5 mm) were cut aseptically into the agar and loaded with 40 µL of dense cultures (1-3 day old) of test isolates grown in MB (28^{o}C, 170 rpm). Plates were incubated (28^{o}C) and observed every 24 h for 72 h for pathogen inhibition zones. *Phaeobacter* (formerly *Roseobacter*) strain 27-4 was used as a positive antagonistic control on each plate because of its broad spectrum inhibitory activity against *Vibrio* pathogens (Hjelm et al. 2004; Bruhn et al. 2005; 2007). Antagonism was classified according to the diameter of inhibition zones as low (5-10 mm), moderate (11-20 mm) or strong (≥ 21 mm).

4.3.3 Phylogenetic identification

Antagonistic isolates were revived on MMA for 24-48 h at 28^{o}C, and colony PCR was performed with universal primers 27F and 1492R (Lane, 1991) under standard conditions. PCR products were purified and sequenced using 27F as a sequencing primer by Macrogen (Seoul, Korea). Sequences were edited with Vector NTI® (Invitrogen) software and submitted to the BLAST algorithm on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/) to determine nucleotide-nucleotide similarity with sequences in the nr/nt database. Isolates were then grouped according to phylogenetic relatedness by partial 16S rRNA gene sequence alignment using MEGA4 (Tamura et al. 2007). For the 16 shortlisted candidates full

sequences were later obtained and submitted to GenBank under accession numbers JX075050-JX075065 (data not shown).

4.3.4 Inoculum preparation

A GFP-labelled transconjugant of *V. owensii* DY05 (DY05[GFP]) was used as a proxy for pathogen growth and attachment in microgrowth coculture and multispecies biofilm assays, respectively (sections 4.3.5 and 4.3.7). DY05[GFP] stably expresses the GFP, and does not differ in growth profile or virulence towards stage 1 *P. ornatus* phyllosomas compared to wild type *V. owensii* DY05 (Goulden et al. 2012; Chapter 3).

DY05[GFP] was cultured on LB20 agar plates (5 g L^{-1} yeast extract; 10 g L^{-1} neutralised peptone; 20 g L^{-1} NaCl; 15 g L^{-1} agar) supplemented with 40 µg mL^{-1} kanamycin and 50 µg mL^{-1} colistin. Probiotic candidates and wild type *V. owensii* DY05 were cultured on marine agar 2216 (MA; Becton, Dickinson and Company) at 28^{o}C for 24 h. For each strain, colony material was suspended in 2 mL phosphate buffered saline (PBS: 8 g L^{-1} NaCl; 0.2 g L^{-1} KCl; 1.44 g L^{-1} Na_2HPO_4; 0.24 g L^{-1} KH_2PO_4; pH 7.2) and absorbance adjusted to OD_{600nm} 0.1 (Nanodrop ND1000). The corresponding total viable counts (expressed as CFU mL^{-1}) were determined for each strain in triplicate in initial experiments by spiral plating (Eddy Jet; IUL) on MA and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL). This information was used to calculate the volume of each OD_{600nm} 0.1 suspension needed to achieve the desired starting concentrations in the assays described below (sections 4.3.5 – 4.3.7).

4.3.5 Microgrowth coculture assay

The relationship between fluorescence and CFU mL^{-1} of *V. owensii* DY05[GFP] monocultures was tested using the Pearson correlation coefficient. Triplicate samples were withdrawn from the microgrowth assay (described below) at 4 h intervals and a strong positive correlation ($p < 0.0001$) between fluorescence and pathogen growth was shown for the first 24 h (Figure A1; Appendix). This showed that the fluorescence signal generated by *V. owensii* DY05[GFP] could be used to indirectly quantify pathogen growth during 24 h coculture with probiotic candidates.

To assess the activity of planktonic candidates, MB was inoculated with PBS suspensions of *V. owensii* DY05[GFP] (initial density 1 x 10^3 CFU mL^{-1}) separately or in combination with PBS suspensions of candidate probiotics (final densities 1 x 10^3, 1 x 10^5, or 1 x 10^7 CFU mL^{-1}) in Nunc™ (NUN137101) black microwell plates (final volume 200 µL). Separate plates were used for each candidate-pathogen combination and all treatments were performed in hextuplicate well sets. Sterile milli-Q water (200 µL) was added to perimeter rows and columns to minimise evaporative loss and plate covers were treated with 0.1% Triton X-100 in 20% ethanol to smooth condensation (Brewster, 2003). Plates were sealed with parafilm and incubated for 24 h (28^{o}C, 170 rpm). Growth of the GFP-tagged pathogen was monitored indirectly by measuring fluorescence (excitation/emission 485/520nm) with a Wallac Victor^2 1420 multilabel counter. Fluorescence values were adjusted by subtracting the average native autofluorescence generated from corresponding controls (wild type *V. owensii* DY05 monocultures or cocultures).

The antagonistic activity of each strain was classified based on their ability to reduce the pathogen fluorescence signal after 24 h relative to the maximum signal reduction recorded for the respective assay. In this way, strain antagonistic activity was classified as low (<50% of max), moderate (50-75% of max), or strong (>75% of max).

4.3.6 Monostrain biofilm production

Maximum biofilm density by monocultures of the pathogen and candidate probionts was quantified using a microwell crystal violet (CV) staining assay modified from O'Toole et al. (1999). MB was inoculated with PBS suspensions of *V. owensii* DY05, DY05[GFP] or candidate probionts (initial density 1 x 10^7 CFU mL^{-1}) in Nunc™ (NUN167008) microwell plates (final well volume 200 µL). Separate plates were used for each incubation period (12, 24, 36, 48, 72 and 96 h; 28^{o}C) and treatments were carried out in hextuplicate well sets. After incubation, wells were washed 3x in 200 µL PBS to remove planktonic and nonadherent cells. Two hundred microlitres of 0.4% CV was added to the wells and incubated at room temperature for 20 min. After staining, CV was removed and the wells were washed in PBS as described. The CV-stained biofilm was solubilised in 200 µL 95% ethanol and absorbance

(OD_{595nm}) was measured using a Wallac Victor^2 1420 multilabel counter. Measurements were adjusted by subtracting background staining generated from a MB control.

4.3.7 Multistrain biofilm assay

Multistrain interactions of exclusion, competition, and displacement were tested using a modified attachment assay (Vesterlund et al. 2005). The sensitivity of the assay was increased by extending the incubation time from 1 h to the time of maximum biofilm formation of the reporter strain DY05[GFP] as determined in the CV assay (t = 48 h and t = 72 h). For each test, MB was inoculated with PBS suspensions of bacterial strains (each at initial density 1 x 10^7 CFU mL^{-1}) in Optical bottom Nunc™ (NUN165305) microwell plates (final well volume 200 µL). Specifically, for the exclusion assay, half volume MB was inoculated with probiotic candidates and incubated for 24 h prior to inoculation with DY05[GFP] (t = 0 h) and fresh MB. For the competition assay, MB was coinoculated simultaneously with DY05[GFP] and probiotic candidates (t = 0 h). For the displacement assay, half volume MB was inoculated with DY05[GFP] (t = 0 h) and incubated for 24 h before inoculation with candidate probionts and fresh MB. Plates were treated to minimise evaporation and smooth condensation as described for microgrowth coculture assay. Separate plates were used for each interaction and incubation period (static at 28^{o}C until t = 48 h or t = 72 h) and treatments were carried out in hextuplicate well sets. After incubation, wells were washed 3x in 200 µL PBS to remove planktonic and nonadherent cells. To prevent dehydration, washed wells were loaded with PBS (200 µL). Biofilm attachment was measured as a function of fluorescence using a multilabel counter (Wallac Victor^2 1420) and fluorescence values adjusted as described above.

Candidates which caused significant signal increase (Student t-test, $p < 0.05$) at any time point were removed from the candidate pool as these were considered pathogen biofilm facilitators. From the remaining observations, the average signal decrease was calculated from the two time points (t = 48 and t = 72) and used to classify the antagonistic activity of each strain (low, moderate, strong) as described above.

4.3.8 *In vivo* protection against *V. owensii* DY05 infection

Selected candidates were tested for inherent pathogenicity towards cultured stage 1 *P. ornatus* phyllosomas using a vector-mediated infection model described previously (Goulden et al. 2012; Chapter 2). Briefly, formalin-disinfected live cultures of the vector organism *Artemia* stage II (nauplii) were enriched with probiotic candidates through filter-feeding in tissue culture flasks (Sarstedt) for 2 h (initial density 1 x 10^7 CFU mL^{-1}). Experimental controls consisted of non-enriched nauplii (negative control) or nauplii enriched with 1 x 10^6 CFU mL^{-1} *V. owensii* DY05 (positive pathogen control). Apparently healthy *P. ornatus* phyllosomas, as assessed by photopositive response and active motility, were sourced from the AIMS larviculture facility (Smith et al. 2009b) distributed to 12-well cell culture plates (1 larva $well^{-1}$) and fed live enriched or non-enriched *Artemia* (t = 0 h) at approximately 3 nauplii mL^{-1}. All treatments were performed in quintuplicate (n = 60) and survival was assessed every 24 h for 5 days.

The protective benefit of selected candidates was initially evaluated in two separate experiments using the same probiotic administration strategy (Strategy 1). In brief, stage 1 *P. ornatus* phyllosomas were fed nauplii enriched with probiotic candidates separately, or in combination (each candidate at 1 x 10^7 CFU mL^{-1}) at t = 0 h. After 24 h, phyllosomas were vector challenged for 6 h with nauplii enriched with 1 x 10^6 CFU mL^{-1} *V. owensii* DY05. Phyllosomas were then transferred to new cell culture plates and again fed nauplii enriched with probiotic candidates (t = 30 h).

Subsequently, the most promising candidates were tested in a third experiment using an alternative administration strategy (Strategy 2) which differed from Strategy 1 by enriching *Artemia* cultures with both the pathogen and probiotic candidates at t = 24 h (pathogen at 1 x 10^6 CFU mL^{-1}; each probiont at 1 x 10^7 CFU mL^{-1}). Encouraging results from the latter experiment were replicated twice in separate experiments to validate observations. Included in all protection experiments were controls consisting of phyllosomas fed non-enriched *Artemia* at each feeding time (negative control), and phyllosomas fed *Artemia* enriched in *V. owensii* DY05 at t = 24 h and non-enriched *Artemia* at t = 0 and t = 30 h (positive pathogen control).

All treatments were performed in quintuplicate (n = 60) and survival was assessed every 24 h for 5 days.

4.3.9 Statistical analysis

Differences between survival curves in the experimental infection models were determined using the product-limit (Kaplan-Meier) estimator and confirmed with an ANOVA. A *post hoc* Dunnett's test was used to compare treatments to the defined control groups. All statistical analyses were performed using the statistical software package JMP®7 (SAS) standardised at significance level $\alpha = 0.05$.

4.4 Results

4.4.1 Antagonistic bacteria

Antagonistic isolates represented 3.3-6.5% of the total bacterial communities of wild *P. ornatus* phyllosomas and zooplankton prey (Table 4-1) cultured on MMA. Interestingly, the frequency of antagonistic isolates recovered from cultured phyllosomas fed wild prey and *Artemia* during an on-board feeding experiment (AIMS; unpublished data) was much lower than wild-caught conspecifics (Table 4-1).

Table 4-1. Frequency of bacteria antagonistic to *V. owensii* DY05 recovered from the culturable communities of wild *P. ornatus* phyllosomas and putative zooplankton prey.

Environmental sample	Communities examined	Culturable bacteria (CFU individual^{-1})	Frequency of antagonists (%)[b]	Confirmed antagonists[c]
P. ornatus phyllosoma				
Wild caught	2	1.9×10^3	6.5	18
Cultured[a]	4	2.9×10^2	1.1	7
Zooplankton				
Salps (*Salpida*)	8	3.8×10^3	5.3	18
Arrow worm (*Chaetognatha*)	6	4.1×10^2	3.3	19

[a] Cultured phyllosomas were taken on board the AIMS RV Cape Ferguson and fed control diets (*Artemia*) and various wild-captured zooplankton (AIMS, unpublished data). Culturable communities of these animals were replica plated and screened for antagonistic isolates as outlined.

[b] Average proportion of antagonistic isolates recovered from culturable communities in replica plate assay.

[c] Isolates picked from replica plating that were antagonistic towards *V. owensii* DY05 in WDAA.

The WDAA confirmed 62 of 149 isolates recovered from replica plating and 29 of 356 AIMS culture collection isolates as antagonistic towards *V. owensii* DY05 (Table 4-1 and 4-2). The number of confirmed antagonistic isolates from wild *P. ornatus* phyllosomas, arrow worms, cultured *P. ornatus* phyllosomas, and salps derived from the replica plate assay were 67%, 58%, 33%, and 26%, respectively. In comparison, the proportion of confirmed antagonistic isolates from culture collections was 0-20% depending on source (Table 4-2), with Appendicularians (20%) and AIMS larviculture water (16%) among the better sources of antagonists.

Table 4-2. Source and number of antagonistic isolates towards *V. owensii* DY05 confirmed in the well diffusion agar assay (WDAA).

Source	Source	No. of isolates tested in WDAA	No. of antagonists
Wild Palinurid phyllosoma			
P. ornatus	AIMS	42	2
P. longipes	AIMS	5	0
P. versicolor	AIMS	18	1
P. penicillatus	AIMS	9	1
Mixed species homogenate	AIMS	23	2
Unknown Palinurid spp.	AIMS	48	1
Cultured Palinurid phyllosoma			
P. ornatus	AIMS	31	3
Zooplankton			
Appendicularians (*Appendicularia*)	AIMS	25	5
Salps (*Salpida*)	AIMS	23	1
Seawater			
AIMS larviculture	AIMS	19	3
Aged (AIMS)	AIMS	36	5
Green (AIMS)	AIMS	19	1
Coral Sea surface water	AIMS	19	1
Other marine organisms			
Montipora sp.	AIMS	13	2
Trypaea australiensis	UNE	11	0
Sycllarus spp.	AIMS	15	1
	Total	**356**	**29**

Antagonism was rated according to the size of the inhibition zones as low (> 5-10 mm), moderate (11-20 mm) or strong (≥ 21 mm) (Figure 4-1a-c). Some isolates demonstrated extensive growth around the wells without exhibiting (detectable) pathogen growth inhibition (Figure 4-1d) and were not included in further *in vitro* screens in this study. Other isolates produced concentric clearing zones (Figure 4-1e), and these isolates were tentatively considered to be antagonistic due to an obvious clearing zone. In general, no appreciable temporal variation in halo sizes were noted over the 72 h monitoring period, yet some antagonistic bacteria were able to populate inhibition zones following elimination of the pathogen (Figure 4-1f).

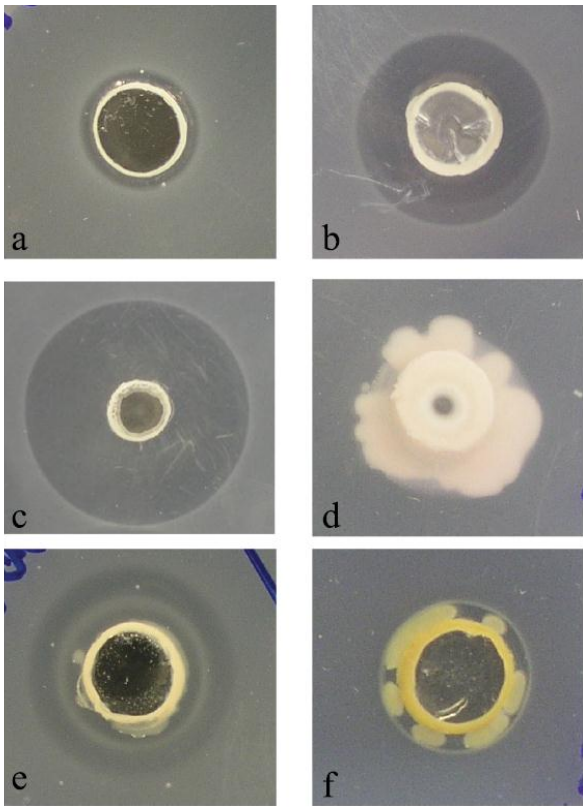


Figure 4-1. Well diffusion agar assay showing wells (diameter 5mm) in marine minimal agar plates (MMA) embedded with *V. owensii* DY05. Antagonism is indicated by clearing zones, classified as (a) low (> 5-10 mm), (b) moderate (11-20 mm) or (c) strong (≥ 21 mm) activity. (d) Example of extensive colonisation around wells and on agar surface without noticeable production of inhibitory substances. (e) Undefined antagonistic activity showing concentric pattern with opaque rings. (f) Colonisation of clearing zones following inhibition of the pathogen. *Phaeobacter* strain 27-4 was used as a quality control microorganism for positive antagonism and was included on each WDAA plate.

4.4.2 Identification of antagonistic isolates and probiotic candidate shortlist

The majority of the 91 antagonistic isolates confirmed by the WDAA belonged to the genera *Pseudoalteromonas* (66 isolates; 1 pigmented and 1 non-pigmented phylotype) and *Vibrio* (16 isolates; 4 phylotypes). The remainder belonged to the *Bacteroidetes* phylum (2 isolates; 1 phylotype), and the genera *Ruegeria* (3 isolates; 2 phylotypes), *Bacillus* (2 isolates; 2 phylotypes), *Psychrobacter* (1 isolate) and *Acinetobacter* (1 isolate). A shortlist of antagonistic strains likely capable of interaction with phyllosoma hosts was selected based on phylogenetic identity, strength of *in vitro* antagonism in WDAA, and environmental origin, with preferences given towards zooplankton-derived isolates, bacteria associated with *P. ornatus* phyllosomas or their environments (natural or artificial). Strains that were closely related to known human pathogens or were difficult to keep in pure culture were excluded from further analysis. In this way, the pool of probiotic candidates was reduced to 16 isolates for further *in vitro* screening (Table 4-3).

Table 4-3. Source and identity of antagonistic isolates shortlisted for candidate probiotics

Taxonomic group	Strain	Source	Antagonistic activity[a]
Vibrio sp. (PT1)	C013	Surface water, Coral Sea	Low
Vibrio sp. (PT2)	Ma31	Aged seawater (AIMS)	Low
Vibrio sp. (PT3)	PP05	Salp (*Salpida*)	Moderate
Vibrio sp. (PT4)	PP25	Salp (*Salpida*)	Moderate
Pseudoalteromonas sp. (PT1)[b]	EPP07	*P. ornatus* phyllosoma, cultured[c]	Strong
Pseudoalteromonas sp. (PT1)[b]	K25	Larviculture water (AIMS)	Strong
Pseudoalteromonas sp. (PT1)[b]	PP107	Arrow worm (*Chaetognatha*)	Moderate
Pseudoalteromonas sp. (PT2)	EPP11	*P. ornatus* phyllosoma, cultured[c]	Moderate
Pseudoalteromonas sp. (PT2)	PP81	*P. ornatus* phyllosoma, wild	Strong
Pseudoalteromonas sp. (PT2)	PP86	*P. ornatus* phyllosoma, wild	Strong
Pseudoalteromonas sp. (PT2)	PP87	*P. ornatus* phyllosoma, wild	Strong
Ruegeria sp. (PT1)	AH10	Appendicularian (*Appendicularia*)	Moderate
Ruegeria sp. (PT1)	K2	Larviculture water (AIMS)	Moderate
Ruegeria sp. (PT2)	EPP04	*P. ornatus* phyllosoma, cultured[c]	Moderate
Bacteroidetes (PT1)	AH26	Appendicularian (*Appendicularia*)	Low
Bacteroidetes (PT1)	PPM04	*P. ornatus* phyllosoma, cultured	Low

[a]Based on inhibition zone in well diffusion assay (see Figure 4-1).
[b]All *Pseudoalteromonas* PT1 isolates showed yellow pigmentation on MMA.
[c] Isolates recovered from a phyllosoma feeding experiment (AIMS unpublished data).
Abbreviations: PT: phylotype.

4.4.3 Coculture assay

Probiotic candidates cocultured with the pathogen at equal starting densities had little or no inhibitory effect on pathogen growth (Figure 4-2a), except for the three pigmented *Pseudoalteromonas* strains (EPP07, K25 and PP107), which demonstrated moderate to strong activity. At a higher initial density (1 x 10^5 CFU mL^{-1}), the candidates generally had increased inhibitory effect (Figure 4-2b), with strong inhibition of pathogen growth observed for the pigmented *Pseudoalteromonas* strains (EPP07, K25 and PP107) and three of the four *Vibrio* strains (C013, Ma31, and PP05) (Figure 4-2b). At the highest initial density (1 x 10^7 CFU mL^{-1}), all candidates belonging to the *Pseudoalteromonas* and *Vibrio* genera, and *Ruegeria* strain K2 caused strong growth inhibition, in some instances resulting in total elimination of fluorescent signals (Figure 4-2c). Irrespective of initial density, the *Bacteriodetes* strains (AH26 and PPM04) had minor impact on pathogen growth during coculture (Figure 4-2a-c).

4.4.4 Monostrain biofilm production

A microwell crystal violet assay was used to study monostrain biofilm production (Figure 4-3). Attachment of *V. owensii* DY05 and DY05[GFP] was detected after 24 h but maximum biofilm density was achieved between 48 and 72 h prior to dispersal. Pigmented *Pseudoalteromonas* strains (EPP07, K25, and PP107) rapidly formed dense biofilms which dispersed after 48 h. Non-pigmented *Pseudoalteromonas* strains (EPP11, PP86, and PP87) were strong biofilm formers reaching maximum density between 36 and 48 h. Strain PP81 differed from other non-pigmented pseudoalteromonads by rapidly forming and maintaining biofilm density for 48 h until sloughing. *Vibrio* strains generally produced low density biofilms with exception of Ma31 which formed a dense and stable biofilm after 12 h. The *Ruegeria* strains (AH10, K2, and EPP04) were strong and stable biofilm formers. *Bacteriodetes* strain PPM04 rapidly formed and maintained a dense biofilm between 12 and 36 h before dispersal, while *Bacteriodetes* strain AH26 was a weak biofilm producer.

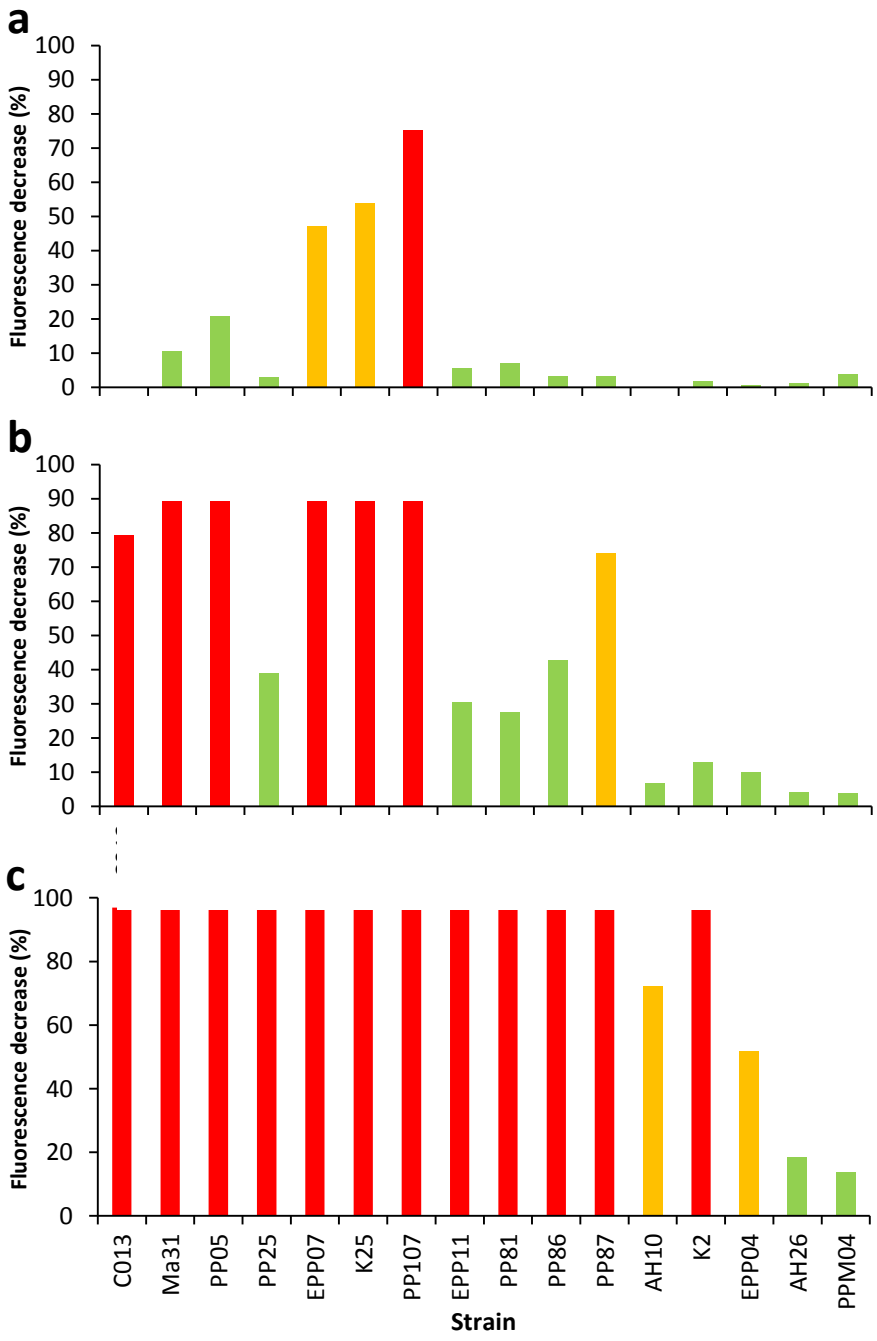


Figure 4-2. Microgrowth coculture assay. Inhibitory effect of probiotic candidates on pathogen growth determined after 24 h coculture, using fluorescence expressed by *V. owensii* DY05[GFP] as a proxy for its planktonic growth. The initial pathogen density was 1 x 10^3 CFU mL^{-1}, while initial probiont densities were (a) 1 x 10^3 CFU mL^{-1}, (b) 1 x 10^5 CFU mL^{-1}, or (c) 1 x 10^7 CFU mL^{-1}. ■: low activity; ■: moderate activity; ■: strong activity.

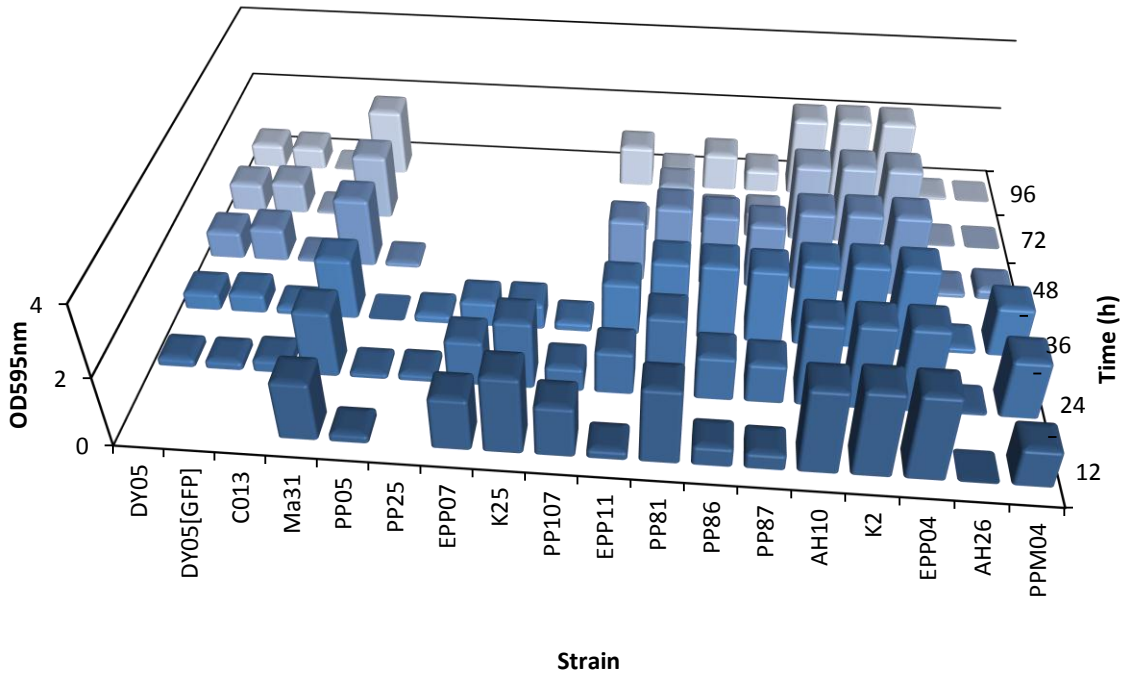


Figure 4-3. Monostrain biofilm formation. A crystal violet-microwell assay was used to assess monospecies biofilm formation of *V. owensii* DY05, *V. owensii* DY05[GFP] and probiotic candidate isolates belonging to *Vibrio* (C013, Ma31, PP05, and PP25), *Pseudoalteromonas* (EPP07, K25, PP107, EPP11, PP81, PP86, and PP87), *Ruegeria* (AH10, K2, and EPP04), and *Bacteroidetes* (AH26 and PPM04).

4.4.5 Multistrain biofilm

A multistrain biofilm assay was used to investigate pathogen biofilm formation in the presence of probiotic candidates under conditions of exclusion, competition and displacement (Figure 4-4). All non-pigmented *Pseudoalteromonas* strains (EPP11, PP81, PP86, and PP87), the pigmented *Pseudoalteromonas* strain EPP07, and *Vibrio* strain C013 caused significant increases (Student's t test $p < 0.05$) in fluorescence signals in at least one multistrain interaction, and hence were regarded as biofilm facilitators and eliminated from the candidate pool. In terms of percentage signal reduction, the remaining 10 candidates were more successful at inhibiting pathogen biofilm by exclusion (52-96%), followed by competition (26-90%) and displacement (0-37%). Under conditions of exclusion, the pigmented *Pseudoalteromonas* strains K25 and PP107, *Vibrio* strains Ma31 and PP05, and all *Ruegeria* isolates (AH10, K2 and EPP04) demonstrated strong inhibition. In the competition assay,

strong inhibitory activity was shown by two pigmented *Pseudoalteromonas* strains (K25 and PP107), *Vibrio* strain PP05 and the *Bacteroidetes* strains (AH26 and PPM04), and weakest activity exhibited by the *Ruegeria* candidates AH10, K2 and EPP04. During conditions of displacement, the strongest inhibitory activity was exhibited by two *Ruegeria* strains (AH10 and K2) and pigmented *Pseudoalteromonas* strain K25.

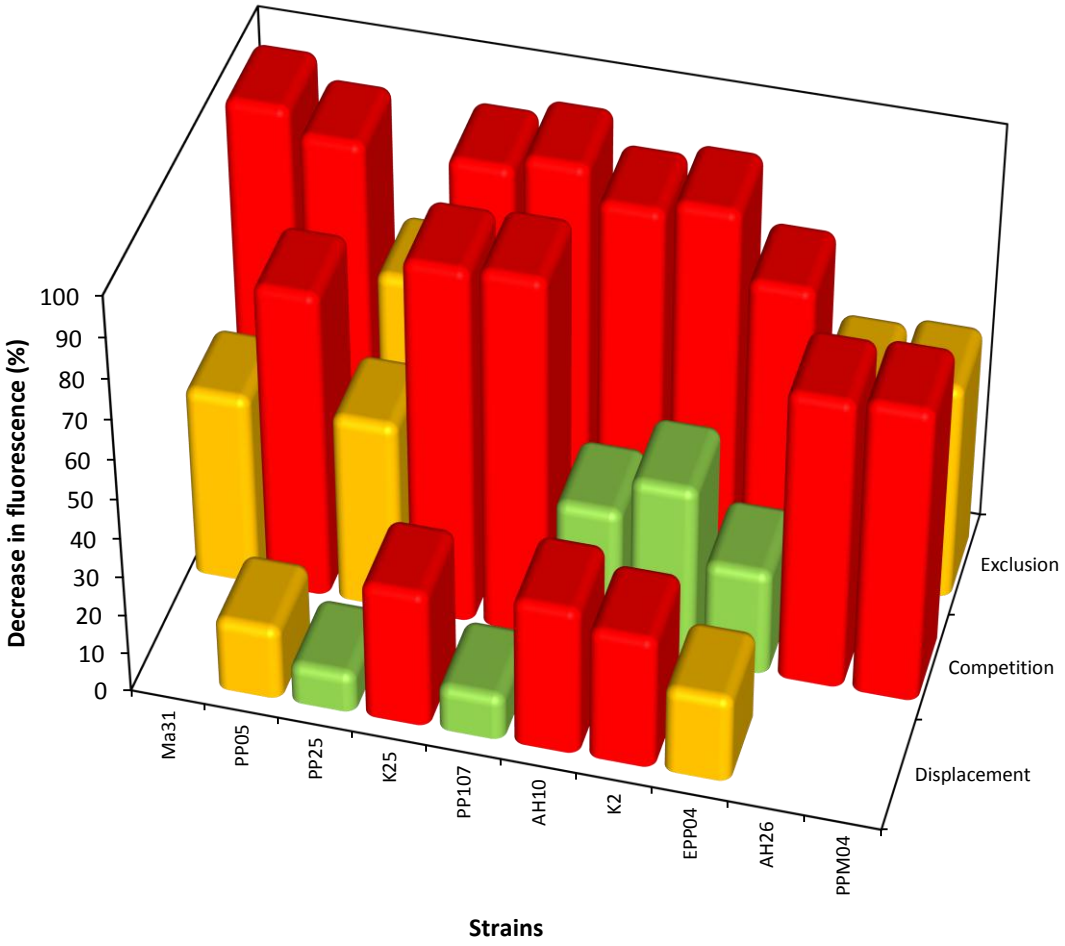


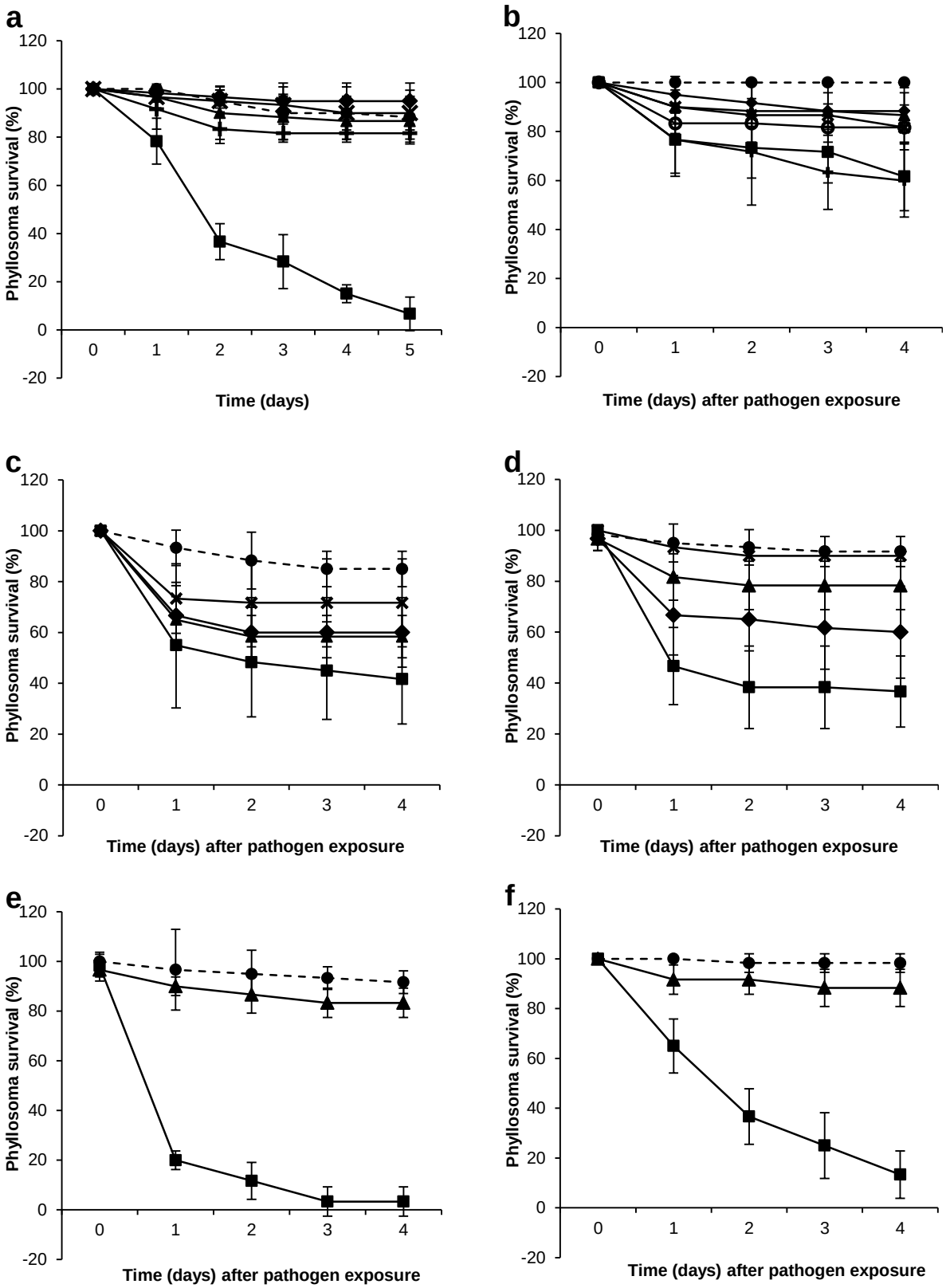
Figure 4-4. Multistrain biofilm interactions. Inhibitory effect of probiotic candidates on pathogen biofilm formation under conditions of exclusion, competition and displacement using fluorescence expressed by *V. owensii* DY05[GFP] as a proxy for pathogen attachment. Strains that appeared to facilitate pathogen biofilm formation are not presented. Columns represent average values from two time points (t = 48 h and t = 72 h). ■: low activity; ■: moderate activity; ■: strong activity.

4.4.6 Pathogenicity testing and *in vivo* protective benefit

Vibrio strain PP05 and *Pseudoalteromonas* strains PP107 and K25 were selected for *in vivo* experimentation based on their overall superior performance in the well diffusion, microgrowth coculture and biofilm assays. In addition, the best performing *Roseobacter* clade isolate (*Ruegeria* strain K2) was included since many strains belonging to this group of bacteria have elicited promising probiotic effects for other aquaculture species.

Pathogenicity testing indicated vectored challenge with PP05, PP107, K25 or K2 did not significantly alter survival (Dunnett's test $p > 0.05$) of *P. ornatus* phyllosomas (stage 1) relative to the unchallenged control (Figure 4-5a) and no anomalous phototactic responses or swimming behaviours were noted, indicating the candidates were non-pathogenic. In comparison, vectored challenge with *V. owensii* DY05 (pathogen control) caused a significant increase (Dunnett's test $p < 0.0001$) in phyllosoma mortality compared to the control (Figure 4-5a).

Figure 4-5. Pathogenicity testing and protective benefit of probiotic candidates on pathogen challenged stage 1 *P. ornatus* phyllosomas. (a) Pathogenicity testing of probiotic candidates. *Vibrio* sp. PP05 (▲), *Pseudoalteromonas* sp. PP107 (♦), *Pseudoalteromonas* sp. K25 (×), or *Ruegeria* sp. K2 (+), unchallenged control (●), *V. owensii* DY05 pathogen control (■). (b) Protective benefit of probiotic candidates towards *V. owensii* DY05 challenged phyllosomas using administration Strategy 1. PP05 (▲), PP107 (♦), K25 (×), K2 (+), a mixture of the four candidates (○), unchallenged control (●), pathogen control (■). (c) Protective benefit of probiotic candidates towards *V. owensii* DY05 challenged phyllosomas using administration Strategy 1. PP05 (▲), PP107 (♦), PP05 + PP107 (×), unchallenged control (●), pathogen control (■). (d) Protective benefit of probiotic candidates towards *V. owensii* DY05 challenged phyllosomas using administration Strategy 2. PP05 (▲), PP107 (♦), PP05 + PP107 (×), unchallenged control (●), pathogen control (■). (e-f) Replicate experiments showing protective benefit of probiotic candidates towards *V. owensii* DY05 challenged phyllosomas using administration Strategy 2. PP05 + PP107 (▲), unchallenged control (●), pathogen control (■). Survival expressed as Means ± SD.


a
b
c
d
e
f
Phyllosoma survival (%)
Time (days)
Time (days) after pathogen exposure
120
100
80
60
40
20
0
-20
0
1
2
3
4
5

The *in vivo* protective effect of the candidates was initially tested using administration Strategy 1, where probiotic candidates were delivered to phyllosomas via *Artemia* before (t = 0 h) and after (t = 30 h) vectored challenge with *V. owensii* DY05 for 6 h (t = 24-30 h). In the first experiment, phyllosoma survival was significantly enhanced for candidates PP05 and PP107 (Dunnett's test $p < 0.05$) compared to the pathogen control (Figure 4-5b). In contrast, survival of phyllosomas treated with K25, K2 or a mixture of the four candidates did not significantly differ (Dunnett's test $p > 0.05$) from the pathogen control (Figure 4-5b), indicating limited or no protective benefit on larval survival. Based on the survival data, PP05 and PP107 were selected as the most promising candidates and their protective benefit singularly or in combination was further investigated. In the second experiment, survival of PP05 or PP107-treated phyllosomas did not significantly differ from the pathogen control. (Dunnett's test $p > 0.05$) (Figure 4-5c). However, the PP05 and PP107 combination resulted in a significant benefit (Dunnett's test $p < 0.01$), enhancing phyllosoma survival relative to the pathogen control by 30% (Figure 4-5c).

Using administration Strategy 2, so that probionts were present simultaneously with the pathogen during vectored challenge (t = 24-30 h), phyllosoma survival was significantly enhanced by 23% for PP107 (Dunnett's test $p < 0.01$), 42% for PP05 (Dunnett's test $p < 0.0001$) and 53% for PP05/PP107 in combination (Dunnett's test $p < 0.0001$) relative to the pathogen control (Figure 4-5d). Due to the gain in survival benefit using PP05/PP107 in combination, the experiment was repeated twice to validate the observations (Figure 4-5e-f) and phyllosoma survival was significantly enhanced by 80% (Dunnett's test $p < 0.0001$) and 75% (Dunnett's test $p < 0.0001$) respectively, compared to the pathogen control (Figure 4-5e-f). It should be noted that survival of PP05/PP107-treated phyllosomas did not differ significantly (Dunnett's test $p > 0.05$) from non-challenged control phyllosomas in each of the three replicated experiments using administration Strategy 2 (Figure 4-5d-f).

4.5 Discussion

The present study has demonstrated antagonistic bacteria recovered from natural prey items of *P. ornatus* phyllosomas were capable of protecting cultured phyllosomas from the serious hatchery pathogen *V. owensii* DY05. The probiotic screening strategy devised in this study

targeted antagonistic activity exhibited by candidate bacteria in both planktonic and attached forms, resulting in the selection of a multistrain combination (*Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107) that conferred a substantial additive survival benefit to pathogen-challenged phyllosomas.

Antagonism is a widespread trait implicated in the competiveness and ecological success of many marine bacterial genera (Long and Azam, 2001; Makridis et al. 2005; Fjellheim et al. 2007; Gram et al. 2010) and is thus considered an important attribute of aquaculture probionts. In the present study, 3.3-6.5% of the culturable bacterial communities of wild *P. ornatus* phyllosomas and zooplankton prey were antagonistic, which is in alignment with the frequencies recovered from global marine environments (Gram et al. 2010) and to that associated with larval turbot *Scophthalmus maximus* (Hjelm et al. 2004) and cod *Gadus morhua* (Fjellheim et al. 2007). Invertebrate aquaculture settings may serve as reservoirs of antagonistic bacteria (Ruiz-Ponte et al. 1999; Jorquera et al. 1999; Vaseeharan and Ramasamy, 2003b; Li et al. 2006; Balcázar and Rojas-Luna, 2007; Prado et al. 2010; Rattanachuay et al. 2010) and this study suggests natural zooplankton prey are another source of inhibitory bacteria which can be acquired or enriched by predating phyllosomas. Interestingly, Makridis et al. (2005) found the culturable bacterial communities of Senegalese sole (*Solea senegalensis*) fed natural polychaete prey harboured over 40% antagonistic bacteria, representing a significant increase compared to animals fed artificial diets.

In the present study, the most readily culturable antagonistic bacteria associated with wild phyllosomas and zooplankton belonged to the genera *Pseudoalteromonas* and *Vibrio*, which together with members of the *Roseobacter* clade, are frequently recovered from environmental and aquaculture settings (Hjelm et al. 2004; Makridis et al. 2005; Fjellheim et al. 2007; Gram et al. 2010; Wietz et al. 2010). Pseudoalteromonads are a metabolically diverse group commonly associated with eukaryotic hosts and produce a range of bioactive compounds central to their colonisation success, including antimicrobial, antifouling and algicidal metabolites (Holmström and Kjelleberg, 1999; Bowman, 2007). Similarly, many *Vibrio* species forge commensal and symbiotic associations with eukaryotic hosts (Thompson et al. 2004) and constitute a large percentage of the autochthonous microbiota of cultured larval

invertebrates including *P. ornatus* phyllosomas (Payne et al. 2007). It is not known by which antagonistic mechanisms these genera inhibited *V. owensii* DY05 in the current study, however broad-spectrum anionic proteins and non-proteinaceous antibiotics produced by *Pseudoalteromonas* spp. (Longeon et al. 2004; Isnansetyo et al. 2008) and aliphatic hydroxyl ethers and andrimid antibiotics (Jorquera et al. 1999; Wietz et al. 2010) synthesised by *Vibrio* spp. are implicated in inhibition of aquatic vibrios.

Interestingly, while *Vibrio* spp. and *Pseudoalteromonas* spp. are not major constituents of microbial communities associated with wild *P. ornatus* phyllosomas, *Roseobacter* clade bacteria including *Sulfitobacter* spp. and *Roseobacter* spp. appear to be relatively dominant (Payne et al. 2008). *Roseobacter* clade bacteria are important primary colonisers (Dang and Lovell, 2000) and many species produce a potent antibacterial, tropodithietic acid (Brinkhoff et al. 2004; Bruhn et al. 2005; Gram et al. 2010) involved in protecting its symbiotic phytoplankton partners from harmful microbial colonisers (Geng and Belas, 2010) and inhibiting aquaculture pathogenic vibrios (Bruhn et al. 2005). It was thus somewhat surprising that antagonistic roseobacters were absent from culturable communities of wild phyllosomas given they also have good cultivation potential (Buchan et al. 2005; Gram et al. 2010). It is possible that members of this clade associated with wild phyllosomas either do produce inhibitory compounds or produce inhibitory compounds that *V. owensii* DY05 is insensitive to (Long and Azam, 2001).

In the present study, the fluorescence-based approach used to monitor the growth of planktonic *V. owensii* DY05 in coculture was not restricted to evaluating the antagonistic effects of probiotic culture supernatants (Gram et al. 1999), but enabled monitoring of growth during interaction with other bacterial cells. Inhibition of *V. owensii* DY05 was dependent on both initial concentration and taxonomic grouping of the candidates and overall the results support previous research trends that antagonists are generally required at higher concentrations than the pathogen for elimination (Gram et al. 1999; Vaseeharan and Ramasamy, 2003b; Jayaprakash et al. 2005). Planktonic forms of all *Vibrio* and *Pseudoalteromonas* strains and the *Ruegeria* strain K2 strongly inhibited pathogen growth at the highest inoculum concentration. In contrast, the other strains (eg. *Bacteroidetes* and the

other *Ruegeria* candidates) showed only moderate or low inhibition of planktonic pathogen growth despite showing antagonistic activity in well diffusion assays. This observation is consistent with several studies suggesting that free-living forms of marine bacteria may be less prone to producing antibacterials (Long and Azam, 2001; Gram et al. 2010). Moreover, some compounds may only be bioactive during certain interactions. For example, Dheilly et al. (2010) found anti-biofilm exoproducts of *Pseudoalteromonas* sp. 3J6 had no antibacterial properties against free-living *Paracoccus* and *Vibrio* strains.

Overall, the strongest biofilm inhibitory activity was seen in the exclusion assay, followed by the competition and displacement assays, respectively. This was probably due to the ability of bacteria such as *Pseudoalteromonas* and *Ruegeria* to rapidly form biofilms on the microwell surface and synthesise compounds with antifouling and antibacterial activity (Holmström et al. 2002; Rao et al. 2005; Bruhn et al. 2007) that resist incoming pathogen propagules. The pigmented *Pseudoalteromonas* strains were the strongest inhibitors of pathogen attachment and pigmentation is known to be linked to production of bioactive molecules in this genus (Holmström et al. 2002; Egan et al. 2002). Some of the *Vibrio* candidates (PP05 and PP25) were poor biofilm formers on the microwell surface yet were among the strongest inhibitors of pathogen attachment suggesting inhibition was probably more related to the potency of the secreted compound rather than the biofilm biomass. Recently, Jiang et al. (2011) discovered an anti-biofilm exopolysaccharide produced by a marine *Vibrio* strain which could inhibit the biofilm formation of a number of Gram-negative and Gram-positive bacteria, and importantly, could disrupt the mature biofilm of a cystic fibrosis isolate of *Pseudomonas aeruginosa.* Attached *Bacteriodetes* strains (AH26 and PPM04) were more successful than their planktonic conspecifics in outcompeting the pathogen, inferring an ecological preference for surface attachment and supporting a growing body of evidence that attached forms are more likely to exhibit antibacterial activity (Gram et al. 2010).

The reduced ability of the probiotic candidates to displace pathogen biofilms could partly be related to cells being buffered against antagonistic compounds. The exopolymeric matrix produced by biofilms can trap or slow the diffusion of antimicrobial compounds leading to increased resistance (Pasmore and Costerton, 2003; Burmølle et al. 2006). Biofilms may also

tolerate antimicrobials through changes in genotypic pathways, including upregulation of genes encoding efflux pumps which facilitate the efflux of antimicrobials (Gillis et al. 2005). Another possible survival strategy for survival in biofilms is the formation of microcolonies reported for *Alteromonas* sp., *P. gracilis*, and *Cellulophaga fucicola* during invasion by antagonistic *P. tunicata* (Rao et al. 2005). The displacement activity that was observed in the present study may not have been exclusively antagonistic action, but could in part be due to other mechanisms including dispersal-inducing diffusible signal molecules (Davies and Marques, 2009) or degradation of matrix polymers and biofilm-regulated quorum sensing factors (You et al. 2007).

An *in vivo* infection model was used to investigate if treatment with probiotic candidates could prevent infection by *V. owensii* DY05 in *P. ornatus* phyllosomas. Phyllosomas were exposed to *V. owensii* DY05 for 6 h as previous studies using the phyllosoma infection model showed that pathogen cells have entered the hepatopancreas at this time point (Goulden et al. 2012; Chapter 3). However, Palinurid phyllosomas are opportunistic carnivores (Suzuki et al. 2008; Smith et al. 2009a; Chow et al. 2010) and some individuals had not consumed all *Artemia* nauplii after 6 h, likely contributing to increased standard deviations relative to the robust and reproducible survival data obtained in a previous study (Goulden et al. 2012; Chapter 2). This may in part explain the discrepancies between the first two protection experiments (Figure 4-5b-c) where a significant protective benefit was conferred by treatment with either *Vibrio* sp. PP05 or *Pseudoalteromonas* sp. PP107 in the first experiment but not in the second.

When the delivery strategy was altered so that the pathogen was added in combination with the probiotic candidates during vectored transmission, the protective benefit towards phyllosomas increased dramatically. Importantly, survival of phyllosomas receiving the combination of PP05 and PP107 did not significantly differ from non-challenged controls across three replicated experiments (Figure 4-5d-f), and this was concomitant with a reproducible survival enhancement (53-80%) relative to pathogen controls. *Pseudoalteromonas* and *Vibrio* species have previously shown good potential as probiotics by enhancing survival of cultured invertebrates (Riquelme et al. 1997; Longeon et al. 2004;

Balcázar et al. 2007) and fish (Austin et al. 1995; Gatesoupe, 1997) following challenge with pathogenic vibrios. However, horizontal gene transfer has contributed significantly to the evolution and dissemination of virulence genes in *Vibrio* genomes (Hazen et al. 2010) so a certain amount of risk is involved in the selection of *Vibrio* probiotic strains. The risks may be considered acceptable given the lack of evidence of probiotic vibrios acquiring virulence traits and the dramatically increased protective effect on phyllosomas when *Vibrio* sp. PP05 was included in the probiotic mixture. The possibility of transfer of virulence traits to PP05 does however exist, and this will have to be considered if disease outbreaks persist or re-emerge.

Multispecies probiotic applications have shown clear advantages over monospecies formulations in improving pathogen resistance also in previous studies (Timmerman et al. 2004). As an example in aquaculture, multispecies application of inhibitor-producing *Vibrio*, *Pseudomonas* and *Bacillus* strains enabled the completion of the pelagic phase of scallop (*Argopecten purpuratus*) larval cultures without antibiotics (Riquelme et al. 2001). At this stage it is not clear which mechanisms are responsible for the additive probiotic effects of PP05 and PP107 and this warrants further investigation.

4.6 Conclusions

Vibrio sp. PP05 and *Pseudoalteromonas* sp. PP107 were prospected from a large pool of antagonistic candidates based on their ability to inhibit planktonic and attached forms of pathogenic *V. owensii* DY05. Used in combination, these bacteria significantly and reproducibly protected *P. ornatus* phyllosomas from infection by *V. owensii* DY05 *in vi*vo. Thus, the use of miniaturised coculture and biofilm assays devised in the study enabled rapid processing of numerous candidates and selection of probiotic bacteria capable of promoting survival. It was shown that natural microbial communities of wild phyllosomas support antagonistic bacteria capable of suppressing pathogens originating from the larviculture ecosystem and affirmed natural prey items as reservoirs of beneficial microorganisms. Further research is required to elucidate probiont-pathogen-host interactions *in situ* thus enabling the design of integrated probiotic biocontrol methods.

4.7 Acknowledgements

I wish to thank Lone Gram for kindly donating *Phaeobacter* strain 27-4 used as a positive antagonistic control in the well diffusion assays. I also acknowledge the zootechnical assistance of Greg Smith, Matt Kenway, Matt Salmon, Grant Milton, Justin Hochen, Katie Holroyd, Jane Gioffre and Michael Clarkson (AIMS). Rochelle Soo, Rose Cobb and Sarah Castine are thanked for their technical assistance (all AIMS). The crew of the AIMS RV Cape Ferguson are also thanked for their assistance. Financial support for the project was provided by AIMS and UNE.

5 Niche specialisation contributes to additive probiotic protection against *Vibrio owensii* infection in ornate spiny lobster (*Panulirus ornatus*) phyllosoma

5.1 Abstract

The development of probiotic mixtures and efficient application protocols for use in marine larviculture relies on a comprehensive understanding of pathogen-probiont-host interactions. In the present study, fluorescently tagged bacterial strains were used to elucidate ecological niches of established probionts *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 during vector-mediated *V. owensii* DY05 infection of ornate spiny lobster (*Panulirus ornatus*) phyllosomas. Both probionts inhibited planktonic pathogen growth in cultures of the common aquaculture live feed organism *Artemia*, which were used for vectored transmission to *P. ornatus* phyllosomas. Within the *Artemia* gut, the pathogen evaded attack by PP107, but was vulnerable to competition with PP05. Following vectored challenge, only PP05 was present in the phyllosoma digestive system, where it was mainly associated with the foregut (proventriculum) setae and midgut gland (hepatopancreas), as well as densely colonising faecal pellets. While *Pseudoalteromonas* sp. PP107 was the overall dominant ectobiont, the probionts also exhibited niche partitioning on the phyllosoma exocuticle. PP107 dominated on the phyllosoma cephalic shield, thorax and inner leg (pereiopod) segments, however PP05 was the primary coloniser of the outer leg segments, locales which may promote ingestion during feeding.

5.2 Introduction

Against a backdrop of stagnating or declining wild fishery supply of spiny lobsters (Palinuridae) (Jeffs, 2010), closed-life cycle aquaculture of the economically important ornate spiny lobster (*Panulirus ornatus*) is on the verge of becoming a reality (Rogers et al. 2010). Nevertheless, a key challenge to refining *P. ornatus* hatchery technology is reducing the incidence of mass mortalities caused by bacterial disease (Bourne et al. 2004; 2007). *Vibrio owensii* DY05 is an emerging enteropathogen causing disease epizootics in the larviculture of

P. ornatus (Cano-Gómez et al. 2010; Goulden, et al. 2012; Chapter 2). Through the use of a green fluorescent protein (GFP) expressing transconjugant of *V. owensii* DY05, we have elucidated the niche preferences and infection cycle of *V. owensii* DY05 in *P. ornatus* phyllosomas, showing that soon after vectored transmission via the live feed organism *Artemia*, the pathogen invades the phyllosoma foregut (proventriculum) and midgut gland (hepatopancreas) tubules, preceding mass proliferation in the hepatopancreas and ultimately systemic infection (Goulden et al. 2012; Chapter 3).

Combating epizootics and increasing hatchery production of *P. ornatus* will be met through developing disease management strategies, including probiotics (Rogers et al. 2010). Earlier studies showed the addition of beneficial bacteria to larval rearing systems of *Jasus edwardsii* phyllosomas lead to increased survival and metamorphosis to puerulus stage (Igarashi et al. 1990), indicating that this approach could supersede current use of antibiotics in spiny lobster larviculture (Murakami et al 2007). We have previously shown that vectored administration of a two-strain (*Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107) probiotic combination via *Artemia* provides additive protection of stage 1 *P. ornatus* phyllosomas from experimental infection with *V. owensii* DY05 (Goulden et al. accepted; Chapter 4). However, further understanding of probiont-pathogen-host interactions is required to develop efficient biocontrol strategies.

Probionts must function in the same ecological niche as the targeted pathogen (Verschuere et al. 2000b) as differential niche specialisation can render promising probionts incapable of protecting hosts against infection (Ruiz-Ponte et al. 1999; Gram et al. 2001; Spanggaard et al. 2001). Visualising the localisation of viable fluorescently labelled probionts in their niches by non-destructive methods and monitoring their niche specialisation *in situ* is an alluring possibility in transparent zooplankton forms (Goulden et al. 2012; Chapter 3). The purpose of the present study was to use FP-expressing strains to elucidate the respective niches of the two probiotic strains, *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107, which, when used in combination, are capable of interfering with the infection cycle of *V. owensii* DY05 in *P. ornatus* phyllosoma (Goulden et al. accepted; Chapter 4).

5.3 Materials and Methods

5.3.1 Larviculture

Maintenance of *P. ornatus* broodstock, production of larvae, and larviculture were achieved according to Smith et al. (2009b) at the Tropical Aquaculture Facility of the Australian Institute of Marine Science (AIMS), Townsville, Australia. Only apparently healthy individuals as assessed by photopositive response were used for experiments.

5.3.2 Bacterial strains, transconjugations and inoculum preparation

Bacterial strains used in this study are listed in Table 5-1. Plasmids carrying FP genes and selection markers were transferred from *E. coli* donor strains to *Vibrio* spp. or *Pseudoalteromonas* sp. by triparental conjugation as described previously (Dunn et al. 2006; Goulden et al. 2012; Chapter 3) using the helper strain CC118λpir or HB101, respectively. Briefly, wild type *Vibrio* and *Pseudoalteromonas* strains were grown in LB20 broth (5 g L^{-1} yeast extract; 10 g L^{-1} neutralised peptone; 20 g L^{-1} NaCl) or half strength marine broth 2216 (Becton, Dickinson and Company), respectively. *E. coli* strains were grown in LB broth (5 g L^{-1} yeast extract and 10 g L^{-1} neutralised peptone) supplemented with 40 µg mL^{-1} kanamycin or 15 µg mL^{-1} chloramphenicol, where appropriate. Agar (1%) was used to solidify media for plating and all incubations were performed at 30^{o}C. To screen for FP expression in *Vibrio* spp., LB20 agar was supplemented with kanamycin or chloramphenicol in addition to 50 µg mL^{-1} colistin. To screen for GFP expression in *Pseudoalteromonas* sp., half strength marine agar was supplemented with chloramphenicol. Fluorescent transconjugant colonies were detected using a blue light transilluminator.

Table 5-1. Bacterial strains used in this study

Strain [plasmid]	Strain characteristics	Relevant plasmid characteristics[a]	Reference
Escherichia coli			
CC118λpir [pEVS104]	Helper	R6Kγ *oriV*; RP4 *oriT*, *trb* and *tra*; Kn^R	Stabb and Ruby (2002).
DH5αλpir [pVSV201]	Donor GFP	*gfp*; Kn^R	Dunn et al. (2006)
DH5αλpir [pVSV208]	Donor RFP	*rfp*; Cm^R	Dunn et al. (2006)
HB101 [prK600]	Helper	RK2-Mob^+ and Tra^+; Cm^R	Kessler et al. (1992)
DH5α [pCJS10]	Donor GFP	*gfp*; Cm^R	Rao et al. (2005)
Vibrio spp.			
V. owensii DY05	Wild type, moribund *P. ornatus* phyllosoma isolate	-	Cano-Gomez et al. (2010)
V. owensii DY05[RFP]	Carrying pVSV208	*rfp*; Cm^R	This study
Vibrio sp. PP05	Wild type, zooplankton isolate	-	Goulden et al. (accepted; Chapter 4)
Vibrio sp. PP05[GFP]	Carrying pVSV201	*gfp*; Kn^R	This study
Vibrio sp. PP05[RFP]	Carrying pVSV208	*rfp*; Cm^R	This study
Pseudoalteromonas sp.			
Pseudoalteromonas sp. PP107	Wild type, zooplankton isolate	-	Goulden et al. (accepted; Chapter 4)
Pseudoalteromonas sp. PP107[GFP]	Carrying pCJS10	*gfp*; CmR	This study

[a]Abbreviations used: Cm^R = chloramphenicol resistance; Kn^R = kanamycin resistance; GFP = green fluorescent protein; RFP = red fluorescent protein.

FP-expressing strains *V. owensii* DY05[RFP], *Vibrio* sp. PP05[GFP] and PP05[RFP], and *Pseudoalteromonas* sp. PP107[GFP] (Table 5-1) showed similar growth profiles compared to respective wild types in a microgrowth assay and the FP were stably expressed in *Vibrio* sp. PP05[GFP] (99%), PP05[RFP] (96%) and *V. owensii* DY05[RFP] (84%) after continuous subculture every 24 h for 7 days in non-selective medium (data not shown). In contrast, expression of GFP in PP107[GFP] dropped to below 79% after 2 subcultures, restricting its projected use to short term (48 h) experiments.

Inocula were prepared by washing 20 h marine broth 2216 cultures using 3 cycles of centrifugation (10 min at 4650 rpm; 10^{o}C) and resuspension in 0.22 µm filtered seawater (FSW). Cell suspensions were adjusted to absorbance OD_{600nm} 0.1 (Nanodrop ND1000) and total viable counts were determined for each inoculum in triplicate by spiral plating (Eddy Jet; IUL) on marine agar (MA; Becton, Dickinson and Company) and enumeration by an automatic colony counter (Flash and Grow v1.2; IUL). This information was used to calculate the volume of each OD_{600nm} 0.1 suspension needed to achieve the desired starting concentrations in the phyllosoma *in vivo* infection experiments described in sections 5.3.3 - 5.3.4.

5.3.3 Confirmation of virulence and probiotic effectiveness *in vivo*

To confirm the virulence of *V. owensii* DY05[RFP] and the probiotic protective benefits of PP05[GFP], PP05[RFP], and PP107[GFP], stage 1 *P. ornatus* phyllosomas were experimentally infected using vectored challenge via instar II *Artemia* nauplii (Goulden et al. 2012; accepted; Chapters 2 and 4). Strain combinations were selected to correspond to treatments used to study pathogen-probiont-host interactions *in situ* (section 5.3.4). Treatments included multistrain applications of (i) wild type strains (*V. owensii* DY05, PP05 and PP107); (ii) wild type pathogen and two differently labelled probiotic transconjugants (PP05[RFP] and PP107[GFP]); and (iii) RFP pathogen transconjugant (*V. owensii* DY05[RFP]) and GFP transconjugants of the probionts (PP05[GFP] and PP107[GFP]). Briefly, *Artemia* (200 nauplii mL^{-1}) were enriched through filter feeding with the probiotic combinations (1 x 10^{7} CFU mL^{-1} of each strain) in tissue culture flasks for 2 h (28^{o}C; 45 rpm) and fed at a final concentration of 3 nauplii mL^{-1} to phyllosomas in 12-well cell culture plates

at t = 0 h and t = 30 h. At t = 24 h, phyllosomas were vector challenged for 6 h with *Artemia* enriched with the pathogen (1 x 10^6 CFU mL^{-1}) together with the probiont combination (1 x 10^7 CFU mL^{-1} of each strain). Phyllosomas were transferred to new cell culture plates following pathogen challenge (t = 30 h). Treatments were performed in quintuplicate (n = 60) and survival was assessed every 24 h for 5 days. Experimental controls consisted of a negative control (phyllosomas fed non-enriched *Artemia*) and pathogen controls fed *Artemia* enriched in *V. owensii* DY05 or DY05[RFP] only at t = 24 h, and non-enriched *Artemia* at t = 0 h and t = 30 h.

5.3.4 Pathogen-probiont-host interactions *in situ*

A vector-challenge experiment was performed as outlined above to investigate the spatiotemporal colonisation of the pathogen (*V. owensii* DY05[RFP]) and probionts (PP05[GFP], PP05[RFP], and PP107[GFP]) during interaction with *Artemia* and the phyllosoma hosts. Treatments consisted of *Artemia* enriched with suspensions of each FP-labelled strain (monostrain treatments) fed to *P. ornatus* phyllosomas at t = 0 h only, or *Artemia* enriched with combinations of the FP-tagged probionts and FP-tagged/wild type pathogen using the administration regimes outlined in section 5.3.3 (multistrain treatments). For each treatment, enriched *Artemia* nauplii cultures and 4 vector challenged *P. ornatus* phyllosomas were removed at 6 h intervals over a 48 h monitoring period, live mounted in FSW and viewed using differential inference contrast (DIC) and fluorescence microscopy (AxioSkop 2 mot plus; Carl Zeiss). Fluorescence was detected using a dual band filter set (59004; Chroma Technology Corp.) and images were captured by an AxioCam MRc5 camera (Carl Zeiss) directed by the multidimensional acquisition module of the AxioVision Rel. 4.8 software (Carl Zeiss).

5.3.5 Statistical analysis

Differences between survival curves were determined using the product-limit (Kaplan-Meier) estimator and confirmed with an ANOVA. A *post hoc* Dunnett's test was used to compare multiple data sets to the defined control group. All statistical analyses were performed using the statistical software package JMP®7 (SAS) standardised at significance level $\alpha = 0.05$.

5.4 Results

5.4.1 Pathogen virulence and probiotic protection

Expression of FP had no significant effect on the virulence or probiotic properties of the bacterial transconjugant strains used in this work (Figure 5-1). *V. owensii* DY05[RFP] caused significant phyllosoma mortality (87%; Dunnett's test $p < 0.0001$) compared to the control, which did not statistically differ from the wild type *V. owensii* DY05 (Dunnett's test $p > 0.05$). In all cases, probiotic treated phyllosomas showed significantly enhanced survival by 79-82% over pathogen-only (*V. owensii* DY05 or DY05[RFP]) controls that did not receive probiotic treatment (Dunnett's test $p < 0.0001$) and survival was not significantly different from unchallenged negative control phyllosomas (ANOVA $p > 0.05$). These traits indicated the pathogen and probiont transconjugants were suitable biomarkers for short term monitoring of bacterial-phyllosoma interactions.

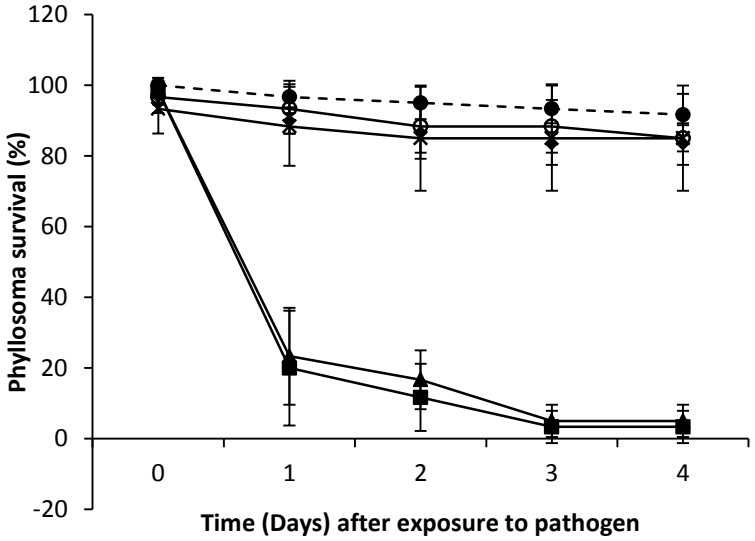


Figure 5-1. Survival of *P. ornatus* phyllosomas vector challenged with *Artemia* nauplii enriched with wild types and fluorescently labelled transconjugants of pathogen *Vibrio owensii* DY05 and probionts *Vibrio* sp. PP05 and *Pseudoalteromonas* PP107 singularly or in relevant combinations. Vectored challenge with non-enriched nauplii (negative control; ●); or nauplii enriched with wild type *V. owensii* DY05 (positive pathogen control; ■), RFP-labelled *V. owensii* DY05[RFP] (▲); wild type *V. owensii* DY05 and wild type probionts (PP05 and PP107) (♦); RFP-labelled *V. owensii* DY05[RFP] and GFP-labelled probionts (PP05[GFP] and PP107[GFP]) (×); wild type *V. owensii* DY05 and differently labelled probionts (PP05[RFP] and PP107[GFP]) (○). Survival expressed as Means ± SD.

5.4.2 Pathogen-probiont-host interactions *in situ*

Interactions of FP-expressing pathogen and probionts were monitored *in situ* using fluorescence microscopy following vectored challenge (*Artemia* nauplii) of stage 1 *P. ornatus* phyllosomas.

The niches of each strain in *Artemia* nauplii were determined after enrichment in monostrain or multistrain suspensions. After monostrain enrichment, probiotic *Vibrio* sp. PP05[GFP or RFP] or pathogen *V. owensii* DY05[RFP] were concentrated in the gut and the degree of bioaccumulation varied between nauplii (Figure 5-2a-b). In contrast, no *Pseudoalteromonas* sp. PP107[GFP] were internalised by *Artemia* nauplii following monostrain treatment, however ectobiotic attachment was observed (Figure 5-2c). Enrichment of *Artemia* nauplii with a mixture of both probionts did not alter their preferred niche, with *Vibrio* sp. PP05[RFP] confirmed as the dominant endobiont and no *Pseudoalteromonas* sp. PP107[GFP] cells seen transiting the *Artemia* gut (Figure 5-2c). In *Artemia* nauplii cultures enriched with both GFP-labelled probionts and RFP-labelled pathogen, single pathogen cells were dispersed amidst a mass of GFP cells (presumably PP05[GFP]) in the nauplius gut (Figure 5-2d). Additionally, there was a lack of *V. owensii* DY05[RFP] cells in the culture suspension surrounding the *Artemia* in this treatment.

The spatiotemporal localisations of the FP strains in *P. ornatus* phyllosomas were monitored *in situ* after vectored challenge with *Artemia* enriched with mono- or multistrain combinations of the probionts and pathogen (sections 5.3.3 and 5.3.4). Six hours after vectored exposure, probiotic *Vibrio* sp. PP05 [GFP] and PP05[RFP] persisted as single or small clusters of cells localised within the phyllosoma proventriculum setae, hepatopancreas, and transiting through the mid- and hindguts (Figure 5-3a-c). The spatiotemporal organisation of this probiont in the digestive system of phyllosomas was not affected when vector challenged in combination with *Pseudoalteromonas* sp. PP107[GFP] and/or pathogen *V. owensii* DY05[RFP]. While fluorescent cells were retained in the proventriculum and hepatopancreas after 48 h, faecal strands showed massive colonisation by the fluorescent *Vibrio* probiont (Figure 5-3d). Hence, it was not clear from this short-term study (48 h) whether residency of probiotic *Vibrio* sp. PP05[GFP] was transient or permanent. In contrast, there was a scarcity of endobiotic

Pseudoalteromonas sp. PP107[GFP] cells in phyllosomas regardless of whether the strain was vector challenged as a monostrain inoculum or in combination with either FP expressing transconjugants of *Vibrio* sp. PP05 and/or pathogen *V. owensii* DY05[RFP]. The small number of endobiotic PP107[GFP] cells that were located were transiting the hindgut. Endobiotic residency by the probionts did not impart any visible structural damage to host tissues and organs.

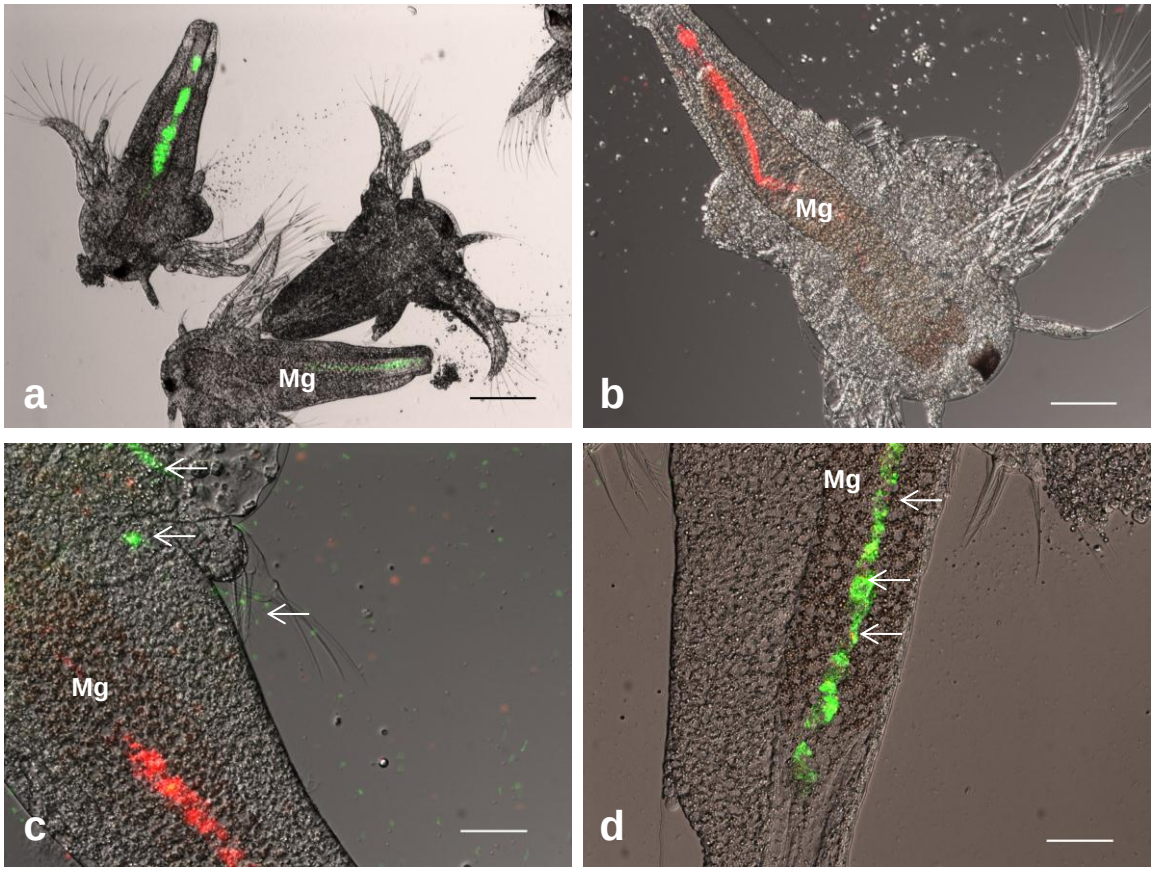


Figure 5-2. Fluorescent protein labelled pathogen *V. owensii* DY05 and probionts *Vibrio* sp. PP05 and *Pseudoalteromonas* PP107 in *Artemia* nauplii cultures after mono- or multistrain enrichment (2 h). (a) Nauplii enriched with PP05[GFP] showing varied bioaccumulation in midgut. Scale bar = 200 µm. (b) Nauplius enriched with *V. owensii* DY05[RFP] showing concentration in posterior midgut. Scale bar = 100 µm. (c) Nauplius enriched with PP05[RFP] and PP107[GFP], showing endobiotic PP05[RFP] in gut and ectobiotic PP107[GFP] confined to carapace and appendages (white arrows). Scale bar = 50 µm. (d). Nauplius gut showing dense concentration of probiotic PP05[GFP] and single *V. owensii* DY05[RFP] cells (white arrows). Note lack of red cells in ambient surrounds. Scale bar = 50 µm. Mg = midgut.

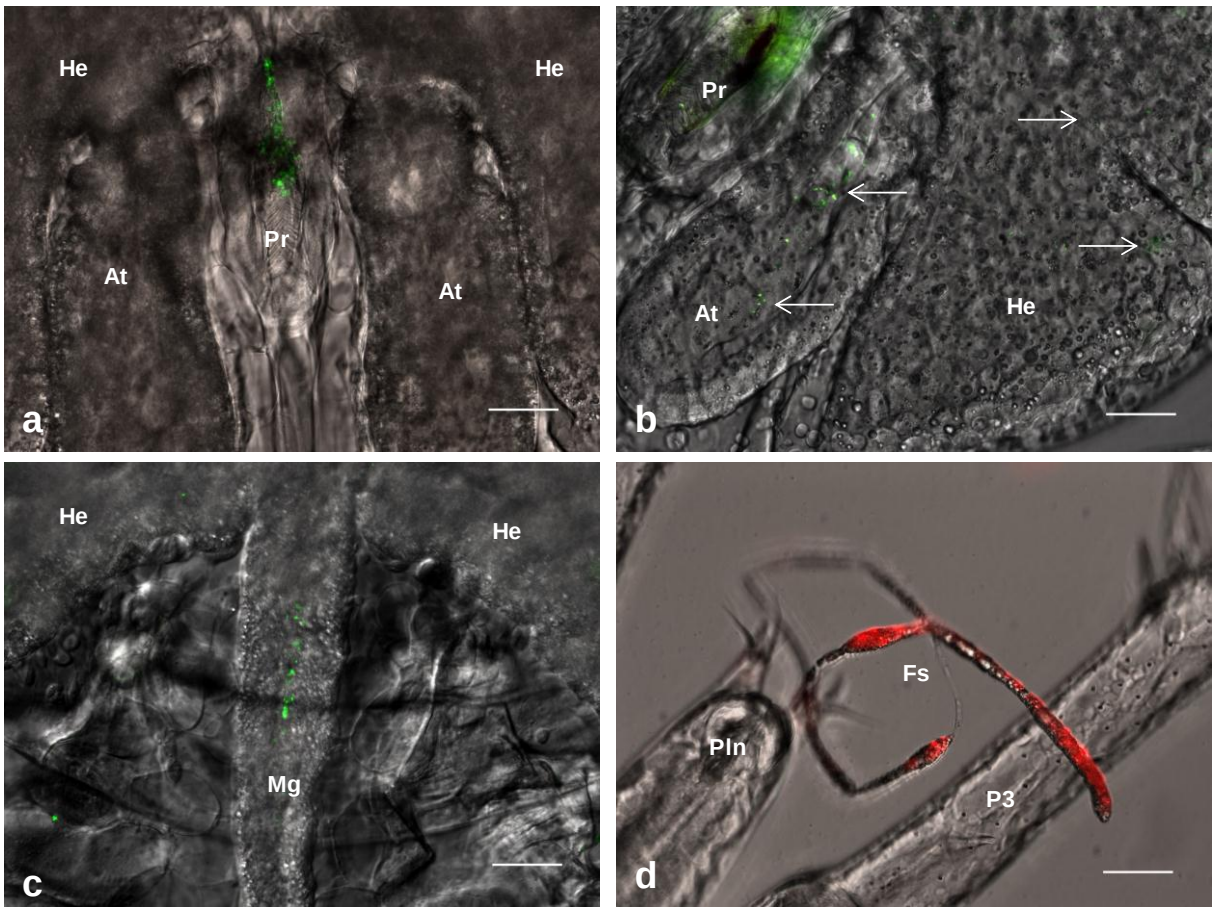


Figure 5-3. Endobiotic localisation of FP-labelled probiont *Vibrio* sp. PP05[GFP or RFP] in stage 1 *P. ornatus* phyllosomas after vectored challenge via *Artemia* nauplii enriched with monostrain inoculum or in combination with probiont *Pseudoalteromonas* sp. PP107[GFP] and/or pathogen *V. owensii* DY05 (wild type and DY05[RFP]). (a) PP05[GFP] colonisation of proventriculum (foregut) setae after vectored challenge with nauplii enriched with PP05[GFP]. (b) Single and aggregate cells of PP05[GFP] in hepatopancreas lobes (white arrows) after vectored challenge with nauplii enriched with PP05[GFP]. (c) Transiting of PP05[GFP] cells through midgut after vectored challenge with nauplii enriched with PP05[GFP]. (d) Prolific colonisation of faecal strand by PP05[RFP] after vectored challenge with nauplii enriched with PP05[RFP]. All scale bars = 50 µm. At = hepatopancreas anterior lobe; Fs = Faecal strand; He = hepatopancreas lateral lobe; Mg = midgut; P = pereiopod (3rd); Pln = pleon; Pr = proventriculum.

Visualisation of single endobiotic RFP-expressing cells (probiont and pathogen) in autofluorescing hepatopancreas tissues was at times difficult using the imagining capturing devices in this study. Autofluorescence also impeded monitoring of DsRed2-tagged *V. fischeri*

in squid (Dunn et al. 2006) and fluorescein-stained *V. harveyi* in crustacean larvae (Soto-Rodriguez et al. 2003b). Signal intensity could be slightly improved when gut contents dissipated and phyllosomas were viewed from the ventral side.

Clear zones of preferential ectobiotic colonisation were observed on phyllosoma exocuticles (Figure 5-4a), although sporadic 'intrusions' into each other's preferential niche was frequently observed. *Pseudoalteromonas* sp. PP107[GFP] was the dominant coloniser of the cephalic shield, thorax (Figure 5-4b), eyestalks, eyes, and inner segments of the pereiopods (legs) and maxillipeds (used to manipulate and shred food). Attachment was often organised on spines, contours, fissures and depressions on the exocuticle but randomly dispersed cells were also observed. The abundance of *Pseudoalteromonas* sp. PP107[GFP] depended more on individual larva rather than time, although colonisation overall appeared to be greater at t = 30 h which coincided with maximum vector and bacterial load in the wells prior to phyllosoma transfer to new 12-well plates. By contrast, the *Vibrio* probiont was the primary ectobiont on the outer pereiopod segments, with cell aggregates in particular, colonising the terminal segment (dactylus) and its terminal spine which is used to impale prey items (Figure 5-4c). Interestingly, in phyllosomas treated with *Artemia* enriched with probiotic monostrain inoculums, *Pseudoalteromonas* sp. PP107[GFP] colonisation ascended to the phyllosoma pereiopod dactylus (Figure 5-4d). In contrast, the spatial distribution of probiotic *Vibrio* sp. PP05[GFP] and PP05[RFP] was conserved, with the cephalic shield, thorax and inner pereiopods remaining relatively devoid of attachment. Ectobiotic attachment by the probionts caused no apparent loss of exocuticle integrity within the monitoring period.

Phyllosoma challenge with the pathogen *V. owensii* DY05[RFP] without probiotic treatment resulted in similar spatiotemporal proliferative patterns as the GFP expressing *V. owensii* DY05 transconjugant previously described (Goulden et al., 2012; Chapter 3). In contrast, six hours after phyllosoma exposure to *Artemia* enriched in *V. owensii* DY05[RFP], PP05[GFP], and PP107[GFP], only one pathogen cell was detected in the proventriculum of a single phyllosoma and none were discovered in the hepatopancreas or other locations. This result strongly indicates most of the pathogen population was eliminated by the probionts prior to phyllosoma ingestion, and occurs during enrichment of the *Artemia* cultures.

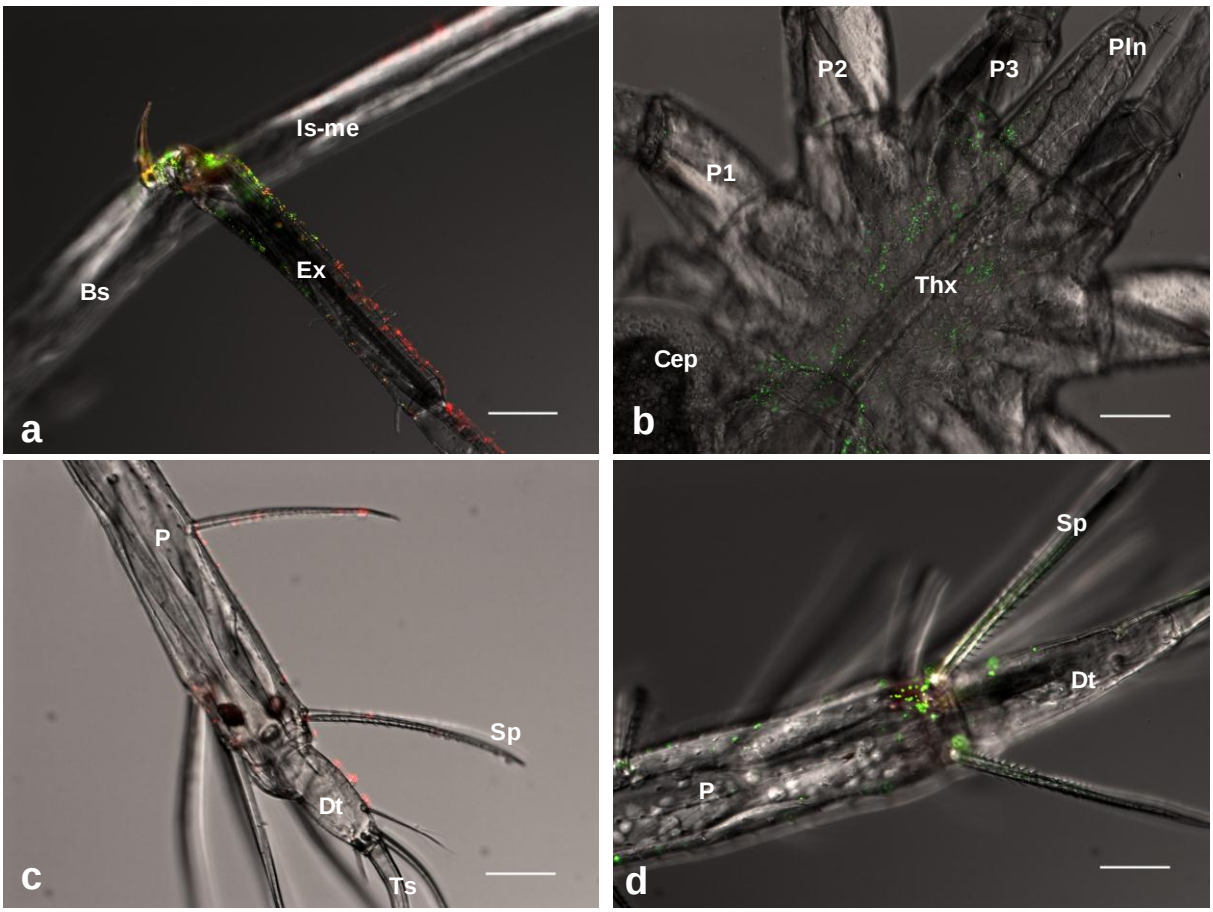


Figure 5-4. Ectobiotic localisation of FP-labelled probionts *Vibrio* sp. PP05[GFP or RFP] and *Pseudoalteromonas* sp. PP107[GFP] in stage 1 *P. ornatus* phyllosomas after vectored challenge via *Artemia* nauplii enriched with monostrain inoculum, in combination, or with pathogen *V. owensii* DY05 (wild type and DY05[RFP]). (a) Exopod of pereiopod demonstrating preferential colonisation by PP107[GFP] on inner segment and PP05[RFP] on outer segment after vector challenge with nauplii enriched with PP107[GFP], PP05[RFP] and wild type *V. owensii* DY05. Scale bar = 50 µm. (b) Prolific attachment of PP107[GFP] on cephalic shield and thorax after vector challenge with nauplii enriched with PP107[GFP], PP05[RFP] and wild type *V. owensii* DY05. Scale bar = 100 µm. (c) Attachment of PP05[RFP] clusters to dactylus and spines of pereiopod after vector challenge with nauplii enriched with PP05[RFP]. Scale bar = 100 µm. (d) Ascending colonisation of PP107[GFP] on dactylus of pereiopod after vector challenge with nauplii enriched with PP107[GFP]. Scale bar = 100 µm. Bs = basis; Cep = cephalic shield; Dt = dactylus; Ex = exopod; Is-me = ischio-merus; P = pereiopod (1-3); Pln = pleon; Sp = spine; Thx = Thorax; Ts = terminal spine.

5.5 Discussion

Early stage spiny lobster phyllosomas require animate prey to stimulate feeding (Johnston et al. 2008; Smith et al. 2009a) and *Artemia* have been used due to their versatility and practicality as a prey item in larviculture (Sorgeloos et al. 2001). While addition of probionts to *Artemia* cultures has previously shown to reduce pathogenic *Vibrio* spp. populations (Verschuere et al. 2000a; Villamil et al. 2003), it can be hypothesised from observations in the present study that niche specialisation by the two probionts contributes to the additive protective effect against *V. owensii* DY05 previously shown by Goulden et al. accepted (Chapter 4). It was observed that ambient planktonic pathogen cells in the *Artemia* cultures were reduced in the presence of the probionts compared to pathogen-only controls, confirming that the probionts can inhibit planktonic growth of *V. owensii* DY05 (Goulden et al. accepted Chapter 4). In the *Artemia* gut, *V. owensii* DY05 evaded attack by *Pseudoalteromonas* sp. PP107 but was exclusively vulnerable to competition with *Vibrio* sp. PP05. Within this niche, pathogen populations were reduced from high density bioaccumulations in pathogen-only controls to isolated cells in *Artemia* enriched with pathogen and probionts. The gut microenvironment appears to be particularly competitive, where different *Vibrio* species inhabiting similar niches may produce distinctive bioactive compounds (Wietz et al. 2010) to enhance ecological success. This is supported by the isolation of many antagonistic and siderophore-producing vibrios from the gastrointestinal tracts of aquatic organisms (Makridis et al. 2005; Fjellheim et al. 2007; Sugita et al. 2012).

Although *Artemia* ingest free living bacteria, the degree of bioaccumulation depends on the bacterial species and strain (Gomez-Gil et al. 1998; Makridis et al. 2000: Verschuere et al. 2000a; Soto-Rodriguez et al. 2003a). In the present study, *Pseudoalteromonas* sp. PP107 was not internalised by *Artemia* nauplii and it is likely the cells produced chemical deterrents and were not grazed by *Artemia*. For example, in a study by Ballestriero et al. (2010), it was demonstrated the bacterivorous nematode *Caenorhabditis elegans* selectively avoided toxic bacterial colonies in a lawn consisting of *E. coli* expressing heterologous genomic fragments of *Pseudoalteromonas tunicata*. Another explanation for the lack of internalised *Pseudoalteromonas* sp. PP107 cells could be due to the inability to adhere to the gastrointestinal epithelia (Orozco-Medina et al. 2009) and hence was rapidly evacuated or

degraded by digestive enzymes. We therefore concur with the views of Orozco-Medina et al. (2009) who suggest the perception of *Artemia* as nonselective filter feeders should be reassessed.

In both *Artemia* and phyllosomas, all observed *Pseudoalteromonas* cells were attached to external surfaces. Pseudoalteromonads have a propensity for surface colonisation (Holmström and Kjelleberg, 1999) and recent genomic studies on epiphytic *P. tunicata* revealed the presence of numerous genes (curli, pili, and capsular polysaccharide) that could mediate adherence to different surface textures and therefore potentially increase host range (Thomas et al. 2008). While *Pseudoalteromonas* sp. PP107 was isolated from arrow worms (*Chaetognatha*), a natural prey item of phyllosomas, we have isolated closely related *Pseudoalteromonas* strains from both wild and aquaculture-reared phyllosomas (Goulden et al., accepted; Chapter 4). It is uncertain whether *Pseudoalteromonas* associated with phyllosomas simply use a hitchhiking strategy to facilitate their dispersal (Grossart et al. 2010) or whether they are true ectosymbionts. Surface associations are recognised as a complex coordination between bacterial and eukaryotic partners (Goffredi, 2010) and higher organisms such as phytoplankton are known to recruit symbiotic roseobacter partners by producing a chemoattractant (dimethylsulfoniopropionate) and in return, the roseobacters protect the host from epibiotic growth by producing a potent antibiotic, tropodithietic acid (Geng and Belas, 2010). Likewise, it is possible that *Pseudoalteromonas* sp. PP107 or similar ecotypes contribute to the unfouled condition of wild phyllosomas (Payne et al. 2008) by producing bioactive compounds (Egan et al. 2002; Holmström et al. 2002). If this were the case, there would be a potential additional benefit of using this probiont in the larviculture system where recalcitrant fouling by filamentous *Thiothrix* spp. occasionally leads to impaired larval function and feeding capacity (Bourne et al. 2007; Payne et al. 2007).

In contrast to *Pseudoalteromonas* sp. PP107, the *Vibrio* probiont (PP05) was mainly internalised in *Artemia* and phyllosomas, though in addition, specific zones on the outer segments of the phyllosoma pereiopods were also colonised by PP05. The establishment of probionts in the digestive tract is a possible means of controlling enteropathogen proliferation in invertebrates and fish (Sugita et al. 1998; Rengpipat et al. 2000; Balcázar and Rojas-Luna,

2007; Li et al. 2007; Boonthai et al. 2010; Avella et al. 2011). The colonisation of the phyllosoma proventriculum setae by PP05 could in theory have contributed to limiting the invasion of the hepatopancreas by *V. owensii* DY05. Importantly, the localisation of PP05 was sustained at least transiently over 48 h using the administration regime in the present study. Furthermore, residence in the phyllosoma hepatopancreas could have provided the main source of faecal pellet colonisation by PP05 (Tang, 2005). Faecal matter can serve as microincubators to increase bacterial abundance and thereby reinoculating ambient water (Beardsley et al. 2011). This strategy would permit cyclic reattachment of PP05 to the outer pereiopod segments of phyllosomas (a preferred ectobiotic niche shown in the present study), and when captured prey items are brought to the oral cavity during mastication (Smith et al. 2009a), the presence of PP05 on the dactylus could promote ingestion during feeding.

A likely explanation for the non-random localisation of ectobiotic *Vibrio* sp. PP05 on the outer pereiopod segments ascending to the dactyl spines is increased nutrient concentrations related to nutrient leaching during sloppy feeding events (Tang et al. 2010). Transient nutrient plumes generated by zooplankton (Lehman and Scavia, 1982) can become rapidly colonised by chemotactic bacteria (Stocker et al. 2008) and such conditions could select for PP05. Evidence in support of this is the finding that the PP05 localisation was independent of the presence of *Pseudoalteromonas* sp. PP107. In contrast, *Pseudoalteromonas* sp. PP107 also colonised the outer limb segments in the absence of PP05, possibly reflecting niche differentiation between the strains where PP05 either directly outcompeted PP107 for nutrients and/or inhibited the growth of PP107 by another mechanism such as production of antibacterial compounds.

5.6 Conclusions

It is hypothesised that niche specialisation exhibited by *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 during vectored administration contributes to the additive survival effect on *P. ornatus* phyllosoma challenged with *V. owensii* DY05. The degree of niche specialisation exhibited by the probionts and relevance in real time larviculture requires further investigation, however the results of this study indicate preemptive conditioning of *Artemia* cultures with probionts represents a strong biocontrol method to avert pathogen ingestion and eliminate vector mediated-transmission during early stage *P. ornatus*

phyllosoma larviculture. Long term biomonitoring of the effects and behaviour of probiotic *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 under commercial scale hatchery conditions, including incorporation into formulated diets for advanced larval stages, is required to build zootechnical confidence. We advocate the use of FP-based *in situ* biomonitoring systems to increase understanding of probiont ecology in aquaculture systems and maximise probiotic efficiency.

5.7 Acknowledgements

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6 Conclusions and future recommendations

6.1 Disease management plan for *P. ornatus* larviculture

With limited potential to enhance productivity of wild Palinurid fisheries against a backdrop of global climatic, economic and demographic challenges, alternative supplies need to be sourced through aquaculture to meet the unquenchable consumer demand. Refining Palinurid larviculture technologies is critical to the commercial viability of these nascent aquaculture sectors, and disease management stands atop of the challenges impeding commercialisation. Bacterial infections are major causes of mass attrition rates in cultured Palinurid phyllosomas (Kittaka, 1997; Diggles et al. 2000; Bourne et al. 2004; Ritar et al. 2006), and until this study, information on epizootic strains, pathologies, infection routes, and counteractive biocontrol strategies for *Panulirus ornatus* was lacking.

In the present study, an emerging bacterium related to the Harveyi-clade vibrios, *Vibrio owensii* DY05 (Cano-Gómez et al. 2010), was identified as a specialised enteropathogen causing disease epizootics in early stage *P. ornatus* larviculture. Pathologies of experimental *V. owensii* DY05 infections were similar to those reported previously in diseased cultured Palinurid phyllosomas (Handlinger et al. 1999; Diggles et al. 2000; Bourne et al. 2004; 2007) which reinforces the epizootiological relevance of the experimental infection models designed by Goulden et al (2012; Chapter 2). It is likely pathogenesis of the bacterium is mediated by a number of virulence factors detected *in vitro*, including haemolysins, proteases, and phospholipases, that have been previously associated with pathogenic vibrios in the Harveyi-clade (Liu et al. 1996; Zhang and Austin, 2000; Sudheesh and Xu, 2001; Soto-Rodriquez et al. 2003a; Austin et al. 2005; Manilal et al. 2009; Ruwandeepika et al. 2011). The bacterium is exquisitely adapted to a pathogenic lifestyle in high host-density larviculture ecosystems, and mediates infectivity by vectored transmission, release from host-associations to a planktonic existence, and possibly exploits the cannibalistic behaviour of its host to perpetuate transfer. A conceptualised snapshot of the infection cycle was made possible using a green-fluorescent protein transconjugant of *V. owensii* DY05 as a biomarker of spatiotemporal colonisation *in situ* (Goulden et al. 2012; Chapter 3). Crucially, this latter study illuminated the vector (*Artemia*) gastrointestinal tract and phyllosoma hepatopancreas as preferential ecological

niches of the pathogen, underpinning the targeted probiotic screening strategies subsequently developed by Goulden et al. (accepted; submitted; Chapters 4 and 5).

Antagonistic bacteria were explored as a biocontrol strategy by Goulden et al. (accepted; Chapter 4) due to their widespread occurrence in natural aquatic ecosystems (Long and Azam, 2001; Gram et al. 2010). *Vibrio* sp. PP05 and *Pseudoalteromonas* sp. PP107 were prospected from a large pool of antagonistic candidates based on their propensity to inhibit planktonic and attached forms of pathogenic *V. owensii* DY05 (Goulden et al. accepted; Chapter 4). Used in combination during *Artemia*-mediated transmission, these bacteria significantly and reproducibly protected *P. ornatus* phyllosomas from *V. owensii* DY05 infection *in vi*vo. The enhanced survival benefit was due in part to niche specialisation exhibited by the probionts during vectored administration, elucidated *in situ* by FP-expressing transconjugants of PP05 and PP107 (Goulden et al. submitted; Chapter 5). Both probionts were capable of inhibiting planktonic *V. owensii* DY05 cells in the ambient *Artemia* culture medium, however within the *Artemia* gut, *V. owensii* DY05 evaded attack by *Pseudoalteromonas* sp. PP107 and was exclusively vulnerable to competition with PP05. Following vectored challenge, only PP05 existed as a phyllosoma endobiont, associated with the proventriculum setae and hepatopancreas, which potentially restricted invasion of the pathogen into the hepatopancreas and augmented its defensive capabilities. The probionts also exhibited niche partitioning on the phyllosoma exocuticle, where the localisation of PP05 on the pereiopodal dactyl may be implicated in promoting ingestion during feeding. The observations show the infection cycle of *V. owensii* DY05 is intercepted during vectored transmission, inferring pathogen ingestion can be averted by preemptive conditioning of *Artemia* cultures in scaled-up phyllosoma hatchery experiments.

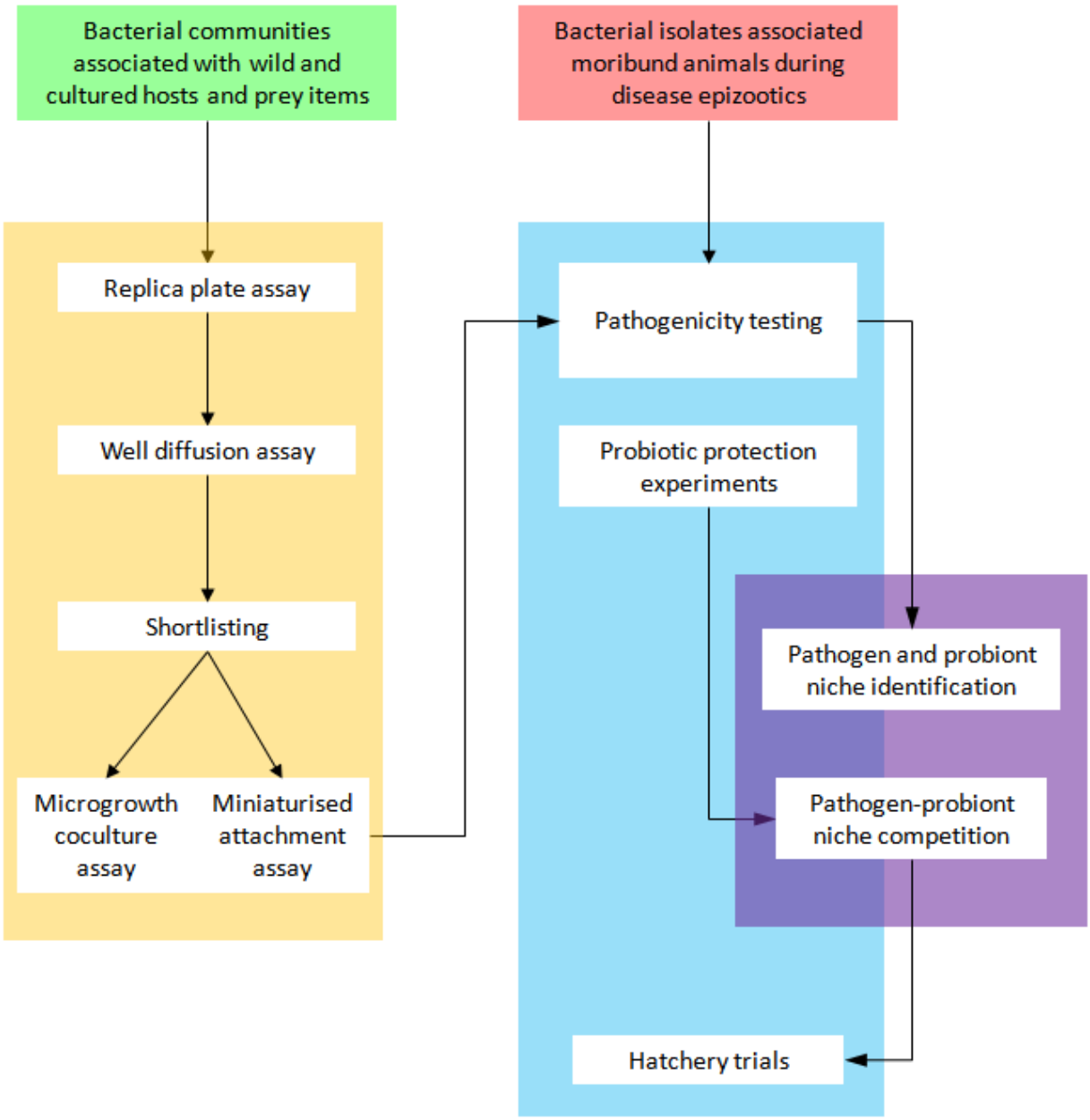

Figure 6-1. Flow chart of a protocol illustrating the first steps in disease management using antagonistic probionts for larviculture of *P. ornatus*. Pathogens are initially identified using *in vivo* (blue box) experimental infection models and niche preferences are determined using *in situ* (purple box) biomonitoring of FP-expressing transconjugants. Bacterial communities associated with the target host are screened for members antagonistic towards the identified pathogen using a suite of *in vitro* (orange box) assays (details Goulden et al. accepted; Chapter 4). Promising candidates are evaluated for inherent pathogenicity towards the host and assessed for their protection capacity *in vivo* and niche preferences *in situ*. Niche competition between pathogen and probiont is confirmed *in situ* prior to hatchery trials.

Overall, this study provides the first step towards combating epizootic pathogens with antagonistic bacteria in *P. ornatus* larviculture. This was highlighted by understanding pathogen niches, infection cycles and localisations of pathogen-probiont interaction *in situ* to maximise probiotic efficacy. Further, the miniaturised coculture and biofilm *in vitro* assays devised for probiotic screening (Goulden et al. accepted; Chapter 4) enabled rapid processing and selection of probiotic bacteria capable of promoting survival *in vivo*. It is important to note that natural microbial communities of wild phyllosomas support antagonistic bacteria capable of suppressing pathogens originating from the larviculture ecosystem and affirm prey items as reservoirs of beneficial microorganisms. Based on the approaches used in the present study, a protocol for an initial disease management plan using antagonistic probionts with integrated *in vitro*, *in vivo* and *in situ* components is proposed for future use in *P. ornatus* larviculture (Figure 6.1) with scope for trans-species applications.

6.2 Future recommendations

While the emphasis of the present study was focused solely on *V. owensii* DY05 because of its recent discovery and quest to unravel its role as a pathogen, it is anticipated the robust infection model described will provide an important diagnostic tool for other emerging pathogens in Palinurid hatcheries. The model would also benefit from supplementary comparative genomic, transcriptomic and proteomic studies (Wu et al. 2008) in order to identify attributes that enable pathogens to become successful in the hatchery ecosystem. This will permit the researcher to identify physiological vulnerabilities in which to direct specific and novel biocontrol artillery. For instance, Hung et al. (2005) reported the compound virstatin causes inhibition of ToxT, the transcriptional regulator of cholera toxin and toxin coregulated pilus in *V. cholerae*, and could protect infant mice from intestinal colonisation by *V. cholerae*. A need to genetically dissect *V. owensii* DY05 and pursue anti-virulence strategies (Natrah et al. 2011) is therefore recommended, especially since other *V. owensii* strains including 47666-1 (formerly *V. harveyi* 47666-1; Cano-Gómez et al. 2010), pathogenic to *Penaeus monodon*, regulate production of extracellular toxins through quorum sensing (Manefield et al. 2000).

Researchers are encouraged to take advantage of transparent zooplanktonic forms to elucidate bacterial-host symbioses *in situ*, and the present study advocates the use of FP-based biomonitoring systems to increase understanding of probiont ecology in aquaculture systems. The *in situ* component of the present study confirmed probiotic treatment of the vector host abolishes infection of *V. owensii* DY05 in phyllosomas under experimental conditions due to niche specialisation and competition; however commercial hatchery scale trials and long term-biomonitoring are needed to validate the results. To ensure dominance of the probiont *Vibrio* sp. PP05 in the phyllosoma proventriculum and hepatopancreas, we suggest continuous replenishment by high and frequent dosages of the probiont (Makridis et al. 2001; Vine et al. 2004a), however it may also be possible to exercise a synbiotic approach using prebiotics to enhance establishment (Ringø et al. 2010). Daniels et al. (2010) recently reported increased stability of intestinal bacterial communities of European lobster (*Homarus gammarus*) larvae supplemented with a dietary prebiotic mannanoligosaccharide and commercial *Bacillus* species. Another immediate challenge will be the design of probiotic-incorporated formulated diets once phyllosomas are weaned from *Artemia*. This is expected to be inherently difficult due to the sensitivity of non-sporulating Gram negative bacteria to storage conditions (Vine et al. 2006). However, Rosas-Ledesma et al. (2012) recently demonstrated high viability of the Gram negative fish probiotic *Shewanella putrefaciens* encapsulated in calcium alginate beads following 1 month of refrigerated storage. Importantly, the authors showed alginate capsule administration of *S. putrefaciens* enhanced the ability of the bacterium to persist and survive in the gastrointestinal tract of sole (*Solea senegalensis*) compared to bacteria administered through commercial fish pellets.

There is a general lack of information on probiotic efficacy in commercial aquaculture systems which deters the sustained use of probiotics and therefore dampens zootechnical confidence (Qi et al. 2009). A greater understanding of probiotic modes of action and how gene expression of host and probiont mutually affect each other could be enhanced through the development of gnotobiotic systems (Tinh et al. 2008a). This approach was not pursued in the present study because the effects of both pathogen and probionts firstly needed to be established in the presence of autochthonous microbiota which colonise phyllosomas in the larviculture ecosystem. Another area of concern during long term use of probionts is the

chance occurrence of the strains developing virulence, which may evolve in the intestinal microbiome when responses by the host are eventually outcompeted by changes in behaviour of the colonising microorganism (Hooper and Gordon, 2001). Some bacterial genomes, including *Pseudoalteromonas* and *Vibrio* species, possess dual function traits (Thomas et al. 2008; Wietz et al. 2010) which could see virulence emerge 'accidentally' through adaptation to new ecological niches (Casadevall and Pirofski, 2007).

It is unlikely that a single biocontrol strategy will efficiently reduce disease prevalence in *P. ornatus* hatcheries and a combination of both prophylactic and curative treatment regimes are needed in the long term. As this research goes to publication, there are many new and exciting developments in biocontrol research which seek to improve disease resistance of the host. For instance, Fu *et al.* (2011) recently engineered recombinant *Bacillus subtilis* spores expressing a white spot syndrome virus (WSSV) envelope protein, which significantly induced phagocytic activity contributing to uptake of the WSSV by haemocytes and resulted in substantially enhanced survival of WSSV-challenged *Litopenaeus vannamei*. A recent review by Harikishan et al. (2011) attests to gaining momentum in the use of botanical extracts to stimulate immune responses in aquatic animals (including shellfish) and augmenting disease resistance. Although controversial, there are also some recent advances in transgenic methods to increase disease resistance in commercially important invertebrates including bivalves and shrimps (Rasmussen and Morrissey, 2007). Finally, and still very embryonic in its development, nanotechnology could revolutionise disease management in aquaculture through the use of nanoscale devices to rapidly detect pathogens and systems that control and monitor the delivery of probiotics (Rather et al. 2011).

Ultimately, the prophylactic *Artemia*-probiotic method proposed in this study should be expanded and developed within an integrated disease management strategy, including curative measures, for different phyllosoma stages and hatchery conditions of the *P. ornatus* larval rearing system. This could include fortifying probiotic arsenals through concoctions of compatible and synergistic microorganisms that exert multiple modes of attack on unfavourable microorganisms. Indeed, the selection of multiple, mutually-compatible microorganisms capable of exerting synergistic benefits forms the basis of the effective

microorganisms concept introduced by Japanese scientist Teruo Higa in the 1980s which has found wide use in agriculture and aquaculture systems in China (Zhou et al. 2009). It can be expected that many selection pressures operating simultaneously would slow the rate of the numerous genetic rearrangements necessary to evolve resistance in a pathogen population. Optimising the microbial structure of the hatchery ecosystem is central to controlling pathogens, however it is anticipated that biocontrol methods will need to continuously evolve during the infinite arms race with emerging pathogens.

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8 Appendix

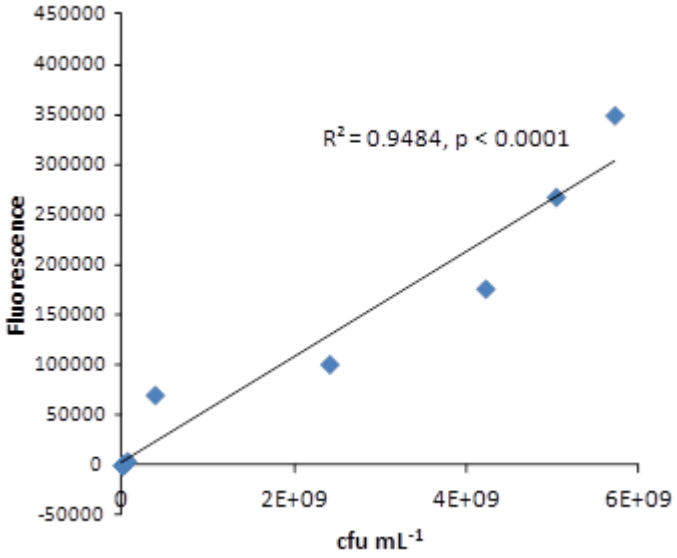

Figure A1. Scatter plot demonstrating correlation between fluorescence and concentration (CFU mL^{-1}) of *V. owensii* DY05[GFP] during 24 h monoculture growth.