Molecular Factors Involved in Suppression of Pathogenic *Vibrio owensii* by Potential Probiotic Bacteria

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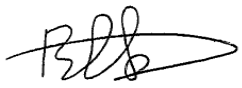
Declaration of Originality

I declare that the substance of this thesis is my own work.I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that the best of my knowledge any help received in the preparation of this thesis, and all sources used, have been acknowledged in this thesis.



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Abstract

In recent decades a rapid increase in the market demand for seafood has led to a significant decline in wild seafood populations. Therefore, the aquaculture industry now represents the best alternative seafood provider. In Australia, the spiny lobster *Panulirus ornatus* has the greatest market demand. However, production from the aquaculture hatcheries together with continuous use of antibiotics has led to an increase in the resistance of bacterial pathogens and the development of particularly resistant strains, thereby rendering antibiotic treatment ineffective. Alternative strategies to confine the spread and virulence of bacterial diseases are in demand due to the risk of further resistance development and the possibility of resistant pathogens evolving into human pathogens. Therefore, in recent years, the research focus has broadened to include probiotics, defined by Fulleret al. (1989) as a live microbial feed supplement that has beneficial affects to the host animals by improving intestinal microbial balance. The use of probiotics as a means of disease control and an alternative to antibiotics has shown to be promising in aquaculture, particularly in fish and shellfish larva-culture.

The current study aimed to identify probiotic bacteria that are antagonistic to the development of Vibriosis, which is a bacterial disease widely spread in Australian's lobster aquaculture industry, caused by members of the genus *Vibrio*. *Vibrio ownsii* is one of the most virulent pathogens, causing severe losses of lobster larva cultures in lobster hatcheries. In the current study we isolated and identified beneficial bacteria (potential probiotics) from a culture collection from corals and shrimps. These potential probiotic strains suppress the lobster pathogen *V. owensii* DY05, as determined in well diffusion assays.

In total, 400 isolates were tested from different species of the corals *Turbinaria* and *Acropora*. A total of 99 isolates were tested from stressed shrimp, both male and female and from the ambient seawater. Of all 499 isolates, 111 were identified as antagonistic to DY05, 80 of them from coral (20% of all isolates) and 31 from shrimp (31% of all isolates). Most of these antagonistic (potential probiotic) isolates produced at least 10 mm in diameter inhibition zone in well diffusion assays.

According to the 16S rRNA gene fragment sequences the coral isolates were assigned to nine genera in four classes. Most of the isolates belong to Gammaproteobacteria (89%), with the other three classesbeingBacilli, Actinobacteria and Alphproteobacteria. The isolates from shrimp that demonstrated antagonism against the pathogen were affiliated with three classes and six genera, with the Gammaproeobacteria predominating (81%) and the other classes beingBacilli and Actinibacteria.On the genes level *Vibrio* species were the dominant phyla, accounting for 62.5% of coral isolates and 74% of shrimp isolates. *Psychrobacter* spp.

were isolated from coral at only 23.7%. Other less common bacterial isolates included *Staphylococcus, Bacillus, Micrococcus, Pseudovirio, Pseudoalteromonas*, *Acinetobacter* sp., *Photobacterium* sp*., Pseudovibrio* sp.and *Brachybacterium* sp. Due to the high occurrence of isolates that are antagonistic against the lobster pathogen *V. owensii* DY05, it can be deduced that coral and shrimp marine environments represent promising sources for isolation of probiotic bacteria.

In carrying the study further, the three bacterial isolatesthat expressed strong and fast antagonistic activity against DY05 were chosen for preliminary characterisation of the observed antagonistic activity. These bacteria were identified to the genus level as *Pseudoalteromonas* sp. strain 80, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34. Preliminary characterisation of antagonistic activity involvedproteolysis assays, which indicated that the three isolates demonstrated activities consistent with gelatinase, caseinase and amylase enzymatic breakdown. Such proteases and small molecules may be responsible of antimicrobial activity.Several methods were used to concentrate and fractionate filtered supernatants to identify or characterise the active molecules. In addition, zymography was used to identify the active fractions of culture supernatants. These fractions were subject to analysis by liquid chromatography / mass spectrometry (LC/MS) to partly identify active antimicrobial proteins. The studied strainswere found to producenumerous protein compounds, some of which were specifically proteolytic enzymes. *Vibrio* sp. strain 34 was found to produce VtpA metalloprotease. *Pseudoalteromonas* sp. strain 80 was found to produce several proteases include metallopeptidase, prolyloligopeptidase, zinc metallopeptidase, iron superoxide dismutase and serine endoprotease. Lastly, *Psychrobacter* sp. strain 62 was found to produce superoxide dismutase and inorganic pyrophosphatase. Cultures were also examined for small active molecules using gas chromatography / mass spectrometry (GC-MS). Several alkenes volatiles were identified; however these volatile alkenes were also present in non-active strains. Therefore, it was concluded that these volatiles were not related to the antimicrobial activity.

Transposon mutagenesis was undertaken in an attempt to identify the genesrelated to biosynthesis of metabolites responsible for pathogen suppression. Successful mutagenesis of *Pseudoalteromonas* sp. strain 80 yielded a mutant, named 7Km, which lacked inhibitory activity against the pathogen and also lost pigmentation. Southern blot analysis confirmed an insertion of the mini-Tn10 transposon in one location in the genome. The gene affected in the mutant was recovered using plasmid rescue and was found to share 100% homology with a

membrane bound transporter proteinof the resistance-nodulation-division (RND) family. The closest homology was to RND protein of *Pseudoalteromonas flavipulchra* JG1, a marine antagonistic bacterium with abundant antimicrobial metabolites. RND proteins were implicated in drug resistance of important pathogens and in the transport of substances from the cell, suggesting that a mutationin such a transporter probably abolished the excretion of pathogen-suppressive substances from *Psedoalteromonas* sp. strain 80 cells.

The current study confirms that the shrimp and coral microbial community represent good sources for the isolation of beneficial bacteria, which may be promising candidates for application as probiotics in aquaculture hatcheries, in particular *Psedoalteromonas* sp. strain 80, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34. The use of a diversity of probiotics theoretically proves advantageous as they have different mechanisms of pathogen inhibition.

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Abbreviations

bp	base pairs
DNA	deoxyribonucleic acid
Kb	kilobase
MA	marine agar
MB	marine broth
LB10	Luria Broth 10 (marine)
µL	microlitre
µg	microgram
mg	milligram
mL	millilitre
hr	hour
rpm	revolution per minunte
NMSC	National Marine Science Centre
NSW	New South Wales
NSWMPA	New South Wales Marine Parks Authority
PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SIMP	Solitary Islands Marine Park
SWST	South-West Solitary Island
HL-ASWS	healthy-looking tissue
M-ASWS	disease margin
D-ASWS	disease tissue
BLAST	basic local alignment search tool
MS	male stressed
FS	female stressed
sp.	specie
spp.	species
SUPA	Sydney University Prince Alfred Macromolecular Analyses Centre
SW	seawater
UV	ultraviolate
Amp	ampicillin
Sm	streptomycin
Tc	tetracyclin
Cm	chloramphenicol

Km	kanamycin
Rif	rifampicin
TLC	thin layer chromatography
X-gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside
GC-MS	gas chromatography mass spectrometry
LC-MS	liquid chromatography mass spectrometry
TCBS	thiosulfate-citrate-bile salts-sucrose agar
SDS	sodium dodecyl sulphate
SDS- PAGE	sodium dodecyl sulphate-polyacrilamide gel electrophoresis
gDNA	genomic DNA
M	molar
V	volts

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Chapter 1

Literature Review

1.1 Introduction

The term 'marine environment' covers a vast number of habitats, from deep-sea regions to the salty water of estuaries. These habitats are different in both biotic and abiotic characteristics (Kis-Papo, 2005). The oceans cover more than 70% of the Earth's surface (Colwell, 2002; Fenical, 1993; Masuma *et al.*, 2001; Proksch *et al.*, 2003). One millilitre of seawater contains somewhere in the order of 10^3 fungal cells, 10^6 bacteria, and 10^7 viruses, including pathogens that cause extensive mortality, and microorganisms that induce host surface fouling (Kubanek *et al.*, 2003). There are many similarities between terrestrial and marine bacteria, but living in the marine environment requires the exploitation of different compounds, necessitating specific adaptations and genotypic changes in marine microorganisms (Běhal, 2003; Imamura *et al.*, 1997; Towse, 2005). Marine microbes have developed unique metabolic and physiological capabilities to be able to survive in extreme environments, and may therefore produce metabolites which are not produced by terrestrial bacteria and fungi (Fenical, 1993). The oceans have proven to be a rich source of a wide variety of potentially active agents that are mostly accumulated in invertebrates such as molluscs, sponges, bryozoans, and tunicates (Proksch *et al.*, 2002). Microbiologists have known that antimicrobial producing marine bacteria are partially responsible for the natural antimicrobial activities of seawater, in addition to playing a most important role in the population dynamics of marine microorganisms (Rosenfeld & ZoBell, 1947).

Preliminary studies of marine bacteria indicated that the bacterial population in seawater is composed mainly of Gram-negative bacteria, while Gram-positive bacteria represented less than 10% of the total. Today the evidence suggests that Gram-positive bacteria are found in higher proportions in biotic and abiotic surfaces, sea sediments, and inner spaces of invertebrate animals (Fenical, 1993; Zheng *et al.*, 2000). These commensal or symbiotic bacteria, in many cases, make up the non-pathogenic microorganisms associated with the host. They chemically protect their microhabitat while defendingtheir host from pathogenic microbes by the production of secondary metabolite compounds (Zheng *et al.*, 2000).

Host-microbe interactions are often qualitatively as well as quantitatively different in aquatic and terrestrial species. In the marine milieu hosts and microbes share the ecosystem, in

contrast, most terrestrial habitat interactions occur at the gut, which represents a moist habitat in an otherwise water-limited environment. In some sense, marine microorganisms have the option of living in association with the potential host's intestinal tract, gills, or skin (Harris, 1993; Verschuere *et al.*, 2000 a).

Most of the important antibiotics in human history were isolated from terrestrial microorganisms, while the suppressive effects of aquatic microorganisms agianst human pathogens has only recently been investageted (Lo Giudice *et al.*, 2007 b, p. 496). Nowadays it has been proved that aquatic bacteria are capable of producing a wide-range of biologically active compounds which might be used in conjunction with those recovered from terrestrial microorganisms (Isnansetyo & Kamei, 2003; Miao & Qian, 2005)(Lo Giudice *et al.*, 2007 b; Nair, 2005; Zheng *et al.*, 2000).

Aquaculture is an important food production industry, providing an alternative source of seafood products to wild fisheries. However, widespread infectious disease in aquaculture hatcheries causes significant economic losses to this industry. Traditionally antibiotics have been used to combat larval loss due to pathogen infection, however,there are increasing concernsabout the development of antibiotic resistance which can render antibiotic treatments ineffective.This has created an urgent need for molecular biology and gene therapy research into the development of new drugs to reduce antibiotics resistance, in addition to enhanced technology in computer-assisted drug design (Munro *et al.*, 1999). New methods for controlling infectious diseases are needed in order to sustain the aquaculture industry. In recent years beneficial microorganisms have been applied in aquaculture industries. Such microbes improve the immune system of cultured marine animals, in addition to producing secondary metabolites that can also improve marine animal's resistance capability (Defoirdt *et al.*, 2007; Zhou *et al.*, 2009). Using probiotic bacteria as a disease controlling measure in aquaculture may replace or improve upon the traditional treatments, such as the use of antibiotics.

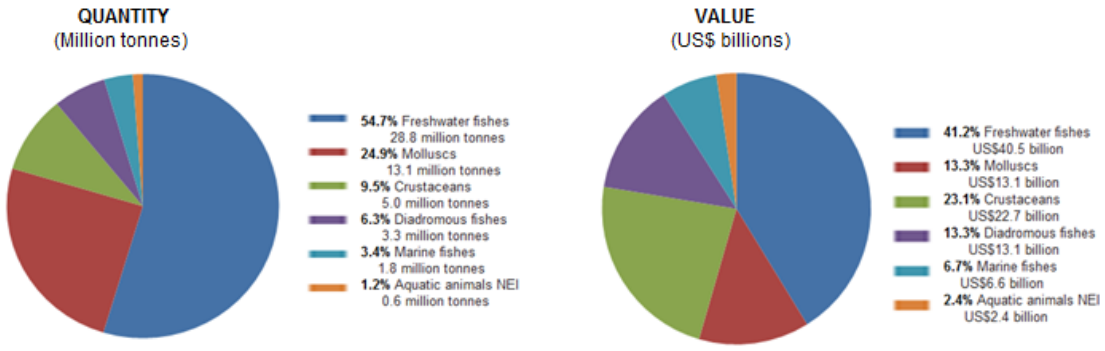
The aims of this work were to identify potential probiotic microbes from culture collections of marine microorganisms that may be used as biocontrol agents against the pathogenic bacteria *Vibrio owensii* thataffects lobster larval culture. Once such potential probiotic microbes were found, molecular analysis was employed to identify the gene(s) involve in antimicrobial production.

1.2 Impact of pathogens on the aquaculture industry

1.2.1 Importance of Aquaculture

The demand for seafood products has increased alongside the world population. However, due to over-exploitation of aquatic environments worldwide there is a growing search for alternative food products (Anand *et al.*, 2011; Martinez-Porchas & Martinez-Cordova, 2012). Aquaculture is being viewed as the only alternative method of enhanced fish production (Anand *et al.*, 2011) and is considered a promising solution to meet the increasing demands for marine products at a global level (Martinez-Porchas & Martinez-Cordova, 2012). Aquaculture is the farming of aquatic organisms including fish, molluscs and crustaceans. "It is an emerging industrial sector which requires continued research and development to maximise efficiency and production" (Toranzo *et al.*, 2005, p. 37).

Worldwide fish consumption has increased gradually over the last five decades, with consumption of fish increasing at an average annual rate of 3.2%, above the 1.6% rise in world population growth per annum. Aquaculture production globally continues to grow slowly. The worldwide fishing production in 2008 was dominated by freshwater fish at 28.8 million tonnes representing 54.7% and worth 40.5 billion US dollars (41.2% value). This was followed by molluscs at 13.1 million tonnes, crustaceans at 5 million tonnes, diadromous fish at 3.3 million tonnes, marine fish at 1.8 million tonnes and other aquatic animals at 0.6 million tones, as shown in Figure 1.1 (FAO, 2010). In 2012, the world aquaculture production reached a peak of 90.4 million tonnes with a 144.4 billion US dollar value, including 66.6 million tonnes of food fish valued at 137.7billion US dollars and 23.8 million tonnes of marine algae, being mainly seaweed valued at 6.4 billion US dollars (FAO, 2014).



Note: NEI = not elsewhere included.

Figure 1.1 Main species clusters aquaculture production worldwide in 2008
adapted from (FAO, 2010)

The Food and Agriculture Organization of the United Nations has estimated that production from aquaculture in 2020 will cover half of the worldwide need for seafood (Moriarty, 1999). However, the spread of infectious disease isconsideredas one of the main restrictions to this development.

1.2.2 Aquaculture and diseases

Populations of all organisms are determined partially or completely by diseases in their environments (Real, 1996)**.** Aquaculture is the fastest growing industry in the production of animal protein worldwide (Bondad-Reantaso *et al.*, 2005; Mohapatra *et al.*, 2013; Subasinghe, 2005), however, the spread of disease effects the development of the aquaculture industry. Diseases are being increasingly documented as a substantial restriction on trade and the aquaculture industry globally, particularly diseases caused by microorganisms such as bacteria, viruses, parasites, fungi, and other emerging and undiagnosed pathogens (Bondad-Reantaso *et al.*, 2005; Defoirdt *et al.*, 2007; Desriac *et al.*, 2010; Muñoz-Atienza *et al.*, 2013; Sanmukh *et al.*, 2012; Subasinghe, 2005). The disease causing agents present in aquaculture tanks also usually exist in natural environments, thoughthey rarely cause mortality in wild fish populations. It is thought that the reason for this is because of the more stressful conditions that are generally present in aquaculture tanks (Toranzo *et al.*, 2005).

Consequently, the spread of diseases in aquaculture is one of the most important causes of serious economic loss in aquaculture of many marine species in several countries (Bondad-Reantaso *et al.*, 2005; Meyer, 1991). For example, according to World Bank statistics, worldwide losses of approximately3 billion US dollars occurred due to shrimp diseases (Subasinghe, 1997). In 1988 more than 20 million trout were lost, costing the trout industry around 2.5 million dollars, and in the same year, channel catfish producers reported losses of over 100 million fish, valued at almost 11million dollars (Meyer, 1991).

Bacterial pathogens are the leading cause of loss due to disease in all types of seafood production, while fungal diseases and external protozoan parasites are responsible for epizootics and large losses of finfish fingerlings, fry and juvenile (larva) shellfish (Meyer, 1991). There have been major global economic losses in cultured fish as a result of opportunistic pathogenic bacteria; in particular Gram-negative bacteria are responsible for epizootics in almost all cultured species (Toranzo *et al.*, 2005).

1.2.3 Vibrio spp.

Family *Vibrionaceae* belong to the class Gammaproteobacteria, members of which are widespread in the marine environment. Genus *Vibrio* are motile, facultativeanaerobic, Gram-negative bacteria (Mansson *et al.*, 2011; Simidu & Tsukamoto, 1985). It is understood that strains belonging to *Vibrio* species can exhibit different virulence patterns ranging from non-virulent to highly pathogenic (HP)(Goarant *et al.*, 2006; Juiz-Rio *et al.*, 2005).

Within the Vibrionaceae family, some species are known pathogens in animal systems including humans. However, species of the family Vibrionaceae are frequently prevalentin the microbiotal community of seawater, fish and plankton (Khandeparker *et al.*, 2011) and represent a major group ofmicroorganisms found in the intestinal flora of aquatic fish and crustaceans (Kita-Tsukamoto *et al.*, 1993; Simidu *et al.*, 1977).

In a review conductedin the West Pacific Ocean, *Vibrio* represented nearly 75% to 100% of the heterotrophic bacterial inhabitants in surface seawater (Simidu *et al.*, 1980). The species *Vibrio anguillarum*, *V. salmonicidaV. ordalii*, and *V. vulnificus* biotype 2 caused the most economically serious diseases in marine culture (Toranzo *et al.*, 2005).

*Vibrio vulnificus*is is naturally present in estuarine environments and may contaminate shellfish, causing potentially fatal sepsis and devastating wound infections (Gulig *et al.*, 2005; Gulig *et al.*, 2009). Few bacterial species belonging to the genus *Vibrio* are recognized to be fish pathogens. Of these, *Vibrio anguillarum* has caused serious losses worldwide in fish farms (Valla *et al.*, 1992). *Vibrio* species are also known to cause disease in humans, and such *Vibrio* species are considered to be seriously pathogenic from the perspective of public health (Nishibuchi & Kaper, 1995). These species currently include *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. metschnikovii* , and the non-O group 1 portion of the *Vibrio cholerae* species (Blake *et al.*, 1980). *V. parahaemolyticus* has been recognized as an agent of gastroenteritis, associated with consumption of seafood; however not all strains of this species are considered to be pathogenic (Nishibuchi & Kaper, 1995). For instance, *V. parahaemolyticus* is one of the most important food-borne disease causative agents in Asia, causing almost 50% of the food poisoning epidemics in Japan, Taiwan, and Southeast Asian countries (Joseph *et al.*, 1982; Wong *et al.*, 2000). However, the relationship between the bacterial isolates from seafood, estuarine environments, and clinical isolates of humansare not clear (DePaola *et al.*, 2003).

Diseases of marine organisms caused by luminescent vibrios such as *Vibrio harveyi* and the other related bacteria including *V. parahaemolyticus* and *Vibrio campbellii* are a serious threat to marine organisms. Severe disease caused by luminescent vibriosis has become a major obstruction topenaeid shrimp aquaculture production in Asia and South America(Austin & Zhang, 2006). Diseases caused by species and strains belonging to *Vibrio* have become a serious threat to the crustacean aquaculture industry such as lobster, crab, and shrimp (Bäck *et al.*, 1974). For example, the American lobster *Homarus americanus* the subgroup *Vibrio fluvialis* has been involved in limp lobster disease (Tall *et al.*, 2003). Another example, more relevant to the research undertaken in this thesis, is *V. owensii* (DY05), a potential pathogen of spiny lobster*Panulirus ornatus* plylosoma, which causes severe larval mortality (Goulden *et al.*, 2012).

1.2.4 Lobster and Disease

Spiny rock lobsters belonging to the family Palinuridaeare an important aquatic resource for many countries (Phillips, 1985). The ornate spiny lobster, *Panulirus ornatus* (Decapoda: Palinuridae), is a tropical species with an Indo-West Pacific distribution. It is most abundant in the north-east of Australia (Jones *et al.*, 2001; Pitcher *et al.*, 1997). Thus, Australia is the world's largest producer and exporter of these animals (Phillips, 1985). Australia has nine species of rock lobster, with six tropical species of the genus *Panulirus* being found in northern areas of Australia (Phillips, 1985). *P. ornatus* is the most abundant of these species in the far north eastern coast of Queensland and the Torres Strait (Pitcher *et al.*, 1997). The Australian ornate rock lobster, *P. ornatus* has a short life cycle compared to other Palinurid lobsters and there is a high demand for the animal in the overseas markets. For these reasons *P. ornatus* was identified as a good candidate for commercialisation as an aquaculture species (Zhang *et al.*, 2009).

Rock lobster species are among the most valuable wild fisheries species. Wild populations are therefore under increasing pressure from overfishing. This pressure has led to research efforts aimed primarily at more effective management of stocks in the ocean to offer greater opportunity for wide dispersal of larvae. The market demand for rock lobster combined with recent research investment has led to an increased interest in the breeding of the tropical ornate rock lobster *P. ornatus*. Nevertheless, a number of limitations have prevented the success of this aquaculture industry, including the long phylosoma phase of *P. ornatus*, spread of infectious diseases, lack of suitable live feed and food-borne diseases (Bourne *et al.*, 2004; Kittaka & Abrunhosa, 1997 ; Payne *et al.*, 2006b).

1.2.4.1 Larval cycle and disease

Rock lobsters of the family Palinuridae representthe most highly valued fisheries sector in Australia, and are nowadays considered a target aquaculture species. However, larval stage diseases such asthe high rate of larval attrition caused by inadequate nutrition and microbial management issues represent the most importantchallenges to overcome for commercial production success(Bourne *et al.*, 2007; Handlinger *et al.*, 1999; Ritar *et al.*, 2006). It is important to understand the relationship between the tropical ornate rocky lobster, *P. ornatus*phylosoma and associated diseases, in order to control infectious diseases that currently prevent aquaculture success (Payne *et al.*, 2007). The extended larval stage of lobster phyllosomas emphasizes problems correlated with disease control. Through the molt phases lobster phyllosomas are similar to other crustacean larvae, which are especially sensitive to microbial infection before the new shell hardens and provides some protection against pathogen invasion (Payne *et al.*, 2006a; Payne *et al.*, 2007).

The development of lobster larvae differs between species in moult frequency and duration as well as morphology (Kittaka & Abrunhosa, 1997). For example, spiny lobsters (Decapoda: Palinuridae) and other closely related species, and coral lobsters (Scyllaridae and Synaxidae) pass through a sequence of unusual phyllosoma larval phases during development that are characteristic of these groups. The tropical rock lobster has a complex life cycle with long migrations and many growth phases as shown in Figure 1.2. The ornate tropical rock lobster diseases, particularly bacterial diseases, are currently considered the main problem in successful larval culture for most aquaculture of crustaceans (Johnston & Aqua, 2006; Payne *et al.*, 2006a).

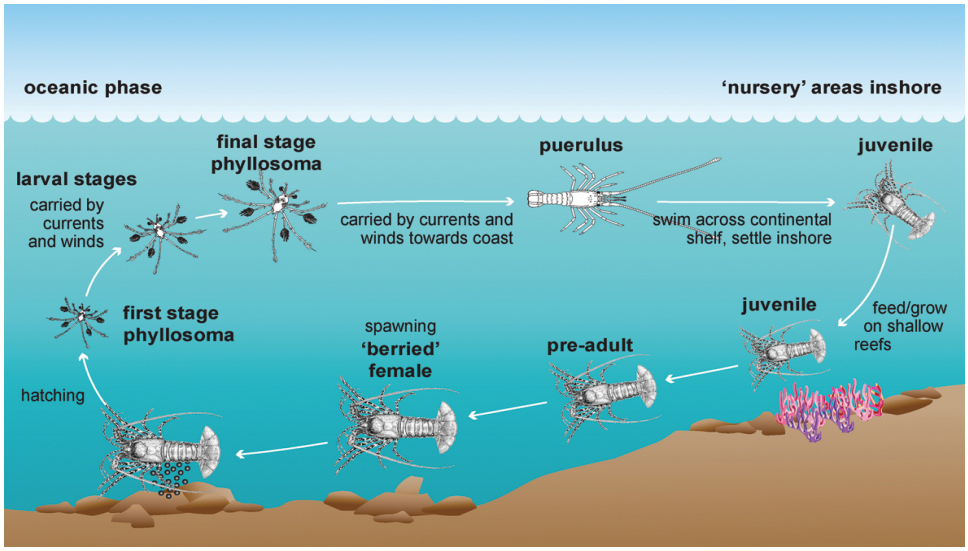


Figure 1.2. Lobster life cycle showing the larval stages (CSIRO 2014).

Vibriosis causes severe losses of phyllosoma in lobster hatcheries. It affects both brine shrimp, raised to feed the lobsters, and the lobster larvae themselves (Diggles *et al.*, 2000). Several important studies investigating the microbiological community of crustacean larval breeding tanks have concentrated on the isolation and identification of diseases pathogens, with species of *Vibrio* being the most frequentlydocumented pathogen (Ferris *et al.*, 1996; Hameed & Rao, 1994). Specific bacterial species including *V. harveyi*, *V. anguillarum*, *V. alginolyticus*, and *V. tubiashii* areinvolved in causing disease in larval and juvenile rock lobster (Diggles *et al.*, 2000). Filamentous bacteria have also been found to be correlated with disease of larval and juvenile rocky lobsters. Filamentous bacteria, such as*Leucothrixmucor,*are usually considered an indicator of stress or bad water quality and have been observed on the eggs and gills of *Jasusedwardsii* (Shields & Behringer, 2004).

Another pathogenic bacterial species affecting lobster is *Leucothrix,* which causes an infestation on the surface of *Homarus americanus* larvae culture (Johnson, *et al.,*1971). "*Leucothrix*-like" bacteria have also been found by Handlinger et al. (1999) to cause disease in *Jasus* species or southern rock lobsters. In a study by Bourne, et al. (2004) many bacterial species associated with phyllosoma larvae were isolated from the microbial community of the *P. ornatus* larval nursing system. These species included *Pseudoalteromonas* sp., *Desulfobulbus mediterraneus*, *Alteromonas* sp., *Pirulella*sp., *V. parahaemolyticus* and an uncultured bacterial species. Species of Vibrio were also reported to proliferate within the hepatopancreas of *P.ornatus* phyllosomas and proliferation of internal bacteria was associated with larval mortality (Webster *et al.*, 2006).

1.3 Biocontrol

1.3.1 Diseases Management and control Strategies

Control of harmful microbes is an important measureto ensure survival and health. Disease caused by microorganisms should be treated in humans, plants and animals (Gram *et al.*, 2010). Management of the health of aquatic species has recently been given a high priority in aquaculture production in many regions throughout the world. This increased focus on animal health has been stimulated by serious economic losses, and environmental impacts of disease

in cultured aquatic organisms. A number of countries have enhanced their laboratory services, control and therapeutic strategies and diagnostic expertise, in order to efficiently control infectious disease in aquaculture(Bondad-Reantaso *et al.*, 2005).

Some progress has been made in the control of disease spread in marine animals by increased awareness, improved research, effective legislation and policy (Bondad-Reantaso *et al.*, 2005).

1.3.2 Traditional methods

Microbial diseases are one of the most significant problems that affect aquaculture commercialization (Natrah *et al.*, 2011). Traditionally the first response to control disease spread in aquaculture is the use of antibiotics (Defoirdt *et al.*, 2007; Kesarcodi-Watson *et al.*, 2008). This is similar in other industries; for example, certain antimicrobial drugs have demonstrated positive effects on the growth of livestock and are used extensively (Acar *et al.*, 2000; Phillips *et al.*, 2004; Wierup, 2001). However, conventional approaches, including the use of antimicrobial drugs and disinfectants, have had limited success in curing or preventing disease in marine organisms (Oliveira *et al.*, 2012; Subasinghe, 1997). The use and misuse of antimicrobial drugs for disease control in aquaculture and agriculture, and as growth promoters in animals, has led to the spread of antibiotic resistance, and therefore results in reduced efficiency of antimicrobial treatment of animal and human diseases (Aarestrup, 1999; Akinbowale *et al.*, 2006; Defoirdt *et al.*, 2007; Hjelm *et al.*, 2004a; Lim *et al.*, 2011; Moriarty, 1997; Witte, 2001). In addition to the spread of resistant pathogens, resistance genes, or genes for virulence are transferred via R plasmids and transposons to other bacteria which have not previously had been exposed to antibiotics (Moriarty, 1999; Witte, 2001). Luminous vibrios isolated from a shrimp breeding farm off Java island, Indonesia, have showed multi antibiotic resistance such as tetracycline, ampicillin, amoxicillin, and streptomycin (Tjahjadi *et al.*, 1994). Thus, β-lactam resistance is now common in *Vibrio* spp. isolated from different locations and sources. It is documented that antimicrobial drugs have been widely overused (Aarestrup, 1999; Schwarz *et al.*, 2001) andtherefore a number of alternative strategies have been suggested to reduce antimicrobial use as a disease control which have previously been applied successfully in aquaculture (Lamari *et al.*, 2014; Subasinghe, 1997; Zhang *et al.*, 2009). These alternative approaches include the use of immunostimulants for the improvement of the innate immunity mechanisms of the host, the use of bacteriophages or phage therapy, the use of probiotic bacteria or vaccines, microalgae

or green–water, short chain fatty acids and poly–β–hydroxybutyrate (Garriques & Arevalo, 1995; Lunestad, 1998; Zhang *et al.*, 2009). The use of probiotics to control diseases in aquacultureis an interesting area, particularly in light of the increasing concern about the development of bacterial resistance with increasing use of chemical methods such as antibiotics and/or disinfectants (Hjelm *et al.*, 2004 b ; Kapareiko *et al.*, 2011; Lim *et al.*, 2011).

1.3.3 Probiotic Bacteria

The aquaculture industry is increasingly interested in the control or reduction of antimicrobial use. Therefore, alternative strategies should be developed to preserve a healthy microbial environment in larval hatcheries. In aquaculture industries the use of probiotic bacteria has become anacceptedalternative method to control the spread of disease (Gomez-Gil *et al.*, 2000). This has resulted in a greater amount of research in order to achieve substantial progress in our understanding and ability to distinguish specific probiotic organisms, in addition to efforts to prove their reputed health benefits (Kapareiko *et al.*, 2011; Senok *et al.*, 2005). It is important to remember that different probiotic strains provide different health benefits (Senok *et al.*, 2005).

1.3.3.1 Definition

Fuller (1989) defines a probiotic ‘‘as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’’ (Fuller, 1989, p. 366). This definition emphasizes the importance of living microbes and of the application of the probiotic to the host as a feed supplement (Irianto & Austin, 2002). (Gram et al.1999, p. 972) proposed that a probiotic is ‘a live microbial supplement, which beneficially affects the host animal by improving its microbial balance’. This is a wider definition that does not restrict probiotics to application in food. Whole microorganisms are usually used as probiotics, but parts of microbial cells have also been observed to be capable of improving the hosts’ health. However, metabolites are excluded as part of the definition, and antibiotics are thus excluded. A proposed definition by Salminen et al. (1999) is based on several factors that include mechanisms of action, viability and non-viability, selection criteria, and scientifically documented health effects. They define probiotics “as viable microbial cultures that influence the health of the host by balancing the intestinal microflora and thus preventing and correcting the microbial dysfunctions”. Verschuere *et al.* (2000 a, p. 657) defined probiotic bacteria as “a live microbial adjunct which has a beneficial effect on the host by modifying

the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". The spectrum of probiotics studied for use in aquaculture has comprised both Gram-positive and Gram-negative bacteria, yeasts, bacteriophages and unicellular algae (Irianto & Austin, 2002). When considering probiotics for marine usage it is important to examine the differences from terrestrial environments. There is a closer relationship between marine animals and their external environment than terrestrial animals and their environment. Potential pathogens are capable of surviving in the water column and may spread independent of the host animal (Hansen & Olafsen, 1999; Verschuere *et al.*, 2000 a). Marine animals are continuously in contact with pathogenic agents through the processes of feeding and drinking (Kesarcodi-Watson *et al.*, 2008).

1.3.3.2 Modes of action

Bacteria are closely linked with all life stages of marine organisms (Bergh, 2000) and marine plants and animals are constantly at risk from a wide variety of pathogenic microorganisms. It seems reasonable then to assume that potential hosts might produce bioactive compounds to prevent microbial attack (Engel *et al.*, 2002). Proksch *et al.* (2003) suggested that microorganisms living in their invertebrate hosts could be the real producers of secondary metabolites often ascribed to the host itself. In order to design protocols to select potential probiotics it is important to understand the mechanisms by which probiotic organisms compete either with pathogens or with other microbes, including other probiotics (Vine *et al.*, 2006). Probiotic isolates have successfully inhibitedpotential pathogens both *in vivo* and *in vitro*bya number of different mechanisms (Balcázar *et al.*, 2006; Jayaprakash *et al.*, 2006). These mechanisms include production of antibacterial and growth-inhibiting compounds, inhibition or suppression of virulence mechanisms, competition with pathogens for adhesion sites, enhancement of water quality, and improvement of host immune response and digestiveenzymes(Balcázar *et al.*, 2006; Irianto & Austin, 2002; Kesarcodi-Watson *et al.*, 2008; Tinh *et al.*, 2008; Verschuere *et al.*, 2000 a; Vine *et al.*, 2006). Additionally, bacteria that are able to improve water quality by mineralizing organic matter or by removing toxic inorganic nitrogen are also considered probiotics. Probiotic bacteria could inhibit virulence gene expression, for example by obstructing quorum sensing (Defoirdt *et al.*, 2007; Zhou *et al.*, 2009).

Antagonisms

The ability of microorganisms to survive in natural environments is related to the ability to produce toxic compounds to deter other organisms, and the capacity to resist the effects of such toxic compounds (Del Sorbo *et al.*, 2000). Some microorganisms present in marine environments inhibit growth of other microorganisms by producing secondary metabolites that have a bactericidal or bacteriostatic effect, such as bacteriocins, bacteriolytic enzymes, toxins, pigments, pheromones, pesticides, siderophores, antitumor agents and antibiotics (Bryers & Characklis, 1982; Burgess *et al.*, 1999; Pandey *et al.*, 2010; Pandey *et al.*, 2011; Verschuere *et al.*, 2000 a). Antagonism between microorganisms is a natural phenomenon by which pathogens in the aquaculture environment may be killed or reduced in number. This phenomenon is called biological control, or biocontrol (Maeda *et al.*, 1997). Bacterial strains have been known to produce a diversity of antibacterial compounds. For example, a *Roseobacter* sp. Strain (BS107) secretes an antimicrobial compound that activelyinhibited*Vibrio anguillarum*growth. The antimicrobial activity was shown to be highest after 48 h of incubation of *V. anguillarum*pathogen in the BS107 supernatant. *In vivo*, filtered extracts of the BS107 strain dramatically improved the survival of scallop larvae (Ruiz-Ponte et al. 1999). Many authors have suggested that marine bacteria of the *Roseobacter* clade be used as a probiotic treatment in aquaculture (Hjelm *et al.*, 2004 a; Planas *et al.*, 2006; Ruiz-Ponte *et al.*, 1998; Ruiz-Ponte *et al.*, 1999) because these bacteria may produce a variety of antibacterial compounds (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005 b; Buchan *et al.*, 2005). One such strain is *Roseobacter* strain 27-4, which has been shown to improve the survival rates of turbot larvae infected with *Vibrio anguillarum,* and also inhibits fish pathogenic bacteria via a sulphur-containing antimicrobial substance (Bruhn *et al.*, 2005 b; Hjelm *et al.*, 2004 b ; Planas *et al.*, 2006).

Aeromonas media strain A199 produces a bacteriocin-like antimicrobial compound (BLIS), which, *in vitro,* displays antagonistic activity inhibiting a widerange of fish and shellfish pathogens. For instance*,* this strain successfully protected the Pacific oyster (*Crassostrea gigas*) larvae from pathogenicity of *Vibrio tubiashii* and inhibited growth of *Saprolegnia* species (Gibson *et al.*, 1998b; Lategan & Gibson, 2003). The active substance was later identified by Lategan et al. (2006) as an indole (2,3-benzopyrrole). This compound demonstrated a wide range of antifungal and antibacterial activity. Furthermore, growth of *Vibrio alginolyticus* has been inhibited *in vitro* using extracellular products from

Lactobacillus brevis. For example, a 10^8 bacteria/ml concentration of inclusive culture of the same species was able to control heavy growth of *V. alginolyticus* in *Artemia* culture water (Villamil *et al.*, 2003).

Ajitha et al. (2004) demonstrated that free cell extracts from lactic acid bacteria strains such as *Lactobacillus acidophilus, Streptococcus cremoris, Lactobacillus bulgaricus*–56*,* and *Lactobacillus bulgaricus*–57 inhibited growth of *Vibrio alginolyticus* in culture media. In addition, the sea water bacterium, *Micrococcus* MCCB 104, isolated from a hatchery water tank, showed extracellular antagonistic activity thatinhibited *Vibrio alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *Vibriofluviallis*, *Vibrio proteolyticus*, *Vibrio nereis*, *V. cholerae*, *Vibrio mediterranei* and *Aeromonas* speciesassociated with *Macrobrachium rosenbergii* larval breedingtanks. This isolate also inhibited the growth of *V. alginolyticus* through co-culture (Jayaprakash *et al.*, 2006).

Competition

Competition between microorganisms for nutrients and space in the aquatic milieu is a strong selective criteriawhich has led to the development of a diversity of effective strategiesinvolving inoculation of specific beneficial microbes into the ecosystem (Burgess *et al.*, 1999). Different bacterial species use different mechanisms to outcompete or dominate other organisms for the same pool of resources (Hibbing *et al.*, 2010).

Competition is a phenomenon where the species was established to reduceor inhibit the bacterial pathogens colonization by competing for nutrients, attachment sites on the mucous membrane, or by producing inhibitory substances includingantimicrobial compounds which prevent growth or destroy putative pathogens (Geovanny *et al.*, 2007; Nair, 2005; Patterson & Bolis, 1997; Vine *et al.*, 2006). For example, competition for availability of energy or nutrients may play a role in establishing the microbial structure of the gut flora or culture water of marine organisms (Tinh *et al.*, 2008).

Competition for attachment sites can serve as the first defence barrier against invasion bybacterial pathogens(Vine *et al.*, 2006). Microbes use a number of mechanisms to assist in attachment or adhesion to intestinal epithelial cells, including specific structural lipoteichoic acids, hydrophobic and steric forces, electrostatic interaction, passive forces, and production

of inhibitory substances (Geovanny *et al.*, 2007). The most well known mechanism is siderophores, which are iron-complex chemical compounds produced by microorganisms (Braun & Braun, 2002). Gatesoupe (1997) found that *Pseudomonas fluorescens*strain AH2,which produces siderophores, has been effectively used as a biological control factor against the fish pathogen *Vibrio anguillarum*. Furthermore, *Vibrio E* secreted siderophores that increased the turbot larvae resistance against *Vibrio splendidus* pathogen, and enhanced larval growth. It also reduced the mortality of rainbow trout (*Oncorynchus mykiss*) that had been infected with *Vibrio anguillarumin vitro*.The authors show the relationship between the production of siderophores and the mode of action of *P. fluorescens* and suggested that competition for free iron is involved in the protective activity (Gram *et al.*, 1999).

The bacterial strain *Bacillus subtilis* UTM 126 exhibits antibacterial activity against several pathogenic species of *Vibrio*, such as *V. parahaemolyticus*, *V. harveyi*and *V. alginolyticus*, (Balcázar & Rojas-Luna, 2007).

Suppression of Virulence

Quorum Sensing (QS)

Bacteria species communicate with each other using chemical signalling molecules called auto inducers (Schauder & Bassler, 2001; Waters & Bassler, 2005).quorum sensing (QS) is a mechanism of bacteria-to-bacteria communication by which bacteria establish specific gene expression in response to the presence or absence of small signalling molecules (Nazzaro *et al.*, 2013; Schauder & Bassler, 2001; Zhu & Sun, 2008).

Bacterial behaviour regulated by QS is involved in a wide spectrum of host-associated phenotypes, including antibiotic biosynthesis, the production of virulence determinants in animal, plant, human, and fish pathogens, symbiosis, swarming, bioluminescence competence and sporulation, secondary metabolism, plasmid transfer, development of fruiting bodies and biofilm formation (Beck von Bodman & Farrand, 1995; Bosgelmez-Tinaz *et al.*, 2007; J. Bruhn *et al.*, 2005 a; Hardman *et al.*, 1998; Hentzer *et al.*, 2002 ; Lewenza *et al.*, 1999; Ni *et al.*, 2009; Schauder & Bassler, 2001; Williams *et al.*, 2000). In addition biofilm formation is considered a pathogenicity trait modulated by quorum sensing (Costerton *et al.*, 1999). Several pathogenic microorganisms of plants and animals are capable of forming a biofilm in certain environments (Choi & Kim, 2009).

Different bacterial species might use different pathways and autoinducers.Gram-positive and Gram-negative bacteria use at least six quorum-sensing pathways, as shown in Table 1.1. Among Gram-negative bacteria the cognate signal molecules: *N*-acyl-homoserine lactones (AHLs) and LuxR-LuxI homologous systemrepresented a patternon most well studied quorum-sensing systems(Eberl, 1999; Hentzer *et al.*, 2002 ; Swem *et al.*, 2009; Waters *et al.*, 2010).

Table 1.1. Different quorum sensing pathways in Gram-positive and Gram-negative bacteria.

Pathway	Signal molecules	Bacteria	References
AHL (A1-1) pathway	Various AHLS	Gram-negative	(Salmond *et al.*, 1995; Zavilgelsky & Manukhov, 2001)
4Qs pathway	PQS and AHLS	Gram-negative	(Diggle *et al.*, 2006)
AI-3 pathway	AI-3 (unknown structure)	Gram-negative	(Kendall *et al.*, 2007; Sperandio *et al.*, 2003)
AI -2 pathway	Two different forms	Gram-negative and Gram-negative	(Chen *et al.*, 2002; Surette *et al.*, 1999)
AIP pathway	Various oligopeptides	Gram-positive	(MDowell *et al.*, 2001)
CAI-1	Hydroxyketons	Gram-negative (*V. cholera*)	(Henke & Bassler, 2004; Higgins *et al.*, 2007)

Adapted from (Ni *et al.*, 2009).

Organisms' immune response to pathogenic bacterialinvasion may be controlled bycell-cell communicationAHLs QS which might effect host protein expression and broader sensing (Chhabra *et al.*, 2003; Mathesius *et al.*, 2003; Telford *et al.*, 1998).

AHLs are produced by a large number of Gram-negative bacterial species belonging to the alpha,beta, and gamma subclasses of proteobacteria, including strains of *Aeromonassalmonicida, Yersinia ruckeri, Aeromonashydrophila, Vibrio vulnificus*, *Vibrio salmonicida, Burkholderia*, *Agrobacterium*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Pseudomonas*,*Erwinia*, *Nitrosomonas*, *Hafnia*, *Obesumbacterium*, *Pantoea*, *Rahnella*, *Ralstonia*, *Rhizobium*, *Rhodobacter*, *Serratia*, and *Xenorhabdus* (Bruhn *et al.*, 2005 a; Eberl,

1999; Freeman & Bassler, 1999; Rasch *et al.*, 2004). The first description of the quorum sensing processing was in a bioluminescent marine bacterium *Vibrio fischeri* (Nealson & Hastings, 1979). Using quorum sensing to regulate population density behaviour makes good biological sense for pathogens, allowing them to keep a low profile of virulence gene expression until they reach an adequate number required for effective attack on the host. Quorum sensingmay well make sense in swapping from the appropriate physiological behaviour of the free–living status to behaviour corresponding to cells in a biofilm or a colony (Bauer & Robinson, 2002).

Quorum sensing inhibition (QSI)

Quorum-sensing mechanisms have a significant role in influencing settlement and biofilm formation. However, it is unclear to what extent quorum sensing molecules effect antibacterial production (Bowman, 2007; Dobretsov *et al.*, 2007). It is reasonable that inhibitors of bacterial quorum sensing might havepharmacological applications as bacterial quorum sensing is implicated in various pathologically relevant events. Firstly, quorum sensing assistsin organizing bacterial community behaviour, nonetheless it is not critical for microbial survival. However, quorum sensing inhibitors can aid in the attenuation of biofilm formation, reduce virulence and increase bacterial sensitivity to antimicrobial treatment. However, they can only be useful as adjuvants in complementing other inhibitory mechanisms, since they do not display bacteriostatic or bactericidal activity (Ni *et al.*, 2009). *Vibrio anguillarum* is a Gram-negative human, fish and plant pathogenic bacterium, which regulates the expression of virulence factors by producing quorum sensing signal molecules calledAHLs. Production of these moleculesmay be prohibited using specific quorum-sensing inhibitors (Rasch *et al.*, 2004). Some quorum sensing inhibitors may specifically block the AHL-regulated systems, and thus the expression of virulence factors, at concentrations where growth of the bacteria is not affected. An example of such inhibitors are the halogenated furanones from the red algae *Deliseapulchra* (Givskov *et al.*, 1996) which specifically block expression of virulence factors in *Erwiniacarotovora, Vibrio harveyi* and *Pseudomonas aeruginosa* (Hentzer *et al.*, 2003; Manefield *et al.*, 2000). These compounds have therefore been suggested as a new treatment for controlling bacterial disease. The halogenated furanone C30 was added to the water during a trout co-habitant challenge experiment with *V. anguillarum* and was reported to substantially reduce mortality in comparison with untreated or negative controls (Rasch *et al.*, 2004).

Immunostimulation

Crustaceans, similar to other invertebrates, do not have specific immunity but display innatewide-spectrum protection mechanisms including encapsulation, phagocytosis, and a number of antimicrobial factors circulating in plasma (Bachère, 2000; Bachère *et al.*, 1995; Roch, 1999; Smith & Chisholm, 1992). As an alternative, crustaceans fight pathogenic microbes through the innate immune system, which is a composite of humoral and cellular immune responses. The humoral response comprises production of antimicrobial peptides (AMPs) (Söderhäll & Cerenius, 1998). Stimulation of host defences using vaccination can enhanceinfectious disease resistance by activating acquired immune responses, in addition to improving innate immune systems by immunostimulation. Vaccines comprise living and dead bacteria, glucans, peptidoglycans, and lipopolysaccharides (Sakai, 1999; Smith *et al.*, 2003). When mortality is often high due to opportunistic pathogens the prophylactic use of probiotics and immunostimulants has many advantages as they can be applied during larval and early fry stages (Gildberg *et al.*, 1995). For example shrimp do not have antibodies and the immune response of shellfish is broadly non–specific. In spite of this fact there is some proof for limited specificity (Browdy, 1998). Stimulation of the non–specific immune system mayenhance the animal's response to challenges from pathogenic bacteria. The use of immunostimulants to control luminescent vibriosis in shrimp is mentioned in several reports, where it is shown that different immunostimulants significantly increased survival when shrimp were infected experimentally with luminescent *Vibrio* spp. (Alabi *et al.*, 1999; Marques *et al.*, 2006; Thanardkit *et al.*, 2002).

Daniels *et al.* (2006) studied the effect of different concentrations of mannan oligosaccharide in the diet on growth and survival of *H. gammarus*. The results showed that the larvae fed with the supplemented diets had higher survival rates to stage IV in comparison to larvae fed with the control diet. The intensive culture of Atlantic halibut, *Hippoglossushippoglossus,* as well as success of many other marine species, is hard to predictas a result of suboptimal growth and poor resistance to disease of juveniles and larvae (Bergh *et al.*, 2001). Opportunistic pathogenic bacteria and down–regulation of the non–specific immune response due to stress in larvae in industrial culture conditions are problems. Therefore, stimulation of the non–specific immune response to deal with microbial problems in juvenile production is attractive (Vadstein, 1997).

Probiotic species

Probiotics are microorganisms or their products, which give health benefits to the host. They are used in aquaculture to control the spread of disease, and to complement or in several cases provide analternativetousing antibacterial drugs(Irianto & Austin, 2002). Despite there being many examples of microorganisms being used for biocontrol of pathogens, until recently only a few commercial probiotics have been used in the culture of larvae of marine organisms (Zhang *et al.*, 2009). According to Vine et al. (2006), probiotics have rarely been tested on a commercial scale. A wide range of yeasts (*Debaryomyces, Saccharomyces* and *Phaffia*), microalgae (*Tetraselmis*), Gram-negative bacteria (*Aeromonas, Photorhodobacterium, Pseudomonas Alteromonas,* and *Vibrio*) and Gram-positive bacteria (*Bacillus, Enterococcus, Carnobacterium, Lactococcus, Lactobacillus, Streptococcus Micrococcus,* and *Weissella*) have been evaluated (Irianto & Austin, 2002). Several bacterial species, including *Bacillus* species and lactic acid bacteria, that comprise a major part of the microflora on skin, gills and intestinal tracts of shrimp, are used as probiotics against fish and shellfish pathogenic bacteria (Rengpipat *et al.*, 2000; Skjermo & Vadstein, 1999). *Lactobacillus* sp., the lactic acid producing bacteria,was one of the first probiotics discovered (Sahu *et al.*, 2008). In warm-blooded animals lactic acid bacteria has been successfully tested as a probiotic bacteria, and this bacteria has also been tested with shrimp to antagonize shrimp pathogens(Gatesoupe, 1999; Skjermo & Vadstein, 1999). *Bacillus* strains, including *Bacillus* strain S11 (BS11) and *Bacillus cereus* have also been shown to reduce mortality of black tiger shrimp *Penaeusmonodon* exposed to *V. harveyi* (Ravi *et al.*, 2007; Rengpipat *et al.*, 2003).

Dang and Lovell (2000) reported that bacteria belonging to the *Roseobacter* subclass were ubiquitous and rapidly colonized marine habitats, and have shown strong antibacterial activity *in vitro* against marine pathogens such as *Vibrio* spp. (Hjelm *et al.*, 2004b). *Roseobacter* spp. have been implicated in improving the survival of prawn *Litopenaeu svannamei* and pathogen-challenged turbot (*Scopthalmusmaximus*) larvae (Balcázar *et al.*, 2007; Planas *et al.*, 2006). Fjellheim *et al.* (2007) and Hjelm*et al.* (2004 a) documented that many *Vibrio* spp.isolated from the intestinal regions of turbot (*Scopthalmusmaximus*) and Atlantic cod (*Gadusmorhua*) in hatcheries were antagonistic to *Vibrio* pathogens. In

particular *V. alginolyticus*, has been revealed to efficiently reduce mortality in Atlantic salmon exposed to *Aeromonas salmoncida* (Austin *et al.*, 1995). Vandenberghe *et al.* (1998) implicated this bacterium in enhancing host resistance to disease caused by *V. harveyi* in the larval stage of cultured *Penaeuschinesis*.

According to Gomez-Gil *et al*. (2000) *V. alginolyticus* is a commonly occurring bacterium which is considered a promising probiotic in shrimp hatcheries. Gatesoupe (1990) discovered *V. alginolyticus* in healthy rotifers and documented a positive relationship between the proportion of *V. alginolyticus* and the survival rate of turbot larvae in the rearing environment. Austin *et al.* (1995)showed that *V. alginolyticus* serotype 1 VIB235 was capable of conferring some degree of protection to shrimp against disease, as detailed in Table 1.2.

Table 1.2. Bacterial probiotics employed in the larval culture of the aquatic organism

Species of bacteria	Target organism	Reference
V. alginolyticus	Shrimp (*P. vannamei*)	(Garriques & Arevalo, 1995)
T. utilis(PM-4)	Shrimp (*P. monodon*)	(Maeda & Liao, 1992)
V. Harvey, Pseudomonas sp., *Nitrobacter* sp., *Nitromonas* sp. *and Bacillus* sp.	Shrimp (*P. Monodon*and *Penaeus penicillatus*)	(Anonymous, 1991)
T. utilis (PM-4)	Crab (*Portususstri tuberculatus*)	(Nogami *et al.*, 1997; Nogami & Maeda, 1992)(Maeda & Liao, 1994)
V. Pelagius	Turbot (*Scophthalmusmaximus*)	(Ringo & Vadstein, 1998)
Bacillus toyoi and Bacillus sp. spores	Turbot via rotifers (*Brachionusplicatilis*)	(Gatesoupe, 1989)(Gatesoupe, 1991)
Lactic bacteria	Turbot via rotifers	(Gatesoupe, 1990)
Lactobacillus plantarum and *L. helveticus*	Turbot via rotifers	(Gatesoupe, 1991)
L. bulgarius Streptococcus lactis	Turbot via *Artemia*	(Garcia-de-la-Banda *et al.*, 1992)
Alteromonas sp.	Oyster (*Crassostreagigas*)	(Douillet & Langdon 1993, 1994)
A. media	Oyster	(Gibson *et al.*, 1998)
Roseobacter sp.(BS107)	Scallop (*Pectenmaximus*)	(Ruiz-Ponte *et al.*, 1999)
Vibrio sp.	Chilean scallop (*Argopecten purpuratus*)	(Riquelme *et al.*, 1997)
Enterococcus faecium SF68	*Anguilla Anguilla*	(Chang & Liu, 2002)
L. rhamnosus ATCC53103	*Oncorhynchus mykiss*	(Nikoskelainen *et al.*, 2001)
Micrococcus luteus A1-6	*O. mykiss*	(Irianto & Austin, 2002)
Carnobacterium sp.	Hg4-03 *Hepialus gonggaensis* larvae	(Youping *et al.*, 2011) Citation
Lactobacillus acidophilus	*Clarias gariepinus*	(Al-Dohail *et al.*, 2011)

Bacillus spp., *Enterococcus* sp. and Lactobacillus spp	*Farfantepenaeus brasiliensis*	(de Souza *et al.*, 2012)
B. stbtilis BT23	*Pemaeus monodon*	(Vaseeharan & Ramasamy, 2003)
A. media, strain A199,	*Crassostrea gigas*	(Gibson *et al.*, 1998)
Pseudomonas fluorescens strain AH2	Fish	(Gram *et al.*, 1999)
Saccharomyces cerevisiae	*Penaeus Íannamei*	(Scholz *et al.*, 1999)
Vibrio alginolyticus	Atlantic salmon	(Austin *et al.*, 1995)
Lactococcus lactis	*Epinephelus coioides*	(Sun *et al.*, 2012)
Pseudoalteromonas isolate PP107	Spiny lobster phllosoma	(Goulden *et al*., 2012)
Vibrio sp. isolate PP05	Spiny lobster phllosoma	(Goulden *et al*., 2012)

Parts of this table were adopted from Gomez-Gil *et al.* (2000)**.**

1.3.4 *In vitro* screening

The first step in probiotic screening is to establish a collection of candidate probiotics. The most widespread method to screen for probiotic bacteria is through *in vitro* antagonism assays, in which known pathogens are exposed to the probiotic strains, or their extracellular secretions, in liquid or solid medium (Figure 1.3)(Balcázar *et al.*, 2006; Geovanny, *et al.*, 2007). Recently, several methods of *in vitro* screening for inhibitory compounds have been described. These methods include the well diffusion method, the double layer method, the disc diffusion method, the cross streaking method and the co culture method (Kesarcodi-Watson *et al.*, 2008). These assays are based on the principle that the probiotic (the producer) secretes extracellular compounds that inhibit the other bacterial strain (the indicator). This inhibitory activity is indicated by inhibition of the indicator growth on agar medium (Kesarcodi-Watson *et al.*, 2008).

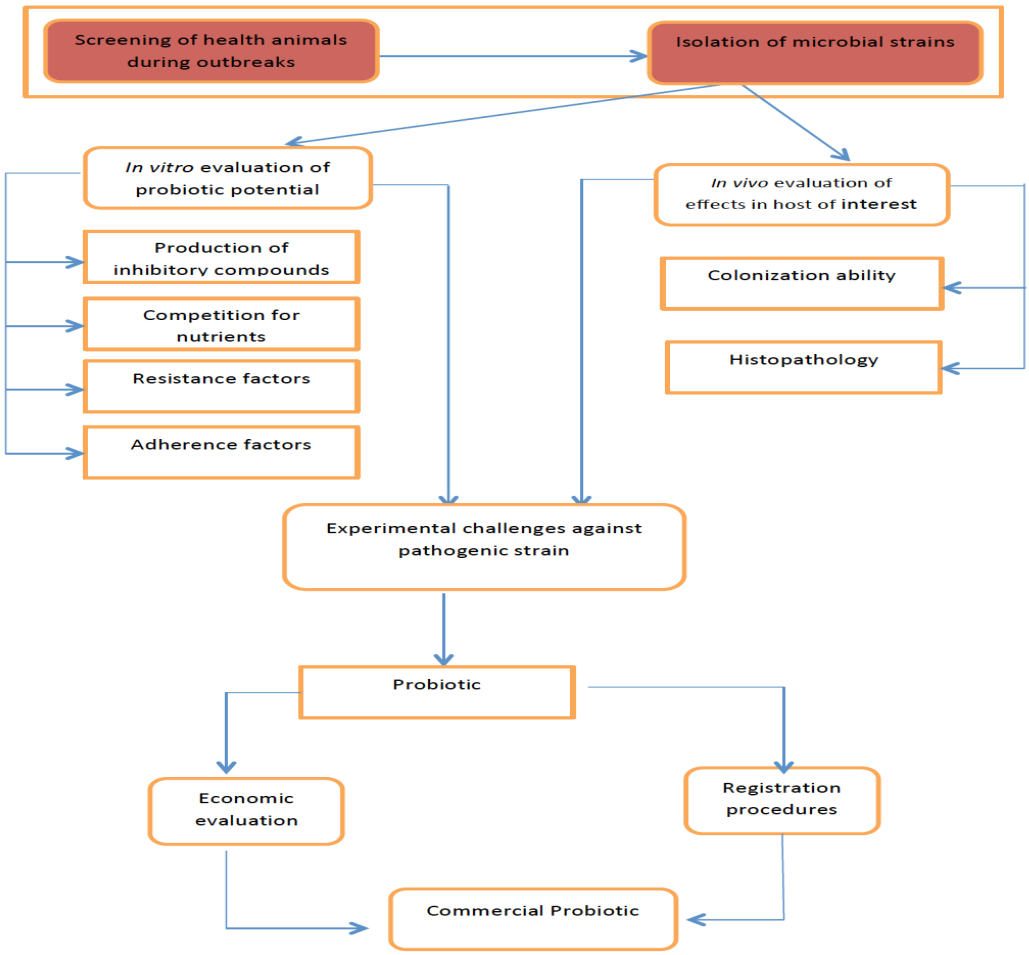


Figure 1.3. Scheme for selection of bacteria as biocontrol agents in aquaculture (Balcázar *et al.*, 2006).

In general, selection of probiotic candidates has usually been a multifaceted process supported bylimited scientific evidence (Gomez-Gil *et al.*, 2000). The selection of probiotics is mostly determined by biosafety considerations, method of administration of the probiotic, the methods of production and processing, and the site in the host body where the probiotics are likely to be active (Veld *et al.*, 1994). Methods to select probiotic microorganisms for use in the larvalculture of marine animals are multifaceted process involving (1) collection of background information, (2) acquisition of putative probiotics (PP), (3) assessment of the pathogenicity of the PP, (4) evaluation of the effect of the PP in larvae, (5) evaluation of the ability of the PP to out-compete pathogenic strains, and (6) an economic cost benefit analysis (Gomez-Gil *et al.*, 2000; Verschuere *et al.*, 2000 a).

1.4 Aims and objectives of this study

P. ornatus also known as the ornate spiny lobster is an aquaculture species with great commercial potential. A significant obstacle to the commercialisation of *P. ornatus* are bacterial diseases that spread during the larval stage with *V.owensii* DY05, isolated during an epizootic of aquaculture-reared ornate spiny lobster emerging as a significant pathogen (Cano-Gómez*et al*., 2010).*V. owensii* DY05 has been demonstrated to be transmitted through live feed vectors (artemia) and to proliferate in the hepatopancreas (midgut gland) of phyllosoma (the larval stage) resulting in extensive tissue necrosis and ultimately major systemic infection (Goulden *et al.,* 2012). In order to identify bacteria from natural prey that were antagonistic to *V. owensii* DY05, Goulden et al. (2012) used a multi-tiered probiotic screening strategy. This study identified *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 two antagonistic bacterial strains from 500 candidates based on their ability to inhibit attached and planktonic forms of pathogenic *V. owensii* DY05. Inoculation of artemia with a combination of *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 was found to provide significant protection to *P. ornatus* phyllosomas against *V. owensii* DY05 infection *in vivo*.

The antagonistic mechanisms through which *Pseudoalteromonas* and *Vibrio* spp. inhibit *V. owensii* DY05 are unknown. Implicated in the suppression of aquatic vibrios are the production of broad-spectrum anionic proteins and non-proteinaceous antibiotics by *Pseudoalteromonas* spp. and aliphatic hydroxyl ethers and andrimid antibiotics by *Vibrio* spp. produce.

The aim of the present study was to further isolate potential probiotic species from marine sources and to study the molecular factors and mechanisms employed by such antagonistic bacteria to suppress the growth or infection by the lobster phyllosoma pathogen *V. owensii* DY05.

A collection of bacterial cultures isolated from coral tissues and shrimp heamolymph was screened to identify potential probiotic candidates antagonistic to the phyllosoma pathogen DY05. Many bacterial isolates from this collection suppressed the pathogen *in vitro*, however, three bacterial isolates were found to be promising candidates for potential use as probiotic bacteria in lobster aquaculture hatcheries. Studies of these isolates were carried out to investigate the mechanisms by which they inhibited the pathogen. They were further subjected to molecular analysis in order to study the genetic background and other traits related to pathogen suppression by the *Pseudoalteromonas* strains.

Isolation and identification of Vibrio owensii DY05 growth-suppressive bacteria fromthe marine environment

2.1 Abstract

The aim of the present study was to identify potential probiotic bacteria with an ability to suppress the growth of the lobster larval pathogen *Vibrio owensii* DY05. Two screening methods were used, the well diffusion and disk diffusion assays, in order to identify such bacterial candidates in strain collections obtained from two distinct sources: coral tissue and ghost shrimp haemolymph. A total of 499 marine bacterial strains were screened: 400 strains from healthy and diseased corals (several *Turbinaria* and *Acropora* spp.) and 99 strains from freshly collected or severely stressed estuarine ghost shrimp (*Trypaea australiensis*). Well diffusion assays identified 111 isolates that showed antagonistic activity against *V. owensii* DY05, while disk diffusion assays identified only 46 of them. It was concluded that the well diffusion assay is a better method of screening for antimicrobial activities, and it was therefore chosen as the main method for pathogen suppression in this study.

16S rRNA gene fragment sequencing was conducted to identify the *V. owensii* DY05-suppressive bacteria and place them in phylogenetic groups. The majority of the coral isolates belong to the classes Gammaproteobacteria (89% of total antagonistic strains), and a few belong to Bacilli (6.2%), Actinibacteria (3.8%) and Class Alphaproteobacteria (1.2%). At the genus level, *Vibrio* spp. were the most highly represented (62.5% of all isolates) followed by *Psycrobacter* (24%), *Staphylococcous* (5%) and *Micrococcus* (2.5%). Other bacterial genera isolated including *Bacillus*, *Photobacterium, Acinetobacter, Pseudovibrio,* and *Brachybacterium* (represented 1.25 % each). Similarly most isolates derived from shrimp were from the class Gamaproteobacteria (81% of the total isolates) followed by class Bacilli (16%) and class Actinibacteria (3.2%). At the genus level, *Vibrio* spp. were predominant with 74%, followed by *Staphylococcus* (13%) as well as *Pseudoalteromonas, Bacillus, Photobacterium* and *Micrococcus* (3.2% each).

The results of this study indicated that some strains showed strong suppressive activity and may be good candidates as probiotics. One of these strains, *Pseudoalteromonas* strain 80, showed the strongest antagonistic activity against *V. owensii* DY05 in both the well and disk diffusion assay. Another two strains, *Vibrio* sp. strain 34 and *Psychrobacter* sp. strain 62, also showed strong pathogen suppression and thus all three strains were selected for further work.

2.2 Introduction

Aquaculture is the farming of aquatic organisms and is the fastest growing food-producing sector in the world. However, the spread of diseases is increasing as aquaculture activities grows and expand (Bondad-Reantaso *et al.*, 2005). Rock lobsters of the family *Palinuridae* are a target aquaculture species and are the most valuable wild fisheries sector in Australia. The extended larval stage of rock lobster acerbates a high rate of phyllosomas mortalitycaused by insufficient nutrition and the spread of microbial diseases, caused particularly by opportunistic pathogens. This presents a significant challenge to the production of commercial scale quantities of post-larvae stage lobster (Bourne *et al.*, 2007; Bourne *et al.*, 2004; Jeffs, 2010). *Vibrio* spp. are abundant in the aquatic environment, and are well documented opportunistic pathogens that are often correlated with larval mortalities in aquatic hatcheries (Forward *et al.*, 2011; Shields, 2011). Several pathogenic *Vibrio* species are known to cause serious disease in invertebratessuch as lobsters, crabs, shrimp, and fish and may even affect humans (Shields, 2011). These species include *V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. anguillarum* (Abraham *et al.*, 1996; Bowser *et al.*, 1981; Brinkley *et al.*, 1976; Forward *et al.*, 2011). *V. owensii* DY05 was selected as a model pathogen in our study since it has been demonstrated to be a significant pathogen causing mid-gut gland infection and mass mortalities of cultured ornate spiny lobster *Panulirus ornatus*phyllosoma (Goulden, *et al.*, 2012).

Antibiotic prophylaxis is commonly used in aquaculture industries to protect against substantial losses to bacterial pathogens such as *V. owensii* DY05 (Goulden, 2012). There is an increasing interest in the use of probiotic in aquaculture to control diseases since application of antibiotics is causing concern over the possible development of antibiotic-resistant bacteria (Hjelm *et al.*, 2004 b). Probiotic bacteria thatare capable of reducing opportunistic pathogens can improve larval health of marine organisms (Hjelm *et al.*, 2004 b ; Kesarcodi-Watson *et al.*, 2008). Understanding the mechanisms of antagonism by probiotic bacteria against pathogens, and the proliferation and/or infection site of marine pathogens is important in the selection of probiotic strains for use in a commercial setting (Hjelm *et al.*, 2004b). Several bacterial species have previously been used as biocontrols in aquaculture hatcheries (Brinkhoff *et al.*, 2004). Examples include *Vibrio alginolyticus*, *V. harveyi*, *Pseudomonas* spp., *Nitrobacter* spp., *Lactobacillusspp*, *Alteromonas* spp., *Roseobacter* spp. and *Bacillus* spp. (Douillet & Langdon 1994; Gatesoupe, 1991; Gibson *et al.*, 1998b).

Many surface-attached bacteria produce antimicrobial substances that obstruct the growth, attachment or survival of competing microbes in order to achieve an advantage in highly competitive environments (Long & Azam, 2001). Antagonistic interactions between bacterial species represent an interesting evolutionary strategy, which confers advantages in competition for space and food in the natural environment (Lo Giudice *et al.*, 2007b), and an effective control of microorganisms residing the same ecological niche (Hentschel *et al.*, 2001). There are few reports investigating inter-species interactions among microbes of the same or related marine habitat, but there is growing evidence to suggest that antagonistic effects, expressed by phylogenetically different bacterial groups, are a widespread feature in marine environments (Bhattarai *et al.*, 2006; Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Long & Azam, 2001; Nair & Simidu, 1987). Many antagonistic marine bacteria are known to stimulate or increase production of antimicrobial compounds in the presence of competitors, and play an important role in preventing attack by bacteria, including potential pathogens (Burgess *et al.*, 1999; Slattery *et al.*, 2001; Trischman *et al.*, 2004).

Antimicrobial activity has been extensively reported for extracts of various groups of marine organisms. Many organisms, such as invertebrates that are associated with coral reef ecosystems, have long been known as the sources of structurally unique natural products, particularly antimicrobial compounds. These organisms include soft corals, sponges, tunicates; shrimp, algae, bryozoans and zooplankton (Kumaran *et al.*, 2012; Long & Azam, 2001; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009; Wiese *et al.*, 2008). For example, sponge-associated bacteria were described a rich source of bioactive metabolites, with more than 6000 metabolites having been recovered from aquatic environments (Dieckmann *et al.*, 2005; Hentschel *et al.*, 2001; Mangano *et al.*, 2009; Santos *et al.*, 2010). Corals are sessile eukaryotic organisms, which represent unexploited sources of diversity of microorganisms of economic importance, and a unique surface for microorganisms' colonization (Nithyanand *et al.*, 2011; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009). Corals harbour complex and diverse microorganisms, including bacteria, archaea, zooxanthellae, and viruses. Coral mucus layers are richer in nutrients than sediments or seawater (Carlos *et al.*, 2013; Karna *et al.*, 2004; Rosenberg *et al.*, 2007; Rosenfeld & ZoBell, 1947; Shnit-Orland & Kushmaro, 2009). Shrimp represent a good source of antimicrobial producing bacteria, and many bacteria with antimicrobial activity have been isolated from shrimp (Austin *et al.*, 1995; Chandrasekaran &Ashok Kumar, 2011; Kumaran *et al.*, 2012). For example, bacteria on the surface of eggs of

the shrimp *Palaeman macrodactylus* produce a metabolite that inhibits fungal infections that would be lethal to the eggs (Gil-Turnes *et al.*, 1989).

The aim of the present work was identify potential disease suppressive bacteria from marine collections that may act as probiotic agents against the lobster pathogen *V.owensii* DY05. In addition, the phylogeny and bioactivity of DY05-supressive strains, demonstrating pronounced antibacterial activity, were investigated.

2.3 Materials and methods

2.3.1 Bacterial strains and culture conditions

A total of 499 marine bacterial isolates from culture collections at the laboratory of L Pereg (UNE, Australia) were used in this study. These isolates originated from the corals *Turbinaria mesenterina, T. redicalis, T. frondens and Acropora solitaryensis* (Godwin, 2007; Harris, 2004) and from males and females of the estuarine ghost shrimp *Trypaea australiensis* (Goulden, 2006).

Coral samples were originally collected in 2004 and 2005 from rocky reefs at depths of 10–20 m at the Solitary Island Marine Park (SIMP) by Godwin et al. (2012). Some isolates were from the seawater adjacent to the coral surface (SW). Coral sources were either healthy ones or Australian Subtropical White Syndrome (ASWS)-affected corals (Figure 2.1) (Dalton *et al*., 2010). Particularly, bacteria were isolated from the coral surface mucus (Figure 2.1) and tissues of healthy corals (H), healthy looking tissue on ASWS-affected corals (HL-ASWS), disease margin (M-ASWS) or diseased tissue on ASWS-affected corals (D-ASWS).

Estuarine ghost shrimps from Coffs Creek and Wooli-Wooli River, NSW, Australia, were either freshly caught or exposed to severe stress that apparently cause them bacteriosis (vibriosis) before bacteria were isolated from their extracted haemolymph (Figure 2.2) using *Vibrio*-selective TCBS agar by Lily Pereg and Evan Goulden (Goulden, 2006).

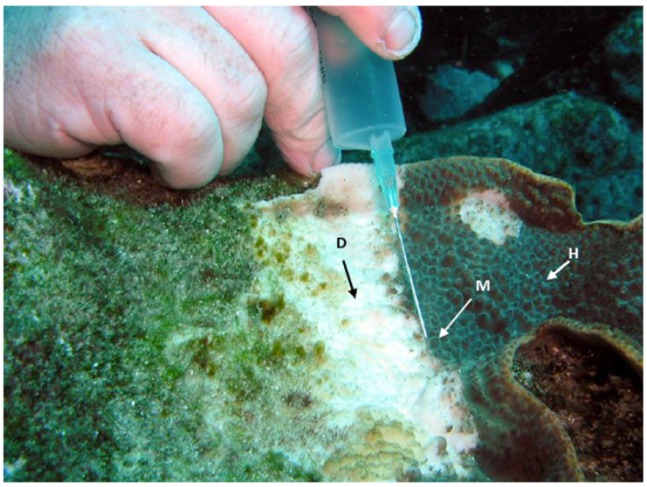


Figure 2.1. *T. mesenterina* colony displaying typical signs of ASWS. Arrows indicate the regions sampled for bacterial community analysis. Dark areas of the coral surface are covered in living tissue; white areas are recently exposed calcium carbonate skeleton. (H on photo) Apparently healthy tissue of disease colony (note – we designated it here as HL-ASWS), (M on photo) Margin of disease lesion (note – we designated it here as M-ASWS), (D on photo) Dead coral skeleton (note- we designated it here as D-ASWS). Healthy tissue from a nearby colony unaffected by disease was also collected (not visible in this photograph, we designated it here H). (Photograph by Steven Smith) (Godwin *et al.*, 2012).

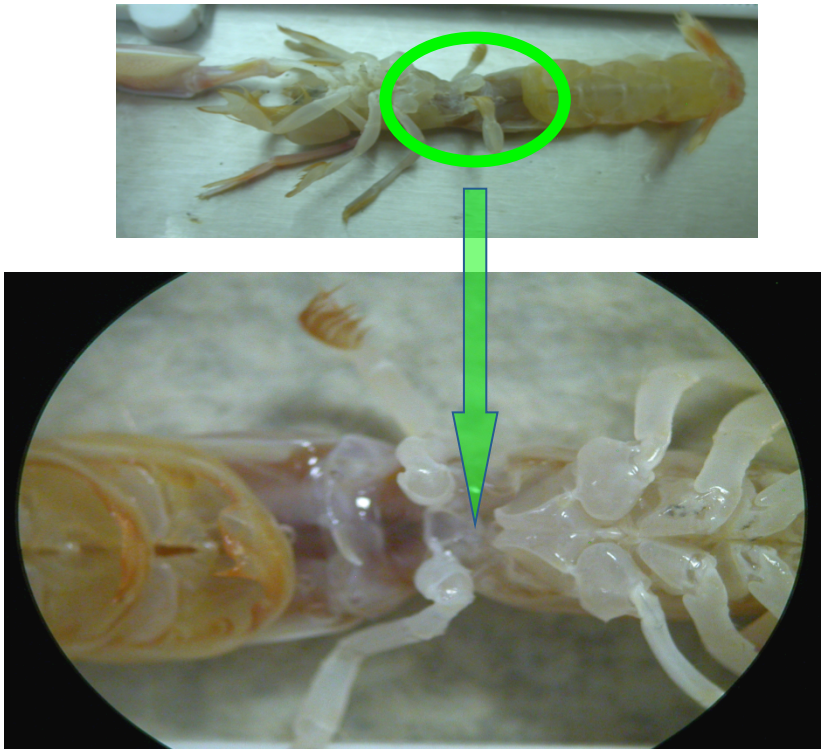


Figure 2.2. Extraction of Haemolymph from Ghost Shrimps. Extraction was performed using a syringe after surface cleaning with 70% ethanol (Photos by Evan Goulden and Lily Pereg).

The test pathogen *V. owensii* DY05 (Cano-Gómez *et al.*, 2010) and the broad spectrum *Vibrio*-suppressive bacterium *Phaeobacter* (formerly *Roseobacter*) strain 27-4 (Hjelm *et al.*, 2004 a) were obtained from Lone Hoj, the Australian Institute of Marine Science (AIMS).

All microorganisms were grown in marine broth 2216 (Difco) for cryopreservation at -80 ^{o}C in 25-50% (v/v) glycerol. Growth media were marine agar 2216 (MA, Difco) or LB10 (10 g/L Tryptone,(OXIOD) 5 g/L yeast extract (OXIOD), 10 g/L sodium chloride (Chem-supply) and 15 g/L of agar (Difco) was added to solidify the medium). Liquid cultures were in marine broth (MB, Becton) or LB10 at 28 ^{o}C, 150 rpm, unless otherwise stated.

2.3.2 In vitro antagonism assays

Two different assays were used as rapid qualitative methods to screen isolates for antimicrobial activities: a well-diffusion assay and a disk diffusion assay.

2.3.2.1 Well diffusion assay (WDA)

The isolates from both culture collections were screened for antagonistic activity against *V. owensii* DY05, a pathogen of the *P. ornatus* phyllosoma (Cano-Gómez *et al.*, 2010; Goulden, *et al.*, 2012), using well diffusion assay (WDA) which was performed as described by Goulden, *et al.* (2012). Briefly, an 18 hr culture of *V. owensii* DY05 was seeded (10 µL/mL) into molten (45 ^{o}C) minimal medium agar (MMA; 0.3% casamino acids; 0.4% glucose; 1% bacteriological agar in filtered seawater). We used 10 mL of the seeded medium per petri dish (85mm in diameter). Following solidification, wells (diameter 6 mm) were cut aseptically into the agar and loaded with 50 µL of dense tested isolate cultures, pre-grown for 18-24 hr period at 28 ^{o}C with 150 rpm shaking. Plates were incubated (28 ^{o}C) for 5 days and inspected every 24 hr for clearing zones signifying antagonistic activity against *V. owensii* DY05. The effect of temperature on the production of growth inhibition zone in well diffusion assays was initially examined at 25, 28 and 37 °C for the first 100 isolates. In all cases 28 °C gave the clearest and largest inhibition zone. Therefore we continued examining other isolates in this assay using this temperature.

The inoculum was sufficient to produce confluent growth on the assay plates and growth inhibition was assessed as the diameter of the zone of pathogen growth inhibition in mm (also called clearing zone).

Assays were performed at least in triplicate for each isolate using *Phaeobacter* sp. 27-4 (Hjelm *et al.*, 2004 a) and in some cases *Pseudoalteromonas* sp. S9.2.2 (an isolate found to inhibit pathogen growth early in this work) as positive antagonistic controls.

2.3.2.2 Disk diffusion assay

Disk preparation protocol was modified from Hayashida‑Soiza *et al.*(2008). The same 18-24 hr tested isolate cultures (see above) were used to produce cell-free supernatants by centrifugation at 14,000 rpm for 30 min at 4°C followed by filter sterilisation (FS; 0.2 µm Sartorius stedim (Minisart)). Sterile filter paper disks (6 mm diameter) were saturated with 50 µL bacterial-free culture supernatant. Paper disks treated with 50 µL of sterile medium were used as control. Disks were placed onto MMA seeded with pathogen as described above. Plates were incubated at 28 ^{o}C for 18-24 hr, monitored and inhibition zones measured as described above.

2.3.3 Identification of antagonistic isolates using 16S ribosomal DNA sequencing

Bacterial isolates were identified by 16S rRNA gene fragment sequence analysis. Genomic DNA was extracted using the freeze-boil method (Pereg-Gerk, 1997) from every bacterial strain, which demonstrated positive *in vitro* antagonistic activity. Briefly, isolates were revived from cryopreserved glycerol stocks by streaking onto MA plates and incubated at 28 ^{o}C for 18-24 hr. Bacterial subculture were grown on MA for 18 hr at 28 ^{o}C. A loop full of fresh colonies was suspended in 50-75 µL of elution buffer (Qiagen) and incubated at -70 ^{o}C for 30 min, then boiled for 2 min at 100 ^{o}C and centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant containing genomic DNA was freshly used.

Preliminary experiments indicated that, while the freeze-boil method was suitable for extracting genomic DNA from most isolates, it was unsuitable for the extraction of genomic DNA from the genera *Pseudoalteromonas* and *Pseudovibrio* (data not shown). For these isolates, genomic DNA was extracted using DNeasy® blood and tissue kit (Qiagen-Hilden, Germany) according to the manufacturer's instructions, using the protocol recommended for Gram-negative bacteria.

2.3.4 PCR amplification of 16S rRNA gene fragment

PCR amplification, sequencing and phylogenetic analysis of 16S rRNA gene fragment from bacterial isolates were performed on genomic DNA extracts using the 16S rRNA gene universal primers 27F (5' GAGCTCAGAGTTTGATCMTGGCTCAG) and 1492R (5' CACGYTACCTTGTTACGACTT) (Valinsky *et al.*, 2002). 16S rRNA gene fragments were amplified from the genomic DNA extracts in PCR reaction mix composed of sterile Milli-Q water (12 µL), 10X PCR buffer (2.5 µL, Qiagen), 50 mM $MgCl_2$ (0.75 µL), 2 mM dNTP mix (2.5 µL), DMSO (2.5 µL), 10 pm of each primer (1.25 µL), Taq DNA polymerase (0.25 µL). PCR was performed using a thermal cycler (PTC-100^{TM}, MJ Research Inc.) using the following conditions: 10 min initial denaturation at 94°C, 35 cycles of 30 s at 94°C (denaturation), 30s at 48°C (annealing), 2 min at 72°C (extension); and final extension for 10 min at 72 °C.

PCR products (of expected size, 1.5 kb) were purified using QIAquick PCR Purification Kit (Qiagen-Hilden, Germany) eluted in 10 mM Tris/HCl (pH 8.0) and quantified on agarose gels against 1 kb standard DNA ladders (New England Biolabs) following the manufacturer's recommendations. Purified PCR products were sequenced by the Australian Genome Research Facility (AGRF, Sydney, Australia) using 8.8 to 43.8 ng of cleaned 16S rRNA gene product and 10 pmol of the forward primer 27F in a final volume of 12 µL.

2.3.5 Classification and identification

The nucleotide sequences were edited (ChromasLite v2.1) and submitted to the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) to determine nucleotide-nucleotide similarity to identify the nearest relative with sequences in the GenBank database. Sequences containing unread bases ('Ns') were trimmed from the 5' and 3' ends and misread bases were corrected where possible and submitted to GenBank database. Classification of isolates to genus level was achieved by submitting their 16SrRNA gene sequences to the RDP Classifier (Wang *et al.*, 2007)

2.4 Results

Potential probiotics bacteria showing various levels of antagonistic activity against the lobster phyllosoma pathogen *V.owensii* DY05 were identified from two marine culture collections, one from corals and the other from shrimp, originated from Northern NSW, Australia.
Bacterial isolates were tested over a range of growth temperatures. It was found that the optimal growth temperatures of all isolates were between 25 ^{o}C and 28 ^{o}C, and all isolates showed a decrease in or no growth at 37 ^{o}C. An isolate was considered suppressive if it showed any size of pathogen-clearing zone.

Out of a total of 499 bacterial isolates tested (400 from corals and 99 from shrimps) 111 isolates (80/400 (20%) of the corals isolates and 31/99 (31.3%) of the shrimp isolates) produced clearance zones, free of *V.owensii* DY05 pathogen growth, using the well-diffusion assay. However, only 46 of these strains showed clearance zones using the disk-diffusion assay. Moreover, the clearance zones using the well-diffusion assay were wider than those obtained in the disk-diffusion assay. Well-diffusion assays produced pronounced inhibition zones of 10-23 mm in diameter after incubation periods of 6 – 24 hr, whereas, clearing zones in disk-diffusion assays were under 10 mm in diameter. Therefore, the well-diffusion assay was selected for further bacterial antagonism test in this project. Examples of the results of both methods are shown in Figure 2.3. Isolates producing clearing zones are summarized in Table 2.3 for coral isolates and Table 2.4 for shrimp isolates.

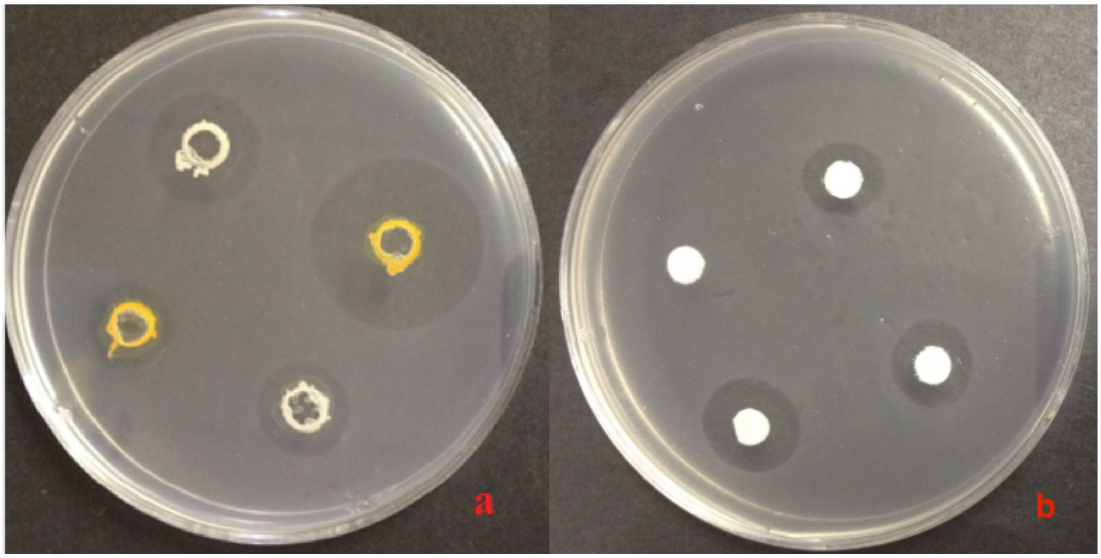


Figure 2.3. Typical *V.owensii* DY05 pathogen-suppression tests: well diffusion assay (a) disk diffusion assay (b). Clearing zones around the wells or the disks indicate that the tested bacterial isolate shows antagonistic activity against the pathogen *V. owensii* DY05.

Antagonistic activity was defined as weak (≤10 mm), moderate (11-20 mm) or strong (≥21 mm) according to the size in diameter of the inhibition zones (Goulden, *et al.*, 2012). The majority of the coral and shrimp antagonistic isolates showed weak activity (58/80 (72.5%), and 20/31 (64.5%) respectively). Other coral antagonistic isolates produced moderate (12/80 (15%)) or strong inhibition zone (10/80 (12.5%)). The reminder of shrimp antagonistic isolates showed moderate activity (10/31 (32.3 %)) and a single strain produced a strong inhibition zone (3.2 %). These results are summarised in Figure 2.4.

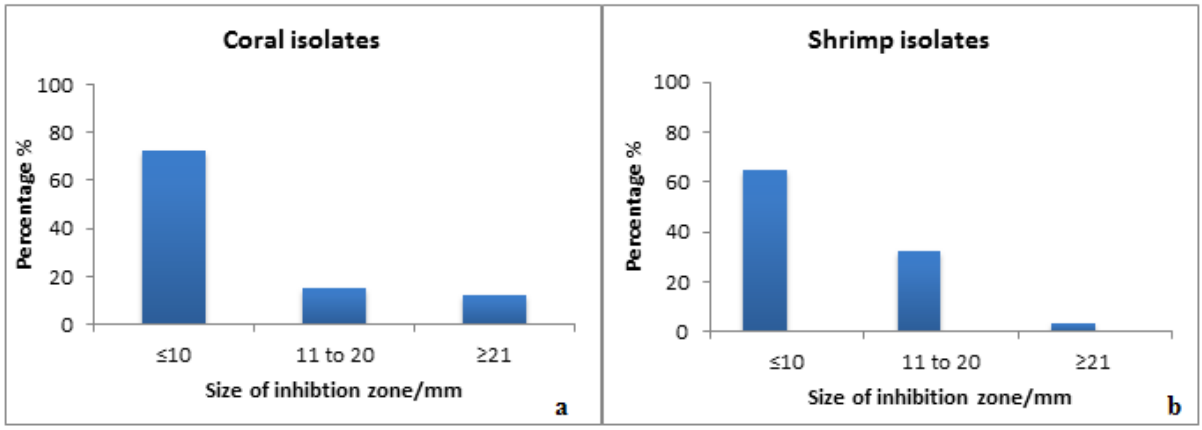


Figure 2.4. Percentage of bacterial isolates showing weak, moderate or strong antagonistic activity against *V. owensii* DY05 in a well diffusion assay. (a) Coral isolates, (b) Shrimp isolates.

2.4.1 **Coral isolates showing antagonistic activity**

Out of the 400 coral isolates from *Turbinaria* and *Acropora*spp., 125 were from healthy coral tissue (H), 57 were from healthy-looking tissue on ASWS-affected corals (HL-ASWS), 89 were from disease margin (M-ASWS) and 129 were from diseased tissue (D-ASWS). In total, 80/400 (20%) DY05-suppressive strains were isolated, with 19/125 (15.2%) from H, 18/57 (31.6%) from HL-ASWS, 20/89 (22.5%) from M-ASWS, and 23/129 (17.9%) from D-ASWS (Figure 2.5).

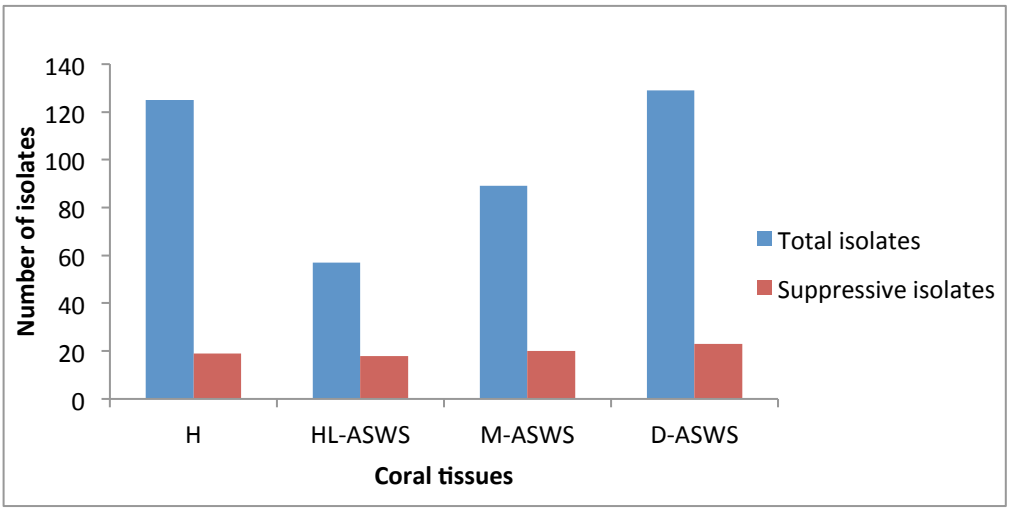


Figure 2.5. Distribution of *V. owensii* DY05-antagonistic isolates originated from coral species. Information is provided on the source of the samples i.e. whether they were obtained from a healthy coral (H), healthy looking tissue on ASWS-affected coral (HL-ASWS), diseased tissue on ASWS-affected coral (D-ASWS).

2.4.1.1 **Shrimp isolates showing antagonistic activity**

Out of the 99 isolates sourced from shrimps of the species *Trypaea australiensis* and their surrounding seawater, 34 were from severely stressed males, 3 from freshly collected males, 42 from severely stressed females and 20 from the surrounding seawater.

All pathogen-antagonistic bacteria identified in this work were from the haemolymph of stressed shrimps (Figure 2.6): 13/34 (38.2%) were from severely stressed male shrimps and 18/42 (42.9%) were from severely stressed females. There were no antagonistic isolates found from freshly collected shrimps (male or females or seawater).

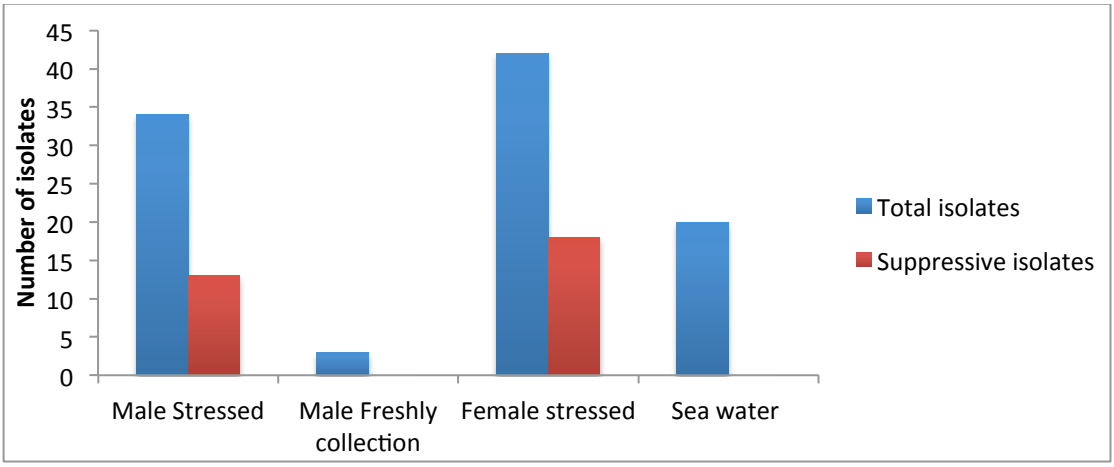


Figure 2.6. Distribution of *V. owensii* DY05-antagonistic isolates originated from shrimp *Trypaea australiensis*. Isolates originated from male and female shrimps that were either stressed,freshly collected or from the surrounding seawater.

2.4.2 Identification of strains

2.4.2.1 Coral isolates

The identity of coral isolates suppressive to *V. owensii* DY05 was determined by sequencing and analysis of the 16S rRNA gene fragment (submitted to BLAST database). RDP classifier was used to classify the bacteria to the genus level and the BLAST to identify species or the closet relative. Antagonistic isolates belonging to 4 classes and 9 different genera were identified, as shown in Figure 2.7.

Gram-negative bacteria comprised 90% of the antagonistic isolates and Gram-positive bacteria 10%. The majority of the isolates were identified as belonging to the classes Gammaproteobacteria within Gram-negative bacteria ((71/80) 88.8% of total antagonistic strains), while other well-represented classes included Bacilli ((5/80) 6.3%), Actinobacteria ((3/80) 3.8%) and single isolate from the class Alphaproteobacteria ((1/80) 1.3%). Within the Gammaproteobacteria, the genus *Vibrio* was the most prevalent, accounting for 50/71 (69.4%) of the isolates. Isolates belonging to genus *Psychrobacter* were also well represented, accounting for 19/72 (26.4%) of the isolates. They were isolated from all different parts of coral tissue similarly to the genus *Vibrio* as summarized in Table 2.1. A single isolate of each of *Photobacterium, Acinitobacter*, and *Pseudovibrio* was identified from bacterial collection from healthy coral.

Within the Gram-positive bacteria the genus *Staphylococcus* was the most predominant, accounting for 4/8 (50%) followed by genus *Micrococcus* 25%. Genus*Bacillus* and *Brachybacterium* represented 12.5 each.

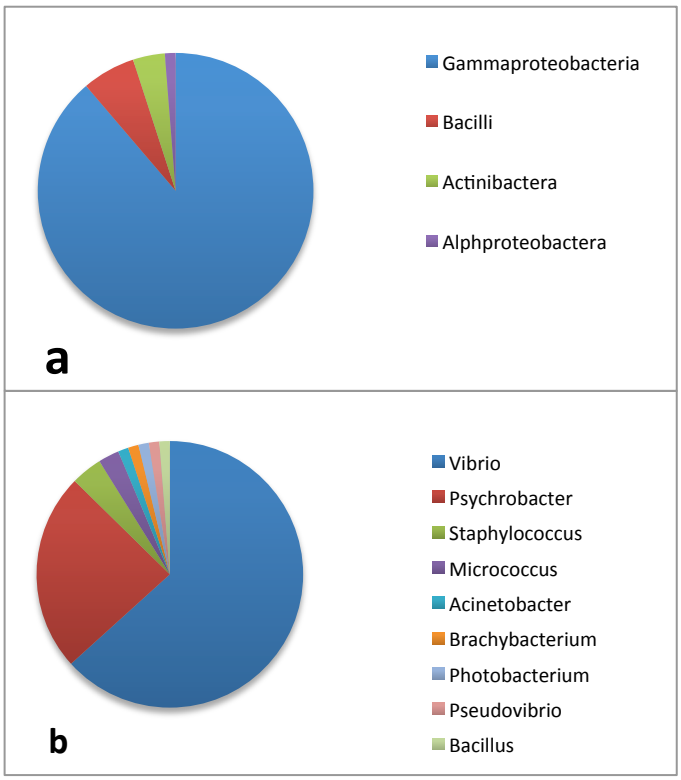


Figure 2.7. Coral Isolates having antagonistic activity against *V. owensii* DOY5 were identified as belonging to 4 major phylogenetic classes (a) and 9 main genera (b).

Table 2.1. The distribution of antagonistic isolates from coral collection identified on the bases of 16S rRNA gene fragment sequencing.

Identification based on 16S rRNA gene sequence	Number of isolates from H	Number of isolates from HL-ASWS	Number of isolates from D-ASWS	Number of isolates from M-ASWS	Total isolates	Percentage of the total isolates
Vibrio spp.	11	8	13	18	50	62.5
Psychrobacter spp.	2	8	7	2	19	23.75
Staphylococcus spp.	3	0	0	1	4	5.0
Micrococcus spp.	0	1	1	0	2	2.5
Bacillus sp.	0	0	1	0	1	1.25
Acinetobacter sp.	1	0	0	0	1	1.25
Photobacterium sp.	1	0	0	0	1	1.25
Pseudovibrio sp.	1	0	0	0	1	1.25
Brachybacterium sp.	0	0	1	0	1	1.25
Total	19	17	23	21	80	

2.4.2.2 Shrimp isolates

The shrimp antagonistic isolates, identified by 16S rRNA gene fragment sequncing, belonged to 3 classes and 6 genera (Figure 2.8). The antagonistic isolates included both Gram-negative and Gram-positive bacteria.

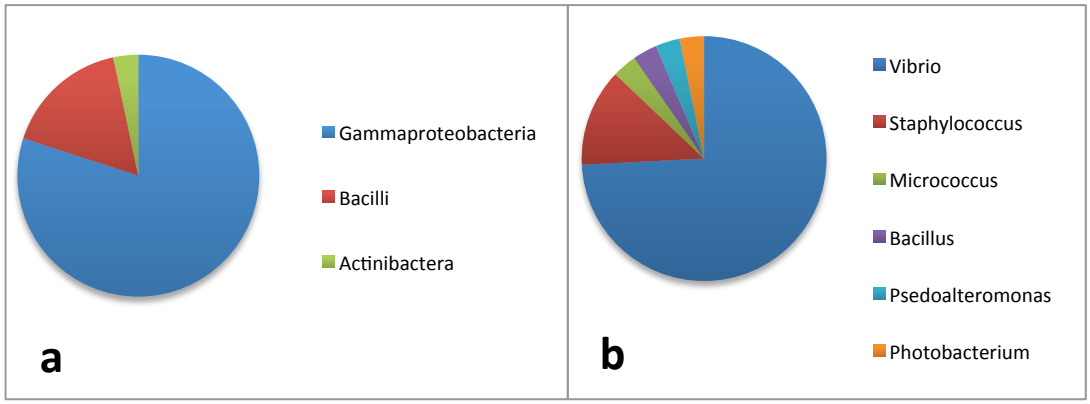


Figure 2.8. Shrimp isolates showing antagonistic activity against *V. owensii* DY05 belong to six genera (b) in three classes (a).

The majority of shrimp bacterialisolates were identified as belonging to the classes Gammaproteobacteria (25/31, 80.7% of a total antagonistic strains), and others were Bacilli (5/31, 16.1%) and Actinibacteria (1/31, 3.2%). Within the Gammaproteobacteria class, on the gene level, the genus *Vibrio* was the most prevalent, accounting 23/25 (92%) of the isolates that were isolated from stressed males and females. One isolate was found belongto the genus *Pseudoalteromonas*, representing 3.2% of the antagonistic isolates. Within the Gram-positive bacteria the genus *Staphylococcus* was the most predominant, accounting for 4/31 isolates (12.9%). Other Gram-positive bacterial isolates belong to the genera*Micrococcus*, *Photobacterium* and *Bacillus,* there was one isolate (3.2%) of each as shown in Table 2.2 and Figure 2.8.

Table 2.2. Antagonistic isolates originated from stressed shrimps.

Identification based on 16S rRNA gene sequence	Number of isolates from MS	Number of isolates from FS	Number of Total isolates	Total percentage
Vibrio spp.	10	13	23	74.19
Staphylococcus spp.	1	3	4	12.90
Micrococcus sp.	1	0	1	3.23
Bacillus sp.	1	0	1	3.23
Photobacterium sp.	0	1	1	3.23
Pseudoalteromonas sp.	0	1	1	3.23
Total	13	18	31	100.01

MS: Male stressed; FS: Female Stressed

2.4.3 Phylogenetic relationships of isolates

The 16SrRNA gene fragments of the bacterial isolates were sequenced and corrected using Chromas 2.1.1 software and submitted to the BALST to determine the identity of the closest relative as shown in Tables 2.3 and 2.4 for the coral isolate and shrimp isolates respectively.

Table 2.3. Isolates from corals exhibiting antimicrobial activity against lobster phyllosoma pathogen *V. owensii* DY05.

Isolate number	Taxonomic Group (Closest 16SrRNA gene BLAST match)	Clearance zone: diameter in mm		% Identity to nearest relative	Nearest accession number
		WDA	DDA		
Healthy coral (H)					
3CC1	*Vibrio* sp.S2-26	10	<10	99%	KM216237
1CC1	*Staphylococcus equorum* strain 179J/2012	10		99%	KM036089
2CC11	*Vibrio mediterranei* strain H025	10		99%	KJ577020
2CC8	*Vibrio coralliilyticus* strain RE98	23		100%	CP009617
2CC7	*Vibrio* sp. VibC-Oc-005	13		99%	KF577010
T2F	*Vibrio* sp.S7-74B	10	<10	99%	KM216245
2CS1	*Vibrio nereis* strain AP508	22		99%	GQ254512
1CS3	*Vibrio* sp. S1653	12	<10	99%	FJ457407
7C(1)	*Vibrio chagasii* strain HNS039	22	<10	99%	JN128273
6C(1)	*Vibrio* sp.S3142	10		99%	FJ457491
9C(4)	*Vibrio* sp. PaH2.24a	10		99%	GQ406770
12C(9)	*Vibrio* sp.S3942	10	<10	99%	FJ457581
1CC4	*Psychrobacter* sp. 2CpBB14	10		99%	JN602232
2CS2	*Psychrobacter piscatorii* strain VSD503	10		100%	KC534182
1CC2	*Staphylococcus* sp. ZWS13	10	<10	99%	KM051419
9C(8a)	*Staphylococcus* pasteuri strain C1PO1	10		99%	JQ689192
7C(6b)	*Acinetobacter* Cai-b1	23	<10	100%	(JX997899
6C(17)	*Photobacterium* sp.h5	22	<10	99%	EF187011
6C(16)	*Pseudovibrio* sp.P1MA3	10	<10	99%	JX477110T
Healthy Looking (HL- ASWS)					
15H(2)	*Vibrio jasicida* (AB562593)	10	<10	99%	AB562593
3.3(14)	*Vibrio coralliilyticus* strain OCN014	10		98%	CP009264
6H(4b)	*Vibrio rotiferianus* strain VSD813	10		99%	KC534399
9HS(1)	*Vibrio* sp. R1M3	10	<10		KC439183
6HS(4)	*Vibrio coralliilyticus* strain OCN014	10		99%	CP009264
9HS(6)	*Vibrio* sp. VibC-OC-067	10			KF576992
4.3(21)	*Vibrio* sp. vibc-co-053	10	<10	100%	KF577062
12HS(3)	*Psychrobacter* sp. SCSWC28	14	<10	95%	FJ461450
9HS(10)	*Psychrobacter celer* strain CUA-856	12		99%	KJ732920
6HS(1)	*Vibrio harveyi* strain 0012KARWAR	10		100%	KC345010
4.3(3)	*Psychrobacter* sp. PM8	23	<10	92%	KC195801

4.3(20)	*Psychrobacter celer* strain 91	21	<10	99%	JF710993
4.3(10)	*Psychrobacterceler* strain 91	10		99%	JF710993
4.3(15)	*Psychrobacter alimentarius* strain N-154	10	<10	100%	KJ735914
4.3(9)	*Psychrobacter* sp. 4Bb	16	<10	99%	HM771253
4.3(19)	*Psychrobacter* sp. PM8	22	<10	97%	KC195801
6H(1)	*Vibrio chagasii* strain HNS039	10		100%	JN128273
9HS(4)	*Micrococcus* sp. 5.22	10	<10	100%	KF681067
Disease coral (D-ASWS)					
15D(5)	*Psychrobacter nivimaris* strain PAMC 27111	10		99%	KJ475192
14D(7)	*Vibriochagasii*	10		99%	HM583983
14D(2)	*Vibrio jasicida*	10	<10	99%	AB562594
14D(3)	*Vibrio* S-3	10	<10	99%	KM201464
12DS(12)	*Vibrio campbellii* ATCC BAA-11	10	<10	96%	CP006606
12DS(3a)	*Vibrio* sp. B8D	10		99%	KF146218
12DS(2)	*Vibrio* sp. S3658	10		99%	FJ457531
6DS(1)	*Vibrio* sp. 2.3.053MS5	10	<10	99%	EU267670
9DS(5)	*Vibrio* sp. VibC-Oc-029	20	<10	99%	KF577114
7D(8)	Uncultured *Vibrio* sp. clone 17H-14	20		100%	KC917724
6DS(8)	*Vibrio* sp. S3942	10		99%	FJ457581
4.2(3)	*Vibrio crassostreae* strain CR-II-12	20		99%	KM014020
4.2(4a)	*Vibrio* sp. S2-26	10	<10	99%	KM216237
4.2(2)	*Vibrio* sp. S2-26	23		99%	KM216237
4.2(8)	*Psychrobacter adeliensis* strain SJ 14	17	<10	99%	NR_104882
4.2(9)	*Psychrobacter piscatorii* strain VSD503	20	<10	100%	KC534182
4.2(10)	*Psychrobacter celer* strain C5B1	10	<10	99%	JX501675
15D(4)	*Psychrobacter nivimaris* strain PAMC 27111	12	<10	99%	KJ475192
9DS(2)	*Psychrobacter adeliensis* strain SJ 14	10		99%	NR_104882
7D(10c)	Uncultured *Psychrobacter* sp. clone CI39	10	<10	99%	FJ695559
1.2(12)	*Brachybacterium* sp. IARI-AB-1	10	<10	100%	JN411299
14.2(4)	Unculturde *Bacillus* sp.clone 264AG8	10		100%	KF836540
4.2(8a)	*Micrococcus luteus*strain UASWS1011	12	<10	100%	KM251315
Disease Margin coral (M-ASWS)					
16M(8)	*Vibrio* sp.ps1	10	>10	100%	EU031648
15M(9)	*Vibrio chagasii* strain CAIM186	10		100%	HM583983
15M(1)	*Vibrio* sp.S2396	10	<10	100%	(FJ457442)
9MS(2)	*Vibrio harveyi*	10	<10	100%	HM771342
9MS(3)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
9MS(4)	*Vibrio* sp. F15C3	10		99%	JX999958
12MS(3)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
6MS(4)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
7MS(8)	*Vibrio* sp. S2-24	10		99%	KM216248
6MS(1)	*Vibrio harveyi* strain CAIM 1792	22		100%	JQ434106
7MS(2)	*Vibrio harveyi* strain CAIM 1792	10		99%	JQ434106
4.1(15)	*Vibrio* sp. VibC-Oc-043	10	<10	99%	KF576999
7MS(12)	*Vibrio jasicida*	10	<10	99%	AB562593
TM(1)	*Vibrio* sp.j21431	10	<10	100%	FJ357689

16M(1)	*Vibrio chagasii* strain HNS039	10		99%	JN128273
15M(4)	*Vibrio* sp.S3661	10		99%	FJ457534
15M(5)	*Vibrio* sp.S7-74B	10		99%	KM216245
16M(3)	*Psychrobacter* sp.189	10	<10	99%	JQ012954
15M(6)	*Psychrobacter* sp.MF2-10b2	10		99%	JF800153
15M(2)	*Staphylococcusxylosus* strain S17	10		99%	KM269001

Table 2.4. Isolates from the shrimp (male and female) *Trypaea australiensis* exhibiting antimicrobial activity against the lobster phyllosoma pathogen *V. owensii* DY05.

Isolate number	Taxonomic Group (Closest 16SrRNA gene BLAST match)	Clearance zone: diameter in mm		% Identity to nearest relative	Nearest accession number
		WDA	DDA		
Shrimp isolates male stressed (MS)					
S6.12.12	*Vibrio* sp.S4074	12		99%	FJ457586
S6.14.14	*Vibrio jasicida*	10		99%	AB562593
S6.5.5	*Vibrio* sp. S7-74B	10		99%	KM216245
S4.6.6	*Vibrio brasiliensis* strain UST010723-012	9		99%	EU834004
SCC6.1	*Vibrio* sp.s2222	10		99%	FJ457425
SCC4.2	Uncultured *Vibrio* sp. clone 2_50	11	<10	99%	KF785711
SCC6.14	*Vibrio hepatarius* strain UST950701-002	10	10	99%	EU834019
SCC16.2	*Vibrio* sp. W-3	9		99%	KC777293
SCC16.1	*Vibrio coralliilyticus*strain ATCC BAA-450	10		99%	NR_117892
S4.2.2	*V. hepatarius* strain UST950701-002	10		99%	EU834019
SCC10.1	*Micrococcus luteus* strain SMR8	10		100%	KF600756
SCC12.14	*Bacillus thuringiensis* strain Dahb4	12	<10	99%	HQ693278
SCC4.1	*Staphylococcus equorum* Subsp.	10		99%	AB975354
Shrimp isolates female stressed (FS)					
S9.2.2	*Pseudoalteromonas sp* NC203	23	11	99%	KF146215
S91.1	*Vibrio* sp.B3K	10	<10	100%	KF146215
S1.9.9	*Vibrio* sp. EJY3 chromosome 1	10		83%	CP003241
SCC2.2	*Vibrio* sp.S3855	10		99%	FJ457563
SCC3.1	*Vibrio* sp.W-13	10		99%	KC777293
SCC1.14	*Vibrio* sp. M-137-19	9		100%	KF746900
SCC.1	*Vibrio* sp. VibC-Oc-012	11		99%	KF576989
CC1.7	*Vibrio* sp. VibC-Oc-012	11		97%	KF576989
CC1.2	*Vibrio harveyi* strain NIOT-sb1	10	<10	99%	KF607055
CC1.1	*Photobacterium damselae* strain SBTB24	10		75%	JX028545
SCC3.14	*Vibrio* sp.BBT47	10	<10	99%	FJ981869
SCC7.14	*Vibrio rotiferianus* strain KM30-12-2	10		99%	JQ670739
SCC2.34	*Vibrio azureus* strain S-41	10		99%	JF412237
SCC2.24	*Staphylococcus equorum* strain BPt-5	9		90%	KJ865579
CCS.1	*Staphylococcus* sp. XQW7	11	<10	99%	KM224521
CC5.1	*Vibrio* sp. VibC-Oc-043	10		100%	KF576999
SCC1.24	*Staphylococcus* sp. XQW7	10		99%	KM224521
SCC6.11	*Vibrio* sp. S2222	10		99%	FJ457425

2.5 Discussion

Vibrio spp. are important bacterial pathogens and the pathogenic effects of certain *Vibrio* spp. are critical for animals reared in aquaculture (Bergh *et al.*, 2001; Samuelsen *et al.*, 2006; Thompson *et al.*, 2004; Toranzo *et al.*, 2005). Members of the *Vibrio* genus may be commensal or symbiotic with different eukaryotic host (Thompson *et al.*, 2004). *Vibrio* species are the most common pathogen and constitute a large percentage of the microbial community associated with cultured larval invertebrates including *P. orantus* (Bourne *et al.*, 2004; Diggles *et al.*, 2000; Payne *et al.*, 2007; Webster *et al.*, 2006).

However, previous studies have suggested that some *Vibrio* species may be used (perform) as biocontrol agents in aquaculture, reducing the need for antibiotics and reducing effluent discharges (Douillet, 2000; Thompson *et al.*, 2003; Vaseeharan & Ramasamy, 2003). Isolation of bacteria that produce inhibitory substances has led to the identification of probiotics for potential use in aquaculture systems (Irianto & Austin, 2002). Antagonistic activity is considered as an important selection criterion for probiotic candidates.

To identify antagonistic bacteria from a culture collection of corals and shrimp two screening assays were used in present study, a well diffusion assay and disk diffusion assay. The well diffusion assay identified a much greater number of bacterial isolates as having probiotic activity, compared to the disk diffusion assay. The well diffusion assay was therefore considered as a more efficient tool for initial screening for antimicrobial production. A possible explanation for the difference between the two assays is that the disk diffusion assay was carried out using filtered supernatant, while the well diffusion assay was carried out using whole culture.The concentration of antibacterial compounds in the filtered supernatant may not be high enough to inhibit the pathogen or produce the same inhibition zone compared with the well diffusion assay as not all the active molecules pass through the filter. On the other hand, the bacteria continuously produce antagonistic substances on an agar plate. In addition it was noticed during the experiment that the antagonistic activity was increased with the presence of pathogen in the liquid culture. Similar results have been documented in previous studies with different explanations for the size of inhibition zones in disk diffusion assay in comparison with other assays. For example, in a study of the antagonistic activity of bacteria and marine fungi by Miao & Qian (2005) two different assays including an antifungal and disk diffusion assay were used. Based on their resultsthey

suggested that bacteria may only produce active antifungal compounds when cultured with target microorganisms on agar plates.Also, not all antimicrobial compounds may be released into the media and therefore may not be present in the filtered supernatant.However, they attributed a small inhibition zone produced using disk diffusion assays to the physical effects of the bacterial extracts and suggest it may not be due to the presence of antimicrobial compounds. It may also be due to the ability of active compounds on the paper disc to diffuse through the agar medium. This may affect the size of inhibition zone produced by the disk diffusion assay, thus leading to a possible underestimation of antimicrobial activity. In another study by Long & Azam (2001) they suggested that the inhibition may not be due to antimicrobial activity, it might be due to aphysiological reaction or bacterium-bacterium communication such as quorum sensing. Shnit-Orland & Kushmaro (2009) also found that whole cultures, but not cell-free supernatants, demonstrate antibacterial activity and raised the possibility that active bacteria may not necessarily secrete compounds to the environment, but may instead be active through alternative mechanisms. Long & Azam (2001) suggested that the percentage of strains identified as antibiotic-producing can be affected by the assay method as well as the species and the number of indicator microbes used in the screening.

In the study of chemical ecology of marine epibiotic bacteria by Brugress *et al.* (1999) they explained the deficiency of the disk diffusion assay in screening of antimicrobial compounds due to a reaction occurring between the chemical components of filter paper and the chemical structure of the antimicrobial compounds. Therefore, they suggested that using different assays to screen for antimicrobial compounds may increase the number of compounds to be discovered.

In brief there are different explanations of the disk diffusion assay efficiency of diagnosing antimicrobial activity of antagonistic bacteria compared with other antimicrobial identification assays. Therefore more than one assay should be used to increased diagnosis of a greater number of antagonistic isolates.

Marine invertebrates present good sources of microorganisms for screening for antimicrobial activity. Bacteria associated with living surfaces and/or particle-associated bacteria displayed a higher degree of antagonistic activity than free-living bacteria (Gram *et al.*, 2010; Hjelm *et al.*, 2004b; Long & Azam, 2001; Long *et al.*, 2005; Miao & Qian, 2005; Nair & Simidu, 1987). The current study was focused on isolation and identification of probiotic bacteria from a coral and shrimp collection. Previous studies found antibacterial activity among and

between coral-associated bacteria, in particular the microbial communities associated with the mucus of healthy corals (Chen *et al.*, 2012; Gantar *et al.*, 2011; Ritchie, 2006; Rypien *et al.*, 2010). Therefore, coral associated bacteria present likely candidates to be screened for antimicrobial production (Chen *et al.*, 2012; Rypien *et al.*, 2010). Godwin (2007) concluded that antimicrobial compounds are produced by the symbiotic bacteria community associated with living tissue of *T. mesenterina* which regulate bacterial densities and inhibit the growth of invasive bacteria, thereby reducing the total number of culturable bacteria in his study on coral disease.

Antagonism is a widespread attribute implicated in the competiveness and ecological success of many aquatic microbes. It is thus considered an important trait of aquaculture probionts (Fjellheim *et al.*, 2007; Goulden, M. Hall*, et al.*, 2012; Gram *et al.*, 2010). Several studies (Burgess *et al.*, 1999; Slattery *et al.*, 2001; Trischman *et al.*, 2004) suggest that antagonism plays a critical role in preventing invasion of bacteria, including potential pathogens. Likewise, the surfaces of the healthy embryos of the lobster *Homarus americanus* are covered almost exclusively by a single Gram-negative bacterium, that produces an antifungal compound highly effective against the fungus *Lagenidium callinectes*, a common pathogen of many crustaceans (Gil-Turnes & Fenical, 1992). In the current study 22.2% of all strains in the culture collections were shown to have antagonistic activity against lobster phyllosoma pathogen *V. owensii* DY05. 72.1% of these strains were from the coral collection and 27.9% from the shrimp collection. This is in agreement with Ritchie (2006), who found that almost 20% of the cultured bacteria from *Acropora palmata* coral in the Caribbean displayed suppressive activity, including towards the causative agent of white pox disease. A study using well-diffusion assays showed that 10 of the 104 isolates (9.6%) released antimicrobial molecules into culture supernatants (Wilson *et al.*, 2010). Burgess *et al.* (1999) found that 35% of surface-associated bacteria isolated from various species of seaweed and marine invertebrates produced antimicrobial compounds. Kelmanet al. (2006) and Motta *et al.* (2004) also demonstrated that the majority (83% and 70% respectively) of marine bacterial species tested exhibited appreciable antimicrobial activity against one or more indicator bacteria.

Most of the antagonistic bacteria identified in the current study were Gram-negative bacteria, while fewer Gram-positive bacteria showed antagonistic activity. This observation is consistent with a previous studies (Lo Giudice *et al.*, 2007 b; Moriarty & Hayward, 1982).

This result may beexplained by the prevalence of Gram-negative culturable bacteriain these aquatic environments (Lo Giudice *et al.*, 2007 b). Fjellheim *et al*. (2010) found only Gram-negative bacteria to have antagonistic properties when attempting to isolate probiotic bacteria with antagonistic activity against *Vibrio anguillarum*. In contrast Lauzon *et al.* (2008)found that 81% of the antagonistic bacteria obtained from cod rearing systems were Gram-positive. Grossart *et al.* (2004) attributed the reason for the predominance of Gram-positive bacteria in the study carried in German Wadden Seamay be related to specific features of the Wadden Sea environment.

It has been known for decades that marine microorganisms produce antibacterial compounds.The current and previous studies (Fjellheim *et al.*, 2007; Grossart *et al.*, 2004; Long & Azam, 2001) have shown that a range of marinebacterial genera produce antimicrobial compounds in *in vitro* tests.We isolated and identified bacteria antagonistic to *V. owensii* DY05 using the well diffusion assay in culture media from four different coral species including 19 isolates of H-ASWS, 17 isolates of HL-ASWS, 23 isolates of D-ASWS and 21 isolates of M-ASWS (Table 2.1). The most abundant antimicrobial-producing bacteria isolated from coral samples were members of Vibrionaceae and Moraxellaceae families belonging to the class Gammaproteobacteria 88.8% of total isolates. Long and Azam (2001) also reported that bacteria belonging to the Gammaproteobacteria (Alteromonadales and Vibrionales) were the most prolific producers of inhibitory substances. In a study on intestinal samples from marine fish all the bacteria found to have inhibitory activity were *Vibrio* species (Makridis *et al.*, 2005).

On the other hand, we found that the shrimp bacterial isolates exhibiting the highest levels of antimicrobial activity belonged to class Gammaproteobacteria and class Bacilli. *Vibrio* were the predominant genus frommale and female stressed shrimps shrimp isolates (74.2% and 92% of total isolates), which is not surprising considering that many of these isolates were selected on TCBS medium, a selective medium for the growth and identification of *Vibrio* (Goulden, 2016).

This results is similar to most earlier studies, that revealed that Gammaproteobacteria are the predominant producers of antimicrobial compounds among marine bacterial isolates (Fjellheim *et al.*, 2007; Long & Azam, 2001). Romanenko *et al.* (2008) also found that most antagonistic bacteria were members of the Gammaproteobacteria and Alphaproteobacteria, and less with Firmicutes, Actinobacteria, and Cytophaga-Flavobacterium-Bacteroides group. Members of the genera Psychrobacter and *Pseudoalteromonas* were found to dominate the microbiota of all shrimp samples regardless of processing procedures or storage conditions (Broekaert *et al.*, 2013). Bacteria belonging to Bacillus and Actinobacteria groups, as well as those isolated from marine environment, are well known for their ability to produce a wide range of antimicrobials and other secondary metabolites (Romanenko *et al.*, 2008).

The results of the current study confirm that shrimp and coral associated bacterial communities represent good sources of antagonistic bacteria. Such strains, particularly *Pseudoalteromonas*sp. Strain 80 may be promising for use as probiotics in lobster hatcheries. The genus *Pseudoalteromonas* is a marine group of bacteria belonging to the class Gammaproteobacteria that has come to attention in the natural product and microbial ecology science fields in the last decade. Pigmented species of the genus have been shown to produce an array of low and high molecular weight compounds with antimicrobial, anti-fouling, algicidal and various pharmaceutically-relevant activities. Compounds formed include toxic proteins, polyanionic exopolymers, substituted phenolic and pyrolle-containing alkaloids, cyclic peptides and a range of brominesubstituted compounds (Bowman, 2007).

Three strains that gave strong antagonistic activity against *V. owensii* in well diffusion assay were renamed and chosen for further work (Table 2.5), as one aim of this study was to choose potential probiotic bacteria from culture collections (coral and shrimp isolates). Different antagonistic bacteria produce antimicrobial compounds at different times when mixed with pathogen. The chosen strains showed strong inhibition zones after 6 hours of incubation.

Table 2.5. The isolates selected for future work and their new names following 16S rRNA gene fragment sequencing.

Original name	New name	Source
S9.2.2	*Pseudoalteromonas* sp. strain 80	Stressed female shrimp
9DS(5)	*Vibrio* sp. strain 34	Diseased region on ASWS-affected coral
4.2(9)	*Psychrobacter* sp. strain 62	Diseased region on ASWS-affected coral

The mechanisms used by the antagonistic bacteria isolated in this study to suppress the pathogen are unknown and were further investigated as presented in Chapters 3-4.

Biochemical characterization of high and low molecular weight substances from antagonistic marine bacteria with potential antibacterial properties

3.1 Abstract

Marine pathogen-suppressive bacterial strains were isolated from cultures established from coral and shrimp using the well and disk diffusion assay as described in Chapter 2. Specific antagonistic isolates that suppressed the lobster phyllosoma pathogen *Vibrio owensii* DY05 were selected for further characterization. The three strains are *Pseudoalteromonas* sp. strain 80, *Vibrio* sp. strain 34 and *Psychrobacter* sp. strain 62 of the Gammaproteobacteria (Chapter 2). The strains were selected due to their relatively high activity among the isolates included in the present study. Further, the strains were selected from either infected shrimp or coral and belonged to either a different species or genera. The strains were cultured through late logarithmic and/or early stationary phase (24 hours) and the culture supernatants were subjected to partial purification, selecting particularly for the fractions with pronounced proteolytic activity. Furthermore, these strains were subsequently screened for antimicrobial molecules with activity related to extracellular enzyme secretion, including protease and amylase activity. It was demonstrated that the three strains are synthesizing and secreting macromolecules with antimicrobial activity against the lobster pathogen. Such diffusible antimicrobial macromolecules were also capable of *in vitro* hydrolysis of casein, gelatin and starch substrates incorporated into agar plates. Several methods were used to extract, concentrate, identify and characterise these macromolecules. The concentrated supernatant that exhibited good proteolytic activity against skim milk was further tested against casein and gelatine embedded polyacrylamide gels using zymogram electrophoresis. Concentrated supernatants that showed strong hydrolysis against milk powder on agar plates also displayed a multiple-bands profile in casein zymogram electrophoresis implicating casein as the protein hydrolysed in the milk powder agar gel reaction. Molecules with molecular weights ranging from 10 kD to 100 kD were partially biologically characterised, with demonstration of anti-vibrio and proteolytic activity. With regard to the proteolytic activity, an optimal growth temperature of 28 °C and optimal pH between pH 7.0 – pH 9.0 was observed for the strains tested, which again correlates with optimal conditions for anti-vibrio activity. The proteolytic activity is therefore strongly implicated as an important mediator of anti-vibrio activity.

The three bacterial strains were further examined for secretion of low-molecular weight molecules that could be implicated in the observed antimicrobial activity, where the methods

of chemical analysis detected both volatile and non-volatile compounds. Lipophilic organic compounds were extracted from filtered supernatants of the bacterial cultures using a series of steps employing organic phases for solvent interface partitioning. The volatile fraction of the subsequent residue was characterized using gas chromatography-mass spectrometry (GC-MS). The studied strains were found to uniformly produce the same linear alkenes with sizes ranging from C11 to C22, including 1-dodecene, 1-tetradecene, 1-hexadecene, 1-octadecene, 1-eicosene and 1-docosene. The same extracted residue was resuspend in acetone and run on a TLC plate in duplicates: one stained with potassium permanganate and the other for bioautography to study antimicrobial activity of separated individual active compounds. Several fractions were shown in TLC plate, some of these fractions showed antimicrobial activity against the pathogen in overlay assay bioautography. The three strains included in this study are suggested to be promising potential probiotic candidates for use in aquaculture industries.

3.2 Introduction

Living organisms, including animals, plants or microorganisms, may produce commercially or industrially important natural products via primary or secondary metabolism (Demain & Sanchez, 2009). Such metabolites are favourably selected for in the course of adaptation to various environmental extremes, providing therefore biochemical or structural features of particular relevance to the organism's niche (Vázquez *et al.*, 2008). Compared with terrestrial microorganisms, marine organisms synthesise and secrete a range of secondary metabolites, most of which are structurally distinct with many more awaiting characterisation, therefore providing impetus for future research (Radhika *et al.*, 2014; Schwartsmann *et al.*, 2001).
Many antimicrobial macromolecules synthesized by microorganisms have already been isolated and reported (Borchmann *et al.*, 2014; Gómez *et al.*, 2008; Tashiro, 2001). Protein itself is the major macromolecule in bacterial cells and constitutes over half of the dry weight (Kirchman *et al.*, 1985).

Marine bacteria frequently excrete extracellular enzymes that facilitate hydrolysis or 'digestion' of ambient macromolecules as nutrient sources (Vázquez *et al.*, 2008). Such extracellular enzymes are strongly represented by the proteases, being the single class of enzyme that mediates the hydrolysis of protein aggregates or peptides by cleaving peptide bonds (Häse & Finkelstein, 1993; Rao *et al.*, 1998). This class includes the aspartate proteases, serine proteases, cysteine proteases and metalloproteases (Häse & Finkelstein,

1993; Rao *et al.*, 1998). Although various proteases of a wide range of specificities can be sourced from plants or animal organs, by far the major source is from microorganisms (Gupta *et al.*, 2002; Kumaran *et al.*, 2012; Sevinc & Demirkan, 2011; Verheijen *et al.*, 1997).

Proteases are one of the most important categories of industrial scale enzymes with commercial proteases making up nearly 60% of the total industrial enzyme demand (Fulzele *et al.*, 2011; Kasana *et al.*, 2011; Rao *et al.*, 1998; Sevinc & Demirkan, 2011; Swamy *et al.*). Proteases are widely employed in leather processing, the food industry, the detergent industry, the bioremediation process, in waste-processing companies, the textile industry, the pharmaceutical industry and even in the film industry (Sevinc & Demirkan, 2011).

Several aquatic microbes are known to synthesise proteases of importance to the biomedical industry (Fulzele *et al.*, 2011). Not only are proteases essential for bacterial growth and proliferation, they are also known to contribute to bacterial virulence. Therefore, proteases are good candidates as diagnostic and indeed therapeutic agents for prevalent infectious diseases (Kaman *et al.*, 2014). Furthermore, research on bacterial proteases and substrates has allowed the development of new compounds for use in proteasein hibition, to reduce the destructive action of the proteases secreted by bacteria during clinical infection (Drag & Salvesen, 2010; Zindel *et al.*, 2013).

Natural substrates, such as skimmed milk, gelatin, fibrin, caseinandelastin, are currently used in the measurement of *in vitro* protease activity. Furthermore, such assays can be adjusted to incorporate the environmental variables affecting protease activity in cleavage of particular peptide bonds. These factors include temperature, pH, and protease stimulators/inhibitors existing in the culture media (Kaman *et al.*, 2014).

Several studies have already described antimicrobial proteins from marine microorganisms (Gómez *et al.*, 2008; Kelecom, 2002). For example, many strains of *Pseudoalteromonas* produce macromolecules with potential antimicrobial action, however the specific nature of these macromolecules has not been defined to date (Gómez *et al.*, 2008). Be this as it may, McCarthy *et al.* (1994) reported that proteolytic activity was prevalent across different *Pseudoalteromonas luteoviolacea* strains.

At the frontier of antimicrobial research, volatile compounds in general might represent a new and powerful source of antibiotics (Romoli *et al.*, 2011). Although such volatile compounds are best known from the plant kingdom, during growth microorganisms also produce an enormous variety of volatile organic compounds; mostly as secondary metabolites against competitors and adversaries, or as quorum sensing (Fernando *et al.*, 2005; Kai *et al.*, 2007; Mackie & Wheatley, 1999; Ryu *et al.*, 2004). In most studies, usually lipophilic VOCs are described, conferring advantages to the respective organism whilst in the gaseous phase. However, those secreted by marine organisms may be expected to be relatively polar, conferring advantages to the organism as solutes.

In the current study specific antagonistic bacterial strains able to inhibit the lobster pathogen *V. owensii* DY05 were isolated from coral tissue and shrimp heamolymph (Chapter 2). Although many pathogen-suppressive bacteria were isolated, only three strains that exhibited strong antimicrobial activity against the pathogen were chosen for further biochemical analysis. The three strains are *Pseudoalteromonas* sp. strain 80, *Vibrio* sp. strain 34 and *Psychrobacter* sp. strain 62 of the Gammaproteobacteria (Chapter 2). The current study includes purification, isolation and partial characterisation of proteins synthesised by these bacterial strains, focusing on proteins with antimicrobial activity as well as analysis of small molecules using methods that employ volatile and non-volatile phases.

3.3 Material and Methods

3.3.1 Bacterial strains

Three antagonistic bacterial isolates that showed strong antimicrobial activity against *V. owensii* DY05 in the well diffusion and disk diffusion assay (Chapter 2) were selected for further characterisation in this chapter. These strains included *Pseudoalteromonas* strain 80; *Psychrobacter* strain 62 and *Vibrio* strain 34.

3.3.1.1 Sample preparation for the identification of antimicrobial active molecules

Bacterial strains were cultured in marine broth (MB 2216 Difco), at 28 °C for 18-24 hr. Cultures were centrifuged at 14,000 rpm for 30 min at 4 °C and the supernatants collected and filter sterilised through a 0.2 µm filter (Sarstedt) (will be referred to as "filtered supernatant"). Bacterial cell pellets were homogenised by sonicationfor three min with 30 sec

cyclesat 2 ampere (cell disruptor). Filtered supernatants and homogenised cells were tested against the target pathogen*V. owensii* DY05 in a well diffusion assay as previously described (Chapter 2, section 2.4.2 - coral isolates).

3.3.2 Concentrated antimicrobial compounds

Filtered supernatant was concentrated using several methods in order to characterise and identify diffusible antibacterial substances. Respectively, the supernatant was freeze-dried, dialyzed, biochemically purified, concentrated and fractionated with organic solvents. The details are given in the following sections.

3.3.2.1 Dialyzed supernatant

The filtered supernatants of the three strains were dialyzed using a semipermeable membrane such as a Colloidon (Thermo Scientific) membrane to separate proteins from smaller molecules and salts, following a protocolmodified from Al-Akl *et al.* (2012). The filtered supernatants were dialyzed against 0.1 M calcium chloride ($CaCl_{2)}$ and deionized distilled water with Spectra/Por 1 dialysis tubing (3000 molecular weight cut off) for 24 hr and the distilled water or calcium chloride was changed three timesand then the dialysate freeze-dried. The freeze-dried powder was extracted in ten volumes (w/v) of 0.1 M phosphate pH 7 buffer for antimicrobial activity testing.

3.3.2.2 Freeze dry method

The filtered supernatant and deionized supernatants were frozen overnight at –70 ^{o}C and immediately dried in a freeze dryer (Dynavac) at -50 ^{o}C for four hr to overnight. The lyophilised supernatants were resuspended in 0.1 M sodium phosphate buffer pH 7 (0.1 M Na_2HPO_4 and 0.1 M Na_2HPO_4).

3.3.2.3 Biochemical purification

Filtered supernatants were concentrated by passage through 3000 NMWL membrane filters (Amicon ULTRA^{-4}) according to the manufacturer's instruction. Samples of 4ml volumes were centrifuged (Beckman Coulter) at 4000 xg for 20 minutes. The supernatant was recovered by pipetting.

3.3.2.4 Concentration of active substances using organic solvents

Organic solvent extraction was performed according to Sakata et al. (2007), with modifications as describedbelow. Approximately 100 ml of supernatant was collected after centrifugation of bacterial culture at 14,000 rpm for 15 min, and then extracted with 10 ml of ethyl acetate (EtAc). The EtAc fraction was evaporated and the residue was resuspended in 30 ml of deionised water (ddH_2O). The resulting solution was further extracted with 10 ml of chloroform ($CHCl_3$). The chloroform fraction was also evaporated and the residue was resuspended in 2-3 ml of methanol.

3.3.3 Antimicrobial related activities

The following tests, related to antimicrobial activity, were performed on whole bacterial cultures, filtered supernatants and concentrated filtered supernatants, prepared using the methods described above.

3.3.3.1 Protease activity against skim milk (skim milk hydrolysis)

Protease activity against skim milk was performed according to a modified protocol fromSyngkon et al., (2010) and for standard casein the protocol fromVijayaraghavan & Vincent (2013) was used. In brief, 50 µL of the whole bacterial cultures, the filtered supernatants or the concentrated supernatants (aqueous) was dispensed into the wells in minimal medium (MMA, described in Section 2.3.2) supplemented with either 1-5% skim milk or 1% casein medium (well diffusion assay). The plates were incubated at 28 ^{o}C for 24-72 hr and checked periodically for hydrolysis of milk or casein. The casein-supplemented medium was flooded with Bromocresol green (BCG) reagent (was prepared by dissolving 0.56% (w/v) succinic acid, 0.1% (w/v) NaOH and 0.028% (w/v) BCG dye. To this reagent, 1% Brij-35 (polyoxyethylene lauryl ether) was added. The pH of the solution was adjusted to 4.15±0.01. This reagent was stored in brown bottle at refrigerator until further use). The reagent was incorporated with casein in the minimal media and gave an opaque color to the medium. The development of a zone of transparency around the well indicated proteolytic activity.

3.3.3.2 Amylase activity against starch (starch hydrolysis)

The three strains were screened for amylase production according to a protocol slightly modified from that described by Jacobs & Gerstein (1960); 50 µL of filtered supernatants or

whole bacterial culturedispensed into the wells on marine agar supplemented with 1% soluble starch in a well diffusion assay. The plates were incubated for 48 hr at 28 °C. After that the culture plates were flooded with 1% Lugol's iodine solution. A clearing zone surrounded by a blue-black starch/iodine reaction indicated positive results.

3.3.3.3 Gelatinase activity against gelatine (gelatine hydrolysis) unexpectedly detected by the Lugol iodine solution

Marin agar supplemented with 4% gelatin (gelatin containing medium), without starch, were also flooded with the 1% Lugol's iodine solution (although this reagent is usually designed to detect starch and is not known to be used to detect gelatin) as described above (instead of the indicated 7.5% $HgCl_2$). Clearing zones against a brown background indicated proteolytic activity.

3.3.3.4 Zymography - enzyme activity assay (Gelatine and Casein hydrolysis)

Substrate-incorporated polyacrylamide gel electrophoresis (zymography) was used to separate and characterize the individual proteolytic enzymes produced by the three antagonistic strains. Ready Gel precast gel (BioRad) was used according to the manufacturer's instructions. Briefly, 25 µL of concentrated supernatants of the antagonistic strain sample was diluted in 15 µl of sample buffer (25% Glycerol, 62.5mM Tris-HCl, pH 6.8, and 0.01% Bromophenol Blue) and the mixture was loaded onto a precast gel. Electrophoresis was run at constant current 20 mA, 100V in 1x running buffer (25mM Tris base, 192mM glycine 0.1% SDS) for 90min or until the tracking dye had migrated to the bottom of the gel. Precision Plus protein kaleidoscope pre-stained standard (BIO-RAD) was included on each gel. Following electrophoresis, gels were incubated in denaturing solution (2.5% Triton X-100) for 30 min at room temperature. Gels were then incubated in development solution (50mM Tris base, 200mM NaCl, 5mM $CaCl_2$ (anhydrous) and ddH_2O) at 37^{o}C for a minimum of 4 hr (highest sensitivity is typically achieved with overnight incubation). Gels were stained with Coomassie Brilliant Blue R-250 staining solution for at least 1hr at room temperature. Gels were de-stained until clear bands appeared against the blue background.

3.3.4 Protease assay and effect of pH and temperature on activity of proteases

Proteolytic activity was assayed following the digestion of azacasein. 400 µL of 1% w/v azacasein added to 400 µL of filteredsupernatant was incubated at 0.1 M Tris/ HCl buffer (pH 8) and 0.5 mM of $CaCl_2 \bullet 2H_2O$ at 20 ^oC for 30 min, andthe reactionwas stopped by adding 800 µL of trichloroacetic acid to a final concentration 5%. After that, the sample was centrifugation at 14000 rpm for 5 min and the absorbance of the supernatant was measured at 340nm. Samples were assayed in triplicate. The effects of pH on protease activity of the filtered supernatants were determined by using the protease assay mention above at different pH ranges. The optimum pH was determination using the following buffer system at 20 ^oC (0.1 M each): Tris/HCl (pH 8-9), KH_2PO_4/Na_2HPO_4 (pH 5-7) and $NaHPO_4/NaOH$ (pH 10-12) (Vazquez & Mac Cormack, 2002).

For determination of the stability of the substances responsible for pathogen growth suppression and proteas activity the flittered supernatants of the studied strains were incubated at different temperatures include 16 ^oC, 28 ^oC, 37 ^oC, 60 ^oC, 80 ^oC and 100 ^oC, for 10 minute at each temperature. Then, the treated supernatants were tested for hydrolysisof milk casien on MMA seeded with pathogen in well diffusion assay (Sakata *et al.,* 1982).

3.3.5 SDS-PAGE separation of protein extracts from bacterial supernatant

According to the protocol modified from Shipp (2011), denatured concentrated supernatant proteins were separated using Mini protein Precast 4-15 % polyacrylamide gels (BIO-RAD). Briefly, 10 µL of each sample was mixed with 15µL of 3.75x sample buffer (10%w/v glycerol, 5%w/v mercaptoethanol and 2.3% w/v sodium dodecyl sulphate (SDS) ddH_2O. Samples were boiled for five min then cooled immediately on ice to avoid any remaining protease activity. Samples were loaded onto the precast gels and electrophoreses performed with 1x running buffer (14.4% glycerol, 3.0% Tris and 1.0%SDS all w/v) at 100V and 20 mA per gel for 90 min or until the tracking dye had moved to the end of the gel. Precision plus protein kaleidoscope prestained standards (BIO-RAD) were included on each gel. Following electrophoresis, gels were stained overnight with Coomassie Brilliant Blue R-250 staining solution. Following staining, gels were washed several times with destain solution (40% methanol, 10% acetic acid, 50% distil water) until dark protein bands appeared.

The major bands observed in the SDS-PAGE separation of supernatants were excised manually from the gel and analyzed using an ORBI-PROC instrument at the University of New South Wales (UNSW) by Dr Anne Poljak. Tryptic peptide sequences derived from these excised bands following trypsin proteolysis were determined using LC-MS analysis as described by (Coumans *et al.*, 2009).

3.3.6 Determination of the antimicrobial protein pattern by Native PAGE

The concentrated supernatants were further subjected to analysis on Native PAGE according to the manufacturer's instructions, using 10% Tris-HCl gel, in order to study the microbial activity of individual bands. 10 µL of each samples were mixed with 15 µL of sample buffer (62.5 mM Tris-HCl, pH8, 25% glycerol and 1% bromophenol blue) and loaded onto the precast gels. Electrophoresis was performed with 1x running buffer (25mM Tris, 192 mM glycine) at a constant current of 20 mA per gel and 100V, at room temperature in a Mini-Gel Electrophoresis Unit. After electrophoresis, the gels were overlaid with minimal medium seeded with 1% of *V. owensii*DY05 pathogen (a layer of approx. 1-2 mm). The overlaid gel was then incubated at 28^{o}C for 24-48hr and the result was examined visually for zones of pathogen clearing on the overlaying medium.

3.3.7 Extraction and identification of small molecular weight compounds

Four hundred (400) mL of three-day old cultures of the respective organism were centrifuged at 14000 rpm for 15 min to remove organisms. The supernatant was filter sterilised, and then washed with an equal volume of ethyl acetate. The organic phase was separated and evaporated in a rotary evaporator. The residue was resuspended in 40 mL of deionised distilled water then washed with an equal volume of chloroform. For identification of volatile compounds, the organic phase was examined undiluted using Gas-Chromatography Mass Spectrometry (GC-MS).

3.3.7.1 Gas Chromatography Mass Spectrometry (GC-MS)

Chemical analyses of volatiles were performed using an Agilent Technologies 7890A GC-System adapted to an Agilent 5975C mass selective detector (insert MSD with triple-Axis detector). This system used an autosampler (Agilent Technologies 7693 – 100 positions), programmed to make 1 µL injections into a split chamber. Using a HP-5MS Agilent column

(30m X 250µm X 0.25µm) the separations were achieved under the following operating conditions: Injector split - ratio 25:1; injector temp - 250˚C; carrier – helium; flow rate - 1.0 mL/min, constant; column temp conditions, 60˚C (no hold), 5 ˚C per minute then @ 250 hold for 15 minutes. MS -70 eV; mass scan range of 30 – 400 m/z.

First identifications were from comparison to an electronic library database (NIST08). Identifications were confirmed by comparing temperature programmed retention indices (AI) (IUPAC, 1997) with published values. Identifications were reinforced by comparison with a second and third library (Adams, 2007; NIST, 2011). Quantification merely used GC-MS operating software, calculated from area under the curve using data with a peak area above 0.1%.

3.3.7.2 Thin-layer chromatography (TLC) and Bioautography

Five microlitres of chloroform-extracted supernatant was loaded onto aluminum backed silicon TLC plates (Merck kieselgel 60 F254) in duplicate. The solvent system was developed with a mobile phase of 50% Hexane, 25% Acetone, and 25% Ethyl acetate (2:1:1, v/v). The solvent chamber was allowed to equilibrate for 30min before plates were inserted. Following separation, the plates were air dried for 3hr to allow solvent to completely evaporate prior to overlay with the target pathogen in agar. One plate was stained using Potassium Permanganate (1.5 g of $KMnO_4$, 10g K_2CO_3, and 1.25 mL 10% NaOH in 200 mL distilled water) and dried and viewed in normal light. The other plate was used for bioautography. The bioautography was prepared in sterile conditions with *V. owensii* DY05 as the target organisms overlay in agar (1% w/w). Minimal medium0.3% agar was seeded with 40 mg/L p-iodonitrotetrazolium dye then inoculated with the test organism. Seeded agar was solidified over the TLC plate at a thickness of not more than 3mm and incubated at 28^{o}C for 20 hr. Zones of inhibition appeared as clearing zones on a red background and were matched to compounds visualized on the TLC plate stained with potassium permanganate (Sadgrove *et al.*, 2013).

3.4 Results

3.4.1 Production, purification and characterisation of antimicrobial substances

The results of preliminary screening for pathogen suppressive isolates indicated that the more active strains included *Pseudoalteromonas* sp. strain 80; *Psychrobacter* strain 62 and *Vibrio* strain 34. To isolate and partially characterise the active antimicrobial compounds from the pathogen-suppressive bacterial strains, culture supernatants were concentrated then the chemical components separated using various solvent partitioning phases and chromatography measures respectively. Antimicrobial-guided fractionation was undertaken using culture supernatants in various stages of purification or fractionation, including dialyzed and freeze-dried supernatants and solvent partitioned extracts. Where concentrated extracts were used they were re-dissolved in methanol or H_2O with an aqueous-triton emulsion; otherwise such extracts were used merely as a whole culture growth supernatant.

All 111 pathogen-antagonistic coral and shrimp bacteria were screened for protease activity (data not shown). However, it is interesting to note that these three more antagonistic bacteria also showed the highest protease activity on the skimmed milk and gelatin agar plates when compared to other antagonistic bacteria. This correlation demonstrates that there may be a relationship between protease and pathogen suppressive activity.

Further biological screening demonstrated that these more active strains secreted macromolecules with both antimicrobial and diffusible (into agar) character. Because these high molecular weight antimicrobial molecules were excluded by a 3 KDa filter, the molecular masses were reportedly in excess of this size. This filter was henceforth used to concentrate and partially clean up the antimicrobial metabolites.

3.4.1.1 Protease and amylase activity

The results of screening for protease and amylase activity of partially concentrated supernatants, using various methods, are listed in the following sections.

Skim milk, Gelatin, Casein and Starch agar

Different substrates were used to investigate extracellular enzymes, including proteases and amylases. Protease activity of antagonistic bacteria was screened on skim milk agar, casein agar, and gelatin agar plates. Both whole cultures and cell-free filtered supernatant were

tested. A clearing halo of hydrolysis around the inoculation well on skim milk agar after a 24hr incubation period indicated bacterial capability to produce caseinase-like protease. The extent of the clearing zone around the wells of the skim milk agar plates gives a comparative, semi quantitative, estimate of the overall alkaline protease activity *in vitro* but does not discriminate between different individual casein proteases.

Figures 3.1 and 3.2 show representative results of the hydrolysis zone of skim milk by whole cultures of all three antagonistic strains, suggesting they all secreted alkaline proteases into the media during growth. Measurements of the hydrolysis zones suggest that *Pseudoalteromonas* sp. strain 80 excretes larger amounts of proteases or more highly active proteases into the media over time, when compared with *Psychrobacter* strain 62 and *Vibrio* sp. strain 34 (Figures 3.1 and 3.2). The results shown in Figure 3.3 indicate that these strains also produced extracellular proteases when growing without the presence of skim milk as the filtered supernatant of a culture grown in LB10 also showed clearing zones of skim milk hydrolysis (Figure 3.3). However, as expected, the sizes of the latter clearing zones stayed almost constant with time.

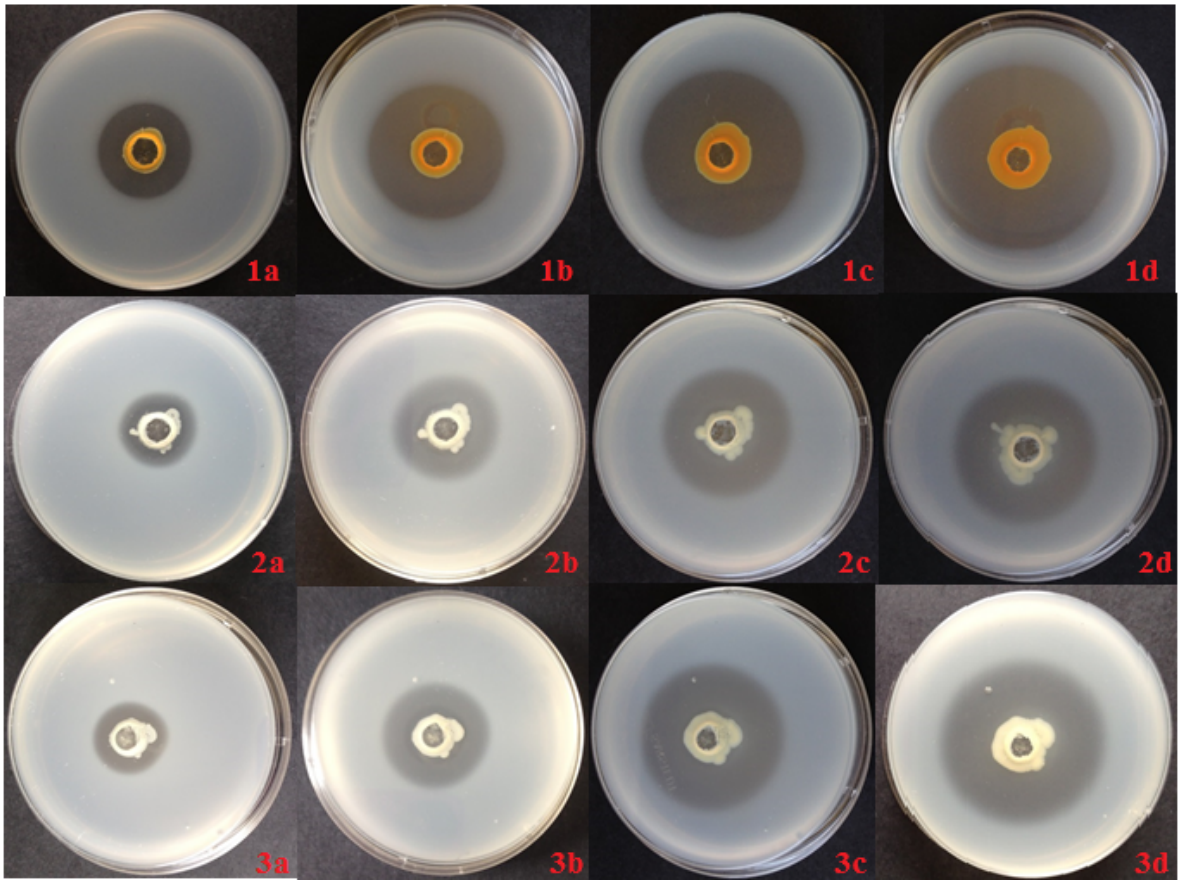


Figure 3.1. Protease activities by whole cultures of three antagonistic strains on MMA plates skim milk agar (1) *Pseudoalteromonas* sp. strain 80 (2) *Psychrobacter* strain 62 (3) *Vibrio* sp. strain 34, after (a) 24hr, (b) 48hr, (c) 72hr, (d) 96hr incubation period.

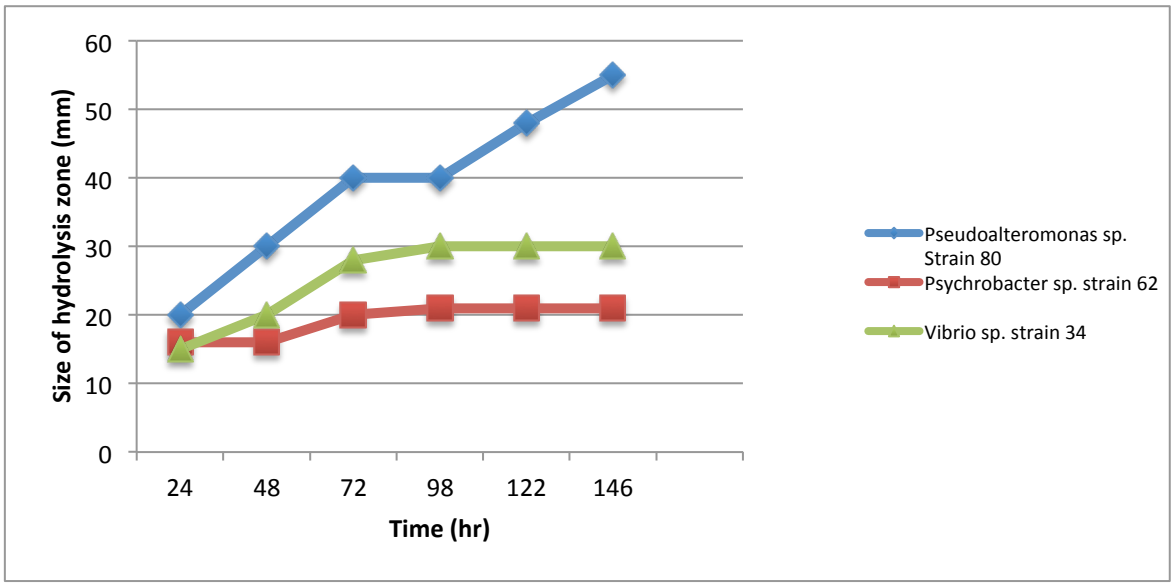


Figure 3.2. Milk casein hydrolysis zone produced by whole cultures over time.

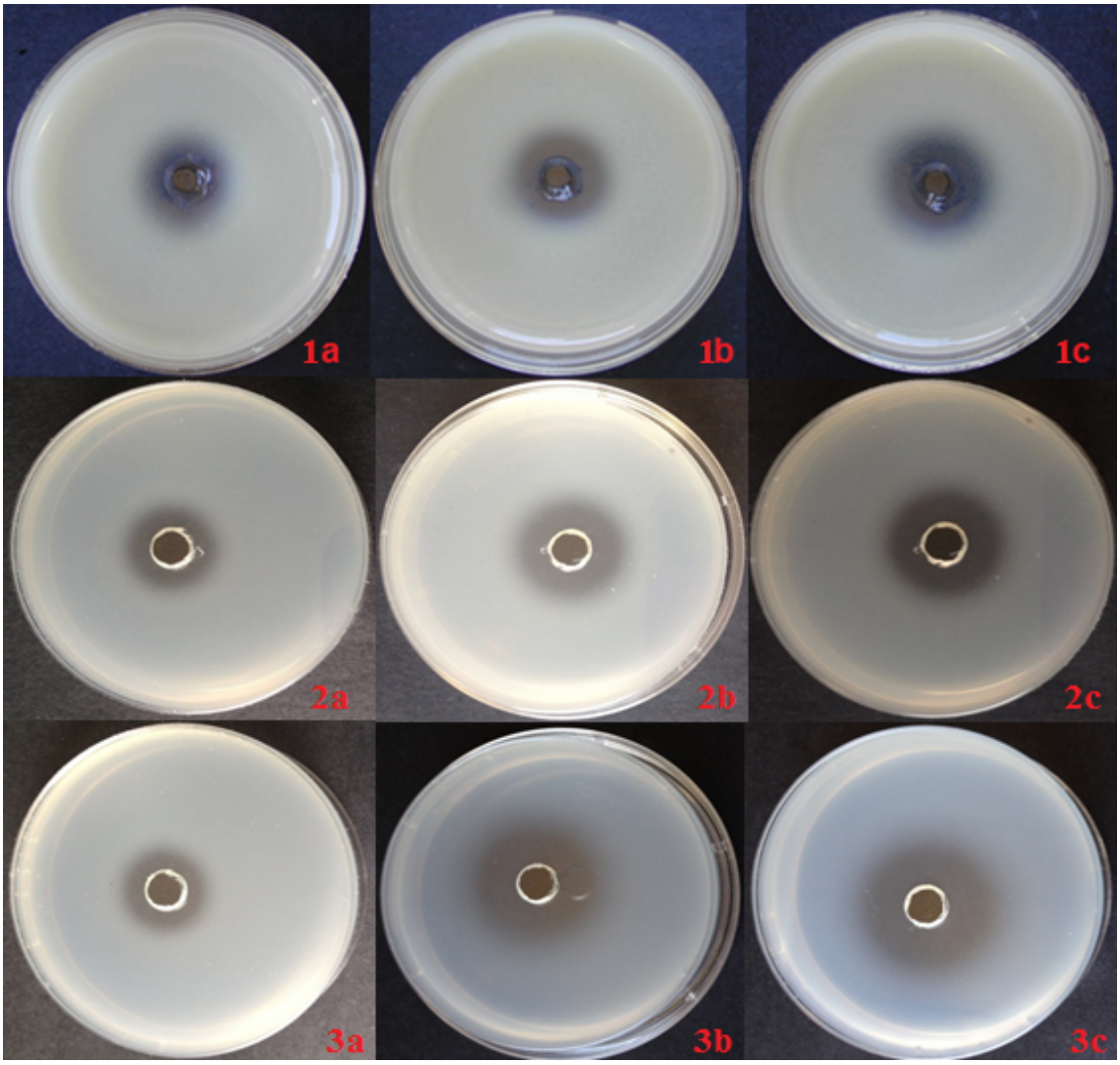


Figure 3.3. Hydrolysis clearing zones of skim milk agar by cell-free filtered supernatants over time. (1) *Pseudoalteromonas sp.*strain 80, (2) *Psychrobacter* sp.strain 62 and (3) *Vibrio* sp. strain 34, incubated for (a) 24 hr, (b) 48 hr and (c) 72 hr.

Amylase and gelatinase production by whole cultures of the three strains, *Pseudoalteromonas* sp. strain 80, *Psychrobacter* strain 62 and *Vibrio* sp. strain 34, was confirmed by the occurrence of a clearing halo around the wells punched into marine agar supplemented with 1% starch or 4% gelatine respectively (Figure 3.4). Cell-free, filtered supernatant gave similar results (data not show).

The three strains investigated here based on their suppression of the growth of the lobster pathogen *V. owensii* DY05, were found positive for protease, amylase and gelatinase-like activities, making these strains good candidates for further more comprehensive investigation on the basis for their pathogen-antagonistic behaviour.

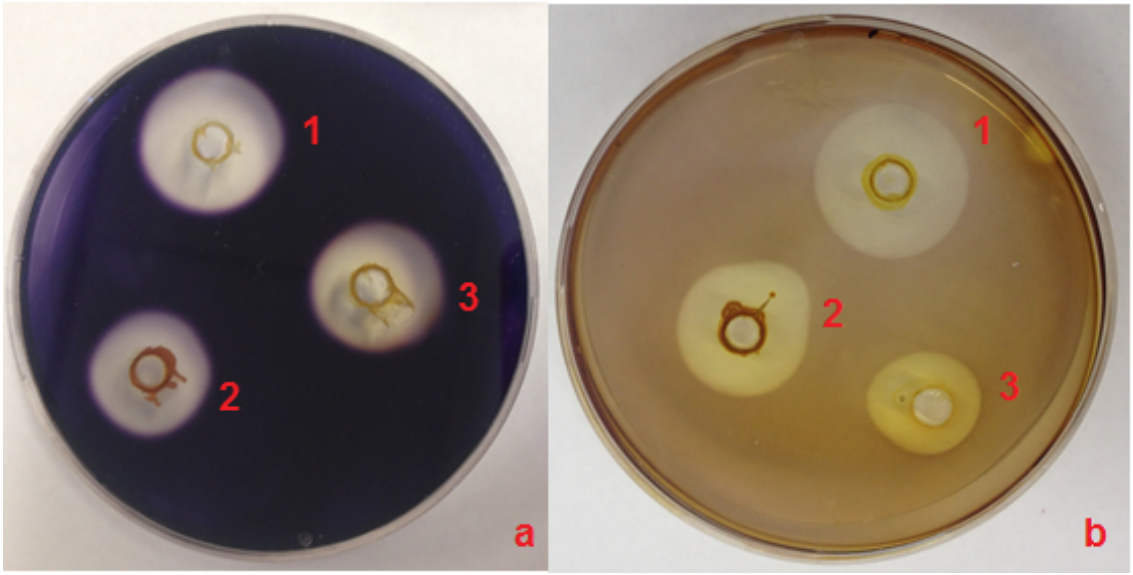


Figure 3.4. Amylase- and gelatinase-like protease activities from whole cultures of three pathogen-suppressive bacteria.Media used were marine solid medium supplemented with (a) starch b) gelatine. Strain tested were (1) *Pseudoalteromonas* sp. strain 80, (2) *Psychrobacter* sp. strain 62 and (3) *Vibrio* sp. strain 34.

Zymogram Electrophoresis

The observed protease activity demonstrated above was further investigated by performing zymography. Culture supernatants concentrated by solvent treatment were separated on a 10%-12% polyacrylamide gel embedded with gelatine or casein substrates, to investigate whether the antagonistic isolates produced different or homogenous extracellular proteases. The enzyme activity was seen on the zymogram gel as a clearing proteolysis zone against a blue background of stained un-degraded gelatine or casein, using the protein detection dye Coomassie Brilliant Blue R-250.

Figure 3.5 shows the presence of unknown proteases, visualized as multiple-bands of hydrolysis on these zymogram gels. These results confirmed that the three antagonistic bacterial strains, *Pseudoalteromonas* sp. strain 80, *Psychrobacter* strain 62 and *Vibrio* sp. strain 34, produced several extracellular proteases in culture supernatants, with some variability across strains. The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins.

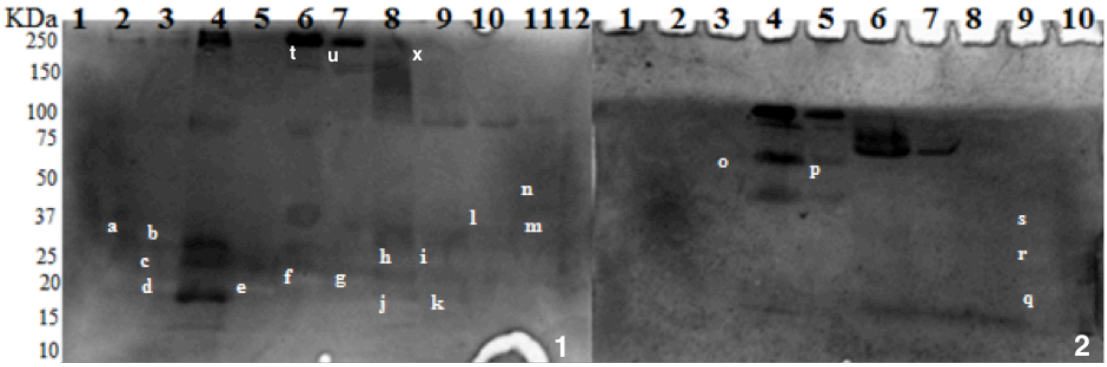


Figure 3.5. Zymogram electrophoretic analyses of various purification steps of proteases produced by pathogen-suppressive bacteria showing the molecular mass of partially purified proteases from these strains. (1) Gelatin zymography of partially purified proteases, Lane 1 molecular markers not visible after treatment so have measured before treatment and position has marked on the left side, lane (2-4) *Vibrio* sp. strain 34, lane (6-10) *Pseudoalteromonas* sp. strain 80 and lane (11-12) *Psychrobacter* sp. strain 62 proteases present in culture supernatant. (2) Casein zymography of the same strains and arrangement, excluding the *psychrobacter* sp. strain 62, were run on another gel and the hydrolysis zones indicated here though distinct to the eye were not all so clear when photographed. Symboles a to z are explained in Appendix 1.

Proteases that were able to hydrolyze gelatin, from both *Pseudoalteromonas* sp. strain 80 and *Vibrio* sp. strain 34, had molecular weights ranging from 10 to150kDa, while *Psychrobacter* sp. strain 62 secreted proteases with smaller molecular weight, ranging from 10 to 37 kDa (some hydrolysis bands on gel were not clear enough when photograph). On the other hand, the molecular mass of the proteases able to hydrolyse casein ranged from 10 to 75 kDa, with no apparent difference between strains at this stage (produce similar pattern of hydrolysis) (Figure 3.5) and Appendix 1.

In zymography, the pattern of proteolysis activity was characterised as multiple-bands of gelatin or casein hydrolysis, as demonstrated in (Figure 3.5, 1 and 2). In casein zymography, proteolysis bands were fainter than those observed with gelatin. Although casein hydrolysis could still be easily visualized with the naked eye, this was not easily captured when photographed (Figure 3.5). Compared to this, a significantly enhanced result was observed on milk agar and gelatin plate agar as shown in Figure 3.1 and casein agar (data not shown). If this effect is a consequence of a lower abundance of casein specific proteolytic macromolecules, this may indicate variability in concentrations. A synergism of interacting macromolecules is another possibility.

3.4.1.2 Determination of growth conditions for optimal protease activity

Several factors were explored as variables to find the optimal growth conditions correlating with the highest extracellular protease activity. The substrate itself was the first such variable, looking at both cell inclusive whole culture and cell free supernatant. The two substrates used were firstly, minimal media supplemented with pathogen, skim milk or the casein standard, and secondly, marine agar supplemented with gelatine or starch. Other variables included temperature, pH and incubation time.

Figure 3.6 shows the changes in the expression of milk casein hydrolytic capacity of bacterial cultures harvested at different stages (age) of their growth. As can be seen cultures of the *Vibrio* sp. strain 34 and *Psuedoalteromonas* sp. strain 80 grown up to 48 hr produced maximal activity whereas cultures of *Psychrobacter* sp. strain 62 grown up to 72 hr produces maximal activity. It suggests that the 24 hr old cultures used for inoculation in the tests presented in Figure 3.1 were in their prime production of extracellular proteases.

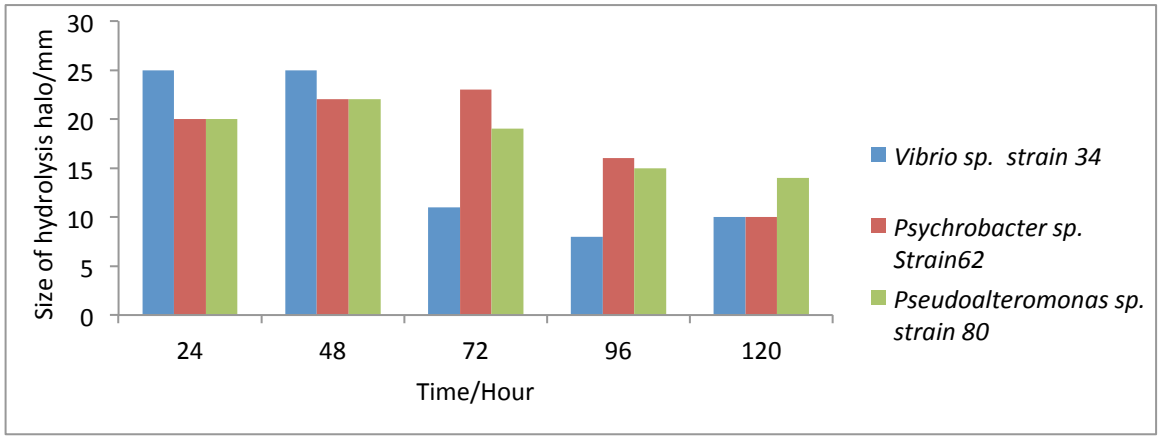


Figure 3.6. The influence of bacterial culture age on extracellular protease activity. Protease activity (zone size of casein milk hydrolysis) was tested with bacterial culture grown for different periods of time before utilisation in well diffusion assay. Aliquots of bacteria were taken each 24 hr and tested on the milk agar for clearing zones. Clearing zones were examined 24 hr after inoculating the wells.

Effects of temperature and pH on cell growth and activity of antimicrobial proteases

The sensitivity to temperature of the enzyme production and activity was studied. Bacterial strains were incubated at different temperatures, 4 ^{o}C, 16 ^{o}C, 28 ^{o}C, 37 ^{o}C, 40 ^{o}C, 50 ^{o}C, and 60 ^{o}C for several days with periodic sampling for protease activity and optical density, an indication of cell growth. Each of the bacterial strains was affected differently by temperatures outside of the 28 ^{o}C optimum. In culturing the various strains, samples of whole cultures and cell-free filtered supernatants were collected each 24 hr and the proteolytic activity was measured on two different substrates (media) for 24-48 hr incubation periods. In all strains, the optimum culture temperature for production of antimicrobial activity was 28 ^{o}C. This temperature was also found to be optimal for expression of proteolytic activity. Culture of *Vibrio* sp. strain 34 produced weak or no growth and no antimicrobial activity outside the 28 ^{o}C optimal temperature before 96 hr of incubation. Cultures of *Psychrobacter* sp. strain 62 produced growth at temperatures in the range between 4-28 ^{o}C, with weak antimicrobial and proteolytic activity at 4 ^{o}C and 16 ^{o}C, appearing only after 92hr of incubation. On the other hand, cultures of *Pseudoalteromonas* sp. strain 80 had weak population growth at the temperatures 4 ^{o}C and 16 ^{o}C, producing no antimicrobial activity. Meanwhile, for the same strain heavy growth was observed at the higher temperatures, being 28 ^{o}C, 37 ^{o}C and 40 ^{o}C, also producing high yields of pigmentation, in particular at 40 ^{o}C; however, 28 ^{o}C was still the best temperature for growth and antimicrobial activity, with enzymatic profile activity for test strains (data not shown).

Cell-free filtered supernatant samples were treated with different temperatures, including 16 ^{o}C, 28 ^{o}C, 37 ^{o}C, 60 ^{o}C, 80 ^{o}C and 100 ^{o}C for 10 minutes each. The treated supernatants were tested in well diffusion assay against the pathogen as well as on skim milk. The zone of inhibition and the hydrolysis zone on milk plates were reduced at 60 ^{o}C and 80 ^{o}C and completely abolished at 100^{o}C.

The effect of pH on the protease (hydrolytic enzymes) activity was measured by incubating the cell-free filtered supernatants at different pH levels, ranging from acid conditions (pH 5) to alkaline (pH 12).

The cell-free filtered supernatants demonstrated some variability between organisms with regard to the optimal pH levels mediating protease activity, but in general the optimal pH was 7-9 as illustrated in Figure 3.7. Individually, the optimal extracellular proteaseactivity of

Psychrobacter sp. strain 62 was found at pH 7 while protease of *Pseudoalteromonas* sp. strain 80 showed optimal activity at pH 8, and *Vibrio* sp. strain 34 major peak at pH 9. This variability across organisms, together with the multiple bands of hydrolysis of gelatin and casein in zymography demonstrate the wide array of proteolytic enzymes.

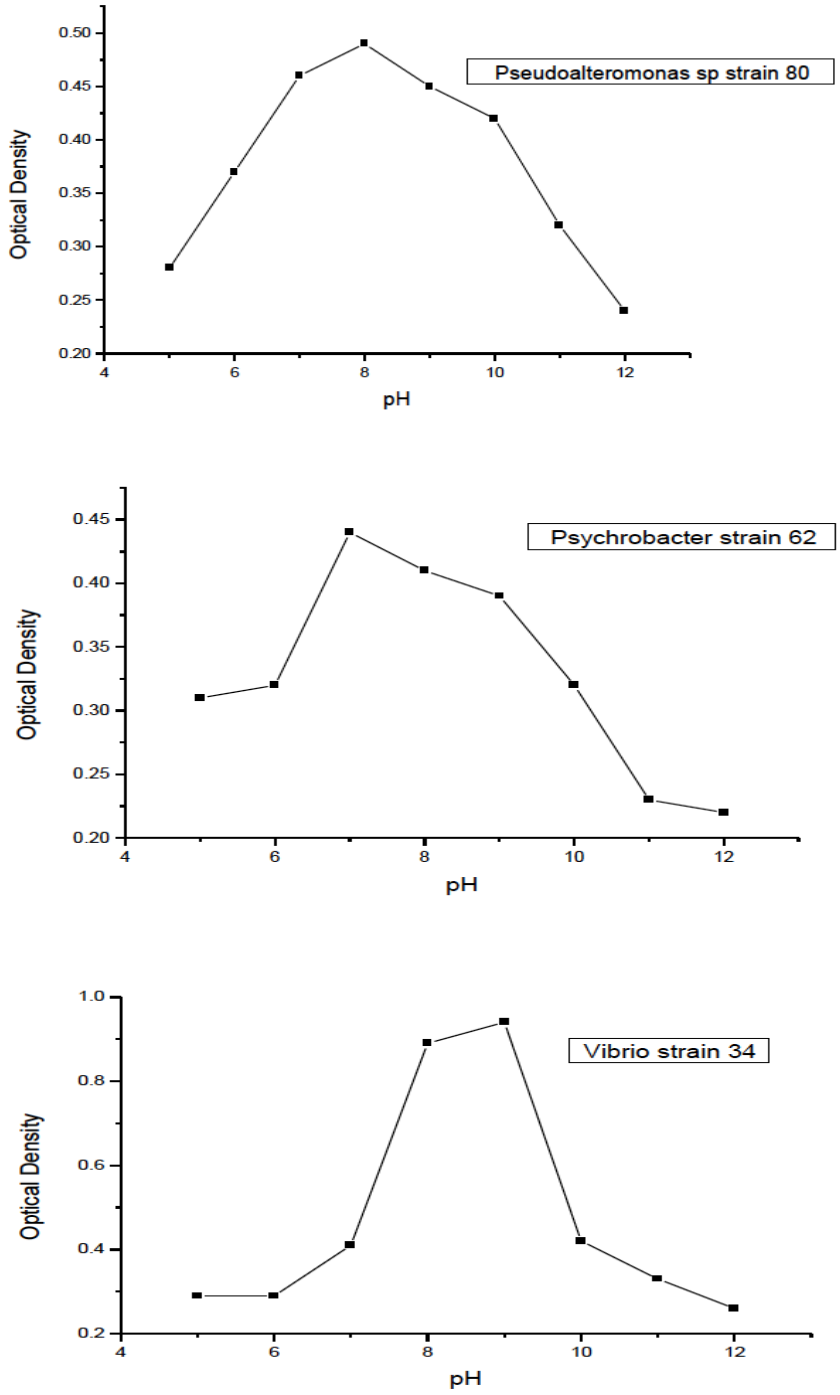


Figure 3.7. Effect of pH on protease activity of *Pseudoalteromonas* sp. strain 80, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34.

3.4.2 SDS-PAGE and Native-PAGE gel electrophoresis

SDS-PAGE was used to determine the approximate molecular weight of partially purified proteins by separation according to the molecular weight of protein sub-units. The organic solvent partitioned and concentrated cell-free filtered supernatants were subjected to 10% Tris-HCl (native page) and 4-15% SDS-PAGE to examine the diversity of macromolecules produced and secreted by the respective organisms.

Several protein bands were revealed by SDS-PAGE (Figure 3.8). Some of these were determined as antimicrobials or 'active' proteins, after overlaying a second native gel, run in parallel, with minimal media agar seeded with the pathogenic *V. owensii* DY05 (data not shown). Clearing zones corresponding to protein bands on SDS/PAGE, indicated in Figure 3.8 as white dots, could be seen with the naked eye but proved difficult to photograph.

Overall, protein bands varied dramatically across bacterial strains, including those apparently 'active' proteins that showed inhibition of *V. owensii* DY05. Accordingly, *Pseudoalteromonas* sp. strain 80 produced active proteins corresponding to the molecular weights of approximately 20, 37, 75 and 100 kDa (Figure3.8 lanes 5 and 6), *Psychrobacter* sp. 62 and *Vibrio* sp. strain 34 were characterised by smaller active proteins at 25 kDa (Figure 3.8 lanes 7 and 10 respectively). The indicated antimicrobial active bands of gel-separated proteins were further characterised using liquid chromatography mass spectrometry (LC/MS) fragmentation and partial protein sequencing. Tentative identification of protein candidates was performed by reference to the Mascot Database.

3.4.3 Liquid chromatography Mass spectrometry: Partial identification of active bands

After staining with Coomassie brilliant blue, the visible protein bands on the SDS-PAGE profile (Figure 4.8), corresponding to bands with demonstrated antibacterial activity separated on native PAGE gels run separately (data not show), were sliced manually from the stained gel, subjected to trypsin proteolysis, and the peptides generated were separated and sequenced by LC-MS. Indicated peptide sequences resulting from the LC-MS analysis were further analysed using the mascot sequence database resulting in the probable identification of some proteins from the same or related microorganisms (Table 3.1) with activities consistent with the active bands of subunit molecular weight identified in Figure 4.8 and indeed in the protease zones previously demonstrated in the zymogram gel (Figure 4.5).

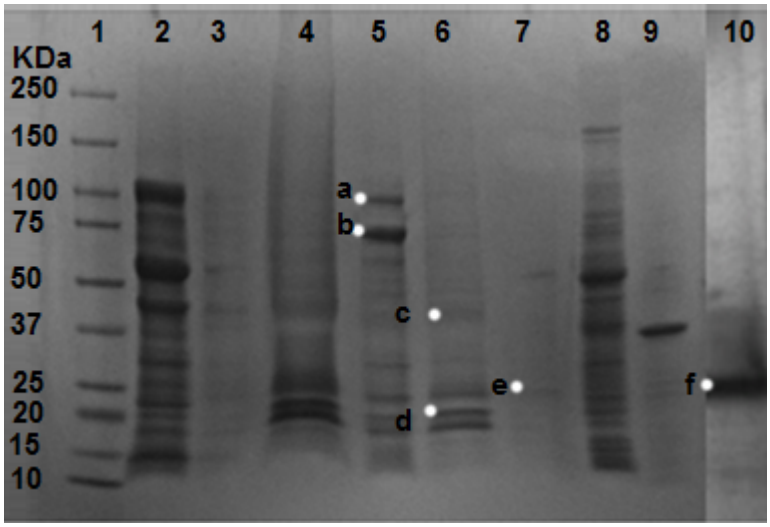


Figure 3.8. Extracellular proteins secreted by pathogen-suppressive bacteria were concentrated from culture supernatants and then separated by 4-15% SDS-PAGE, and visualized with Coomassie brilliant blue R-250. Lane (1) protein standard ladder with molecular weight indicated, lanes (2-6) different concentrated methanol supernatants of *Pseudoaltromonas* sp. strain 80, lanes (7-8) methanol and triton extracts of *Psychrobacter* sp. strain 62 respectively, lane (9-10) methanol and triton concentrated supernatant of *Vibrio* sp. strain 34 respectively. Zones corresponding to antimicrobial activity are indicated by white dots.

Table 3.1. Identity of tentative pathogen-suppressive bacterial proteins.

Band No.	Gi Mascot reference	Bacterial species	Score (emPAI)	Estimated MW (KDa)	Tentative assignment from Mascot		Matches (%.Coverage)
					Protein hit	Bacterial species	
a	gi|392542479	*Pseudoalteromonas* sp. strain 80	828 (0.47)	100	Metallopeptidase	*Pseudoalteromonas piscicida* JCM20779	18(1)
b	gi|409203420 gi|392544290	*Pseudoalteromonas* sp. strain 80	472 (0.29) 355 (0.05)	75	prolyloligopeptidase zinc metallopeptidase	*P. piscicida* JG1 P. piscicida JCM20779	10(2) 10(0)
c	gi|392542673	*Pseudoalteromonas* sp. strain 80	244 (0.16)	25	Iron superoxide dismutase	*P. piscicida* JCM 20779	4(1)
d	gi|392543363	*Pseudoalteromonas* sp. strain 82	624 (0.49)	20	Serine endoprotease	*P. piscicida* JCM20779	13(3)
e	gi|400288460 gi|400287504	*Psychrobacter* sp. strain 62	457 (1.91) 212 (0.17)	25	superoxide dismutase inorganic pyrophosphatase	*Psychrobacter* sp. PAMC21119	11(1) 5(1)
f	gi|189309494	*Vibrio* sp. strain 34	368	25	VtpA (Metalloproteases)	Vibrio tubiashii RE22	9(0)

The entire table is presented in Appendix 2. A selection of tentative protein identification based on sequences identified from stained protein bands (Figure 3.8) was accomplished using MASCOT software that correlated the uninterrupted MS/MS data with sequences in a database (http://ccc.chem.pitt.edu/wipf/Agilent%20LC-MS%20primer.pd). The entire table is presented in Appendix 2.

A short list of protein matches in the Mascot domains database is shown in (Table 3.1). Four protein bands from *Pseudoalteromonas* sp. strain 80 wild type were referenced here (a, b, c and d). Of particular relevance are band a, 100 kDa matching18 (1) the metallopeptidase of *Pseudoalteromonas piscicida* JCM20779 from mascot database. Band b 75kDa matched 10(2) with prolyloligopeptidase and Zinc metalloproteinase from *P. piscicida* JCM20779 matching 10(0). Band c 25 kDa matching 2(0) the Iron superoxide dismutase from *P. piscicida* JCM2077. Band d 20 kDa matching 17(3) Serine endoprotease from *P. piscicida* JCM20779. Band e 25 kDa from Psychrobacter sp. strain 62 matching 10(0) to superoxide dismutase and inorganic pyrophosphatase matching 2(1) of *Psychrobacter* sp. PAMC 21119. Band f from *Vibrio* sp. strain 34 matching 9(0) to VtpA (metalloprotease) from *Vibrio tubiashii* RE22. It should be noted that these matches though mostly plausible in terms of putative functionality are all tentative only and remain to be confirmed in future structure/function analyses.

3.4.4 Gas Chromatography Mass Spectrometry (GC-MS)

In an attempt to identify possible diffusible small molecules contributing to probiosis the culture supernatants were extracted into organic phases using a series of steps described previously (material and method, secation 3.3.8) and the concentrated extracts were firstly examined for interesting components by GC/MS. Because this method is limited to small compounds that are capable of going into the gaseous phase, the study also used bioautography (where small molecules are separated by TLC and then the separated components probed for antibacterial activity following overlay with the target organism and subsequent stain with a metabolisable dye). At this stage bioautography was employed as a means to elucidate chromatographic behaviour of active compounds in the solvent extracts (Table 3.2 and Figure 3.9). This provided a general guide as to the relative polarity of active compounds that will guide fractionation and purification of the active small molecules in subsequent studies.

Analysis of volatile components in GC-MS (Table 3.2) revealed that the fraction of bacterial culture supernatants capable of going into the gaseous phase isare generally dominated by linear alkenes terminating in a single double bond. Alkenes ranged in size from C11 to C22. None of these components were observed in the control (supernatant alone before bacterial inoculation), indicating that the organisms themselves elaborated these alkenes.

Table 3.2. Alkene volatiles extracted from filtered supernatant of three studied strains.

Compound	AI	Pub.AI	62	34	80	Control
1-dodecene	1191	1189	8.9	10.6	13.5	0
1-tetradecene	1391	1393	26.5	28.1	27.4	0
1-hexadecene	1591	1590	31.4	29.7	28.8	0
1-octadecene	1792	1793	20.1	17.6	18.0	0
1-eicosene	1992	1990	9.7	10.8	8.5	0
1-docosene	2193	2195	3.4	3.2	3.7	0

AI–Arithmetic Index. Pub.AI – Published Arithmetic Index for comparison. Relative abundance of components from (62) *Psychrobacter* sp. strain 62, (34) *Vibrio* sp. strain 34, (80) *Pseudoalteromonas* sp. strain 80 are in percent (%). It is not clear if these volatiles are involved in pathogen suppression.

The whole extract was subjected to further investigation employing TLC-autography and bioautography. Figure 3.9 (a) autography dyed with potassium permanganate and (b) bioautography overlay assay with the minimal media seeded with pathogen showed the presence of several solvent fractions with inhibitory compound in the solvent extracted material.

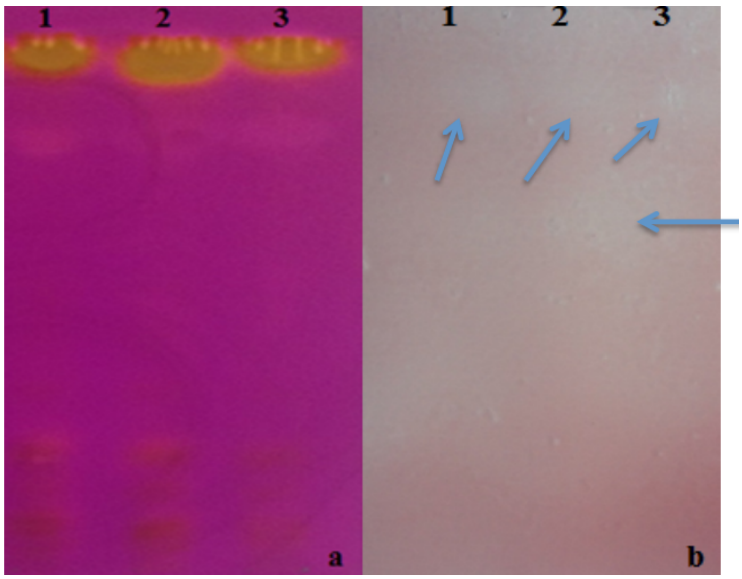


Figure 3.9. (a) TLC plates dyed with potassium permanganates and (b) bioautography plate. Lane1*Pseudoalteromonas* sp. strain 80 lane 2; *Psychrobacter* sp. strain 62 and lane 3*Vibrio* sp. strain 34. The blue indicated to the bands of inhibition.

3.5 Discussion

Marine bacteria are a rich source of natural products. Many compounds that derive from these microbes have antimicrobial activity. The present study involves screening of marine probiotic bacteria to isolate and partially identify diffusible macromolecules with such antimicrobial activity, specifically against the pathogenic species *V. owensii* DY05, which is known to threaten the lobster hatchery industry. Most marine probiotic strains belong to the families Moraxellaceae and Vibrionaceae, but fewer come from other families, such as those mentioned in Chapter 2. In the current study three antagonistic or potentially probiotic strains demonstrated strong antagonism against *V. owensii* DY05 and were also able to hydrolyse casein, gelatine and starch on solid media, possibly attributable to diffusible extracellular enzymes. Investigation for such potential protease activity was undertaken by isolation and partial purification of individual molecules. The three bacterial strains that demonstrated the greatest inhibitory activity against *V. owensii* were *Pseudoalteromonas* sp. strain 80, P*sychrobacter* sp. strain 62, and *Vibrio* sp. strain 34. These strains were investigated for macromolecules and small molecules with antimicrobial activity. In addition, these molecules were investigated for proteins that were able to demonstrate activity similar to enzymes, such as protease or amylase. In order to identify and isolate these molecules, in the first instance concentrated organic extractions of cell free supernatants were examined for proteolytic and antimicrobial activity. In addition, the effect of pH and temperature on the protease production and activity was also taken into account.

Organic solvents have been widely employed to partially purify antimicrobial substances (Isnansetyo & Kamei, 2003; Sakata *et al.*, 2007; Vynne *et al.*, 2011). Differential organic extractions using solvents of progressively increasing polarity serve as a starting point for purification of the active molecules (Fábregas *et al.*, 1991). In the current study the chosen solvents were methanol and triton, which resulted in a high yield and well-resolved electrophoretic separation on polyacrylamide gel, without prior use of more lipophilic solvents during extraction.

3.5.1 Effects of temperature and pH on protease activity

Protease activity was tested in various pH conditions ranging widely from 5 to 12, achieved using different buffer systems as described in the materials and methods. The highest proteolytic activity was obtained using a neutral to slightly alkaline pH, ranging from pH 7 to pH 9 (Figure 3.7). *Psychrobacter* sp. strain 62 was characterised by extracellular enzymes with optimal activity at pH 7, while such enzymes from *Pseudoalteromonas* sp.strain 80 and *Vibrio* sp. strain 34 were most active in slightly alkaline conditions of pH 8 and pH 9 respectively. This agrees with optimal pH measures in previous studies, with a neutral pH optimum observed for protease from psychrotolerant bacteria (Vazquez & Mac Cormack, 2002) and between pH 7 and pH 9 for protease activity from *Pseudoalteromonas*sp. P96-47 (Vázquez *et al.*, 2008).

In another study, the optimal pH of 8.5 was demonstrated for CP1 protease activity of the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76 (Sanchez-Porro *et al.*, 2003). Karbalaei-Heidari et al., (2009) isolated protease from*Halobacillus karajensis* strain MA-2, with a wide ranging active pH of 5-10, but with optimum casein hydrolysis at pH 8.5. Adinarayana *et al*. (2003) found the optimal protease activity of *Bacillus subtilis* PE11 at pH10. Protease activity from *Haloalkaliphilic bacterium* strain S-20-9 functioned at an optimum pH of 9 (Joshi *et al.*, 2008). The optimal pH of serine protease extracted from the protease-producing bacterium *Vibrio metschnikovii* J1 was found within a wide range in the alkaline spectrum from 9 to 12, with an optimum at pH 11 (Jellouli *et al.*, 2009). Thus, a wide range of optimal pH environments can affect such protease activity, which is reflective both of the type of bacteria and the type of proteases secreted.

Results here showed that probiotic bacteria produced more than one active compound according to the peaks of enzyme activity differentiated by optimal pH. Furthermore, this was reinforced by demonstrated different bands of activity with proteolytic activity on zymogram gels. Vázquez *et al*. (2008) in their study of the effect of pH and temperature on the P96-47 protease isolated from *Pseudoalteromonas* sp. also demonstrated more than one peak.

In the current study, the optimum temperature for extent of protease secretion was found to be at 28 ^{o}C but the protease activity itself was optimal within a wider range at 28-37 ^{o}C. The activity was slightly reduced if temperatures were raised to 60-80 ^{o}C and activity ceased entirely when the samples were boiled at 100 ^{o}C for 10 minutes. A study on the optimal temperature of protease secretion and of enzyme activity from the moderately halophilic

bacterium *Halobacillus karajensis* strain MA-2 demonstrated optima of 34 ^{o}C and 55 ^{o}C respectively (Karbalaei-Heidari *et al.*, 2009). The higher temperature of 55 ^{o}C is also the optimal temperature of protease activity of enzymes produced by *Salinivibrio* sp.strain AF-2004 (Karbalaei-Heidari *et al.*, 2007). In the study by Sanchez-Porro *et al*. (2003) it was also found that 55 ^{o}C is the optimum temperature for CP1 protease activity. The optimal temperature and pH for production and activities are therefore variable and depend on the species and culturing conditions.

3.5.2 Proteas and amylase activities

Skim milk, gelatin, and casein agar assays allow primary semi-quantitative assessment of protease activity from culture supernatants, while zymography gives an indication of the number of proteases present in the crude extract (Sanchez-Porro *et al.*, 2003; Sandhya *et al.*, 2005; Vermelho *et al.*, 1996). In the current study, skim milk, casein and gelatine agar plate assays followed by zymography were used to investigate protease activity of bacterial cultures of marine probiotic bacteria and their cell free supernatants, while starch agar medium was used to investigate amylase production or amylase type activity.

The findings are that probiotic bacteria produce extracellular enzymes, particularly protease enzymes that are capable of hydrolysis of gelatine, milk casein and acasein standard. In addition the presence of the amylase enzyme was indicated by hydrolysis of starch in a starch agar plate. It was observed that milk hydrolysis (on milk plates) progressively increased during the incubation period using cell inclusive applications (whole culture). This proceeded in an accumulated fashion over time until all the milk in the plate was hydrolysed. This observation was particularly pronounced using whole culture of the studied strains as shown in Figures 3.1. By contrast, using cell free supernatants the maximum milk hydrolysis was observed in supernatants after 48hr cultures (Figure 3.2). The same pattern was observed for gelatin and starch hydrolysis, confirming that bacteria actively secrete and progressively accumulate extracellular protease and amylase while growing on the milk agar plate, as well as secreting such enzymes into the liquid media. Vermelho et al. (1996) found that hydrolysis continuously increased during the first to the fifth day, then reached its plateau after another ten days (Vermelho *et al.*, 1996).

In the current study several media were used in order to select a suitable culture medium for enzyme production and activity. Minimal media supplement with 1% casein was found to be

the best medium for secretion of caseinase enzymes, while the marine agar supplemented with 1% starch suited amylase activity and 4% gelatine was best for protease activity. Thus, the results demonstrated that the composition of the culture medium strongly affected extracellular enzyme activity, which is generally what was expected. As found in this study the amount of protease produced varied greatly with the bacterial strains and culture media used. This was also demonstrated by a previous study on gelatinase like proteases from *P.aeruginosa, S. marcescens* and *M. luteus,*where the nature of the protein substrates (media) also influenced the character of secreted extracellular proteases (Vermelho *et al.*, 1996). There is still no consensus on a quantitative correlation between the zone of casein hydrolysis and the amount of the protease present (concentration). Vermelho *et al*. (1996) found that there was a correlation between the size of the inhibition zone and the amount of the protease. However,Vázquez *et al*. (2008) contended that there was no such correlation between the amount of protease present and the size of the halo around the colonies on skim milk agar.

In the current study, to the best of our knowledge iodine was used instead of $HgCl_2$ for the first time. Here iodine was used as an indicator for clearing zones on gelatine plates, to indicate gelatinas activity. The appearance of a clear zone against a brown background indicated the occurrence of proteolytic activity in a similar manner both qualitatively and quantitatively to that detected by the usual proteinase detection system with $HgCl_2$ (Jacobs & Gerstein, 1960) in (Bairagi *et al.*, 2002). We are not yet sure how to interpret this serendipitous finding but our investigations have established that this reagent may be better for the detection of gelatinase activity on gelatine agar plates than the original $HgCl_2$ reagent and this warrants further investigation.

In general, bacterial species produce extracellular enzymes specific to the growth phase. In the current study, the protease production started early in cell cultures of probiotic strains and showed maximum activity at the end of the exponential phase. This is in agreement with previous studies. Furthermore, protease secretion started at the beginning of the stationary phase of growth, reaching its maximum in the late stationary phase (48-72 hr) for almost all the isolates (Vázquez *et al.*, 2008). The moderate halophile, *Pseudoalteromonas* sp. strain CP76 demonstrated maximum enzyme production at the end of the exponential growth phase (Sanchez-Porro *et al.*, 2003). Using *Halobacillus karajensis* strain MA-2,

Karbalaei-Heidari *et al*. (2009) found thatthe maximum protease production occurred at the mid-stationary phase.

3.5.3 Hyrolytic enzyme production

"Zymography is a technique for studying hydrolytic enzymes on the basis of substrate degradation" (Vandooren *et al.*, 2013). Zymogram electrophoresis results showed probiotic strains synthesised and produced more than one proteolytic macromolecule with antimicrobial activity according to the multiple bands of hydrolysis on zymogram gels. This is demonstrated in Figure 3.4. This result agrees with other studies that found bacteria produce more than one antimicrobial enzyme. For example, Trejo-Estrada *et al*. (1998) found that *Streptomyces violaceusniger* strainYCED-9 produced three antimicrobial compounds with antifungal activity. Proteolytic hydrolysis of the partially characterized culture supernatant proteases of *Pseudoalteromonas* sp. strain P96-47 demonstrated a multiple-band profile on gelatine-zymography as the multiple protease secretion is common in aquatic bacteria(Vazquez & Mac Cormack, 2002). Despite this ambiguity in interpreting multiple band profiles, in the current study and previous studies, some exceptions have been reported, such as a study by Martínez-Rosales & Castro-Sowinski (2011) on the Antarctic bacterial isolates, suggesting the presence of only one band per isolate satisfactorily confirms that a single extracellular protease is secreted. Our observation of more zones of activity on gelatine zymography by comparison with casein zymography (Fig 3.4) is consistent with the observation that gelatine is susceptible to degradationby a wide range of protease enzymes (Heussen & Dowdle, 1980). In other studies only a single band was demonstrated for a homogenous protease enzyme. For example, *Pseudomonas aeruginosa* san-ai yielded only a single band protein profile on SDS-PAGE which copolymerized with casein (Karadzic *et al.*, 2004). A homogenous protease enzyme was also isolated from *B subtilis*PE-11 (Adinarayana *et al.*, 2003).

Previously it was found by zymography that casein hydrolysis bands were more faint than gelatine hydrolysis bands under the same conditions (Wilson *et al.*, 2010). In the current study, while the hydrolysis band in zymography aremore visible as clearing zones against gelatine, the proteolysis of casein produced much fainter bands. Indeed we have found difficulty photographing clearing zones on casein gel although they were obvious to the eye. Despite being faint in zymography, the casein hydrolysis on the agar plate itself produced a

wide and clear halo. Production of molecules with protease activity was however, related to bacterial species and protein substrates. More than likely multiple hydrolysis bands on the zymography gels are a consequence of the number of different proteases secreted.

3.5.4 Extracellular protein production

Gram-negative bacteria are known to secrete a wide range of proteins into the extracellular environments. These proteins have a variety of functions, such as cytotoxicity, haemolysis, protein phosphorylation, proteolysis and protein dephosphorylation. For example, *Vibrio vulnificus* produce protease, cytolysin, and phospholipase with various biological activities. These enzymes may be involved in the bacterial virulence (Wu *et al.*, 2001). Many compounds found in vibrios have also been isolated from other more distantly related bacteria (Mansson *et al.*, 2011).

In the current study SDS-PAGE was used to study the protein diversity of the chosen isolates. SDS-PAGE is the most widespread method used for qualitatively analysing protein mixtures. It is specifically useful for following protein purification. The method is based on the separation of proteins according to sub-unit molecular mass (Walker, 1996). In the present study the SDS-PAGE 4-15% and native PAGE 10% Tris-HCl were used to separate protein mixtures derived from different organic treatments of the potential probiotic bacteria included in this study. The results showed multiple protein bands separated on native PAGE and SDS-PAGE according to the molecular weight. A high diversity of extracellular proteins was found between the isolates. In general a variety of molecular weights of these molecules was estimated from between 150 kDa to 10 kDa. *Pseudoalteromonas* sp. strain 80 secreted proteins ranging in size from 100-20 kDa and the active bands are 75-20 kDa. *Psychrobacter* sp. strain 62 secretedproteins ranging between 150-25 kDa and the active anti-bacterial protein was 25 kDa in size. The molecular weight range of the protein fraction of *Vibrio* sp. strain 34 was 50- 10 kDa and the active protein was again at 25 kDa. In the previous study by Zhang & Austin (2000) a group of *Vibrio harveyi* isolates were recovered from diseased shrimp in China. These isolates showed a highly diverse protein profile, with sizes ranging from 76 kDa to 27 kD, although most strains shared a 34 kDa active protein. Liu *et al*. (1997) extracted a 38 kDa extracellular protease from luminous *Vibrio harveyi* strain 820514, with the size confirmed using SDS-PAGE.

Many of the strains belonging to the genus *Pseudoalteromonas* have been found to produce proteins and soluble high molecular weight molecules with antimicrobial activities (Bowman, 2007). Wilson *et al*. (2010) suggested that the antimicrobial molecules that were identified from *Pseudoalteromonas* spp. are highly polar and may be protein. By removing the antimicrobial activity by proteolysis digestion, this study confirmed that the antibacterial substances are proteins.

Several other studies have demonstrated mainly larger sized molecules involved in pathogen suppression, which suggests that our results are unusual in that the isolates are secreting such antimicrobial compounds of a relatively small size. For example, James et al. (1996) identified a high molecular weight protein (190 kDa) with antibacterial activity from marine bacterium D2 (James *et al.*, 1996; Longeon *et al.*, 2004). In another study it was found that *Pseudoalteromonas issachenkonii* produced proteases that reduced the biofilm of bryozoan *Bugula neritina* (Dobretsov *et al.*, 2007). Likewise, the antimicrobial protein P-153 at 87 kDa produced from *Pseudoalteromonas* sp. X153 strongly inhibited pathogens involved in dermatological diseases of both fish and human (Longeon *et al.*, 2004). Rearing of scallop in co-culture with *Pseudoalteromonas*sp. X153 significantly reduced scallop mortality, but due to reasons unknown it also slightly reduced the scallop growth rate (Longeon et al., 2004). Lastly, antibacterial proteins were purified from *Alteromonas* strainsthat were nearly 100 kDa in size (Barja et al., 1989; McCarthy et al., 1994).

3.5.5 Identity of extracellular proteins

The results obtained thus far indicate that the studied strains are producing several active proteins. These proteins were visualized as discrete proteases on the zymography gels. Protein bands from SDS-PAGE gels corresponding to inhibition zones in overlay assay (zymography) were excised and sent to Dr Anne Poljak at UNSW for LC-MS analysis. Several unique sequences were identified by comparison with the mascot database as shown in Table 3.1.

Metalloproteases are produced by all species of plants, animals, and microorganisms. For example, Arctic sea-ice bacterium *Pseudoalteromonas* sp. SM495 produce metalloprotease E49 (He *et al.*, 2012). Some metalloproteases that are secreted to the periplasm or outside the cell are called extracellular metalloproteases. According to Wu & Chen (2011) the bacterial

extracellular metalloproteases (BEMPs) are a large group of metal-containing proteases secreted by heterotrophic bacteria. Here we show that the bacteria *Pseudoalteromonas* sp. strain 80 secrete several biologically active compounds that matched with published sequences from related bacteria from the Mascot database. These included metallopeptidase, prolyloligopeptidase, zinc metalloprotease, iron superoxide dismutase and organic pyrophosphatase, although these matches are tentative only. With respect to the possible probiotic effects, proteases from strain *P.issachenkonii* have been found to be effective in reducing biofouling (Dobretsov *et al.*, 2007).

Furthermore we have shown that *Vibrio* sp. strain 34 appears to produce a protein tentatively matched to the VtpA (*Vibrio* metalloprotease) metalloprotease from strain *Vibrio tibiashii* RE22. Several studies reported that *Vibrio* species produced extracellular metalloproteases that were involved in bacterial pathogenicity (Norqvist *et al.*, 1990). Delston *et al*., (2003) isolated zinc-containing metalloprotease haemagglutination from *Vibrio tubiashii*. The VtpA was considered as an important factor in *V. tubiashii* toxicity, however, the exact role of its enzyme in the pathogenicity of this bacteria has yet to be considered (Hasegawa *et al.*, 2008). A known coral pathogen *Vibrio coralliilyticus* was shown to secrete a zinc-metalloprotease involved in coral disease (Gharaibeh *et al.*, 2013).

Judged by comparison with results from the mascot database *Psychrobacter* sp. strain 62 secretes proteins with sequences tentatively matched to superoxide dismutase and inorganic pyrophosphatases already known from *Psychrobacter* sp.More specifically, PAMC21119 was isolated from permafrost soil on Barton Peninsula, King George Island, Antarctica (Kim *et al.*, 2012). One strain from the *Psychrobacter* sp.,is a residential bacteria with antagonistic activity in the gut of fast-growing groper *Epinephelus coioides*(Sun *et al.*, 2011). Castellano *et al*. (2008) have isolated Superoxide dismutase (SOD) from *Pseudoalteromonas haloplanktis* (PhSOD) with high specific activity and good thermostability. SOD is a universal metal enzyme, exhibiting a crucial role in the cell resistance mechanism against the reactive oxygen species (ROS) but there is no confirmation of their possible role in probiosis. Based on their bacterial antagonist properties, *Pseudoalteromonas* strains are candidates for application as probiotics.

3.5.6 Small antimicrobial molecules

The vast majority of bacteria, from both terrestrial and marine environments, have the ability to synthesise a wide range of metabolites, some of which are volatile organic compounds (VOCs) formed via primary and/or secondary metabolic pathways (Bruce *et al.*, 2000; Minerdi *et al.*, 2009; Papaleo *et al.*, 2013; Vining, 1990). Prominent volatiles commonly observed from bacteria include alcohols, alkenes, alkanes, and ketones, followed by pyrazines, esters, sulfides and lactones (Effmert *et al.*, 2012).

The focus of this experiment presented here was to investigate and partially characterise the role of small molecules with antimicrobial activity, secreted by the probiotic strains included in the present study. The solvent extracted residues derived from the filtered supernatant of bacterial cultures were submitted to GC-MS analysis to identify the character of volatile molecules present. Several volatile alkenes were identified, but generally no variation was observed between the strains. It is not clear if these small alkenes were involve in the observed antimicrobial activity.

The occurrence of a similar GC-MS profile across strains is not surprising, as previous studies found that different bacteria can produce the same volatiles. In one such study by Papaleo *et al*. (2013) such similarities were demonstrated between the four strains studied, which included two *Pseudoalteromonas* strains (TB41 and TAC125) and two *Psychrobacter* strains (TB47 and TB67). In that particular study it was hypothesized that the different clustering and inhibition patterns observed may have been attributed to a difference in the relative abundance of one or more of the VOCs produced by the different strains (Papaleo *et al*., 2013). However, such speculation is not necessary in the current study, as it was not shownthat the volatiles characterized in Table 3.2 contributed to antimicrobial activity in any capacity.

To further investigate the possible role of non-volatile small molecules, the residue from the air-dry chloroform extract was re-suspended in acetone and the components were separated by thin-layer chromatography (TLC). The chemical nature of components listed in Table 3.2, being of a more lipophilic tendency, means that they would have travelled with the solvent front, together with other unknown components, using that particular solvent system. A stain of this TLC demonstrated components migrating along the TLC plate at varying Rf values,

some as low as Rf 0.1 and as high as Rf 0.8. These visualized components provide the basis for further examination of corresponding antimicrobial activity. Indeed, some of these separated compounds visualized in the stained TLC demonstrated antimicrobial activity in the bioautography. Thus, although previous studies have corroborated that volatiles were involved in antimicrobial activities, the current study implicates larger and slightly more polar non-volatile compounds are involved.

Antimicrobial volatiles derived from aquatic or marine bacterial strains are not often described but, interestingly, Romoli *et al.* (2011) demonstrated that volatiles secreted by Antarctic bacteria were able to inhibit the growth of the*Burkholderia cepacia* complex (Bcc) strains. In this regard, further studies should elucidate the role of antimicrobial volatiles with particular emphasis on the habitat of the isolated strain.

Vibrio spp. are common inhabitants of aquatic environment and are found free living as well as associated with various marine organisms such as squids, shrimps, corals, fish, molluscs, sea grasses and sponges (Pandey *et al.*, 2010) There are a few reports on *Vibrio* spp. producing antimicrobial substances (Hjelm *et al.*, 2004 b ; Isnansetyo *et al.*, 2009; Long & Azam, 2001; Towse, 2005). In a study by Pandey *et al*. (2010) gas chromatography / mass spectrometry (GC-MS) was employed to examined volatile compounds in a crude cell extract of *V. parahaemolyticus* which inhibited the growth of other bacterial fish pathogens. That particular study revealed the presence of indole, phenyl acetic acid, n-(3-methyl-1, 2, 4-oxadiazol-5-yl)-1-pyrrolidine carboximidamide, pyrrolopyrazines, tetramethyl pyrazine and other important phenolic compounds. Although these compounds have known antimicrobial activities, the concentrations of these components were not reported. In subsequent studies aimed at elucidating the chemical character of volatiles it may be important to utilize solid phase microextraction (SPME), as this method also reveals the smallest volatile components which cannot be visualized if dissolved into a solvent. Using the solvent extraction methodology in the current study, volatile components with relative sizes comparable to the solvent itself are lost via evaporation or are co-eluting with the solvent during GC-MS analysis. Using SPME this difficulty is overcome.

Pigmented species of *Pseudoalteromonas* have been shown to produce antimicrobial (including antifungal) substances and these include *P. luteoviolacea, Pseudoalteromonas*

rubra, Pseudoalteromonas aurantia, Pseudoalteromonsas citrea, Pseudoalteromonas tunicata, P. piscicida, and *P. issachenkonii* (Egan *et al.*, 2002; Holmström & Kjelleberg, 1999; Isnansetyo & Kamei, 2003; Ivanova *et al.*, 2002; Kalinovskaya *et al.*, 2004).

P. tunicata produces yellow pigment YP1 that comprises a 2,2-bipyrrole-ring scheme with an unsaturated 12-carbon alkyl chain (Frank *et al*., 2005). YP1 is a pigment belonging to the tambjamine class of substances, which has been isolated from eukaryotic sources in the aquatic environment and has previously been shown to display antibacterial activities (Franks *et al.*, 2006). However, the inhibition zone observed here on the TLC did not correspond to the pigmented components. This suggests that the biosynthetic pathway for the pigment compound may parallel that of the antimicrobial compound, but is not necessarily the antimicrobial compound itself.

In conclusion the potential probiotic bacteria investigated here produce diffusible small molecules as well as macromolecules such as metalloproteases. Some of these molecules have antimicrobial activity and therefore warrant further investigation, with such investigation concentrating particularly on structural elucidation and structure/function relationships.

Analysis of Pseudoalteromonas sp.strain 80 genes and traits involved in growth suppression of pathogenic Vibrio

4.1 Abstract

The yellow-pigmented *Pseudoalteromonas* sp. strain 80 suppresses the growth of *V. owensii* DY05 in well diffusion assays, probably by excreting extracellular antimicrobial substances into the media. The genetic and phenotypic background of this pathogen-suppression ability was unknown. To identify genes related to pathogen suppression, potentially antimicrobial synthesis, we first produced non-suppressive Tn10 transposon-induced mutants and then analysed the gene and traits affected. The mini-Tn10 cassette used carries kanamycin resistance, which is useful for selection of Tn10-transformed cells. In addition, producing the rifampicin-spontaneous *Pseudoalteromonas* sp. mutant strain 80-Rif2 assisted in the selection of transformed *Pseudoalteromonas* recipient cells in the background of Tn10 *E. coli* donors (kanamycin resistant). Out of 35,000 pigmented and non-pigmented rifampicin/kanamycin resistant colonies of the transformed strain 80-Rif2, one light-colored colony showed lack of pathogen suppression (no inhibition zone). This mutant, named 7Km, lost the inhibitory activity against the *V. owensii* DY05 pathogen. Southern blot analysis confirmed that the genome of 7Km contains an insert of the mini-Tn10 transposon in a single chromosomal location. The gene affected by the Tn10 insertion was analysed using TAIL-PCR and plasmid rescue techniques. The latter rescued a 5.5 kb *Sph*I DNA fragment from 7Km, containing the Tn10 and flanking chromosomal sequences. BlastX search indicated that the protein affected in 7Km shares 100% homology with a membrane bound transporter proteinof the resistance-nodulation-division (RND) family. The closest homology was to RND protein of *Pseudoalteromonas flavipulchra* JG1, a marine antagonistic bacterium with abundant antimicrobial metabolites. RND proteins were implicated in drug resistance of important pathogens and in the transport of substances from the cell, suggesting that a mutation in such a transporter probably abolished the excretion of pathogen-suppressive substances from *Psedoalteromonas* sp. strain 80 cells.Phenotypical and biochemical properties associated with the mutation were also examined and it was found that the mutation had greatly reduced the protease, including gelatinase and caseines, as well as amylase activities.The mutant also excretes modified non-volatiles molecules that did not suppress the pathogen on bioautography. However the mutant produce the same volatile small molecules as the wild type.

4.2 Introduction

Aquatic bacteria are a rich source of potentially useful antimicrobial molecules, importantly the genus *Pseudoalteromonas* of the class Gammaproteobacteria, which was first described by Gauthier *et al*. (1995). This genus exists in association with eukaryotic aquatic organisms, such as invertebrates and algae. It was suggested that *Pseudoalteromonas* species protect their host by colonization (Burkholder *et al.*, 1966; Egan *et al.*, 2001; Egan *et al.*, 2002; Franks *et al.*, 2005). The genus contains numerous species that produce a wide range of biologically active compounds (Bowman, 2007; Franks *et al.*, 2005; Gauthier *et al.*, 1995; Holmström *et al.*, 2002; Holmström & Kjelleberg, 1999; Rao *et al.*, 2005). Pigmented *Pseudoalteromonas* species possess a broad range of bioactivity associated with the secretion of extracellular compounds (Holmström & Kjelleberg, 1999).

Some of the genes coding for specific enzymes involved in antibiotic biosynthesis are located in clusters on the bacterial chromosome or on plasmids (Hopwood & Merrick, 1977; Kirby & Hopwood, 1977; Martin & Liras, 1989). The mechanisms of the antimicrobial production and secretion by beneficial bacteria, such as *Psedoalteromonas,* can be studied using mutagenesis. The generation of mutations by transposon insertion can be a powerful analytical technique (Huisman *et al.*, 1987). Transposable genetic elements are found in most sequenced genomes and in many organisms for which the entire genome sequence is not yet available (Ton-Hoang *et al.*, 2005). The insertion of a number of kilobases of transposon DNA inside thetarget gene allows accurate mapping of the site of the mutation (Belas *et al.*, 1984).

Transposon mutagenesis was employed to identify gene(s) controlling antimicrobial activity of *Pseudoalteromonas* sp. strain 80. Transposable elements provide genetic markers that enable correlating genetic and phenotypic information (Bardarov *et al.*, 1997; Hayes, 2003; Kleckner *et al.*, 1977). Mini-transposon is derived from transposons Tn10 and Tn5, in which the naturally occurring functional segments of DNA have been rearranged to create shorter mobile elements (Lorenzo *et al.*, 1998). A mini-Tn10 transposon was used in this study to search for phenotypes and rescue genes involved in pathogen suppression.

Reported here is the cloning and molecular characterization of Tn10 transposon mutant of *Pseudoalteromonas* sp. strain 80, 7Km, which has lost the pathogen suppressive ability, and the genetic analysis of this mutant.

Transposition is the recombination reaction that mediates the relocation of mobile (DNA) segments from one genomic location to another location on the same or different chromosomes. These segments are called insertion sequences, transposons, inversions, deletions, transposable elements and chromosome fusions (Berg & Howe, 1989; Choi & Kim, 2009; Craig, 2002; Davies *et al.*, 1999; Goryshin *et al.*, 2000; Hayes, 2003; Richardson *et al.*, 2006). The movement of transposable elements is highly regulated and can profoundly influence gene expression (Hayes, 2003). Transposition occurs via one of two mechanisms: cut-and-paste transposition (e.g., Tn5 and Tn10) (Reznikoff, 2008), or replicative transposition (e.g., Tn3, Mu, and many IS) (Shapiro, 1979). It involves an ordered series of events: (1) sequence-specific binding of transposase to the terminal inverted repeats (IRs) present at the ends of the transposon, (2) cleavage of both strands of DNA at each end of the transposon, (3) synapsis of the ends by transposase–transposase interactions, (4) capture of the target DNA and (5) strand transfer to insert the element into the target (Richardson *et al.*, 2006). In general, transposon-based gene integration is independent of previously recognised mechanisms for the integration of DNA molecules and does not require homologous sequences for transfer to the chromosome (Choi & Kim, 2009; Vizváryová & Valková, 2004). Therefore, transposons can be widely utilized for the creation of random insertional mutagenesis, which might be exploited and applied further for the characterization of essentiality and the functions of genes associated with host-pathogen relationship (Choi & Kim, 2009; Wilson *et al.*, 2007).Tn10, the composite bacterial transposon comprising of two IS10 elements (R and L) plus internal sequences including antibiotics resistance, can move into and out of chromosomes or plasmids in a non-replicative fashion (Haniford & Chaconas, 1992; Kleckner *et al.*, 1996). Using Tn10 for generation of mutations by transposon insertion can be a powerful analytical technique. Historically derivatives of bacterial transposon Tn10 were described that were useful for detrmining the functional limits and regulatory sites of bacterial genes (Way *et al.*, 1984).

4.3 Materials and Methods

Attempts were made in this project to generate mutants from a range of antagonistic bacteria as listed below (Section 4.3.1). However, only one mutant affected in pathogen-suppression was obtained from*Pseudoalteromonas* strain 80 mutagenesis and most of the Materials and Methods will be described for this mutant only.

4.3.1 Bacterial strains, plasmids and culture media

Bacterial strains and plasmids are listed in Table 4.1. Complete growth medium used was LB10 (see Section 2.3.1) and 15 g/L of agar (Difco) was added to solidify the medium. Solid and liquid LB10 media were supplemented were required with 100 µg/ml ampicillin and 100 µg/ml or 85 µg/ml kanamycin. *Pseudoalteromonas* strain 80, *Pseudoalteromonas pscicida*-like isolate pp107, *Psychrobacter* strain 62, *V. owensii* DY05 and *Vibrio* strain 34 were grown on marine broth (MB) or marine agar (MA) (Difco). MB and MA were supplemented with 20 µg/mL rifampicin (Sigma) for growing *Pseudoalteromonas* Rif-resistance mutants 80-Rif2. Minimal medium agar (see chapter two section 2.3.1.) was used to test the abilityof transposon-induced mutants to suppress the pathogen.

Table 4.1. Bacterial strains and plasmids used in this study.

Strains / plasmids	Description	Reference
E. coli Sm10λ pir	Donor of mini Tn10 Km^R and Amp^R Sm^S, mobRP4, π-preplicase (pir)	(Egan *et al.*, 2002; Herrero *et al.*, 1990)
V. owensii DY05	A pathogen of *P. orantus* phyllosoma (isolated from moribund stage 3 phyllosomas during epizootic in the AIMS larval rearing system)	(Cano-Gómez *et al.*, 2010)
Vibrio sp. strain 34	Isolated from coral tissue (*Turbinaria mesenterina*), East coast, Australia (see Chapter 2)	This work
Pseudoalteromonas sp.strain 80	Isolated from shrimp (*Trypaea astraliensis*), East coast, Australia (see Chapter 2)	This work
Psychrobacter sp. strain 62	Isolated from coral tissue (*Turbinaria mesenterina*), East coast, Australia (see Chapter 2)	This work
Pseudoalteromonas pascicida-like isolate PP107	Isolated from culture collection of wild *P. orantus* phyllosomas	(Goulden, 2012)
Pseudoalteromonas strain 80-Rif2.	Spontaneous Rif resistant mutant	This work

E. coli DH5α lamda pir 3	a λpir 3 dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1	(Metcalf *et al.*, 1996)
E. coli BW20767	Mu-1kan::Tn7 integrant leu-63::IS10 recA1 creC510 hsdR17 endA1 zbf-5 uidA(DMluI):pir/ thi	(Metcalf *et al.*, 1996)
E.coli TG1	supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rK mK –)	(Wain-Hobson *et al.*, 1985)
E. coli BMH8117	(lac pro), nalA thi sup E F' lac pro Iq- Z- Y	(Oehler *et al.*, 1990)
pLOF/Km	Plasmid containing mini-Tn10 Km^R Amp^R	(Herrero *et al.*, 1990)
pTZ19R	f1, pBR322, lacZ gene, multiple cloning site (MCS) from $pCU19Amp^R$	Sequencing vector from Pharmacia

4.3.2 Antibiotic sensitivity test

All bacteria used in this work as recipient or donors strains for Tn10-transposon mutagenesis were tested for sensitivity to six antibiotics. Two to three single colonies of 24 hr pure culture were used to streak each of sterile MA plates supplemented with appropriate concentration of antibiotics to final concentrations of 10 µg/ml of tetracycline (Tc, Sigma), 20 µg/ml of rifampicin (Rif, Sigma), 50 µg/ml of chloramphenicol (Cm, Roche), 100 µg/ml of ampicillin (Amp, Roche), 50 µg/ml of kanamycin (Km, Sigma) or 50 µg/ml of streptomycin (Sp, Sigma). Plates were incubated at 28 ^oC for 24-48 hr periods and colony growth observed.

4.3.3 Generation of Tn10-induced mutants impaired in pathogen suppression

4.3.3.1 Generation of Rifampicin and streptomycin spontaneous mutants

Spontaneous antibiotic resistance bacteria were generated from *Pseudoalteromonas* sp. strain 80, *P. piscicida*-like isolate PP107, and *Vibrio* strains 34 and 62. Antibiotic pressure was applied against streptomycin and rifampicin. In brief, strains were grown at 28 ^oC with shaking in marine broth without antibiotics (two flasks of each strain with 100 ml of culture in each 1 L flask). After 24 hr incubation, antibiotics were added to a final concentration of 20 µg/ml rifampicin in one flask and 100 µg/ml streptomycin in the second flask, which were then incubated for another 48 hr in order to generate spontaneous mutant resistance to these antibiotics. 100 µL of each culture were spread on two marine agar plates, one supplemented with 20 µg/ml rifampicin and the other with 100 µg/ml streptomycin. The plates were incubated at 28 ^oC for 24 hr. Antibiotic-resistant mutants were kept at -80 ^oC in 25-50% glycerol stocks. The 16S rRNA gene frament of 80-Rif2, a mutant of *Pseudoalteromonas*

strain 80, was amplified and sequenced. Once its identity was confirmed, the strain was used in the generation of Tn10-induced mutants impaired in pathogen (DY05) suppression.

4.3.3.2 Tn10 transformation of Pseudoalteromonas sp. 80-Rif2

Pseudoalteromonas sp. 80-Rif2 strain mutagenesis was carried out using the mini-Tn10 transposon system. Mini-Tn10/Km cassette was provided on the plasmid pLOF/Km (Figure 4.1) and the donor strain was *E. coli* Sm10 λpir [pLOF/Km] (Table 4.1). For restriction map analysis of pLOF/Km, plasmid DNA was isolated from a culture of *E. coli* Sm10 λpir [pLOF/Km], grown in 100 mL of LB10 broth containing 100 µg/mL ampicillin and 100 µg/mL kanamycin, using the Plasmid Midi kit (QIAGEN) according to the manufacturer's instructions. The plasmid DNA was eluted with 250 µL of TE buffer (10 mM Tris-HCl, pH 8.5) and stored at -20 ^{o}C.

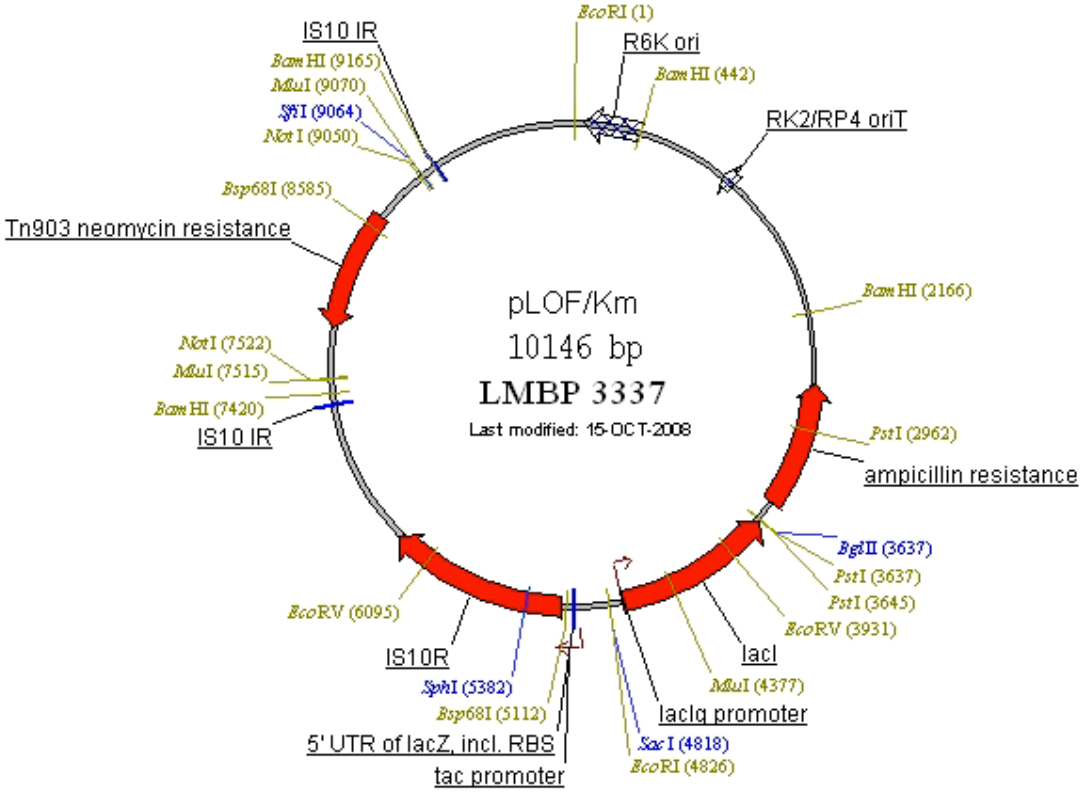


Figure 4.1. Restriction enzyme map of pLOF/Km plasmid. The plasmid pLOF/Km is a Tn10 based transposon vector delivery plasmid; it carries a Km resistance indicator gene inserted at the single *Not*I site of the pLOF vector. The transposition system consists of the IS10R transposase gene located outside the mini-Tn10 element and driven by the tac promoter, and *Mlu*I-*Sfi*I-*Not*I-*Mlu*I sites between the inverted repeats of the Tn10 transposable element. (Obtained from: http://bccm.belspo.be/catalogues/files/lmbp-plasmids/p3337.pdf) April 2014

4.3.3.3 Restriction map analysis of the plasmid pLOF/Km

In order to confirm the identity of the plasmid and to design probes and primers for analysis of Tn10-induced mutants a restriction analysis of pLOF/Km (Figure 4.1) was conducted. Restriction map was constructed by first digesting a pLOF/Km with a set of restriction enzymes, then separating the fragments using agarose gel electrophoresis alongside the DNA ladder λ-Hind III to determine the sizes of fragments. The restriction enzymes, listed in Table 4.2, were used according to the manufactures' instructions.

Table 4.2. Restriction enzymes used to digest genomic DNA and plasmids.

Restriction enzyme	Target sequences	Company
BglII	AGATCT	Promega
SphI	GCATGC	Promega
NoteI	GCGGCCGC	Promega
EcoRI	G AATTCCTTAAG	Biolab
EcoRV	GATATCCTATAG	Promega Biotech
BamHI	GGATCC	Promega Biotech
PstI	CTGCAG	Promega Biotech
HindIII	AAGCTT	Promega Biotech

4.3.3.4 Tn10 transposon mutagenesis and selection of mutants

The recipient strains *Pseudoalteromonas* sp. 80-Rif2 was transformed with pLOF/Km carrying a mini-Tn10 transposon with a kanamycin resistance marker.Transfer of the mini-Tn10 transposon to recipient strains was performed by conjugation, following a protocol modified from Egan *et al.* (2002). One mL LB10 cultures of the recipient strain and *E. coli* Sm10 [pLOF/Km] donor were centrifuged at 10,000 g, at 4 ^{o}C for 10 min. The pellet was resuspended in antibiotic free LB10 broth. Fifty µl of the donor suspension were placed in the centre of antibiotic-free LB10 agar and allowed to dry then covered with 50 µl of recipient suspension. The conjugation plates were incubated overnight at 28 ^{o}C. A loop full of the mixed culture was suspended in 200 µL sterile LB10, spread on ½MA (MA powder (Difco) 27.5 g, agar-agar (Bacto) 9g, sodium chloride (chemist supply) 9g in 1L distiled water) supplemented with 20 µg/ml Rif and 100 µg/ml Km and incubated for at least 48 hr at 28 ^{o}C.

To identify the transformants that had lost the ability to produce antimicrobial compounds, Rif and Km-resistant random mutants of 80-Rif2 (35000 trans conjugants) were screened by transferring each single colony using a toothpick onto MMA medium seeded with 1% *V. owensii* DY05. 25 colonies were transferred onto each plate and the plates were incubated at 28 ^{o}C for 24 hr. Rif/Km mutants with reduced ability to inhibit the pathogen (showed no clearing zones) were tested using well diffusion assay to confirm the loss of pathogen suppression.

4.3.4 Mutant analysis using Southern blot hybridization

4.3.4.1 Preparation of DIG labelled probes

Probes were prepared by digesting 1 µg of pLOF/Km plasmid with the restriction enzyme *Bgl*II (10 u/µL), *Not*I (10 u/µL), *Eco*RI (20 u/µL), *Sph*I (10 u/µL) or *Eco*RV (11 u/µL). A typical digestion reaction consisted of: 5 µL H_2O, 2 µL 10xTA buffer, 2 µL 1 M NaCl, 10 µL plasmid (1 µg) and 1 µL restriction enzyme and was made up to 100 µL with TE buffer.
*Bgl*II digestion was used to prepare a 10.15 Kb probe of the linearised plasmid. *Not*I, and *Sph*I/*Eco*RV digestions of plasmid pLOF/Km were separated using agarose gel electrophoresis and selected fragments for probe preparations were purified using a gel extraction kit (Qiagen). The expected sizes of the probes were 1.5 kb *Not*I (including the Km resistance gene and other sequences from within the Tn10) and 0.7 kb *Sph*I/*Eco*RV (outside of the Tn10). Digested DNA was extracted with phenol/chloroform, precipitated with ethanol, air dried and resuspended in 17 µL of distilled water. Digested DNA (15 µL) was boiled for 10 minutes, and then cooled immediately on ice, and labelled with DIG. The labelling reaction consisted of 15 µL digested plasmid, 2 µL hexanucleotide mix (Roche), 2 µL DIG labelling mixture (Roche), and 1 µL (2 u/µL) Klenow enzyme (Roche). Reaction mix was incubated for 24 hours at 37 ^{o}C to increase the amount of labelled DNA. The reaction was stopped by adding 2 µL of 0.5 M EDTA, pH 8.0. DNA was precipitated with 2 µL of 4 M LiCl, and 60 µL of 100% ethanol at -20 ^{o}C. The precipitated DNA was held at -70 ^{o}C for at least 30 min then centrifuged at 12,000 rpm for 15 minutes at 4 ^{o}C. The pellet was washed with ice cold 70% ethanol, spun for 1 min, and resuspended in 50 µL TE buffer.

4.3.4.2 Extraction and restriction digest of bacterial genomic *DNA*

Genomic DNA (gDNA) was extracted from bacterial strains using a Promega Genomic DNA purification kit according to manufacturer's instructions. Restriction digests were carried out using 1 µg of gDNA from wild-type *Pseudoalteromonas* sp.strain 80, *Pseudoalteromonas* strain 80-Rif2 or Tn10-induced mutant 7Km using 1-2 µL of restriction endonucleases *SphI* (10 u/µL), *NotI* (10 u/µL), *EcoRI* (20 u/µL),or *BglII* (10 u/µL) (New England Biolabs). Fifty µL digestion reactions were incubated for approximately 23 hr at 37 ^{o}C to ensure complete digestion.

Linearised pLOF/Km served as control. The pLOF/Km plasmid vector was digested with *SphI* and *Bgl*IIfor 5 hr at 37 ^{o}C. DNA fragments were separated using agarose gel electrophoresis (0.8% w/v). Samples were run against a 1 kb DNA ladder (New England Biolabs) and a λ DNA-*Hind*III standard, and both undigested and linearized pLOF/Km plasmid were included as controls.

4.3.4.3 Transfer of DNA from agarose gel to nylon membrane

Gels were pre-treated for transfer of DNA. Two washes with 0.25 M HCl, for 15 min each, depurinated the DNA fragments. Two washes with 0.5 M NaOH / 1 M NaCl, for 15 min each, denatured the DNA to facilitate hybridization of the probe. Finally, two washes with 0.5 M Tris- HCl / 1 M NaCl (pH 7.4), for 15 min each, equilibrated the pH of the gel. The gel was rinsed for 2 min in distilled H_2O after each set of washes.

The DNA was transferred from the agarose gel to a positively-charged nylon membrane (Roche Applied Science). The gel was placed on a stack of 3 sheets of 3 MM blotting paper (Whatman), on top of a glass plate, placed across a dish containing 20 × SSC (3 M NaCl, 300 mM trisodium citrate, pH 7). The nylon membrane was wet with distilled H_2O then rinsed in 20 × SSC and placed flush on top of the gel. The membrane was topped with 3 sheets of 3 MM paper and a weight stack of paper towel.

Transfer was allowed to proceed for 4.5 h. After transfer the nylon membrane was placed between two pieces of 3 MM paper covered in foil and baked overnight at 70 – 80 ^{o}C to bind the DNA to the membrane.

4.3.4.4 Hybridization of the DNA probe

The nylon membrane pre-hybridized in a hybridization bag with 20 mL of hybridization solution which consist of 50% formamide, 5x SSC, 2% blocking stock (from 10% sterile stock solution), 0.1% Na-N-laurylsarcosine, and 0.02% SDS, and incubated overnight (16 hr) at 37 ^{o}C. The DIG-labelled DNA probes were boiled for 10 min to denature the DNA to facilitate hybridization and immediately transferred to ice-water for a further 5 min. The DIG-labelled probes were diluted 1:1000 in hybridization solution. The pre hybridization solution was removed from the hybridization bag and replaced with 10 mL of diluted probe per 100 cm^2 of membrane. The membrane was incubated with the probe overnight at 37 ^{o}C.

4.3.4.5 Washing nylon membrane and detection of DNA probe

The nylon membrane was washed at 68 ^{o}C in 2 × SSC / 0.1% SDS two times for 5 min and 0.1 × SSC / 0.1% SDS two times for 15 min to remove un-hybridized DNA probe. This was followed by further washing at room temperature for 1 min in washing buffer (0.1 M maleic acid, 0.15 M NaCl, 0.3% Tween 20, pH 7.5). The membrane was then incubated with gentle rocking at room temperature for 30 min in buffer 2 (1% (w/v) blocking reagent (Roche Applied Science) in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) to decrease background in the Southern blot.

The membrane was incubated in 50 mL of buffer 2 containing 10 µL anti-DIG-AP conjugate (Roche Applied Science) with gentle rocking at room temperature for 30 min. The membrane was washed two times for 15 min in washing buffer, followed by incubation for 5 min in buffer 3 (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5) to equilibrate the membrane. The membrane was placed into a hybridization bag and 1 mL of CSPD (diluted 1:1000 in buffer 3) was massaged into the membrane and the excess removed. The sealed hybridization bag was placed in a pre-warmed film cassette and incubates in the dark for 15 min at 37 ^{o}C, then exposure for 30 min, 60 min and 2 hr at room temperature to Fuji X-ray film. The film was developed under safe light in dark room for 2 min in developer solution, wash with water for 1 min, then transfer to fixer solution for 5 min. Finally, washed with water for few minutes and left to air dry. The membrane was stripped of their probe and kept wet in sealed in bag in 2 X SSC, or incubated at 37 ^{o}C with new probe.

4.3.5 Analysis of the mutated gene using TAIL-PCR

TAIL-PCR uses three nested specific primers in consecutive reactions together with an arbitrary degenerate (AD) primer having a lower *T*m (melting temperature), so that the relative amplification efficiencies of specific and nonspecific products can be thermally controlled. In the primary reaction, one low stringency PCR cycle is conducted to create one or more annealing sites for the AD primer in the targeted sequence. Specific product is then preferentially amplified over nonspecific ones by swapping of two high-stringency PCR cycles with one reduced-stringency PCR cycle (Table 4.3). This is based on the principle that in the high-stringency PCR cycles with high annealing temperature only the specific primer having a higher *T*m can efficiently anneal to target molecules, whereas the AD primer is much less efficient for annealing because of its lower *T*m. The semi nested PCR amplifications help to achieve higher specificity. By two rounds of TAIL-PCR reactions, specific products that are primed at one end by specific primers and the other end by AD primer are amplified to levels visible on agarose gel (Liu *et al*., 2004).

4.3.5.1 Primer design

Three adjacent primers were designed complementary to each end of the Tn10 sequence between *Bam*HI sites, using the free software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/). The sequences and locations of the specific primers are shown in Figure 4.2.

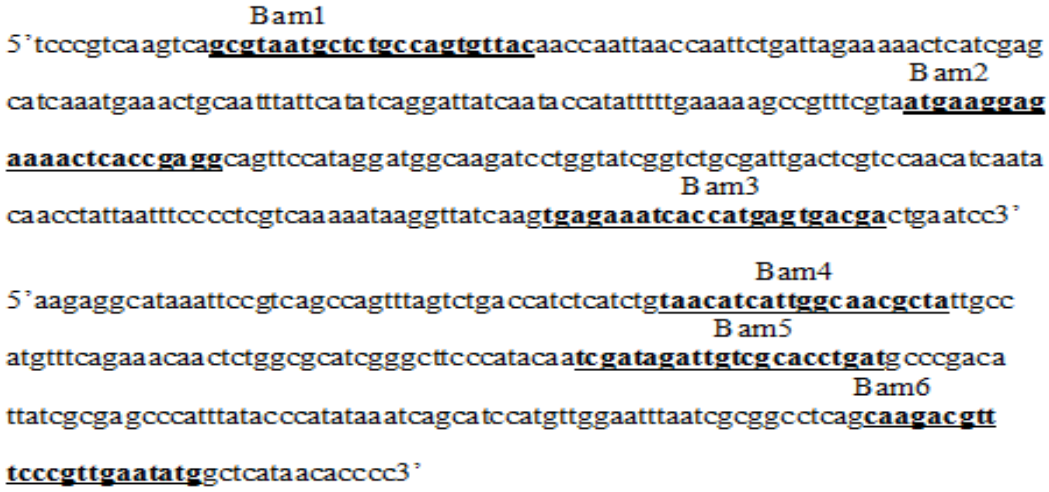


Figure 4.2. Primers used for thermal asymmetric interlaced PCR (TAIL-PCR) Bam1, Bam2 and Bam3 are left Tn10 transposon specific primer. Consequently, Bam4, Bam5 and Bam6 are right Tn10 transposon specific primers.

Arbitrary degenerate (AD) primers were designed to be 15-16 nucleotides in length with an average Tm of approx. 45-46 ^{o}C. Degenerate base are introduced in the primers with 128-256 times degeneracy. The degenerate primers used are given in Table 4.3 (Clarke & Wilkson, 2003).

Table 4.3. Sequences of specific and arbitrary degenerate primers used for TAIL-PCR.

Specific primers				
Name	Sequence	Length	Temperature ^{o}C	
Bam1	cctcactttctggctggatgat	22-mer	60-63	
Bam2	acaccttcttcacgaggcagac	22-mer	60-63	
Bam3	cgactgtgctggtcattaaacg	22-mer	60-63	
Bam4	acaccttcttcacgaggcagac	22-mer	60-63	
Bam5	cgactgtgctggtcattaaacg	22-mer	60-63	
Bam6	acactgatgaatgttccgttgc	22-mer	60-63	
Arbitrary primers (Clarke & Wilkson, 2003)				
Name	Sequence 5'-3'	Length	Temperature ^{o}C	Degeneracy
AD1	ngtcgaswganawgaa	16-mer	46	128'
AD2	gtncgaswcanawgtt	16-mer	46	128'
AD3	wgtgnagwancanaga	16-mer	45	256'
S = C or G W = A or T				

The TAIL-PCR cycling conditions were derived from (Liu *et al.*, 2004). Cycling conditions for the primary, secondary and tertiary PCR reactions are shown in Table 4.4.

Table 4.4. Reaction parameters for the primary, secondary and tertiary TAIL-PCR.

Reaction	File No.	Cycle No.	Thermal condition
Primary	1	1	93°C (3 min), 95°C (1 min)

	2	5	94°C (30 s), 62°C (1 min), 72°C (2.5 min)
	3	1	94°C (30 s), 25°C (3 min), ramping to 72°C over 3 min, 72°C (2.5 min)
	4	14	94°C (15 s), 65°C (1 min), 72°C (2.5 min), 94°C (15 s), 65°C (1 min), 72°C (2.5 min), 94°C (15 s), 29°C (1 min), 72°C (2.5 min)
	5	1	72°C (5 min)
Secondary	6	11–12	94°C (15 s), 62°C (1 min), 72°C (2.5 min), 94°C (15 s), 62°C (1 min), 72°C (2.5min), 94°C (15 s), 29°C (1 min), 72°C (2.5 min)
	5	1	72°C (5 min)
Tertiary	7	12–14	94°C (40 s), 45°C (1 min), 72°C (2.5 min)
	5	1	72°C (5 min)

TAIL-PCR was used to amplify the 5′-flanking regions of the gene of 7Km mutant modified from (Liu *et al.*, 2004)

4.3.5.2 Primary PCR

The reaction mix for the primary PCR consisted of 2.5 µL 10X PCR buffer, 2.0 µL 25 mM$MgCl_2$ (final concentration 2.0 mM), 2.0 µL of 2mM mix dNTPs, 2.5µL specific primer Bam1 or Bam4, 2.5µL AD1 primer (50 pmol, final concentration 2 µM), *Ex-Taq* DNA polymerase 0.75–0.8 U (0.16 µl), ddH_2O to 25 µL. 1.0 µL of the 3.5-5.5 kb *Sph*I fragments of 7Km mutant (30-35 ng) DNA template was added. If one AD failed (e.g. AD1), another one was tried (e.g. AD2) and so on(Liu *et al.*, 2004). The template of genomic DNA was prepare by digesting the genomic DNA with *Sph*I separating on agarose gel, cutting out the gel piece containing the 3.5-5.5 kb fragment and eluting the DNA fragment from the gel. Primary PCR amplification was carried out in a thermocycler (Eppendorf Mastercycle) using the conditions given in (Table 4.4).

4.3.5.3 Secondary PCR

The reaction mix for the secondary PCR consisted of 2.5 µL 10X PCR buffer, 2.0 µL 25 mM$MgCl_2$, 2.0 µL of 2mM mix dNTPs, 2.5 µL specific primer Bam2 or Bam5 (5 pmol), 2µL of the same AD primer (40 pmol) which was used in primary reaction, and Ex-TaqDNA polymerase, and the volume was adjusted with ddH2O to 25µL. The product of the primary PCR reaction was diluted 1:20 in H_2O and 1 µL used as template in the secondary reaction. Secondary PCR amplification was carried out using the conditions given in (Table 4.4) (Liu *et al*., 2004).

4.3.5.4 Tertiary PCR

The reaction mix for the tertiary PCR consisted of 2.5 µL 10X PCR buffer, 2.0 µL 25 mM$MgCl_2$, 2.0 µL, of 2mM mix dNTPs, 2.0 µL, Specific primer Bam3 (left site) or Bam6 (right) site (6.25 pmol), 1.5 µL of the AD primer which was used successfully in the primary reaction (30 pmol), *Taq* DNA polymerase 0.6 U, adjusted with ddH_2O to 25 µL. One µL of the secondary PCR product was diluted with 10 µL of H_2O and 1 µL of the diluted DNA was added to each reaction. Tertiary amplification was performed using thermal conditions as summarized in Table 4.4(Liu *et al*., 2004).

4.3.5.5 Purification and sequencing

Tertiary PCR products were separated on a 0.8% agarose gel. Target bands were excised and purified using a gel purification kit (Qiagen) according to the manufacturer's protocol. Purified DNA was sequenced by the Australian Genomic Research Facility (AGRF, Sydney, Australia). The nucleotide sequences were edited using Chromas Lite v2.1 and nearest relative sequences identified using a BLAST search.

4.3.6 Analysis of the mutated gene using plasmid rescue technique

The gene rescue technique includes the digestion of the mutant's genomic DNA with selected restriction enzymes to generate genomic fragments that include Tn10/Km flanked by genomic DNA, then cloning these fragments into a linearize vector, transforming *E. coli* with the plasmids and selecting for Km resistant colonies. Plasmid DNA is then extracted from Km resistant isolates and the cloned inserts sequenced to reveal the identity of the interrupted gene.

4.3.6.1 Preparation of *E. coli* competent cells

The competent cells of several *E. coli* DH5α were prepare according to standard protocols (Sambrook *et al.*, 1989).

4.3.6.2 Digestion of genomic DNA and vector

*Sph*I restriction enzyme digests of 1µg of genomic DNA of 7Km mutant, or the plasmid vector PTZ19R, were carried out according to the manufacturer instructions. This enzyme linearizes the vector (restriction site located in the multiple cloning site of the vectors) and is not cutting inside the Tn10 transposon cassette (and thus expected to cut the genomic DNA of 7Km outside the Tn10 sequences). Digestion reactions were incubated for 5 hr at 37 ^{o}C. Digestion was confirmed by agarose gel electrophoresis. Two microliters of the digests were run on 0.8% agarose gel in 1x TAE buffer (40 mM Tris, 1mM EDTA, 20 mM glacial acetic acid) at 60 volts for 3 h along side Lambda-*Hind*III DNA standards to confirm that the genomic DNA was completely digested. The restriction enzyme was inactivated at 65 ^{o}C for 20 min before ligation.

4.3.6.3 Ligation

The DNA fragments generated from the Tn10-induced mutant 7Km by cutting the bacterial genomic DNA with *Sph*I were cloned into the vector PTZ19R in a 10µL ligation reaction consisting of 2 units of T4 DNA ligase (New England Biolabs) and the manufacturer's buffer. The ligation reaction was performed at 16 °C overnight and inactivated by incubating at 65 °C for 20 min (according to manufacturer's instructions).

4.3.6.4 Transformation of *E. coli* and selection of recombinant plasmid

100 µL of competent cells of *E. coli* DH5α were mixed with 10 µL of the ligation reaction and incubated for 20 min on ice. Cells were heat shocked for 5 min at 37 ^{o}C then placed on ice for 2 min. 900 µL of LB medium was added to each reaction and incubated for 1-2 hr at 37 °C. 100 µL of each culture was spread onto two plates containing selective LB agar supplemented with 100 mg/mL ampicillin and 25 mg/mL Km.Plasmid DNA was extracted from colonies that grew on selective medium and analysed by restriction analysis, using the restriction enzyme *Sph*I to confirm the presence of a 5.5 kb fragment. Primers used to sequence the cloned fragment from the vector and from the Tn-10 into the genomic DNA

were Bam3 and Bam6 (Table 4.3).Further primers were designed to sequence further into the genomic DNA using sequences derived from Bam6 (Bam6-seq1 - TGGTGATGACGCTGAGAAGT and Bam6-seq2 - CGGTGAAGTATGCCTTTGAA)

4.4 Results

4.4.1 Rifampicin and streptomycin mutants

The approach taken in this work to investigate and identify the gene(s) responsible for producing inhibitory compound(s) in *Pseudoalteromnas* sp. strain 80 was to construct mutants that lost their ability to suppress the pathogen and compare them to the wild-type strain 80. The mutagenesis was carried out using a mini-Tn10 transposon, which was carried on the pLOF/Km plasmid vector. The mini-Tn10 transposon contains a kanamycin-resistance gene to simplify the selection of transformed cells. Therefore it was required to test that strain 80 was sensitive to Km before starting the mutagenesis procedure. It was also necessary to produce a Rif-resistant mutant of strain 80 to enable the selection against the *E. coli* donor. Antibiotic sensitivity test against six known antibiotics (Amp 100 µg/ml, Sm 50 µg/ml, Tc 10 µg/ml, Cm 50 µg/ml, Km 100 µg/ml, Rif 100 µg/ml) revealed that Sm and Rif could be useful for selecting Tn10-transformed cells since the *E. coli* donor of the transposon was sensitive to these antibiotics.

Antibiotic pressure for the generation of spontaneous mutants was done against Sm (25 µg/ml and 50 µg/ml) and Rif (40 µg/ml and 100 µg/ml). Three antagonistic bacteria, which gave strong inhibitory activity against *V. owensii* DY05, including *Vibrio* sp. strain 34*,* *Psychrobacter* sp. strain 62*,* and *Pseudoalteromonas* sp.strain 80, were used for producing Rif and Sm resistant mutant strains. Two of each spontaneous Rif and Sm resistance mutants were studied morphologically and confirmed for maintaining antimicrobial activity against the pathogen in well diffusion assay using wild type as positive control. The Rif mutants were found to be more stable and thus more reliable for transposon mutagenesis. The identities of two Rif mutants of each strain were confirmed by sequencing of the 16S rRNA gene fragment (Australian Genome Research Facility Ltd) and submission to the blast and Clustalw2. Consequently, Rif mutant from all three strains, including *Vibrio* sp. strain 34*,* *Psychrobacter* sp. strain 62 and*Pseudoalteromonas* sp. strain 80, were subjected to the transposon mutagenesis. However, it was only successful in yielding non-suppressive

mutants with 80-Rif2 mutant of *Pseudoalteromonas* sp. strain 80, so only the work with this strain is reported in this chapter.

4.4.2 Generation of mutants affected in pathogen-suppression

Transposon mutagenesis was undertaken to determine the gene(s) responsible for the antimicrobial activity of *Pseudoalteromonas* sp.strain 80. The 80-Rif2 mutant (a spontaneous mutant of strain 80 resistant to Rif) was first transformed with a pLOF/KM carrying mini-Tn10 transposon containing a kanamycin-resistance marker.The Tn10 transposable element is located between the IS10IR elements (Figure 4.1). This is from base-pair7339to base-pair 9241 of the plasmid sequence (approx. the 1.9 kb fragment between the *Bam*HI sites, with a small number of bp flanking these sites, Figure 4.1).

Thirty-five thousands (35000) Rif/Km-resistant, Tn10-induced, transformants were obtained on Rif+Km selective medium. Most transformants were yellow and a few were orange (248 transformants) or non-pigmented (one transformant). All transformants were tested for suppression of the lobster phyllosoma *V. owensii* DY05 pathogen and for antibiotic sensitivity. All yellow and orange transformants retained antimicrobial activity, while the non-pigmented transformant showed lack of DY05 suppression (Figure 4.3). This non-pigmented mutant, designated 7Km (Figures 4.3 and 4.4), was tested against six antibiotics (Amp 100 µg/ml, Sm 50µg/ml, Tc 10µg/ml, Cm 50 µg/ml, Km 100 µg/ml, Rif 100µg/ml) and was found to be resistant to Km (100 µg/mL) and Rif but sensitive to the other antibiotics. The sensitivity to Amp, which is carried on the pLOF/Km plasmid outside the Tn10, indicated that only the mini Tn10 transposon integrated into the bacterial genomic DNA and not the entire pLOF/Km plasmid. The same Tn10-transfomation method was attempted also with *pseudoalteromas pacicida*-like isolate PP107, *Psychrobacter* sp. strain 62 and *Vibrio* sp. strain 34, but unfortunately no mutant lacking the pathogen inhibitory activity was produced. Therefore this chapter will concentrate on the characterization of the mutant of strain 80-Rif2, namely 7Km.

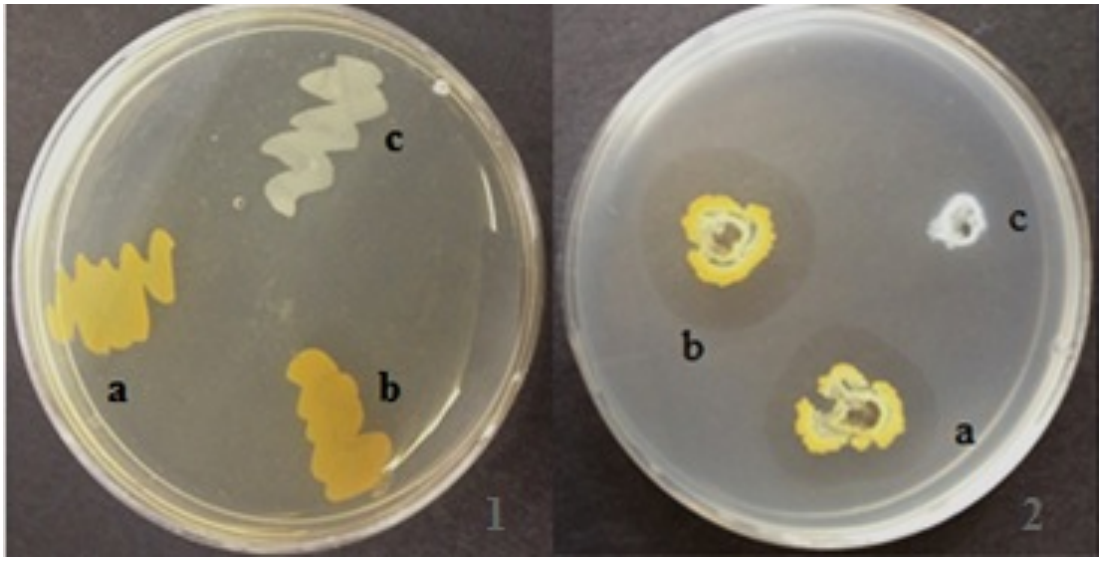


Figure 4.3. (1) Morphological characteristics on MA after 24 hr incubation period and(2) Antimicrobial activity against the *V. owensii* after 24 hr incubation period (a) *Pseudoalteromonas* sp. strain 80 WT, (b) *Pseudoalteromonas* strain80-Rif2 mutant and *Pseudoalteromonas* strain7Km mutant.

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CLUSTAL 2.1 multiple sequence alignment


80WT_H12 TGCACATGCAGTCGAGCGGTAACATTTCTAGCTTGCTAGAAGATGACGAGCGGCGGACGG 60
7Km_H12  TGCACATGCAGTCGAGCGGTAACATTTCTAGCTTGCTAGAAGATGACGAGCGGCGGACGG 60
Rif2_G03 TGCACATGCAGTCGAGCGGTAACATTTCTAGCTTGCTAGAAGATGACGAGCGGCGGACGG 60
 ************************************************************

80WT_H12 GTGAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACCATTGGAAACGATGGCTAA 120
7Km_H12  GTGAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACCATTGGAAACGATGGCTAA 120
Rif2_G03 GTGAGTAATGCTTGGGAACATGCCTTGAGGTGGGGGACAACCATTGGAAACGATGGCTAA 120
 ************************************************************

80WT_H12 TACCGCATAATGTCTACGGACCAAAGGGGGCTTCGGCTCTCGCCTTTAGATTGGCCCAAG 180
7Km_H12  TACCGCATAATGTCTACGGACCAAAGGGGGCTTCGGCTCTCGCCTTTAGATTGGCCCAAG 180
Rif2_G03 TACCGCATAATGTCTACGGACCAAAGGGGGCTTCGGCTCTCGCCTTTAGATTGGCCCAAG 180
************************************************************

80WT_H12 TGGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCCTAGCTGGTTTGA 240
7Km_H12  TGGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCCTAGCTGGTTTGA 240
Rif2_G03 TGGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCCTAGCTGGTTTGA 240
************************************************************

80WT_H12 GAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT 300
7Km_H12  GAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT 300
Rif2_G03 GAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGT 300
************************************************************

80WT_H12 GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC 360
7Km_H12  GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC 360
Rif2_G03 GGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC 360
 ************************************************************

80WT_H12 CTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCTAGCT 420
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Rif2_G03 CTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCTAGCT 420
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80WT_H12 GTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG 480
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Rif2_G03 GTGACGTTACTGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG 480
************************************************************

80WT_H12 AGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTACGCAGGCGGTTTGTTAAGC 540
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Rif2_G03 TGATAGAGGGTGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATA 660
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80WT_H12 CCGATGGCGAAGGCAGCCACCTGGGTCAACACTGACGCTCATGTACGAAAGCGTGGGGAG 720
7Km_H12  CCGATGGCGAAGGCAGCCACCTGGGTCAACACTGACGCTCATGTACGAAAGCGTGGGGAG 720
Rif2_G03 CCGATGGCGAAGGCAGCCACCTGGGTCAACACTGACGCTCATGTACGAAAGCGTGGGGAG 720
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80WT_H12 CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGGAGCTGGGG 780
7Km_H12  CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGGAGCTGGGG 780
Rif2_G03 CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGGAGCTGGGG 780
         ************************************************************

80WT_H12   TCTTCGGACAACTTTTCCAAAGCTAACGCA 810
7Km_H12    TCTTCGGACAACTTTTCCAAAGCTAACGCA 810
Rif2_G03   TCTTCGGACAACTTTTCCAAAGCTAACGCA 810
*****************************a
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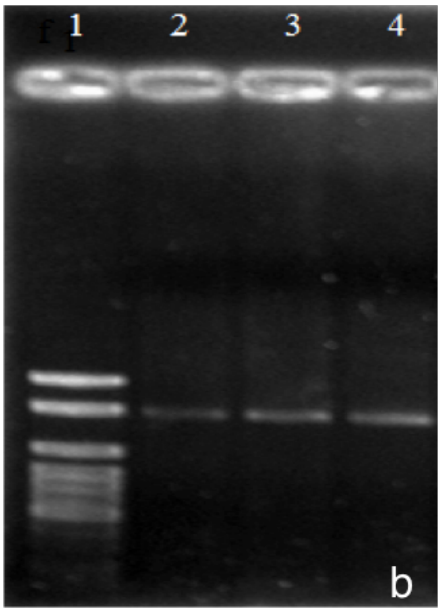

Figure 4.4. (a) ClustalW2 alignment of nucleotide sequences of the 16SrRNA gene fragments of *Peudoalteromonas*sp. strain 80, WT, and the mutants fragment 80-Rif2 and 7Km **(b)**Amplification the 16S rRNA gene from *Peudoalteromonas* sp. wild type and the mutants. (1) 1kb DNA ladder, (2) 80-Rif2 mutant, (3) 7km mutant, (4) WT.

4.4.3 Biochemical traits of the *Pseudoalteromonas* sp.strain 80and mutants80-Rif2 and 7Km

4.4.2.1 Protease and amylase activities

Testing the whole culture and filtered supernatant samples of the *Pseudoalteromonas* sp. strain 80 and the mutants80-Rif2 and 7Km for extracellular enzyme activity (as described in Chapter 3, Sections 3.3.2 and 3.3.4) revealed that while the wild typeproduced enzymes that degraded skim milk, starch and gelatin the mutant lost (or was significantly affected in) these protease and amylase activities (Figure 4.5).

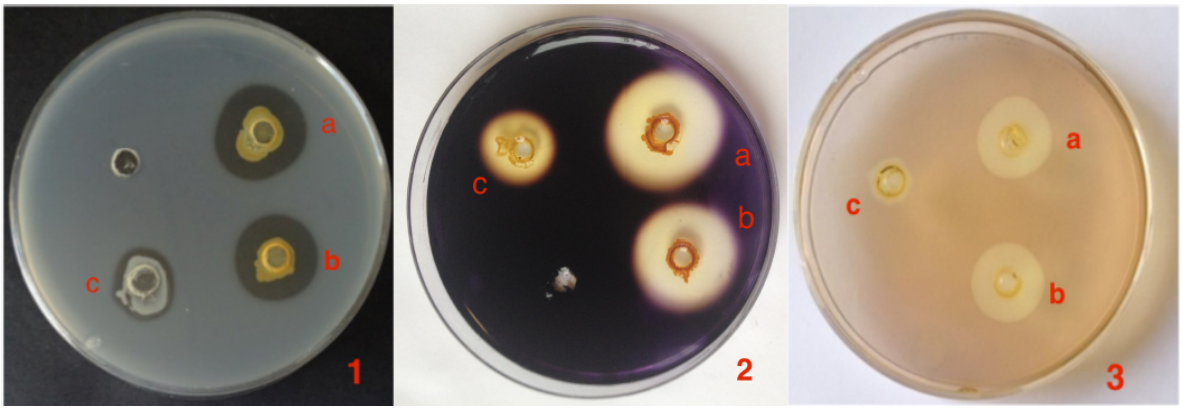

Figure 4.5. Protease and amylase activities (1) Protease activity of whole culture samples on skim milk, (2) amylase activity of whole culture samples on starch medium, and (3) protease activity offiltered supernatant on medium containing gelatin. (a)*Pseudoalteromonas* sp. strain 80, (b) 80-Rif2 mutant, (c) 7Km mutant.

4.4.2.2 Analysis of the antimicrobial molecules using GS-MS

Volatile and non-volatile small molecules were extracted as described in Chapter 3 (Section 3.3.7) and submitted to the GC-MS and TLC autography followed by bioautography.

It is clear that the 7km mutant produced the same alkene volatiles as the wild type and the 80-Rif2 mutant as presented in Table 4.5. Because the mutant demonstrated no pathogen suppressive activity it can be suggested that these alkene volatiles are not involved in pathogen suppression in this case. However on the bioautography plate bands of inhibition were observed (bands of inhibition indicated here though distinct to the eye were not all so clear when photographed), corresponding to non-volatile compounds that supressed organism growth. Such suppressive compounds derived from *Pseudoalteromonas* sp. strain 80 wild

type but not the 80-Rif2 mutant as showed in Figure 4.6 a and b. This confirmed that these molecules should be further investigated as prime candidates in pathogen suppression.

Table 4.5. Alkene volatiles extracted from filtered supernatant of the *Pseudoalteromonas* sp. strain 80 and the mutants 80-Rif2 and 7Km.

Compound	AI	Pub .AI	7Km	Rif2	80	Control
1-dodecene	1191	1189	10.2	12.9	13.5	0
1-tetradecene	1391	1393	27.1	27.5	27.4	0
1-hexadecene	1591	1590	31.1	28.2	28.8	0
1-octadecene	1792	1793	19.1	18.2	18.0	0
1-eicosene	1992	1990	8.9	9.6	8.5	0
1-docosene	2193	2195	3.5	3.6	3.7	0

The methods are described in Chapter 3, Section 3.3.8.1.

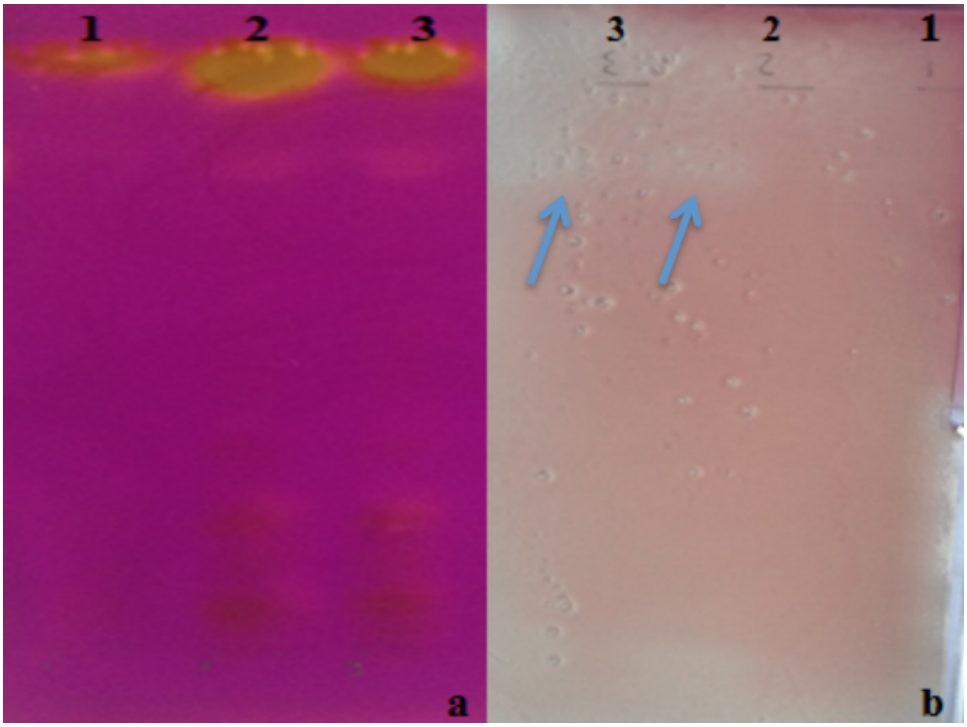

Figure 4.6. Separation of small molecules on TLC plate (a) autography dyed with Potassium pemanagante. (b) bioautography of (1), 7Km mutant, (2) 80-Rif2 mutant, (3) *Pseudoalteromoans* sp. strain 80 wild type. The methods are described in Chapter 3, Section 3.3.7. The blue arrows indicates the bands of inhibition of the *Pseudoalteromonas* sp strain 80 and the 80-Rif2 mutant.

4.4.3 Confirmation of transformation using Southern Blot analysis

4.4.4 Southern Blot Hybridization analysis

To confirm that 7Km mutant (that lost the antimicrobial activity) contained integrated Tn10 transoposon in its genome Southern blot analysis was performed on 7Km and the parent strains 80 and 80-Rif2. Genomic DNA was extracted from all three strains and analysed by agarose gel electrophoresis. The suitability of the DNA for further analysis was demonstrated by a single band of high molecular weight DNA from each of the extractions (Figure 4.7a). Plasmid DNA and genomic DNA, digested with several restriction enzymes including *Not*I or *Bam*HI (cutting within the mini-Tn10) as well as *Bgl*II, *Eco*RI, *Eco*RV or *Pst*I (do not cut within the mini-Tn10), are presented in Figure 4.8a. The *Bgl*II-linearized plasmid probes prepared from pLOF/Km is presented in Figure 4.7b. The 1.5 kb *Not*I and 0.7 kb *Eco*RV/*Sph*I probes are not shown.

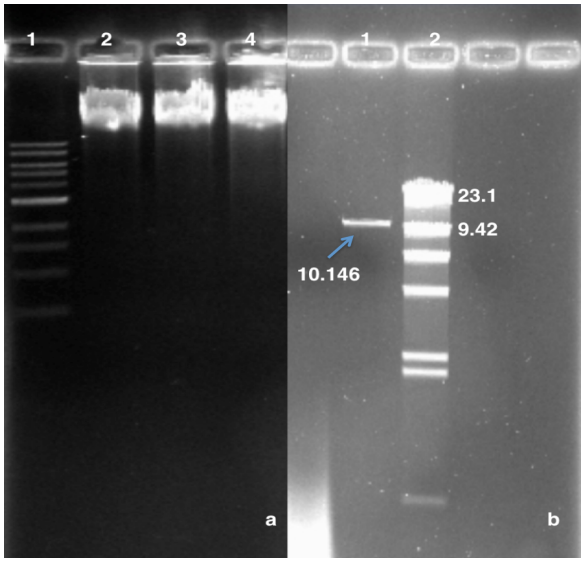

Figure 4.7. Gel analysis of (a) extracted genomic DNA from WT, and 80-Rif2 and 7Km mutants (lanes 2-4 respectively) (b) Linearized pLOF/KM plasmid digested with restriction enzyme *Bgl*II.

The entire-plasmid (*Bgl*II-linearized 10.15 kb plasmid, Figure 4.7b) was used as a probe to confirm that the Tn10 integrated into a single site in the genome of the 7Km mutant. Figure 4.8 shows that the mini-Tn10 transposon had integrated at a single chromosomal location. The probe did not hybridise with wild type DNA as shown in Figure 4.8,confirming the Tn10 presence only in the 7Km mutant.

The 0.7 kb probe, which was prepared by digesting pLOF/Km with *Eco*RV+*Sph*I, contains

sequences from outside the Tn10 transposon. Indeed, this probe did not hybridize to 7Km chromosomal DNA, confirming that only the Tn10 and not the entire plasmid got integrated into the chromosome (Figure 4.9). The probe did hybridize with control plasmid DNA containing this fragment (Figure 4.9, lanes 2&3), as expected.

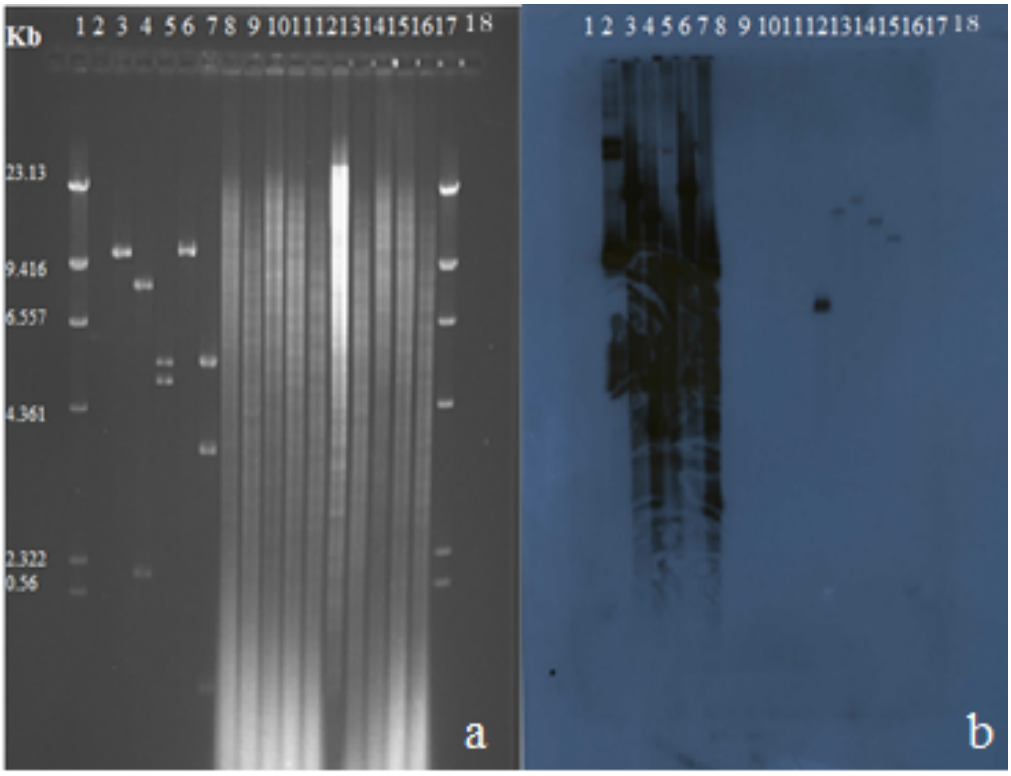

Figure 4.8. (a) Gel analysis prior to Southern blot. Lane 1and 18, λ HindIII DNA standard ladder; lanes 3-7, pLOF/KM plasmid digested with *Sph*I, *Eco*RV, *Eco*RI, *Bgl*II, and *Bgl*II+*Eco*RI; lanes 8-12 genomic DNA of*Pseudoalteromonas* sp. strain 80 wild type digested with *Sph*I, *Eco*RV, *Eco*RI, *Bgl*II, and *Bgl*II+*Eco*RI; Lanes 13-17 genomic DNA of 7Km digested with following restriction enzymes:*Sph*I, *Eco*RV, *Eco*RI, *Bgl*II, and *Bgl*II+*Eco*RI (b) Southern blot hybridization with 10.15 kb, *Bgl*II probe (linearized-plasmid).

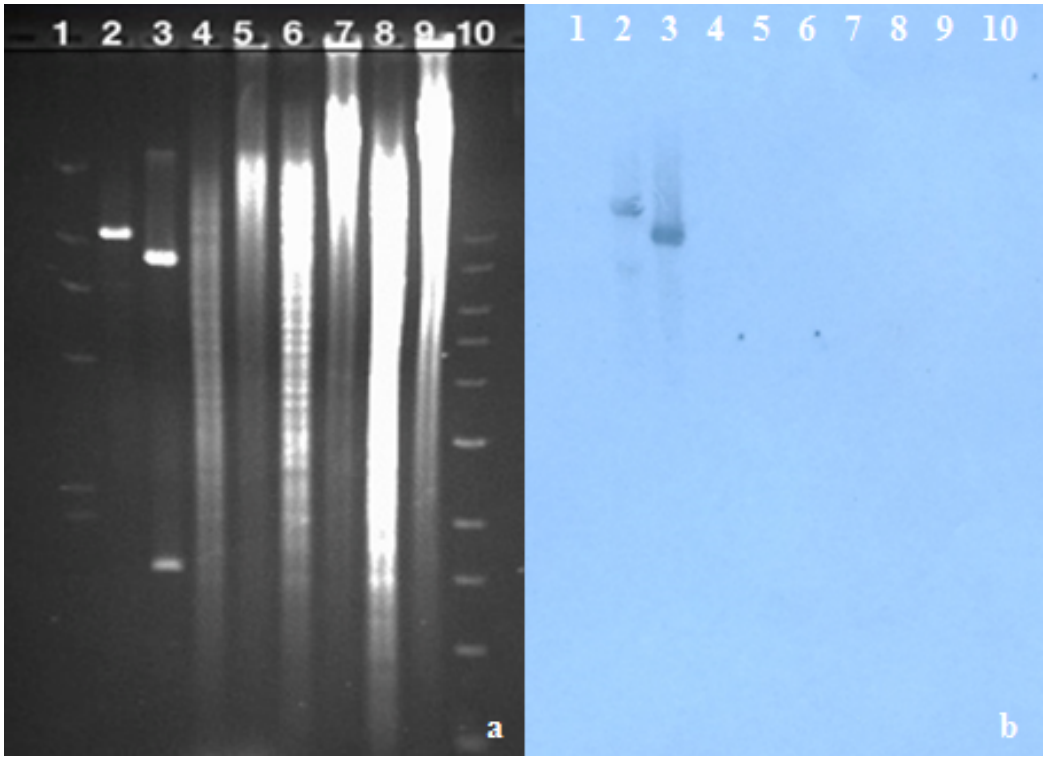

Figure 4.9. (a) Gel analysis prior to Southern blot. Lane 1, λ *Hind*III DNA standard ladder; lanes 2-3 pLOF/KM plasmid digested with *Bgl*II and *Not*I; lanes 4-5, gDNA of wild type strain 80 digested with *Bgl*II and *Not*I; lanes 6-7, gDNA of 80-Rif2 mutant digested with *Bgl*II and *Not*I; lanes 8-9, gDNA of 7Km mutant digested with *Bgl*II and *Not*I; lane 10, 1kb ladder. (b) Southern blot hybridization with *Eco*RV+*Sph*I 0.7 kb probe.

Southern blot analysis of the 7Kmmutant, 80-Rif2 mutant and wild-type strain 80 was also performed using the DIG-labelled 1.5 kb *Not*I probe, which contains Tn10 sequences. No bands were visible forthe genomic DNA of the wild-type strain 80 or 80-Rif2 mutant (Figure 4.10), confirming they do not contain Tn10 sequences. However, as expected, the genomic DNA of 7Km showed hybridization bands with the DIG-labelled 1.5 kb *Not*I probe (Figure 4.10). The probes hybridised to the *Bgl*II digested 7Km DNA (*Bgl*II does not cut inside the Tn10), confirming an insertion of the Tn10 into the chromosome in a single location (Figure 4.10, lane 10). It also hybridised to the *Not*I-digested 7Km DNA, but, as expected, only to the internal 1.5 kb *Not*I present on the Tn10 (Figure 4.10, lane11). All together, the Southern blot results presented in Figures 4.8to 4.10 confirmed that the mini-Tn10 transposon integrated into the genome of 7Km mutant at a single site.

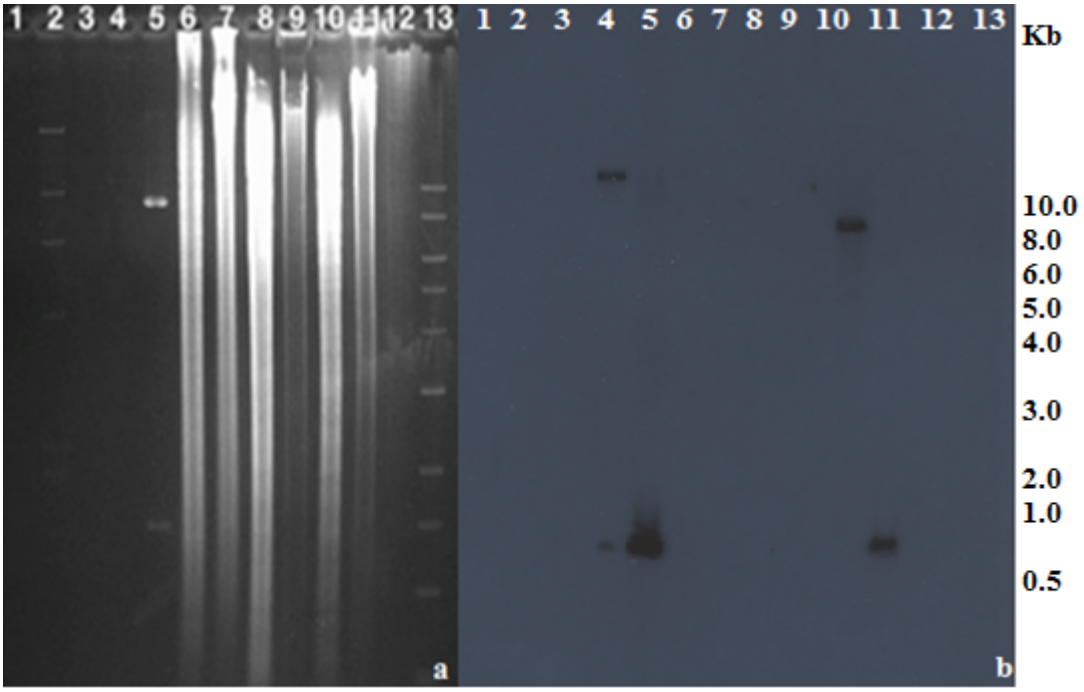

Figure 4.10. (a) Gel analysis of digested genomic DNA prior to Southern blot. Lane 1 loading buffer; lane 2 λ *Hind*III DNA standard ladder; lane 3 empty well; lanes 4 -5 pLOF/KM plasmid digested with *Bgl*II and *Not*I; Lanes 6-7 genomic DNA of strain 80 digested with *Bgl*II and *Not*I; Lanes 8-9 genomic DNA of 80-Rif2 digested with *Bgl*II and *Not*I; Lanes 10-11 genomic DNA of 7Km digested with *Bgl*II and *Not*I; Lanes 13 1kb ladder. (b) Southern blot hybridized with1.5kb *Not*I probe.

4.4.5 Attempting to identify the mutated gene using Tail PCR

TAIL-PCR utilizes three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled. Selection of an optimal specific primer for the primary TAIL-PCR is important for successful amplification.
Sequences flanking mini-Tn10 insertions were amplified using a thermal asymmetric interlaced polymerase chain reaction protocol described by (Liu & Whittier, 1995).

Figure 4.11 presents the expected gradual development of a specific PCR product as the process is progressing from the primary to the tertiary PCR reactions. Unfortunately, sequencing of the purified fragments did not yield any sequence that made sense. Therefore, we have proceeded with an attempt to rescue the Tn10 together with flanking chromosomal sequences from the affected gene in the mutant 7Km.

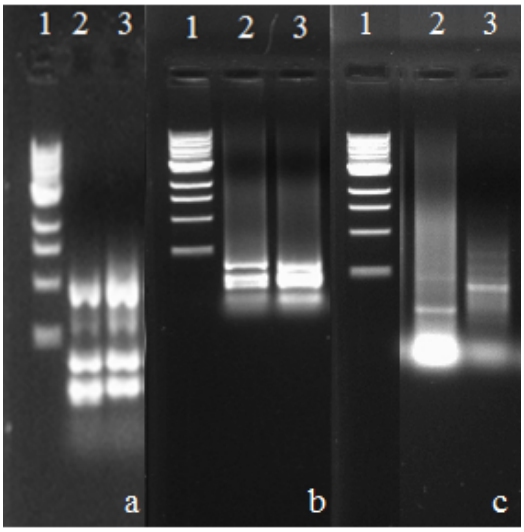

Figure 4.11. Results of TAIL-PCR used to amplify the flanking regions of the Tn10 in 7km mutant. (a) Primary reactions, lane 1 1Kb ladder, lane 2 amplification using specific primer Bam1, lane 3 amplificatin using Bam4 (b) Secondary reactions, lane 1 1Kb ladder, lane 2 amplification usung Bam2, lane 3 amplification using Bam5 (c) Tertiary reactions, Lane 1Kb ladder, lane 2 amplification using Bam3, lane 3 amplification using Bam6. In all reaction the arbitrary primer AD1 was used.

4.4.6 Partial identification of the gene affected in the mutant 7Km

The identification of the gene disrupted by the transposon in 7Km was made possible using the plasmid rescue technique. The genomic region containing the transposon and flanking chromosomal DNA of the 7Km mutant were obtained by *Sph*I digestion and cloning the digested genomic DNA into pTZ19R vector (in the *Sph*I site on the multiple cloning site). Selection of Amp+Km resistant colonies resulted in pTZ19R plasmids containing a 5.5 kb *Sph*I fragments (Figure 4.12). This is in agreement with Southern blot analysis that indicated that the *Sph*I fragment of approx. 5.5 kb contained the Tn10 transposon (Figure 4.8).

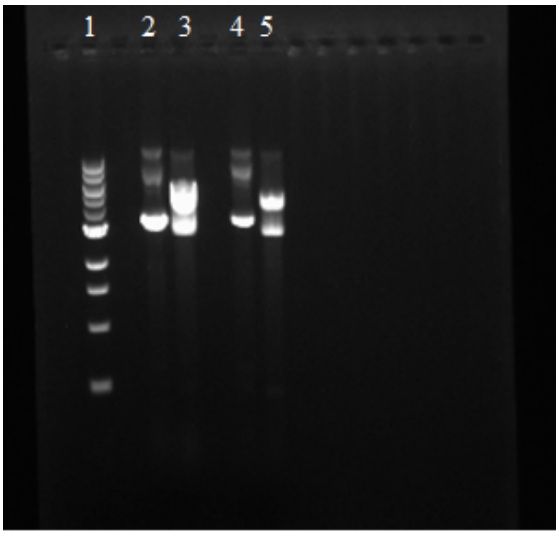

Figure 4.12. Gel analysis of pTZ19R candidates carrying the 5.5 kb *Sph*I fragment containing the Tn10 from the mutant 7Km. Lane 1, 1kb ladder; lanes 2 and 4, uncut candidate plasmids 1 and 2 respectively; lane 3 and 5, *Sph*I digested candidates 1 and 2 respectively, showing the presence of two fragments (the smaller one in the size of the pTZ19R vector and the larger one is the 7Km 5.5 kb insert containing the Tn10).

Alignment of the sequences obtained with the primers pTZ1233, pTZ1212 (vector origin, sequence from vector towards the *Sph*I insert of 7Km genomic sequences) as well as Bam3, Bam6 (Tn10 origin, sequence outwards from the Tn10), Bam6-seq1 ans Bam6-seq2 (drived from 7Km genomic sequences obtained with Bam6) (Appendix 3), allowed the construction of the physical map of the rescued plasmid and the location of the Tn10 in the genomic DNA of 7Km (Figure 4.13).

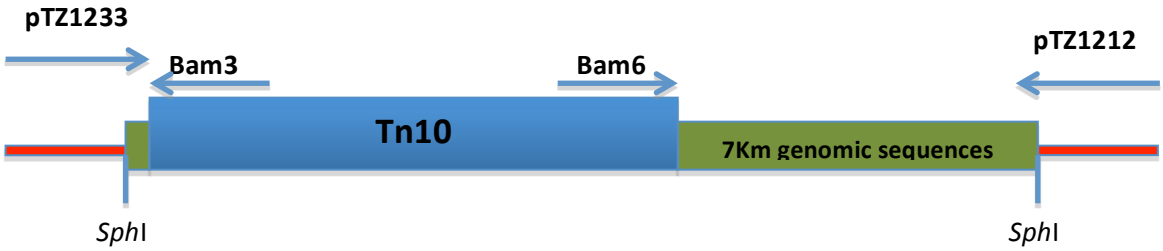

Figure 4.13. Physical map showing the location of the Tn10 inside genomic sequences of the mutant strain 7Km. Thin lines outside the *SphI* sites indicate the vector; Full alignment is shown in Appendix 3.

Sequence and blastX analyses revealed that the interrupted gene is likely to be a membrane bound transporter protein, with 100% homology to a resistance-nodulation-division (RND) transporter of*Pseudoalteromonas flavipulchra* JG1 (Table 4.6), a marine antagonistic bacterium with abundant antimicrobial metabolites (an unpublished shotgun sequence fragment from genomic sequencing). Other homologous proteins are shown in Table 4.6.

Table 4.6. Proteins with high homology to sequences interrupted by Tn10 and rescued from *Pseudoalteromonas* mutant 7Km.

Protein	Organism	% Identity to nearest relative	Nearest accession number
RND transporter	*Pseudoalteromonas flavipulchra*	100% (to nucleotides 2-523 of rescued 7Km genomic sequences)	WP_010605844
Hypothetical protein	*P. flavipulchra*	99% (nucleotides 1307-2254)	WP_010605842
Memberane protein	*P. flavipulchra*	98% (nucleotides 507-1295)	WP_010605843

BlastX results can be found in the following URL:

http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Get&ALIGNMENTS=100&ALIGNMENT_VIEW=Pairwise&CDD_SEARCH_STATE=0&DATABASE_SORT=0&DESCRIPTIONS=100&DYNAMIC_FORMAT=on&FIRST_QUERY_NUM=0&FORMAT_OBJECT=Alignment&FORMAT_PAGE_TARGET=&FORMAT_TYPE=HTML&GET_SEQUENCE=yes&I_THRESH=&LINE_LENGTH=60&MASK_CHAR=2&MASK_COLOR=1&NEW_VIEW=yes&NUM_OVERVIEW=100&OLD_BLAST=false&PAGE=Translations&QUERY_INDEX=0&QUERY_NUMBER=0&RESULTS_PAGE_TARGET=&RID=BSPM5WR6013&SHOW_LINKOUT=yes&SHOW_OVERVIEW=yes&STEP_NUMBER=&WORD_SIZE=3&DISPLAY_SORT=3&HSP_SORT=3&CONFIG_DESCR=2,3,4,5,6,7,8#alnHdr_498291686

Discussion

The genus *Pseudoalteromonas* is divided into two groups: pigmented and non-pigmented species. Pigmented species of the genus have been known to produce a range of low and high molecular weight active molecules with antimicrobial, anti-algicidal, fouling, and various pharmaceutically relevant activities. There is increasing evidence that the genus *Pseudoalteromonas* plays important ecological roles in the marine environment, and is a good resource for new bioactive compounds (Bowman, 2007). However, so far only a few strains have been well studied (Huang *et al.*, 2011). In the present study transposon mutagenesis was used to generate a mutant lacking the ability to produce compounds with antimicrobial activity against the hatchery pathogen *V. owensii* DY05. The bacterium *Pseudoalteromonas* sp. strain 80 has been shown to be antagonistic to the growthof lobster phyllosoma pathogen *V. owensii* DY05. To investigate gene(s) essential for antimicrobial production of *Pseudoalteromonas* sp. strain 80, genetic analysis was performed using transposon mutagenesis. The sensitivity test confirmed that the recipient *Pseudoalteromonas* sp. Strain 80 and the donor *E.coli*Sm10λ pir were sensitive to the same antibiotics. Therefore, rifampicin mutant of strain 80, namely 80-Rif2 strain, was produced using antibiotic pressure and its Rif resistance stability allowed its use in transposon mutagenesis.

Out of 35,000 kanamycin-resistant tranformants of strain 80-Rif2 only one non-pigmented mutant showed no antimicrobial activity. The chosen rifampicin mutant (80-Rif2) and the wild-type strain 80 produce yellow pigments, which give the bacterial colony a strong yellow appearance in particular on marine agar. It is believed that there is a correlation between the pigments and antimicrobial activity. Previous transposon study by Egan *et al*. (2002) correlated the antifouling production with pigmentation and discussed the importance of the pigment for the physiology and survival. Another study has shown that *Pseudoaltermonas tunicata* secreted an antibacterial protein in the presence of other competitors that contributed a competitive advantage to the bacterium during surface colonization (Rao *et al.*, 2005). Indeed our study shows that the mutant 7Km, which has lost its pigmentation, also lost its ability to suppress the growth of the pathogen, suggesting it has lost its ability to produce antimicrobial compounds. Vynne *et al*. (2011) suggested thatsome antimicrobial compoundsproduced by *Pseudoalteromonas* spp.can interfere with community interactionsdespite being unrelated to pigmentation.

The analysis of mutants affected in the expression of specific genes is perhaps the most powerful method to study the role and the mechanism of action of molecules that may contribute to pathogenicity and virulence of microbe (Tascon *et al.*, 1993). This preliminary analysis may lead to the identification of genes involved in antimicrobial production. Mutagenesis of strain 80-Rif2 resulted in three different types of pigment mutants including, yellow, orange and white phenotypes. Analysis of the antimicrobial activity properties of the three different pigmentation phenotypes revealed that the non-pigmented mutant differed from the wild type, the 80-Rif2 and other kanamycin (yellow and orange) mutants by losing the ability to inhibit the pathogen. Meanwhile the yellow and orange mutants still showed fully inhibiting activity toward *V. owensii* DY05. The observations indicated that there is a correlation between the pigmentation and the antimicrobial activity of *Pseudoalteromonas* sp. Wild-type strain 80. This observation agreed with Egan *et al*. (2002) who, in their study on antifouling activity of *Pseudoalteromonas tunicate,* demonstrated that the loss of antifouling capabilities correlates with the loss of yellow pigment production either directly or indirectly. The data describing the antifouling properties of the transposon mutants suggest that the production of fouling inhibitors is linked to the synthesis of yellow pigment or that fouling inhibitors and pigment are jointly regulated in *P. tunicata.* Therefore, genes disrupted to cause a change in pigmentation will also provide information regarding the identity and/ or regulation of antifouling components in this organism.

Due to their ability to produce single, stable and random insertions into the target genome, transposons are useful tools for genetic manipulation. High efficiency of mutagenesis allows a large number of colonies to be produced that contain random insertions within a specific gene, thus resulting in the loss of function of that gene. The use of transposon technology has previously been restricted to *E. coli* (Egan, 2001). De Lorenzo *et al*. (1990) have been successfully developed a method for manipulations introducing mini Tn5 into chromosomes of target Gram-negative bacteria. Herrero *et al.* (1990) developed a simple stable insertion cloning protocol for insertion of foreign genes into recipient chromosomesusing a modified mini-Tn10 version of the Tn10 transposon which has a kanamycin-resistance marker that allows for easy selection of mutants. We successfully generated transposon mutants of *Pseudoalteromonas* sp. strain 80 lacking the ability to inhibit lobster pathogen *in vitro* using a suicide vector pLOF mini-Tn10 system. However, Egan (2001) suggested three reasons for the low frequency of specific non anti-bacterial mutants generated by transposon mutagenesis

when they isolated one non anti-fungal mutant (FM3) from screening approximately 45000 transconjugants. The reason suggested were: “Firstly, it is possible that during mutagenesis a saturation point is reached whereby all genes in the pathway have been mutated. This would suggest that the pathway for the production of the anti-fungal compound is quite short, consisting of only one gene. A second possibility is that the production of the compound is linked to essential genes or cellular metabolites, in which case a mutation in any other gene would result in lethal phenotype. A third possibility is the insertion of the transposon into so-called “hot-spots”. Hot-spots are specific DNA sites where a transposon will preferentially insert” (Egan, 2001, p. 117).

The DIG-labelled linearized probe constitutes the kanamycin resistancegene mini Tn10 transposon from plasmid pLOF/KM. The probe is thus designed to confirm successful insertion of the mini Tn10 into the genome of 7kmputative transformants. It is expected that a band would be present in the genomic DNA of transformants, whilst being absent from the wild type, and the 80-Rif2 mutant, which lacks the mini Tn10. Hybridization with this probe will allow further conclusions to be drawn as to the nature of the mini Tn10 insertions. When digesting the genomic DNA with specific restriction enzymes, the number of bands present reveals how many insertion events have occurred in the genomic DNA of each transformant. It is most favourable that a transformation system produces single insertion events at a single locus, as this will minimise any difficulties with later recovery of the tagged sequences. The band sizes indicate randomness of integration and may also give some idea as to whether tandem repeats have occurred (depending upon which restriction enzymes are used). It is important that a successful transformation results in random integration in the host genome to increase the chances of tagging *Pseudoalteromonas* sp.strain 80 inhibition genes.

Here we succeeded in generating a mutant lacking the ability to inhibit the lobster phyllosoma pathogen *V. owensii* DY05. The gene affected in the mutant was found to share 100% homology with a membrane bound transporter proteinof the RND family, particularly to protein of *Pseudoalteromonas flavipulchra* JG1, a marine antagonistic bacterium with abundant antimicrobial metabolites. This finding may explain the phenotypes associated with the mutation in 7Km, as biochemical analyses of this mutant showed it is impared in milk casein and gelatine hydrolysis as well as in starch hydrolysis. It also excreted modified range of non-volatiles and extracellular ptoreins when compared to the wild type using TLC and

bioautagraphy-plates and GC-MC analysis. This suggests that while the wild type strain 80 most likely exports proteases and amylases into the medium, the 7Km mutant is impaired in this function due to a mutation is an RND membrane bound transporter. On the other hand, the mutant produced similar volatiles as the wild type strain 80, implicating a different excretion pattern of volatiles, independent of the RND transporters. RND proteins were implicated in drug resistance of important pathogens and in the transport of substances from the cell(Bazzini *et al.*, 2011; Nikaido & Takatsuka, 2009), suggesting that a mutation in such a transporter probably abolished the excretion of pathogen-suppressive substances from *Pseudoalteromonas* sp. strain 80 cells.

5.1 General discussion

The spiny lobster *Panulirus ornatus* is a scarce and an important commercial seafood product particularly in South East Asian countries and consequentlya potential candidate for Australian aquaculture (Cano-Gómez, 2012). However, infectious diseases threaten the viability of this lobster industry. Of particular consequence is the condition known as Vibriosis, caused by pathogenic species belonging to the *Vibrio* genus. The highly virulent *Vibrio owensii* DY05, an emerging bacterium related to the Harveyi-clade, has been isolated from affected lobsters *Panulirus ornatus*(Cano‐Gómez *et al.*, 2010) and is identified as a specialized enteric pathogen causing disease epizootics in early stage *P. ornatus*larviculture (Cano-Gómez, 2012; Goulden, *et al.*, 2012). *Vibrio* spp. are widespreadin marine environments, including estuaries, sediments, marine coastalwaters, and aquaculture farms (Hervio‐Heath *et al.*, 2002; Sawabe *et al.*, 2013; Thompson *et al.*, 2004; Urakawa *et al.*, 2000). In the marine milieu *Vibrio* spp. are the main common pathogens and establish a highly percentage of the microbial community associated with culturing larval invertebrates, including *P. orantus* (Diggles *et al.*, 2000; Sharma *et al.*, 2012; Thompson *et al.*, 2004; Toranzo *et al.*, 2005). However, recent studies have revealed that whilst some *Vibrio* species are pathogenic, there are many others that can serve as biological control agents (Gomez-Gil *et al.*, 2000).

The first aim of the current study was for the isolation and identification of probiotic bacteria from cultures collected from marine environments. Cultures were collected from two marine environments, including severely stressed estuarine ghost shrimp (*Trypaea australiensis*) and several species of coral *Turbinaria* and *Acropora.* Subsequently isolated bacterial organisms were subject to *in vitro* screening for suppressive activity (antagonism) of secreted metabolites against the known lobster pathogen *V.owensii* DY05. Beneficial bacteria were isolated from different parts of coral and shrimp such as the H, LH-ASWS, D-ASWS and M-ASWS regions. Although bacterial communities were similar both across regions and between coral and shrimp, the only significant difference was observed between the coral and shrimp where *Phychrobacter* spp. were isolated from coral collection only.

Marine invertebrates represent good sources of microorganisms for screening for antimicrobial activity. Justifiably, bacteria associated with living surfaces or particle-associated bacteria showed more antagonistic activity than free-living bacteria (Gram *et al.*, 2010; Hjelm *et al.*, 2004 b ; Long & Azam, 2001; Long *et al.*, 2005; Miao & Qian, 2005;

Nair & Simidu, 1987). Thus, it is hypothesized that whilst corals harbor diverse microbial communities the bacteria itself may exhibit synergism that protects coral from invading detrimental microbes via secretion of antagonistic metabolites. Therefore, coral associated bacteria present likely candidates for production of substances that could be further employed in aquaculture systems. Previous studies isolated antimicrobial producing bacteria from coral-associated bacteria, particularly the microbial communities' colonization mucus of healthy corals(Chen *et al.*, 2012; Gantar *et al.*, 2011; Karna *et al.*, 2004; Knowlton & Rohwer, 2003; Ritchie, 2006; Ritchie & Smith, 2004; Rohwer *et al.*, 2002; Rypien *et al.*, 2010; Shashar *et al.*, 1994). On the other hand, several studies have also isolated beneficial bacteria from shrimp, for example *Vibrio* spp. and *Bacillus* spp. were isolated from shrimp culture or the intestine of different penaeid species and have since been used successfully as probiotics (Luis-Villaseñor *et al.*, 2011; Rengpipat *et al.*, 2000; Rengpipat *et al.*, 2003; Tanasomwang *et al.*, 1998).

Two *in vitro* screening methods were employed in the current study to isolate and identify probiotic bacteria from marine cultures. These methods were the well diffusion and disk diffusion assays. The results indicated that the well diffusion assay is more reliable for probiotic screening as the pathogen and the beneficial bacteria are grown in the same conditions and the method facilitates direct contact between the two growing organisms, as in the hatchery environment. At this stage it is not known if antimicrobial substances are actively secreted when probiotic organism come into contact with the pathogen; however there is little evidence that this is occurring because antimicrobial compounds were secreted without such stimulus, as cell free supernatants were also able to demonstrate such antimicrobial activity against the lobster pathogen. Apparent differences in activity across the assays may be attributed to some other factors, such as in the case where live cultures were used in the well diffusion assay. Here it is likely that the growing organisms are actively secreting such antimicrobial compounds as part of normal metabolism, raising concentrations to above those used in a disk diffusion assay. In addition, when cell free culture supernatant is used a greater volume of liquid is applied when compared to a disk diffusion assay. Thus, where inhibition was not observed in the disk diffusion assay yet observed in the well diffusion assay (live cultures or cell free), this result may be attributed to an increased load of the antimicrobial compound, which enhanced inhibition of the pathogen to the point of visible detection by the observer.

The preliminary results showed promising antibacterial activity against the lobster pathogen.

It was found that coral and shrimp represented good sources for isolation of antimicrobial producing probiotic bacteria. In all we isolated 111 suppressive strains from both coral and shrimp, with most of these isolates belonging to the Gammaproteobacteria family and with *Vibrio* species representing the main suppressive isolates. The reasons may relate back to the high prevalence of culturable *Vibrio* species, ie., *Vibrio* species generally make up a substantial proportion of the culturable bacteria (Godwin, 2007) associated with all the particular types of corals sampled in the current study.

Antagonistic activity is considered as an important criterion for selection of probiotic candidates (Pan *et al.*, 2008). Therefore, in the current study *in vitro* antagonistic techniques were utilized as a primary step for probiotic screening, following other studies where this technique has been used (Ravi *et al.*, 2007). Antagonistic bacteria are generally considered good candidates for the ongoing development of a biocontrol tool (Gram *et al.*, 2010; Long & Azam, 2001). Probiotic bacteria represent a good alternative strategy to reduce the use of antibiotics or chemicals in the aquaculture hatchery. Although many previous studies have demonstrated that *in vitro* antagonistic effects of selected bacterial strains can occur against larviculture pathogens, researchers are cautious about concluding that the same inhibitory activity will be observed *in vivo*, i.e., in a larval rearing tank. Thus, only in a few cases have such conclusions been drawn, mostly in relation to shellfish larvae (Tinh *et al.*, 2008).

The second aim of the current study was to partially identify the metabolites responsible for the antimicrobial activity of the three studied bacteria (probiotic), which were selected with some diversity in mind, also due to their relatively high activity among the test strains. The bacterial strains were assigned to the class of Gammaproteobacteria and to the genus level using 16SrRNA fragment sequencing. After sequencing the three strains were assigned to *Pseudoalteromonas* sp. Strain 80, *Vibrio* sp. strain 34 and *Psychrobacter* sp. strain 62. For preliminary characterization of metabolites responsible for pathogen inhibition, filtered culture supernatants from the three species were subjected to partial purification. Steps were taken to investigate an approximate size of the antimicrobial molecules, employing methods such as SDS-page, gas and thin layer chromatography. However, the mechanism of these inhibitory substances and their molecular structures will be more comprehensively investigated in future studies.

Furthermore, antimicrobial metabolites were subsequently screened for activity related to extracellular enzyme secretion, including protease and amylase type activity. It was

demonstrated that these diffusible antimicrobial macromolecules are also capable of *in vitro* hydrolysis of casein, gelatine and starch substrates incorporated into agar plates. It was also demonstrated that more than one metabolite was responsible for this activity, which is evident from the multiple-bands profile in zymogram electrophoresis using either casein or gelatine as the protein substrates. Several studies have already documented this type of hydrolysis activity with secretion of metabolites from specific bacterial strains, producing inhibitory effects on the growth or survival of other strains. The character of such activity was demonstrated to be consistent with that of proteases, lysozymes, hydrogen peroxide or bacteriocins (Balcázar *et al.*, 2006; Fuller, 1989; Gatesoupe, 1999; Irianto & Austin, 2002).

In order to identify the active molecules from the studied strains two techniques were used including GS-MS to investigate the chemical character of small volatile molecules, followed by TLC with an agar overlay to investigate the polarity of active components and elucidate solvent systems suitable for subsequent purification in future studies. LC-MS was used for investigation of molecules too large to be visualised in the gaseous phase, in particular proteins or proteases. Results from the GC-MS demonstrated uniformity across all strains including the mutant that lost inhibition activity against pathogen. It is clear that these smaller volatile compounds are therefore not involved in the pathogen inhibition. However, in the current study it was demonstrated that GC–MS analysis of solvent extracted enriched small compounds is a useful method to chemically characterize the lower molecular weight compounds produced by the studied bacteria. In the current study it was demonstrated that the chosen strains were biosynthesizing linear alkenes (n-C_{12} to n-C_{22}) with only even numbered carbon chains and the single double bond positioned on the terminal carbon.

Although the observed volatiles in the current study were not of any importance in pathogen inhibition, clearly gas chromatography has proven a useful tool for analysis of secreted small molecular weight compounds (Niessen, 2012). In other studies GC-MS has been used for the identification of bacterial volatiles from cultures of cyanobacteria, for profiling rhizobacterial volatiles and for analyzing volatiles associated with infections of *Neisseria meningitides* (Bunge *et al.*, 2008). Recent studies have obviously confirmed that bacteria also secret the volatile molecules through competition with other organisms in order to influence populations and communities (Bunge et al., 2008; Kai et al., 2007).

However, in cases where the particular metabolites responsible for inhibition are too large to

be visualised in the gaseous phase, such as in the current study, the bioautographic technique is a useful method to provide rapid elucidation of chromatographic aspects of the active metabolite so that purification and subsequent characterisation can be performed. In the current study this latter step was not an objective, therefore the identity and chemical structure of active metabolites is not yet known. In another study, the antimicrobial compounds produced by the different strains of *P. luteoviolacea* have been suggested to be due to two categories of compounds. The first being cell bound polyanionic macromolecules, which are partially diffusible in culture media. These are supposed to be more specifically acidic polysaccharides. The second class is not diffusible (Holmström & Kjelleberg, 1999). In the current study antagonistic metabolites were diffusible through the media, meaning they may belong to the former group of compounds, however the polarity of the former is much lower than expected from a polysaccharide.

In the probiotic bacteria it is important to identify the gene responsible for expression of the metabolite that demonstrated inhibition of the pathogen in the current study. Therefore, the third aim of this study was to investigate or identify the gene responsible for biosynthesis of pathogen suppressive metabolites, using transposon mutagenesis (generated mutant(s)). In this regard, the generation of mutants by transposon insertion is considered a powerful analytical technique. In particular, the mini-transposon Tnl0 is useful for defining the functional limits and regulatory sites of bacterial genes (Way *et al.*, 1984).

Because transposon mutagenesis is considered a suitable method to study the genes responsible for antimicrobial production it was chosen for the current study, where we used pLOF/Km carrying a Tn10 mini-transposon to generate the mutant that lost the activity against the pathogen. This vector has proven to be a useful tool for generating single, random, and stable transposon insertions, at a workable frequency (Tascon *et al.*, 1993). The marine bacterium *Pseudoalteromonas* sp. strain 80 was successfully mutated, which produced a mutant that lost the inhibitory activity toward *V.ownsii* DY05. In addition, the mutant became white (non-pigmented), which may be due to a defect in the biosynthetic pathway that produces the pigment metabolite or to that transporting it to the exterior of the cell. This observation leads us to the conclusion that the secretion of antimicrobial compounds correlated with pigment production and/or excretion. This result was similar to a previous study by Egan *et al*. (2002) where it was confirmed that inhibitory activity was also

correlated with pigment production. However, from bioautographic analysis in the current study we speculate that the inhibitory compound itself may not be the same compound as that responsible for pigmentation.

Here we made further progressin identifying the gene affected in the mutant, which was found to share 100% homology with a membrane bound transporter proteinof theRND family, particularly to protein of *Pseudoalteromonas flavipulchra* JG1. This species is a marine antagonistic bacterium with abundant antimicrobial metabolites. Our findings suggest that such RND transporters are involved with the secretion ofproteinases and amylases out of the cells, since the RND-mutant 7Km is impared in milk casein and gelatine hydrolysis as well as in starch hydrolysis. It also excreted modified range of non-volatiles and extracellular ptoreins when compared to the wild type using TLC and bioautagraphy-plates aand GC-MC analysis. The gene sequencing results are strongly supported by the presence of *P. flavipulchra* JG1 transmembrane proteins in the extracellular material identified in the supernatant of *Pseudoalteromonas* strain 80 (Appendix 2). RND proteins were implicated in drug resistance of important pathogens and in the transport of substances from the cell (Nikaido and Takatsuka, 2009; Bazzini et al., 2011), suggesting that a mutation in such a transporter probably abolished the excretion of pathogen-suppressive substances from *Pseudoalteromonas* sp. strain 80 cells.

RND family transporters are known catalyze the active efflux of many antibiotics and chemotherapeutic substances. They are widespread, particularly among Gram-negative bacteria, and have been shown to play a major role in conferring drug resistance to several bacterial pathogens, such as *Burkholderia cenocepacia* (Bazzini *et al.*, 2011)

RND proteins have large periplasmic domains, and form complexes with outer membrane channels and periplasmic adaptor proteins. An example is AcrAB-TolC complex of *E. coli*.This membrane protein pumps out a wide range of drugs and was shown to capture even substrates that cannot cross the plasma membrane, includingseveral beta-lactams, suggesting that the capture of the substrates may occur from the periplasm (Nikaido & Takatsuka, 2009). The *Pseudomonas aeruginosa* MexAB-OprM complex also supportsthe model suggesting these efflux systems form a channel for the extrusion of substrates/drugs from within the cell envelope back into the external environment ((Bazzini *et al.*, 2011) and references within).

RND efflux systems were also suggested to have roles in bacterial pathogenesis, host colonization and persistence of bacteria in the host, as well as in homeostasis of metal ion (Ma *et al.*, 2009; Piddock, 2006). While the sequence of the putative RND transporter in 7Km shares hology with a sequence of an unpublished genomic sequence generated by shotgun sequencing from *Pseudoalteromonas flavipulchra* JG1, it is the first time to our knowledge that phenotype of this putative protein have been described. Further studies of the phenotype will lead to the description of a new transporter protein, important in pathogen-suppression and possibly secretion of extracellular antimicrobial substances.

5.2 Further study

The results of this study suggest that bacteria isolated from shrimp and coral may be useful as potential probiotics against the lobster pathogen *V. owensii*. However, there is still much that is unknown regarding the mechanisms of antagonism employed by different bacterial strains, and the genes involved in the production of the antimicrobial metabolites. Avenues for future investigation include:

1- Further investigations are necessary to evaluate whether the effect of the active molecules is via inhibition of growth or death of pathogenic *V. owensii*.
2- Although the elution of the pigment compound did not correspond to inhibition zones in bioautography, to confirm this further experimentation could be conducted to extract the pigment, followed by chemical characterisation and testing for antimicrobial activity. This may also reveal if pigmentation is due to a single compound or a mixture of compounds. It is likely that this pigment will demonstrate antimicrobial activity consistent with that observed in the current study.
3- It may be worthwhile to carry out PCR experiments to reveal the altered genes in the mutant, to narrow the range of possible genes responsible for biosynthesis of active metabolites.
4- Although the current study seeks to identify bacterial strains that could be used in probiotic strategies to enhance aquaculture, another possible strategy is that the metabolite itself be produced in abundance, perhaps by splicing the gene into a plasmid or by using the original organism itself. The metabolite alone could be introduced to infected larviculture, rather than introduction of the whole organism.

5- The method employed to examine small molecular weight compounds is not suitable for analysis of compounds with lower boiling points, similar to the boiling points of the solvents themselves. In subsequent studies another method could be employed, such as solid phase micro extraction (SPME) followed by GC–MS analysis. This will clarify if more gaseous type metabolites are being secreted.
6- No doubt research should continue onward to the process of purification of the active metabolites for further structural and functional characterization, perhaps using comprehensive two dimensional nuclear magnetic resonance spectroscopy (2D-NMR).
7- Investigations of the active metabolite should focus on a non-volatile component using purification steps informed by TLC-bioautography performed in the current study. In this regard it was revealed that the inhibitory compounds are present in the solvent extracted material from the wild type but not the mutant. The solvent system used will inform the process of purification in subsequent studies where inhibitory concentrations will be calculated and the main component identified.
8- Probiotic bacteria produce a wide range of biologically active extracellular molecules including proteins, including enzymes, small volatiles and non-volatile. The antagonistic strains studied here produced a range of extracellular molecules, but in the future study should include analysis of cell-bound molecules.
9- The antimicrobial activity of extracted molecules depends on several factor such as bacterial species itself, the growing conditions and solvents used for their extraction. Future work should address the influences of such variables on microbial metabolite secretion patterns and should assess the potential use of volatile organic compound (GC-MS) profiles as indicators for the status of microbial metabolism, seeking perhaps a correlation between smaller metabolites and the other active ones.

“It will be most important to elucidate the exact chemical structure of the antibacterial compounds produced by the probiotic bacteria that now appear useful for commercial applications in the medical or agricultural industries. In addition, knowledge of the differences between the mutant compound and the wild-type compound would benefit any future developments that may involve making chemical variations of the natural compound to improve activity and/ or stability. In terms of molecular biology, most recombinant-DNA techniques can be easily applied to marine bacteria. Thus, genetic engineering may be

employed to further increase the production of the inhibitory compound either through stimulating the production in the original organism or by cloning the genes into a new host organism" (Egan, 2001).

Further identification of probiotic organisms and ilucidating their mode of action would be significant for the reduction of use of antibiotics in modern aquaculture and other food industries.

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Appendix

6.1 Appendix 1 - Active band numbering on zymogram gels

Active band numbering on zymogram gels, according to the molecular weight of the protein ladder of casein and gelatine.

Band number	Bacterial species	Molecular weight (KDa)	
		Gelatine hydrolysis	Casein hydrolysis
a	*Vibrio* sp. strain 34	28	
b	*Vibrio* sp. strain 34	27	
c	*Vibrio* sp. strain 34	25	
d	Vibrio sp. strain 34	20	
E	*Vibrio* sp. strain 34	18	
f	*Vibrio* sp. strain 34	25	
g	*Pseudoalteromonas* sp. strain 80	20	
h	*Pseudoalteromonas* sp. strain 80	20	
i	*Pseudoalteromonas* sp. strain 80	20	
j	*Pseudoalteromonas* sp. strain 80	15	
k	*Pseudoalteromonas* sp. strain 80	15	
l	*Pseudoalteromonas* sp. strain 80	25	
m	*Pseudoalteromonas* sp. strain 80	27	
n	*Pseudoalteromonas* sp. strain 80	38	
o	*Vi brio* sp. strain 34		50
p	*Vibrio* sp. strain 34		53
q	*Pseudoalteromonas* sp. strain 80		17
r	*Pseudoalteromonas* sp. strain 80		22
s	*Pseudoalteromonas* sp. strain 80		37

6.2 Appendix 2 - Identity of tentative pathogen-suppressive bacterial proteins- the full list

Band	Gi	Protein name	Score	emPai	Sp. Picked by Mascot	No. of unique peptide (sequence of unique peptide)	Peptide score
Methanol concentration of*Psychrobacter* strain 62 (8) band 1							
1(1)	gi|400288460	superoxide dismutas	457	1.91	[Psychrobacter sp. PAMC 21119	3	44
Methanol concentration of Vibrio strain 34 (4) band 2							
2(2)	gi|189309494	VtpA	368		Vibrio tubiashii RE22]	4	48
Methanol concentration of *Psychrobacter* strain 62 (1) band 3							
3(1)	gi|400288460	superoxide dismutase	248		Psychrobacter sp. PAMC 21119	2	46
3(8)	gi|400287504	inorganic pyrophosphatase	98		[Psychrobacter sp. PAMC 21119	3	40
3(9)	gi|189309494	VtpA	98		Vibrio tubiashii RE22	4	35
***Vibrio* strain 34 filtrated supernatant band (4-7)**							
4(6)	gi|88854480	30S ribosomal protein S18	102		marine actinobacterium PHSC20C1	9	31
5(2)	gi|323358931	ribosomal protein L7/L12]	190	0.59	Microbacterium testaceum StLB037	7	28
5(6)	gi|323357347	30S ribosomal protein S11	145	25	[Microbacterium testaceum StLB037	7	33
6(3)	gi|260774946	flagellin protein FlaC	646	0.74	[Vibrio coralliilyticus ATCC BAA-450	8	34
7(2)	gi|323356847	glycerol-3-phosphate dehydrogenase	454	0.29	[Microbacterium testaceum StLB037	5	35
7(10	gi|323359444	enolase	92	0.07	[Microbacterium testaceum StLB037	7	35
Methanol concentrationof *Vibrio* strain 34 (6) band 8							
8(1)	gi|409204290	curli production assembly/transport component CsgG	484	1.53	[Pseudoalteromonas flavipulchra JG1	4	32
8(12)	gi|93006911	OmpA/MotB protein	97	0.32	[Psychrobacter cryohalolentis K5	9	28
9(9)	gi|392542600	bacterioferritin (cytochrome B-1) (cytochrome B-557)	381	1.8	Pseudoalteromonas piscicida JCM 20779	4	37
10(4)	gi|409203106	OmpA-like transmembrane domain-containing protein	781	4.5	[Pseudoalteromonas flavipulchra JG1]	4	21
10(7)	gi|392543358	ABC transporter auxiliary component	551	0.62	[Pseudoalteromonas piscicida JCM 20779	5	22
10(11)	gi|392542101	ABC transporter ATP-binding protein	411	1.04	[Pseudoalteromonas piscicida JCM 20779	7	31
10(13)	gi|392541359	ribosome releasing facto	326		[Pseudoalteromonas piscicida JCM 20779	2	41
10(20)	gi|39254115	outer membrane protein W	253	0.97	[Pseudoalteromonas piscicida JCM 20779]	3	29
10(21)	gi|409204264	disulfide bond formation protein [Pseudoalteromonas flavipulchra JG1]	246	0.7	[Pseudoalteromonas flavipulchra JG1]	2	38
10(26)	gi|392542011	protein prenylyltransferase domain-containing protein	207	0.26	[Pseudoalteromonas piscicida JCM 20779]	2	37
10(27)	gi|392541855	alanine dehydrogenase	202	0.08	[Pseudoalteromonas piscicida JCM 20779	3	38

10(42)	gi|392541296	ATP-dependent Clp protease proteolytic subunit	108	0.32	[Pseudoalteromonas piscicida JCM 20779]	4	36
11(3)	gi|392543363	serine endoprotease	859	1.22	[Pseudoalteromonas piscicida JCM 20779]	4	51
11(4)	gi|392543358	ABC transporter auxiliary component	821	2.74	[Pseudoalteromonas piscicida JCM 20779]	7	20
11(9)	gi|409203106	OmpA-like transmembrane domain-containing protein	609	3.77	[Pseudoalteromonas flavipulchra JG1]	7	24
11(10)	gi|392541288	peroxiredoxin 2 (thioredoxin peroxidase 1) (thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA) (PRP)	533	2.1	[Pseudoalteromonas	3	32
11(12)	gi|392540352	molecular chaperone GroEL	458	0.25	[Pseudoalteromonas piscicida JCM 20779]	8	39
11(13)	gi|409204264	disulfide bond formation protein	457	1.45	[Pseudoalteromonas flavipulchra JG1]	4	31
11(19)	gi|392544006	adenylate kinase [Pseudoalteromonas piscicida JCM 20779]	402	0.31	[Pseudoalteromonas piscicida JCM 20779]	3	25
11(19)	gi|392544006	adenylate kinase	402	0.31	[Pseudoalteromonas piscicida JCM 20779]	2	50
11(24)	gi|392541855	alanine dehydrogenas	325	0.17	[Pseudoalteromonas piscicida JCM 20779]	2	51
11(27)	gi|392542984	disulfide bond isomerase	299	0.27	[Pseudoalteromonas piscicida JCM 20779	3	27
11(29)	gi|409203019	ThiJ/PfpI family protein	239	0.88	[Pseudoalteromonas flavipulchra JG1]	2	22
11(34)	gi|392541359	ribosome releasing factor	258	0.36	[Pseudoalteromonas piscicida JCM 20779]	3	23
11(47)	gi|392544140	30S ribosomal protein S4	178	0.14	[Pseudoalteromonas piscicida JCM 20779	4	46
11(53)	gi|294141548	adenylate kinase	156	0.31	[Shewanella violacea DSS12	2	50
11(57)	gi|409202328	delta-aminolevulinic acid dehydratase	143	0.09	[Pseudoalteromonas flavipulchra JG1]	4	23
11(59)	gi|429555107	chaperonin GroL	137	0.13	[Sinorhizobium meliloti GR4]	2	65
11(62)	gi|392541356	30S ribosomal protein S2	125	0.12	[Pseudoalteromonas piscicida JCM 20779]	3	52
11(69)	gi|77359198	elongation factor Tu	104	0.08	[Pseudoalteromonas haloplanktis TAC125]	2	66
12(1)	gi|392541855	alanine dehydrogenase	1209	2.94	[Pseudoalteromonas piscicida JCM 20779]	4	21
12(2)	gi|392540596	flagellin	1076	3.55	[Pseudoalteromonas piscicida JCM 20779]	2	25
12(3)	gi|409201774	leucine dehydrogenase	1037	2	[Pseudoalteromonas flavipulchra JG1]	10	36
12(5)	gi|409200697	hemolysin-type calcium-binding protein	813	2.15	[Pseudoalteromonas flavipulchra JG1]	9	26
12(6)	gi|392543363	serine endoprotease	695	0.49	[Pseudoalteromonas piscicida JCM 20779	2	25
12(7)	gi|392540595	flagellin	683	0.94	[Pseudoalteromonas piscicida JCM 20779	3	53
12(10)	gi|409203106	OmpA-like transmembrane domain-containing protein	563	2.59	[Pseudoalteromonas flavipulchra JG1]	6	27
12(12)	gi|392308792	alanine dehydrogenase	515	0.62	[Pseudoalteromonas citrea NCIMB 1889]	2	36
12(13)	gi|392540444	phosphotransacetylase	487	0.33	[Pseudoalteromonas piscicida JCM 20779]	3	54
12(16)	gi|392553736	elongation factor Tu	426	0.44	[Pseudoalteromonas spongiae UST010723-006]	2	22
12(18)	gi|392540855	phosphopentomutase	416	0.55	[Pseudoalteromonas piscicida JCM 20779]	9	30
12(19)	gi|409204191	phosphopentomutase	392	0.8	[Pseudoalteromonas flavipulchra JG1]	2	25
12(24)	gi|409199986	hemolysin-type calcium-binding region	286	0.27	[Pseudoalteromonas flavipulchra JG1	10	20
12(27	gi|409200735	putative alkaline phosphatase	262	0.23	[Pseudoalteromonas flavipulchra JG1]	2	49

12(40)	gi|409200361	NADH:flavin oxidoreductase	189	0.18	[Pseudoalteromonas flavipulchra JG1	8	30
12(41)	gi|88860184	citrate synthase	181	0.07	[Pseudoalteromonas tunicata D2	4	24
12(42)	gi|392542536	peptidyl-prolyl cis-trans isomerase	180	0.14	[Pseudoalteromonas piscicida JCM 20779]	2	34
12(45)	gi|392541151	outer membrane protein W [Pseudoalteromonas piscicida JCM 20779]	165	0.31	[Pseudoalteromonas piscicida JCM 20779]	2	20
12(54)	gi|392541994	homogentisate 1,2-dioxygenase	131	0.07	[Pseudoalteromonas piscicida JCM 20779	2	30
12(56)	gi|392540592	Flagellar capping protein	125	0.07	[Pseudoalteromonas piscicida JCM 20779	5	24
12(61)	gi|409199698	peptidase M14, carboxypeptidase A	113	0.08	[Pseudoalteromonas flavipulchra JG1]	3	21
12(66)	gi|392541394	putative metal-dependent dipeptidase	97		[Pseudoalteromonas piscicida JCM 20779	3	56
12(70)	gi|392542673	iron superoxide dismutase	84		[Pseudoalteromonas piscicida JCM 20779	8	33
12(77)	gi|392543167	branched-chain amino acid aminotransferase	69	0.1	[Pseudoalteromonas piscicida JCM 20779	2	33
Methanol concentration of *Pseudoalteromonas piscicida* like isolate pp107 (2) bands (13-16)							
13(2)	gi|392540596	flagellin	695	1.13	[Pseudoalteromonas piscicida JCM 20779]	5	21
13(20)	gi|400287375	nitrogen regulatory protein P-II	194	0.64	[Psychrobacter sp. PAMC 21119	2	44
13(26)	gi|392543363	serine endoprotease	150	0.07	[Pseudoalteromonas piscicida JCM 20779	6	47
13(53)	gi|71066623	6,7-dimethyl-8-ribityllumazine synthase	78		[Psychrobacter arcticus 273-4]	2	43
13(56)	gi|121634398	zinc-binding alcohol dehydrogenase [Neisseria meningitidis FAM18]	76		[Neisseria meningitidis FAM18]	6	31
13(58)	gi|392543671	LasA protease	73	0.08	[Pseudoalteromonas piscicida JCM 20779	3	35
13(59)	gi|33207911	histone 3	72		[Neopisosoma mexicanum]	7	22
14(11)	gi|392540485	leucine dehydrogenase	386	0.4	[Pseudoalteromonas piscicida JCM 20779	2	85
14(16)	gi|392544375	aminopeptidase B	231	0.15	[Pseudoalteromonas piscicida JCM 20779]	2	48
15(2)	gi|400289198	glutamine synthetase	455	0.36	[Psychrobacter sp. PAMC 21119	2	21
15(9)	gi|392543389	TonB-dependent receptor	278	0.1	[Pseudoalteromonas piscicida JCM 20779	2	53
15(10)	gi|71065418	TonB-dependent receptor	260	0.24	[Pseudoalteromonas piscicida JCM 20779	2	22
15(11)	gi|400286730	aldehyde dehydrogenase	207	0.26	[Psychrobacter sp. PAMC 21119	2	22
15(17)	gi|340356822	aldehyde dehydrogenase	172	0.06	[Sporosarcina newyorkensis 2681	2	40
15(21)	gi|71066489	multifunctional fatty acid oxidation complex subunit alpha	114	0.04	[Psychrobacter arcticus 273-4]	2	38
16(7)	gi|7839585	DnaK	261	0.05	[Psychrobacter sp. St1	5	34
16(8)	gi|148653897	multifunctional fatty acid oxidation complex subunit alpha	257	0.13	[Psychrobacter sp. PRwf-1]	4	40
16(16)	gi|254491227	chaperone protein DnaK	105		[Methylophaga thiooxidans DMS010]	2	26
Triton extracted of *Peudoalteromonas* strain 80 Band (17-18)							
17(1)	gi|409201415	TonB-dependent receptor:Cna B-type]	2681	2.04	[Pseudoalteromonas flavipulchra JG1	6	28
17(2)	gi|409200596	putative Outer membrane protein with a TonB box	1521	1.03	[Pseudoalteromonas flavipulchra JG1]	5	23
17(3)	gi|409201970	TonB-dependent receptor plug	1429	0.72	[Pseudoalteromonas flavipulchra JG1]	6	20
17(4)	gi|409201347	TonB-dependent receptor plug	1414	1.31	[Pseudoalteromonas flavipulchra JG1]	6	22

17(5)	gi|409199921	TonB-dependent receptor plug	1253	0.57	[Pseudoalteromonas flavipulchra JG1]	3	40
17(7)	gi|409201294	TonB-dependent receptor plug	999	0.47	[Pseudoalteromonas flavipulchra JG1]	7	30
17(9)	gi|392542479	metallopeptidase	828	0.47	[Pseudoalteromonas piscicida JCM 20779]	8	20
17(10)	gi|409203198	Outer-membrane heme receptor	697	0.43	[Pseudoalteromonas flavipulchra JG1]	4	46
17(12)	gi|409201592	Outer membrane TonB-dependent receptor	620	0.28	[Pseudoalteromonas flavipulchra JG1]	3	34
17(14)	gi|409201199	TonB-dependent receptor	581	0.23	[Pseudoalteromonas flavipulchra JG1]	4	28
17(16)	gi|392554235	TonB-dependent receptor plug	495	0.2	[Pseudoalteromonas undina NCIMB 2128	2	53
17(18)	gi|392542126	TonB-dependent receptor	327	0.1	[Pseudoalteromonas piscicida JCM 20779]	2	59
17(24)	gi|409201076	polysaccharide biosynthesis/export protein [Pseudoalteromonas flavipulchra JG1]	141	0.07	[Pseudoalteromonas flavipulchra JG1]	2	36
17(25)	gi|392544638	TonB-dependent receptor	130	0. 03	[Pseudoalteromonas piscicida JCM 20779	2	21
17(28)	gi|392543822	TonB-dependent receptor plug	120	0.03	Pseudoalteromonas piscicida JCM 20779	3	39
17(33)	gi|392541364	N-acetylglucosamine-regulated TonB-dependent outer membrane receptor	93	0.04	[Shewanella sp. HN-41	7	34
18(5)	gi|392544416	TonB-dependent receptor domain-containing protein	1018	1.08	[Pseudoalteromonas piscicida JCM 20779	6	44
18(6)	gi|409203198	outer-membrane heme receptor	1015	1.04	[Pseudoalteromonas flavipulchra JG1]	4	43
18(8)	gi|409203572	tonb-dependent siderophore receptor	788	0.49	[Pseudoalteromonas flavipulchra JG1]	5	23
18(16)	gi|409199709	Outer membrane receptor for ferric siderophore	498	0.24	[Pseudoalteromonas flavipulchra JG1]	4	32
18(17)	gi|409203420	prolyl oligopeptidase	472	0.29	[Pseudoalteromonas flavipulchra JG1]	8	21
18(18)	gi|409200829	enterobactin receptor protein	456	0.36	[Pseudoalteromonas flavipulchra JG1]	2	42
18(21)	gi|409203625	lipoprotein	436	0.26	[Pseudoalteromonas flavipulchra JG1]	5	31
18(25)	gi|392544290	zinc metallopeptidase	355	0.05	[Pseudoalteromonas piscicida JCM 20779]	2	41
18(27)	gi|392541598	TonB dependent outer membrane receptor	307	0.13	[Pseudoalteromonas piscicida JCM 20779]	4	30
18(30)	gi|409199921	TonB-dependent receptor	244	0.09	[Pseudoalteromonas flavipulchra JG1]	5	42
18(35)	gi|392542704	putative lipoprotein	150	0.05	[Pseudoalteromonas piscicida JCM 20779]	2	66
18(37)	gi|77359198	elongation factor Tu	125		[Pseudoalteromonas haloplanktis TAC125	2	61
18(41)	gi|94538598	nef protein	80		[Human immunodeficiency virus 1]	4	31
Triton extracted of *Vibrio* strain 34 one Band							
19(1)	gi|260775435	outer membrane protein OmpU	870	2.04	[Vibrio coralliilyticus ATCC BAA-450]	2	22
19(4)	gi|260775306	predicted deacylase	657	0.75	[Vibrio coralliilyticus ATCC BAA-450]	3	30
19(4)	gi|260775306	predicted deacylase	657	0.75	[Vibrio coralliilyticus ATCC BAA-450]	2	24
19(11)	gi|260777198	putative outer membrane protein	186	0.19	[Vibrio coralliilyticus ATCC BAA-450]	2	20
19(13)	gi|157144958	fructose-bisphosphate aldolase	158	0.18	[Citrobacter koseri ATCC BAA-895	2	59
19(14)	gi|260779367	dihydrodipicolinate synthase	143		[Vibrio coralliilyticus ATCC BAA-450]	2	24
19(15)	gi|238757655	Outer membrane protein A	129	0.09	[Yersinia aldovae ATCC 35236]	6	23
19(17)	gi|401763772	porin	106		[Enterobacter cloacae subsp. cloacae ENHKU01]	2	54
Triton extracted of *pseudoalteromonas* piscicida like isolate pp107 (3) band (20-21)							

20(7)	gi|409203606	outer membrane channel protein	594	0.48	[Pseudoalteromonas flavipulchra JG1]	2	26
20(8)	gi|392542704	putative lipoprotein	552	0.33	[Pseudoalteromonas piscicida JCM 20779]	3	53
20(11)	gi|392543555	type VI secretion protein EvpB	442	0.4	[Pseudoalteromonas piscicida JCM 20779]	6	32
20(12)	gi|392544616	peptidase S9 prolyl oligopeptidase	424	0.18	[Pseudoalteromonas piscicida JCM 20779]	3	32
20(13)	gi|400289198	glutamine synthetase	376	0.28	[Psychrobacter sp. PAMC 21119]	2	31
20(18)	gi|26991722	glutamine synthetase, type I	302	0.2	[Pseudomonas putida KT2440] >gi|148550122|ref|YP_001270224.1| glutamine synthetase [Pseudomonas putida F1] >gi|386014316|ref|YP_005932593.1| protein GlnA [	2	48
20(19)	gi|339489437	polynucleotide phosphorylase/polyadenylase	301	0.14	[Pseudomonas putida S16] >gi|338840280|gb|AEJ15085.1| polynucleotide phosphorylase/polyadenylase [Pseudomonas putida S16]	3	28
20(24)	gi|392543521	protease IV, a signal peptide peptidase	262	0.21	[Pseudoalteromonas piscicida JCM 20779]	9	21
20(24)	gi|392543521	protease IV, a signal peptide peptidase	262	0.21	[Pseudoalteromonas piscicida JCM 20779]	3	21
20(43)	gi|260775435	outer membrane protein OmpU	174	0.29	[Vibrio coralliilyticus ATCC BAA-450] >gi|260608616|gb|EEX34781.1| outer membrane protein OmpU [Vibrio coralliilyticus ATCC BAA-450]	2	30
20(41)	gi|28867471	phosphoenolpyruvate carboxykinase	136		[Pseudomonas syringae pv. tomato str. DC3000] >gi|213970742|ref|ZP_03398867.1| phosphoenolpyruvate carboxykinase [Pseudomonas syringae pv. tomato T1]	2	28
21(1)	gi|392541992	OmpA family Oar-like outer membrane protein	1214	0.75	[Pseudoalteromonas piscicida JCM 20779]	3	25
21(2)	gi|392542938	Outer membrane TonB-dependent receptor	1068	0.48	[Pseudoalteromonas piscicida JCM 20779]	3	29
21(6)	gi|409201347	TonB-dependent receptor	549	0.3	[Pseudoalteromonas flavipulchra JG1]	3	26
21(9)	gi|392542610	TonB-dependent receptor, plug	275	0.07	[Pseudoalteromonas piscicida JCM 20779	7	31
21(21)	gi|93005633	glycine dehydrogenase	99		[Psychrobacter cryohalolentis K5] >gi|122415699|sp|Q1QCL7|GCSP_PSYCK RecName: Full=Glycine dehydrogenase [decarboxylating]; AltName: Full=Glycine cleavage	4	36
21(25)	gi|392543968	TonB-dependent receptor	82	0.04	[Pseudoalteromonas piscicida JCM 20779	4	26
21(26)	gi|392545639	TonB-dependent chitooligosaccharide receptor	79	0.03	[Pseudoalteromonas rubra ATCC 29570	2	30
Triton extracted of Psychrobacter strain 62(1) band 22							
22(1)	gi|400286450	Acetyl-CoA C-acetyltransferase	930	1.55	[Psychrobacter sp. PAMC 21119]	3	41
22(5)	gi|308047966	(EF-1A/EF-Tu) [	500	0.16	Ferrimonas balearica DSM 9799] >gi|307630156|gb|ADN74458.1| translation elongation factor 1A (EF-1A/EF-Tu) [Ferrimonas balearica DSM 9799]	5	53
22(12)	gi|71064945	D-3-phosphoglycerate dehydrogenase	359	0.24	[Psychrobacter arcticus 273-4]	2	34
22(13)	gi|93005649	4-aminobutyrate aminotransferase	345	0.22	[Psychrobacter cryohalolentis K5]	5	20

22(16)	gi|400288526	succinyl-CoA synthetase subunit beta	321	0.58	[Psychrobacter sp. PAMC 21119]	4	27
22(18)	gi|148653880	serine hydroxymethyltransferase	295	0.23	[Psychrobacter sp. PRwf-1] >gi|172048576|sp|A5WH82|GLYA_PSYWF RecName: Full=Serine hydroxymethyltransferase; Short=SHMT; Short=Serine methylase >gi|148572964|	2	29
22(26)	gi|400288190	aspartate aminotransferase	263	0.25	[Psychrobacter sp. PAMC 21119	5	54
22(29)	gi|71066078	aspartate-semialdehyde dehydrogenase	258	0.26	[Psychrobacter arcticus 273-4]	2	49
22(32)	gi|71064890	isovaleryl-CoA dehydrogenase	250	0.16	[Psychrobacter arcticus 273-4]	8	22
22(34)	gi|400287054	molecular chaperone DnaK	221	0.1	[Psychrobacter sp. PAMC 21119]	3	51
22(36)	gi|400289082	oxidoreductase, aldo/keto reductase	217	0.18	[Psychrobacter sp. PAMC 21119]	2	58
22(41)	gi|400287487	3-ketoacyl-(acyl-carrier-protein) reductase	201	0.14	[Psychrobacter sp. PAMC 21119	7	27
22(44)	gi|344345138	translation elongation factor Tu	189	0	[Marichromatium purpuratum 984] >gi|343803229|gb|EGV21139.1| translation elongation factor Tu [Marichromatium purpuratum 984]	8	22
22(51)	gi|400288741	adenylosuccinate lyase	168	0.13	[Psychrobacter sp. PAMC 21119]	2	25
22(54)	gi|71065388	S-adenosylmethionine synthetase	165	0.28	[Psychrobacter arcticus 273-4] >gi|109892655|sp|Q4FTH7|METK_PSYA2 RecName: Full=S-adenosylmethionine synthase; Short=AdoMet synthase; AltName: Full=MAT;	4	57
22(60)	gi|71066049	30S ribosomal protein S1	153	0.05	[Psychrobacter arcticus 273-4] >gi|71039034|gb|AAZ19342.1| SSU ribosomal protein S1P [Psychrobacter arcticus 273-4]	7	33
22(64)	gi|71064707	osmolarity response regulator	141	0.12	[Psychrobacter arcticus 273-4] >gi|93004966|ref|YP_579403.1| osmolarity response regulator [Psychrobacter cryohalolentis K5] >gi|71037692|gb|AAZ18000.1	3	61
22(70)	gi|71066060	glyceraldehyde-3-phosphate dehydrogenase	132	0.06	[Psychrobacter arcticus 273-4] >gi|71039045|gb|AAZ19353.1| d-Glyceraldehyde 3-phosphate dehydrogenase [Psychrobacter arcticus 273-4]	4	32
22(71)	gi|400287969	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	130	0.07	[Psychrobacter sp. PAMC 21119]	4	28
22(73)	gi|71065958	aromatic amino acid aminotransferase	127		[Psychrobacter arcticus 273-4]	2	28
22(74)	gi|400288704	acyl-CoA dehydrogenase domain-containing protein	127	0.16	[Psychrobacter sp. PAMC 21119]	2	21
22(77)	gi|148653385	30S ribosomal protein S1	125	0.05	[Psychrobacter sp. PRwf-1] >gi|148572469|gb|ABQ94528.1| SSU ribosomal protein S1P [Psychrobacter sp. PRwf-1]	7	32
22(78)	gi|71065216	UDP-glucose/GDP-mannose dehydrogenase	119	0.12	[Psychrobacter arcticus 273-4] >gi|71038201|gb|AAZ18509.1| putative UDP-glucose/GDP-mannose dehydrogenase [Psychrobacter arcticus 273-4]	7	20
22(90)	gi|93006013	acyl-CoA dehydrogenase-like protein	106		[Psychrobacter cryohalolentis K5] >gi|92393691|gb|ABE74966.1| acyl-CoA dehydrogenase-like protein [Psychrobacter cryohalolentis K5]	4	42
22(92)	gi|152997910	xylose isomerase domain-containing protein	102	0.05	[Marinomonas sp. MWYL1] >gi|150838834|gb|ABR72810.1| Xylose isomerase domain protein TIM barrel [Marinomonas sp. MWYL1	5	20
22(93)	gi|71066187	dihydroorotase	101	0.08	[Psychrobacter arcticus 273-4] >gi|71039172|gb|AAZ19480.1| dihydroorotase [Psychrobacter arcticus 273-4]	4	21

22(98)	gi|71065383	chaperonin clpA/B	89	0.03	[Psychrobacter arcticus 273-4] >gi|71038368|gb|AAZ18676.1| putative chaperonin clpA/B [Psychrobacter arcticus 273-4]	2	65
22(100)	gi|400289028	extracellular solute-binding protein	88	0.08	[Psychrobacter sp. PAMC 21119]	4	28
22(106)	gi|71065002	tryptophan synthase subunit beta	85	0.07	[Psychrobacter arcticus 273-4] >gi|71037987|gb|AAZ18295.1| tryptophan synthase, beta chain [Psychrobacter arcticus 273-4]	2	33
22(108)	gi|71065744	prephenate dehydratase	83	0.09	[Psychrobacter arcticus 273-4] >gi|71038729|gb|AAZ19037.1| prephenate dehydratase [Psychrobacter arcticus 273-4]	8	22
Methanol concentrationof Peudoalteromonas strain 80 (2) band (23-26)							
23(1)	gi|409203106	OmpA-like transmembrane domain-containing protein	766	4.5	[Pseudoalteromonas flavipulchra JG1]	2	30
23(8)	gi|392542574	putative glutathione-regulated potassium-efflux system protein	259	0.4	[Pseudoalteromonas piscicida JCM 20779]	2	34
23(10)	gi|392541626	6,7-dimethyl-8-ribityllumazine synthase	196	0.77	[Pseudoalteromonas piscicida JCM 20779] >gi|409201474|ref|ZP_11229677.1| 6,7-dimethyl-8-ribityllumazine synthase [Pseudoalteromonas flavipulchra	5	34
24(2)	gi|409203106	OmpA-like transmembrane domain-containing protein	740	3.14	[Pseudoalteromonas flavipulchra JG1]	4	24
24(9)	gi|392542673	iron superoxide dismutase	244	0.16	[Pseudoalteromonas piscicida JCM 20779] >gi|409201808|ref|ZP_11230011.1| iron superoxide dismutase [Pseudoalteromonas flavipulchra JG1]	5	33
24(11)	gi|392543358	ABC transporter auxiliary component	207	0.12	[Pseudoalteromonas piscicida JCM 20779	6	35
24(13)	gi|392541151	outer membrane protein W	193	0.5	[Pseudoalteromonas piscicida JCM 20779]	7	22
24(22)	gi|315127161	ribosome releasing factor	94	0.18	[Pseudoalteromonas sp. SM9913] >gi|315015675|gb|ADT69013.1| ribosome releasing factor [Pseudoalteromonas sp. SM9913]	3	22
25(1)	gi|409204290	curli production assembly/transport component CsgG	840	7.31	[Pseudoalteromonas flavipulchra JG1]	3	27
25(4)	gi|392543363	serine endoprotease	624	0.49	[Pseudoalteromonas piscicida JCM 20779]	2	26
25(5)	gi|392543358	ABC transporter auxiliary component	572	1.05	[Pseudoalteromonas piscicida JCM 20779]	4	21
25(5)	gi|392543358	ABC transporter auxiliary component	572	1.05	[Pseudoalteromonas piscicida JCM 20779]	4	27
25(12)	gi|392541288	peroxiredoxin 2 (thioredoxin peroxidase 1) (thioredoxin-dependent peroxide reductase 1) (Thiol-specific antioxidant protein) (TSA) (PRP)	350	0.52	[Pseudoalteromonas piscicida JCM 20779] >gi|409202127|	4	57
25(14)	gi|409204264	disulfide bond formation protein	274	0.7	[Pseudoalteromonas flavipulchra JG1]	3	40
25(18)	gi|392542673	iron superoxide dismutase	231	0.16	[Pseudoalteromonas piscicida JCM 20779] >gi|409201808|ref|ZP_11230011.1| iron superoxide dismutase [Pseudoalteromonas flavipulchra JG1]	3	28
25(21)	gi|392541151	outer membrane protein W	204	0.97	[Pseudoalteromonas piscicida JCM 20779]	4	28
26(2)	gi|39254185	alanine dehydrogenase [Pseudoalteromonas piscicida JCM 20779]	849	1.34	>gi|409199930|ref|ZP_11228133.1| alanine dehydrogenase [Pseudoalteromonas flavipulchra JG1]	5	34
26(7)	gi|39254059	flagellin	504	0.61	[Pseudoalteromonas piscicida JCM 20779] >gi|409201675|ref|ZP_11229878.1| flagellin [Pseudoalteromonas flavipulchra JG1]	3	47

26(9)	gi|392543245	delta-aminolevulinic acid dehydratase	465	0.31	[Pseudoalteromonas piscicida JCM 20779]	5	30
26(10)	gi|392543363	serine endoprotease	442	0.22	[Pseudoalteromonas piscicida JCM 20779]	8	39
26(16)	gi|409200444	beta-lactamase	232	0.34	[Pseudoalteromonas flavipulchra JG1]	3	36
26(17)	gi|409204191	outer membrane protein; signal peptide	221	0.29	[Pseudoalteromonas flavipulchra JG1]	2	33
26(18)	gi|392541694	malate dehydrogenase	216	0.22	[Pseudoalteromonas piscicida JCM 20779] >gi|409201405|ref|ZP_11229608.1| malate dehydrogenase [Pseudoalteromonas flavipulchra JG1]	2	45

6.3 Appendix 3 – CLUSTAL format alignment by MAFFT (v7.214)

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CLUSTAL format alignment by MAFFT (v7.214)

Bam6            CTTCTTCACGAGGCACACCTCAACGCTATTCTGACCTTGCCTTCACGACTGTGCTGGTCA
Seq1            ------------------------------------------------------------
Seq2            ------------------------------------------------------------
PTZ1212         ------------------------------------------------------------


Bam6            TTAAACGCGGCGGCCGCGGCCTACGCGGCCACGCGTATTCAGGCTGACCCTGCGCGCTCA
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Bam6            CAGGGCTTTATTGATTCCATTTTTACACTGATGAATGTTCCGTTGCGCTGCCCGGATTAC
Seq1            ------------------------------------------------------------
Seq2            ------------------------------------------------------------
PTZ1212         ------------------------------------------------------------


Bam6            AGCCGGATCCGGGATCATATGACAAGATGTGTATCCACCTTAACTTAATGATTTTTACCA
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PTZ1212         ------------------------------------------------------------


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Seq2            ------------------------------------------------------------
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PTZ1212        ------------------------------------------------------------


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Bam6           TATGCCTTTGAAACCGTCGGTGAAGCTATTTTACTGACCACATTTATTCTGGCTGGCCGC
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Seq2           -------------------------------------------TTTATTCTGGCTGGCGGC
PTZ1212        ------------------------------------------------------------


Bam6           TTCGCAGTGTTGGCCTATTCCACCTTTATGATCAATGCGCAAATGGGACTGCT-ACTGC
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PTZ1212       ------------------------------------------------------------

Bam6          ------------------------------------------------------------
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PTZ1212       ------------------------------------------------------------

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Bam6           ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
Seq1          ------------------------------------------------------------
Seq2          ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
Seq1          ------------------------------------------------------------
Seq2          ------------------------------------------------------------
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Bam6          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Seq1          ------------------------------------------------------------
Seq2          ------------------------------------------------------------
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Seq2          ------------------------------------------------------------
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Bam6           ---------------------------------------------
Seq1           ---------------------------------------------
Seq2           ---------------------------------------------
PTZ1212        TATTGGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAG
```

End of the Tn10 sequences are shown in **bold**.

*BamH*I sites are highlighted.

***Sph*I** site in shown in bold and highlighted.

* indicates the start of the reading frame with 100% identity to RND transporter of *P. flavipulchra*. This homology runs following the Tn10 sequences from nucleotide 2 (indicated with a star) to 523.