MOLECULAR CHARACTERISATION OF THE *VIBRIO MIDAE* SY9 EXTRACELLULAR ALKALINE SERINE PROTEASE AND ITS ROLE IN THE PREVIOUSLY OBSERVED PROBIOTIC EFFECT ON THE GROWTH OF *HALIOTIS MIDAE*

by

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I dedicate this thesis to my darling wife Sue.

Now faith is being sure of what we hope for and

certain of what we do not see.

Hebrews 11:1

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ABSTRACT

Abalone are marine gastropods that command a very high market price, particularly in the Far East where they are a highly sought after sea food delicacy. South Africa has a rapidly developing abalone aquaculture industry, based on the cultivation of *Haliotis midae* in land-based race-way systems. The relatively slow growth rates of abalone represent a major constraint on the abalone aquaculture industry. However, there is mounting experimental evidence showing that the health and physiology of aquacultured species can be improved through the prophylactic use of probiotic bacteria. Previous research by Macey and Coyne (2005) showed that *H. midae* fed a high protein artificial diet, ABFEED® S34, supplemented with the bacterium *Vibrio midae* SY9 have enhanced digestion, growth and immune responses. Probiotic microorganisms are thought to function in a variety of ways, which include the secretion of extracellular enzymes that may enhance digestion in the host organism. However, most of the investigations conducted on probiotic microorganisms for aquacultured species have failed to elucidate the exact mode of action.

In this study, the predominant *V. midae* SY9 extracellular alkaline protease, VmproA, was investigated in an attempt to determine the role of VmproA in the growth enhancing probiotic effect observed by Macey and Coyne (2005) for abalone fed *V. midae* SY9 supplemented feed.

The *V. midae* SY9 gene, *vmproA*, encoding the protease was cloned from a previously constructed genomic library and characterised. Nucleotide sequencing and analysis indicated that *vmproA* encodes a protein, VmproA, which has high similarity to a *Vibrio alginolyticus* extracellular detergent resistant alkaline serine protease. Furthermore, during the course of this investigation it became apparent that VmproA may represent an extracellular alkaline detergent-stable, member of the proteinase K-like subfamily of the subtilase superfamily of serine proteases.

The detergent-stable protease gene, *vmproA*, was targeted for gene mutagenesis through *vmproA* gene duplication and disruption, resulting in the construction of the mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2, respectively. *VmproA* gene duplication and disruption did not significantly influence the growth of the mutant strains in batch culture in comparison to *V. midae* SY9. *V. midae* SY9Pro2 produced and secreted VmproA and had equivalent levels of extracellular protease activity to *V. midae* SY9 when cultivated in a high protein medium. However, insertional inactivation of *vmproA* resulted in a loss of VmproA production and secretion. This also resulted in a significant reduction in the extracellular protease levels produced by *V. midae* SY9Mut2 in comparison with that of *V. midae* SY9.

The effect of dietary supplementation with either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 on *H. midae* growth performance was investigated in a growth trial. The basal diet of ABFEED® S34 weaning chips was separately supplemented with the *V. midae* SY9 strains by vacuum impregnation so as to achieve a viable cell concentration of greater than 10^8 CFU g^{-1} ABFEED®. After 180 days, *H. midae* receiving either the *V. midae* SY9Pro2 or *V. midae* SY9Mut2 supplemented diets displayed significantly ($P<0.05$) enhanced growth parameters compared to abalone fed either the basal diet or the *V. midae* SY9 supplemented diet. However, there was no significant difference ($P>0.05$) between animals fed the *V. midae* SY9 supplemented diet or the control diet. *In situ* alkaline protease levels within the crop/stomach and intestinal digestive tract regions were significantly enhanced ($P<0.05$) in *H. midae* fed *V. midae* SY9 supplemented ABFEED® S34 compared to animals fed the basal diet or the basal diet supplemented with either *V. midae* SY9Pro2 or *V. midae* SY9Mut2.

In situ hybridization and immunohistochemistry were employed to examine the *in vivo* localisation of dietary supplemented *V. midae* SY9 cells and VmproA within the *H. midae* digestive tract. *V. midae* SY9 was chromosomally tagged with the mini-Tn10-*gfp-kan* transposon and the resulting strain, *V. midae* SY9::Tn10.52, contained a single chromosomal copy of a *gfp* gene. *V. midae* SY9::Tn10.52 was incorporated into ABFEED® S34 weaning chips by means of vacuum impregnation at a final concentration of greater than 10^9 CFU g^{-1} ABFEED®. Juvenile *H. midae* were fed either the basal diet of unsupplemented feed or *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 for 14 days. *In situ* histological analysis of whole abalone sections, probed with specific DNA probes to the *gfp* gene, indicated that *V. midae* SY9::Tn10.52 could be localised to the crop/stomach and intestine of abalone fed the probiotic supplemented diet. Similarly, polyclonal anti-VmproA antibodies localised VmproA to the crop/stomach and intestine of *H. midae*. The chromosomally tagged probiotic cells were observed to be associated with the walls of the crop/stomach, and adhered to feed and/or other particulate matter within the gut of abalone fed the probiotic supplemented diet. Likewise, VmproA appeared to be associated with feed and/or other particulate matter within abalone crop/stomach and intestinal regions.

TABLE OF CONTENTS

NOMENCLATURE

ABBREVIATIONS

A	adenine
Amp	Ampicillin
Amp^r	Ampicillin resistant
ATP	adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
C	cytosine
CHB	church hybridisation buffer
cfu	colony forming units
Ci	Curie
cm	centimeter(s)
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
dATP	deoxy-adenine 5'-triphosphate
dCTP	deoxy-cytosine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxy-guanine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicyclic acid
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP)
dTTP	deoxy-thymine 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis[β-aminoethylether]-N,N,N',N'-tetraacetic acid
g	gram(s)
G	guanine
hrs	hour(s)
k	kilo
K	thousand
kb	kilobase(s)
kDa	kilodalton(s)
kg	kilogram(s)
Km	Kanamycin
Km^r	Kanamycin resistance

l	liter(s)
LA	Luria agar
LB	Luria broth
m	meter
M	molar
mA	milli-Amperes
MA	marine agar
MB	marine broth
MCS	multiple cloning site
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mM	millimolar
mol	mole(s)
MOPS	(3-[N-morpholino]propane-sulphonic acid)
mRNA	messenger RNA
ng	Nanogram(s)
nm	Nanometer(s)
NSS	nine salts solution
O/N	overnight
OD	optical density
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
P-MBM	peptone marine basal medium
PCR	polymerase chain reaction
per. comm.	personal communication
pNPP	4-Nitrophenyl disodium orthophosphate
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second(s)
SDS	sodium dodecyl sulphate
Sm	Streptomycin
Sm^r	Streptomycin resistance
S.E.	standard error
SSC	sodium chloride tri-sodium citrate buffer
sp.	species

SSC	sodium chloride tri-sodium citrate buffer
SSS	sterile sea salts
STE	sodium chloride tris-EDTA buffer
T	thymine
TAE	tris-acetate-EDTA buffer
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
UV	ultraviolet
V	volts
v	volume
W	Watt(s)
w	weight
WBA(B)	wash buffer A or B

SYMBOLS

A_{600}	absorbance at a specified wavelength (i.e. 600 nm)
α	alpha
β	beta
$\varnothing$	diameter
Δ	delta
λ	lambda
μ	micro
μCi	microcurie
μg	microgram(s)
μl	microlitre(s)
μm	micrometer(s)
μM	micromolar(s)
M_r	relative molecular mass
%	percentage
Ψ	psi
°C	degrees Celcius

CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

1.1 The biology of *Haliotis midae*

Abalone are marine invertebrates that belong to the phylum mollusca, which includes the chitons, snails, clams, oysters, mussels, squids, and octopuses (Bevelander, 1988). Molluscs are soft-bodied, non-segmented, invertebrates that have a mantle cavity covering the gills, a definitive anterior head and a large muscular foot. Abalone are members of the class Gastropoda. These are molluscs that have one-piece shells or no shell at all, and move using their large muscular foot which is also used for attachment to submerged rocks (Stearns, 1869; Bevelander, 1988).

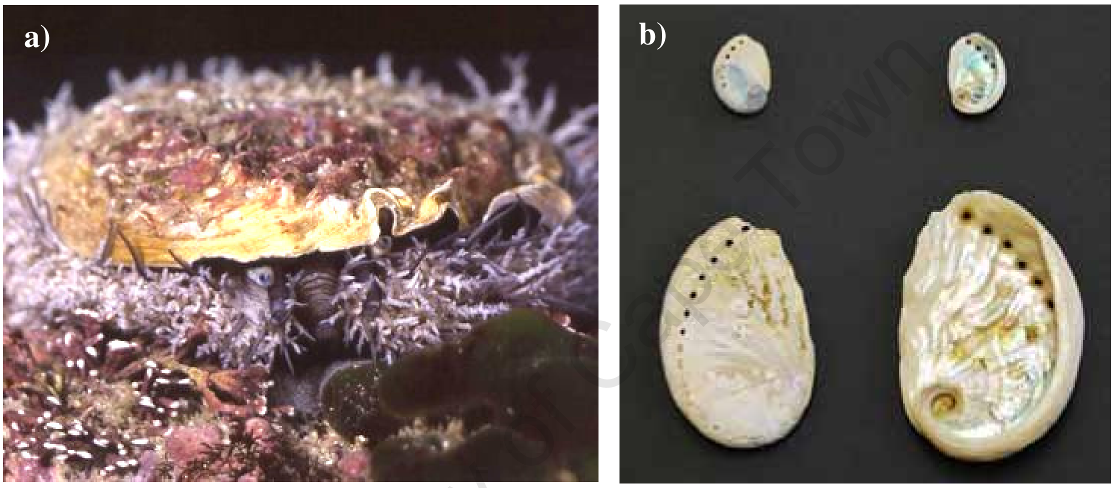


Figure 1.1 *Haliotis midae* (Photograph by Rob Tarr, Marine and Coastal Management, South Africa) (a) and *H. midae* shells (Photograph by Patrick Smart) (b) showing the shell's flat, ear-shape with the respiratory pores along the left side of each shell.

Abalone belong to the family Haliotidae and the genus *Haliotis*, meaning "sea ear", as they have a flattened shell whose general shape resembles that of a human ear (Stearns, 1869; Bevelander, 1988). The shell has a distinctive row of respiratory holes along its left side (Fig. 1.1 b) (Bevelander, 1988; Branch *et al.,* 2007). As the animal grows and the shell increases in size, new pores are made and the older ones are filled in and incorporated into the shell. The respiratory current exits the animal through these pores and in the process also removes faeces and urine (Bevelander, 1988).

There are approximately 100 species of abalone that are broadly distributed around the world including Australia, Japan, southern Africa and the coast of the northeastern

Pacific Ocean (Lindberg, 1992; Bevelander, 1988). Most of the world's abalone species are found in the central and south Pacific, and parts of the Indian Ocean. None of these are considered large abalone species, as the larger species are generally found in temperate waters and the smaller species in tropical and arctic regions (Imai, 1977). Abalone are generally found in the intertidal and subtidal regions at depths exceeding 30 meters (Bevelander, 1988). They occur in crevices or colonise exposed shallow reefs, rocks and boulder surfaces (Branch and Branch, 1982; Lindberg, 1992).

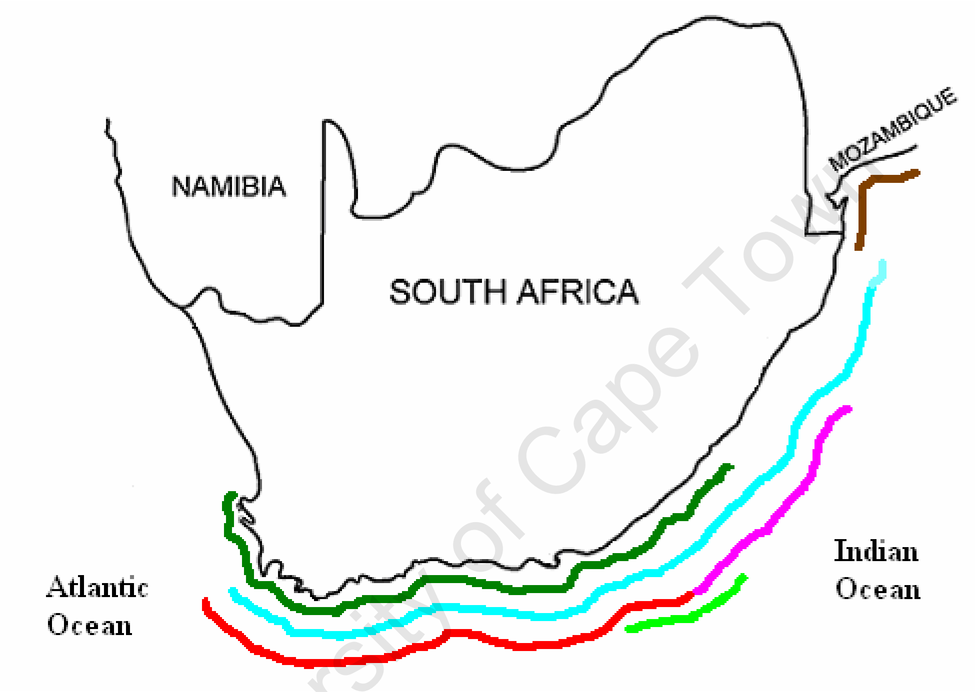


Figure 1.2 A map of Southern Africa, showing the distribution along the coastline of the six endemic abalone species, (■) *Haliotis midae*; (■) *Haliotis pustulata*; (■) *Haliotis spadicea*; (■) *Haliotis parva*; (■) *Haliotis queketti*; and (■) *Haliotis speciosa* (figure adapted from http://web.uct.ac.za/depts/zoology/abnet/safrica.html).

There are six haliotid species endemic to southern African waters [*Haliotis midae* (Fig. 1.1 and 1.2), *Haliotis parvum* L., *Haliotis spadicea*, *Haliotis queketti, Haliotis speciosa,* and *Haliotis pustulata*] (Fig. 1.2). However, only *H. midae*, known locally as 'perlemoen', is commercially exploited (Muller, 1986; Hecht, 1994). *H. midae* is the largest, reaching up to 200 mm in shell length in the wild, and the most abundant of the southern African abalone species. This species occurs naturally from St. Helena Bay (32° 45'S; 18° 10'E) on the west coast of southern Africa to just north of

Port St. Johns (31º 40'S; 29º 35'E) on the east coast of southern Africa (Simpson, 1994).

1.2 Abalone nutrition

Abalone are herbivorous animals that live on, and are generally found in association with, macroalgae (Mai, 1998; Branch *et al.,* 2007). The greatest densities of adult *H. midae* are found in beds of kelp, *Ecklonia maxima* (Branch *et al.,* 2007). *E. maxima* is the predominant algal species along the southwest coast of southern Africa and forms the main source of food in the diet of wild *H. midae* adults. Abalone generally feed on drifting pieces of seaweed which they trap by rapidly clamping down their muscular foot as the kelp drifts past. Wild abalone will feed on a range of different algae in an opportunistic manner, with at least two different algal species being present in the gut region at almost any stage of their adult life (Barkai and Griffiths, 1986).

1.3 The abalone digestive system

The digestive system of the abalone (Fig.1.3) consists of a buccal region and the digestive tract comprising the oesophagus, crop, stomach, intestine, digestive diverticulum and anus (Bevelander, 1988).

The buccal region is made up of a short round snout with a vertical mouth cleft and the buccal cavity. Within the buccal cavity is the long rasp-like tongue called the radula. The radula is used to scrape and grind food into easily ingestible-sized pieces that are then swallowed. The paired salivary glands secrete mucous into the buccal cavity, and together with the buccal pouches function by lubricating the radula, entangling fine food particles and aiding the movement of food into the oesophagus (Campbell, 1965; Bevelander, 1988). The oesophagus is divided into anterior, middle and posterior regions and is lined with ciliated columnar cells (Bevelander, 1988). The walls of the middle region of the oesophagus are covered with papillae and are made up of numerous folds that increase the surface area and capacity of this region. The oesophagus widens and leads directly into the first part of the stomach, which is referred to as the crop (Bevelander, 1988). The crop is primarily responsible for storing food, but is also the first part of the digestive tract where absorption takes place (Bevelander, 1988; McLean, 1970). The crop is joined to the stomach, and collectively they are referred to as the crop/stomach region.

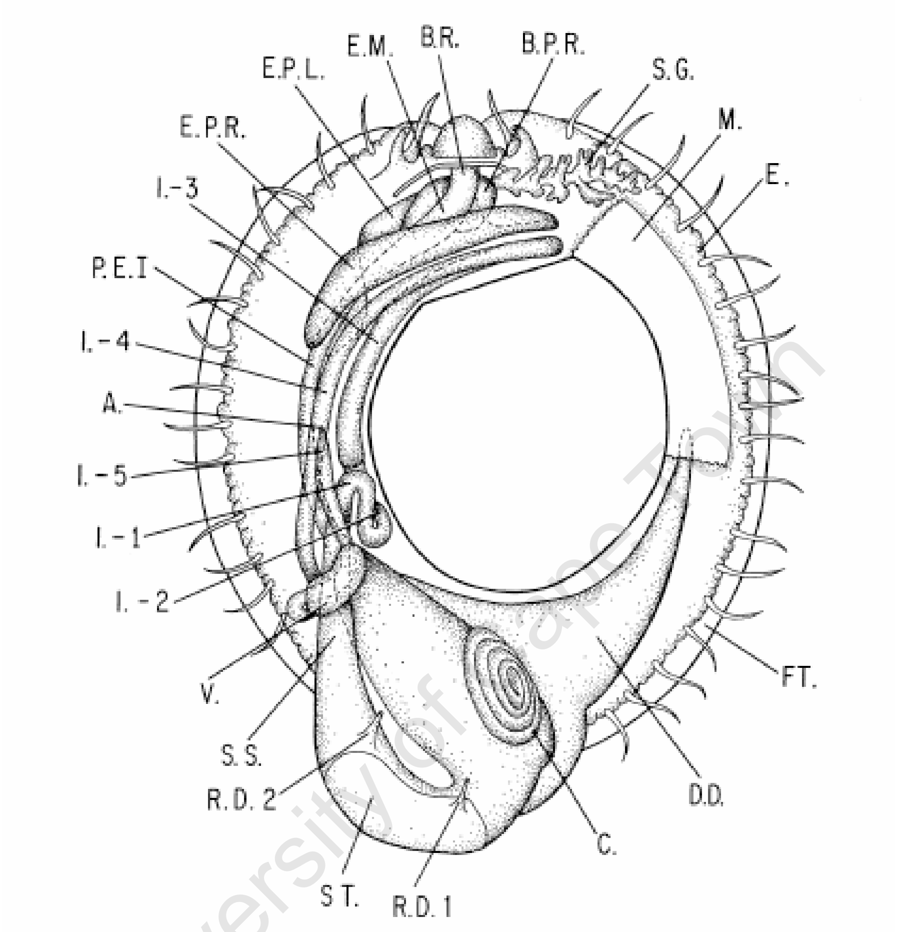


Figure 1.3 A detailed diagram of the digestive system of the Black Abalone, *Haliotis cracherodii* Leach, showing the complex and convoluted nature of the abalone digestive tract. Abbreviations used in this figure are as follows: A., anus; B.P.R., right buccal pouch; B.R. buccal region; C., caecum; D.D., digestive diverticulum (partially removed from oesophagus and stomach); E. epipodium; E.M., mid-oesophagus; E.P.L., left oesophageal pouch; E.P.R., right oesophageal pouch; F.T., foot; I-1 through 5, 5 regions of the intestine; M., mantle; P.E.I, postoesophagus region; R.D.1 and 2, right ducts of digestive diverticulum; R.S.M., right shell muscle; S.G., salivary gland; S.S., style sac; ST., stomach; and V., ventricle (Campbell, 1965).

The crop/stomach region is the primary site of abalone extracellular enzymatic digestion and a range of hydrolytic enzymes, including alginate lyases, laminarinases, agarases, carrageenases and proteases, have been detected in abalone digestive gland samples (Tsujino and Saito, 1962; Nakada and Sweeny, 1967; Erasmus *et al.,* 1997;

Edwards and Condon, 2001). Abalone have been shown to secrete these hydrolytic enzymes into the lumen of the gut and the expression of these enzymes is influenced by the make-up of their diet (Erasmus *et al.,* 1997; Garcia-Esquivel and Felbeck, 2006). The digestive diverticulum, also known as the hepatopancreas or digestive gland, is the primary organ involved in the storage and secretion of digestive enzymes (Campbell, 1965; Bevelander, 1988; Picos-García *et al.,* 2000; Garcia-Esquivel and Felbeck, 2006). This very large gland is comprised of two secretory cell-types, α-duct and β-crypt cells, which secrete digestive enzymes into ducts leading to the stomach, via the caecum, and postoesophagus (Campbell, 1965; Bevelander, 1988).

The long and highly convoluted intestinal tract is the main site of nutrient absorption and is divided up into five distinct regions; each specialized for the organization of fecal matter (Campbell, 1965). The intestine ends in the rectum from which the faeces are expelled in a mucous sheath. The rectum lies between the two gills within the mantle.

1.3.1 Abalone protein digestion

Abalone are unable to synthesize 10 of the 20 L-amino acids required for protein production and somatic growth. Therefore, abalone growth is dependent on the quantity and proportion of these essential amino acids in their diet. As a result, protease enzyme activity and protein digestion within the digestive tract is a key factor in the efficient utilization of ingested protein sources and growth of abalone (Serviere-Zaragoza *et al.,* 1997).

Abalone have significant levels of digestive proteolytic activity due to the production of a range of proteolytic digestive enzymes, including trypsins, chymotrypsins, non-specific proteases and aminopeptidases (Edwards and Condon, 2001; Garcia-Carreño *et al.,* 2003). Proteolytic enzyme activity was detected throughout the digestive tract, including the digestive gland, crop/stomach, and intestinal regions and rectal fluids, of juvenile and adult *Haliotis fulgens* (Serviere-Zaragoza *et al.,* 1997; Hernández-Santoyo *et al.,* 1998; Picos-García *et al.,* 2000). The optimal pH range for protease activity within *H. fulgens* digestive gland and crop/stomach samples was within the acidic range of 4-6, and acidic to alkaline range of 6-8, respectively, while the intestinal and rectal extracts were within the alkaline range of 7-11 (Serviere-Zaragoza *et al.,* 1997). This is in agreement with studies that showed the optimal pH for proteolytic, amylolytic and lipolytic digestive enzymes varied between the digestive tract regions of *Haliotis rufescens* (McLean, 1970). These investigations suggest that several different protease enzymes, with different pH optimal reaction conditions, are produced within the various regions of the digestive tract by abalone in response to the composition of their feed at that point in time.

1.4 Commercial abalone fishery

Abalone have been harvested from the sea for food since ancient times (Bevelander, 1988). There is documented evidence of a traditional Japanese abalone fishery, involving divers harvesting wild stocks of abalone, dating back to around 30 A.D. (Miyamoto, 1962 (in Hahn, 1989b)). Globally, there are approximately 15 species of abalone that are commercially exploited, and the main abalone producers are Mexico, United States (California), Australia, New Zealand, South Africa and Japan (Bevelander, 1988). Abalone are a highly prized and sought-after seafood delicacy, particularly in the Far East where abalone products are used in traditional cuisine and ceremonies (Britz, 1995). The high demand for abalone, together with disease outbreaks amongst wild populations and illegal poaching, has placed strain on the natural abalone populations (Hauck and Sweijd, 1999).

The South African abalone fishery began in Gansbaai in 1949 and was based on the collection of subtidal stocks of *H. midae*. However, the natural abalone resources along the South African coastline have been put under tremendous pressure due to over-fishing and abalone poaching (Bevelander, 1988; Sales and Britz, 2001). The abalone poaching fishery has always been a factor in the management of the natural South African abalone stocks, but since the mid-1990s there has been a significant increase in the levels of abalone poaching. Despite attempts to control poaching, this activity has spiraled out of control and has resulted in the almost complete collapse of the wild abalone populations along the South African coastline. As a result of the continued decline in the natural abalone population, the authorities closed the recreational abalone fishery in 2003 (Troell *et al.,* 2006). However, this was to no avail and as a result of continued poaching, and in a final attempt to save the wild abalone stocks, the South African commercial abalone fishery was closed in 2007.

With the world-wide supply of abalone by commercial abalone fisheries continuing to decline and the high demand and price for abalone, the only feasible option for meeting the world's abalone requirement is aquaculture-based cultivation of abalone species for sale on the international market (Britz, 1990).

1.5 Global abalone aquaculture industry

Aquaculture is the systematic cultivation of aquatic organisms including finfish, crustaceans, molluscs, and algae (FAO, 2000). Capture fisheries and aquaculture supplied approximately 110 million tonnes of food fish to the world in 2006, providing roughly 16.7 kg (live weight equivalent) per capita (FAO, 2009). Of the 110 million tonnes, 47% was produced by inland fresh water and marine aquaculture (FAO, 2009). Despite the fact that the majority of aquaculture production is freshwater finfish, crustaceans and molluscs, aquaculture of marine organisms, or mariculture, contributed 36.5% of the production and 35.7% of the total value of the global aquaculture industry in 2002 (FAO, 2004). Aquaculture is the fastest developing food production sector with a global production of 51.7 million tonnes (47% of the world's fish food supply) recorded in 2006 which represented an increase in production of 11.2% from 2004 and 21.9% from 2002 (FAO, 2009). China remains the largest global fisheries producer, with reported production figures of 17.1 and 34.4 million tonnes from capture fisheries and aquaculture, respectively, in 2006. The worldwide aquaculture production sector has become an important activity and economic contributor. Worldwide aquaculture has grown at an average rate of nearly 7% per annum since the early 1950s, when production was reported to be less than 1 million tonnes, to approximately 51.7 million tonnes, providing 40% of the world's aquatic harvest (including capture fisheries), with a value of US$78 billion in 2006 (FAO, 2007; FAO, 2009). World aquaculture production is over-shadowed by the Asia-Pacific region, which provides 89% of production in terms of quantity and 77% in terms of value to the global aquaculture industry. The Asia-Pacific region is in turn dominated by China, which provides 67% of production and 49% of value to the global aquaculture industry. Despite 40 years of research and development, aquaculture production in Africa has produced relatively little by comparison to the rest of the world and increased by only 12.7% in the period between 1970 and 2006, and presently represents a contribution of 1.5% of production and 1.8% of value to the global aquaculture industry (Brummett *et al.,* 2008; FAO, 2009). Despite the desperate need for food security and economic growth of the continent, the further development of aquaculture in Africa has been largely impeded by ineffective governance and management, political instability and a lack of foreign investment and technology (Brummett *et al.,* 2008). The most important species cultured in Africa are the Nile tilapia and sharptooth catfish. African aquaculture is led by Nigeria with an annual production of approximately 85,000 tonnes of freshwater finfish species, while Black Tiger Shrimp (*Penaeus monodon*) and abalone (*H. midae*) are being successfully cultured in Madagascar and South Africa, respectively (FAO, 2009).

Aquaculture of molluscan species comprises 65% of the annual global molluscan harvest, and is worth approximately US$11 billion per annum. Aquaculture production of molluscs accounts for 27% of the total aquaculture produce (more than 50 million tones) and 15% of the total value of global annual aquaculture production (FAO, 2009). However, of all the molluscan species, only gastropods and bivalves (mussels, oysters, scallops and clams) are extensively and intensively cultured world-wide within the aquaculture industry (Landau, 1991). Abalone are the most commercially important gastropod in the aquaculture industry, fetching prices of approximately US$34 - US$36 per kg for small cocktail sized (approximately 50 mm shell length) animals (Landau, 1991; Mai, 1998; Stanford, 2004). World-wide production of aquacultured abalone has increased dramatically in recent years, with 8,600 metric tonnes of farmed abalone being harvested in 2002 (Gordon and Cook, 2004).

The initial stages in the development of the world-wide abalone aquaculture industry began when Japanese scientists began investigating controlled spawning of *Haliotis gigantea* and *Haliotis discus hannai* (as reviewed in Hahn, 1989b). In the modern abalone aquaculture industry, the animals are either cultured onshore (in tanks or raceways; Fig. 1.4) or offshore (in a container, such as a cage, or ranched in the ocean). Although land-based abalone aquaculture requires pumping sea water onto land and through the tanks, it does enable the farmer to control aspects of the environmental conditions (temperature, dissolved oxygen and photoperiod) in order to maintain the health of the animals and maximise abalone growth (Preece and Mladenov, 1999).

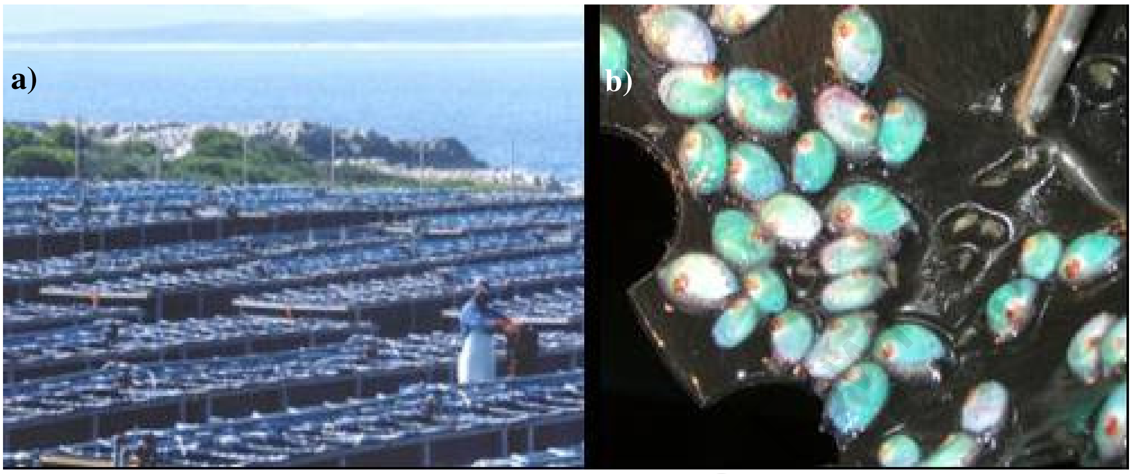


Figure 1.4 A typical South African raceway based abalone aquaculture farm (a), depicting the grow-out phase. The abalone are kept on solid plastic supports within plastic mesh baskets suspended in raceways that are aerated and supplied with sea water. The animals are fed (b) a diet of freshly harvested kelp (*Ecklonia maxima*) and/or a high-protein artificial feed (http://www.abfeed.com/mari-about.htm).

1.5.1 Abalone aquaculture in South Africa

Although a South African abalone fishery has existed since 1949, the local abalone aquaculture industry only became commercially viable once *H. midae* was successfully spawned in captivity in 1981 (Genade *et al.,* 1985; Genade *et al.,* 1988). The decline in the natural stocks of abalone due to over fishing and poaching, together with a high demand and favourable foreign exchange rates, has resulted in the South African abalone aquaculture industry being the only viable solution to meet the demand for *H. midae* on the international market (Sales and Britz, 2001).

The South African abalone aquaculture industry has grown to be the largest abalone producer outside Asia (FAO, 2004). At present there are approximately 22 land-based abalone aquaculture farms in South Africa, with at least 14 farms at a commercial production level, exporting abalone to the Far East (Troell *et al.,* 2006;

ten Doeschate and Coyne, 2008). The abalone aquaculture facilities are distributed along the South African coastline, from Port Nolloth on the West coast to East London on the East coast, with the majority of the farms located in the vicinity of Hermanus in the Western Cape (Troell *et al.,* 2006) (Fig. 1.5). Most of the abalone aquaculture facilities in South Africa have a production structure comprised of three separate phases, (i) hatchery producing spat (<8 mm); (ii) intermediate nursery phase (8 - 26 mm); (iii) and the final growout phase to cocktail market size (50 - 80 mm). When the animals reach cocktail size, they are harvested and exported to the Far East markets, either as a live product or canned or frozen (Vosloo and Vosloo, 2006).

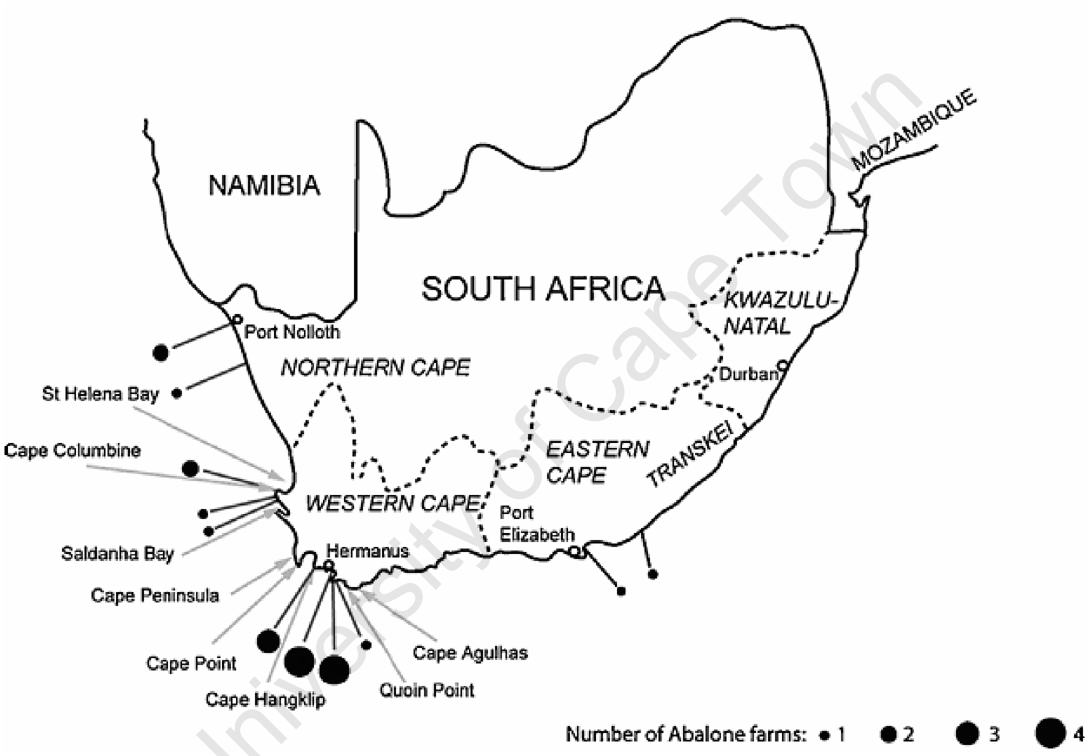


Figure 1.5 The distribution of abalone aquaculture farms along the South African coastline (Troell *et al.,* 2006).

1.6 Challenges associated with the abalone aquaculture industry

As with many other intensive agricultural practices, stressful conditions such as deterioration of environmental conditions and water quality, and disease outbreaks pose some of the major challenges facing the aquaculture industry as a whole (Balcázar *et al.,* 2006).

These problems are exacerbated by the nature of intensive agriculture, with large numbers of animals confined in a small area in order to maximize production. However, high stocking densities can have a negative impact on the growth and health of cultured species. Mgaya and Mercer (1995) showed that the growth rate of juvenile European abalone, *Haliotis tuberculata*, decreased as the stocking density increased. Similar results were obtained in studies of other aquacultured marine species, including shrimp (Allan and Maguire, 1992), clams (Hadley and Manzi, 1984), scallops (Parsons and Dadswell, 1992) and oysters (Holliday *et al.,* 1993).

Another major constraint on the abalone aquaculture industry is the relatively slow growth rate (14-40 mm year^{-1}) of these commercially important animals (Britz, 1995). Although the growth rate of abalone within abalone aquaculture facilities is faster than that of wild abalone (Cook, 1990), improving the growth rate of cultured abalone will result in significant increases in revenue for abalone farmers and investors. Interest in improving the growth rate of cultured abalone has led to species-specific optimization of culture conditions through increasing water temperatures and optimizing feed conversion (Cook, 1990; Britz and Hecht, 1997; Harris *et al.,* 1998b). Artificial formulation feeds have also been developed and shown to increase abalone growth rates (Fleming *et al.,* 1996) which has also positively impacted the industry. The major advantages of these artificial diets are that the feed can be manipulated to optimize nutritional value for different abalone species and culture conditions, the feeds are readily available from commercial suppliers and can be stored for long periods of time. Dry, pelleted, artificial formulation abalone feeds are being successfully used in Japan, China, Australia, New Zealand and South Africa (Bautista-Teruel and Millamena, 1999).

In South Africa, research into the development of a specific artificially formulated feed for the culture of *H. midae* began in 1989 at Rhodes University (Shipton, 1999). There are currently two locally manufactured formulated feeds, ABFEED® (Marifeed (Pty.) Ltd., Hermanus, South Africa) and Midae Meal™ (Taurus Products, (Pty) Ltd.) available to South African abalone aquaculture facilities, although international formulation feeds have been investigated for use in the culture of *H. midae* (Troell *et al.,* 2006). ABFEED® S34 is a high-protein artificial feed, containing approximately 34% fishmeal or soy bean meal as the protein source, starch, vitamins and minerals (Table 1.1). This feed was developed specifically for the cultivation of the South African abalone *H. midae*. A lower protein (approximately 26% protein content) alternative, ABFEED® K26, has been developed for larger cultured abalone (>50 mm shell length) (Troell *et al.,* 2006) and contains a small amount of dried kelp (*E. maxima*) as an attractant. Midae Meal™ is an all-seaweed based dried formulation feed that contains a mixture of dried seaweed species, including *Laminaria* spp., *E. maxima*, *Gracilaris* spp., *Gelidium* spp. and *Porphyra capensis* (Troell *et al.,* 2006).

Table 1.1 Proximate analysis of the artificial formulation feed - ABFEED® S34 (adapted from http://www.abfeed.com/mari-about.htm)

Ingredient	Content (%)
Protein	33 - 35
Carbohydrates	53
Fat	1.2
Crude fibre	0.9
Ash	5.0
Moisture	approx. 10.0
Vitamin and mineral mix	Unknown

An alternative approach to increasing the growth rate of abalone is through selective breeding programmes, where fast growing individuals are identified and hybridized to potentially produce faster growing offspring. This approach relies on the identification of suitable genetic markers, such as microsatellite loci, that will enable researchers to identify the genetic characteristics of faster growing individuals. Abalone species have been shown to be relatively easily hybridized and future investigations should focus on the development of hybrids that have other improved qualities, besides increased growth rates (Leighton and Lewis, 1982; Hahn, 1989a).

Disease outbreaks as a result of pathogenic bacteria, fungi and viruses, are becoming increasingly common within aquaculture facilities, and are seen as one of the major limitations affecting the successful development of the industry (Verschuere *et al.,* 2000). In response to disease problems, aquaculture farmers have routinely made use of broad-spectrum antibiotics to control the proliferation of potentially pathogenic microorganisms and improve the health and production of the cultured animals

(Gram *et al.,* 1999; Van den Bogaard and Stobberingh, 2000; Gullian *et al.,* 2004). However, the wide-spread use of antibiotics in terrestrial agriculture and aquaculture has resulted in the emergence of antibiotic-resistant bacteria within these environments (Schwarz *et al.,* 2001; Akinbowale *et al.,* 2006). The emergence of antibiotic-resistant pathogenic bacteria has resulted in massive production collapses within the Asian shrimp aquaculture industry (Karunasagar *et al.,* 1994). Shrimp production in the Philippines dropped by 55% between 1995 and 1997 as a result of disease problems, and an industry that was worth US$760 million is now only worth US$240 million (FAO, 2007). The annual global loss in revenue as a result of disease in the shrimp aquaculture industry is estimated at US$3 billion (Farzanfar, 2006).

Disease outbreaks as a result of bacterial and viral infections have also had a negative impact on the abalone aquaculture industry. Several *Vibrio* species have been identified as the causative agent in many outbreaks. A pathogenic *Vibrio alginolyticus* strain was isolated from moribund juvenile Red abalone *H. rufescens* (Elston and Lockwood, 1983), whilst a pathogenic *V. alginolyticus* strain was responsible for large-scale mortalities of the Taiwanese small abalone *H. diversicolor supratexta* (Liu *et al.,* 2001). *Vibrio carchariae* was identified as the cause of mortalities of *H. tuberculata* (Nicolas *et al.,* 2002) and *Haliotis* (*Sulculus*) *diversicolor supratexta* (Nishimori *et al.,* 1998). In 2006 viral infections by a herpes-like virus was identified as the likely cause of mass mortalities of cultured and wild abalone populations that have almost completely destroyed the Australian abalone industry (Gavine *et al.,* 2009).

The prophylactic use of probiotic bacteria is becoming widely accepted as an alternative to the use of antibiotic treatments, in an attempt to improve the health, disease resistance and growth of various aquacultured species (Austin *et al.,* 1995; Balcázar *et al.,* 2006; Gatesoupe 1999; Kesarcodi-Watson *et al.,* 2008; Verschuere *et al.,* 2000).

1.7 Probiotics for aquaculture

The original concept of probiotic microorganisms was probably formed in the early part of the 1900s by Elie Metchnikoff, who suggested that consuming fermented milk products could improve human health by modifying the gut microbiota (Metchnikoff, 1907 as reviewed by Gatesoupe, 1999 and Zhou *et al.,* 2009). Today, there is a lot of interest in probiotics and the health benefits associated with regular consumption of probiotic strains (Lee and Salminen, 1995; Guarner and Schaafsma, 1998), although the actual mode of probiotic action has not been conclusively elucidated (Holzapfel *et al.,* 1998). Probiotics are investigated and used for improved health, better digestion and utilization of feed, improved disease resistance and immunity, increased production and enhanced growth in the animal production sector of terrestrial agriculture (Fuller, 1992; Burr and Gatlin, 2005).

Fuller (1989) initially defined pobiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Kesarcodi-Watson *et al.,* 2008). However, as a result of the aquatic environment and its constant contact with aquatic animals, host-microbe interactions are not limited to the fairly stable environment of the gastrointestinal tract, as they are in the case of humans and terrestrial animals. The majority of the microbes associated with and within aquatic animal species are only transiently present (Fig. 1.6) (Moriarty, 1990). As a result, any changes in environmental conditions such as temperature or salinity can result in a significant shift in the microbial community associated with aquatic animals (Hamid *et al.,* 1978; Sakata *et al.,* 1980; Lésel, 1990; Ringø and Strøm, 1994). A broader and more appropriate definition of probiotics within the context of aquaculture was made by Verschuere *et al.* (2000) who defined a probiotic 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." Irianto and Austin (2002a) comment that, "a probiotic is an entire or component(s) of a microorganism that is beneficial to the health of the host". Perhaps these definitions need to be combined in the case of probiotics within aquaculture environments. Thus, a probiotic for aquaculture may be defined as an entire or component(s) of a microorganism added, or fed, to aquacultured host species, or the culture environment, that has a beneficial effect on the host organism 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.

1.7.1 Modes of action of probiotics for aquaculture

There are many studies on probiotic microorganisms for the aquatic environment that have demonstrated a particular effect, following probiotic treatment of cultured aquatic species (summarized in Table 1.2; Kesarcodi-Watson *et al.,* 2008). Most of the early research conducted on probiotics for use in aquaculture, and the suitability of selected strains for use in aquaculture environments, was not the result of in-depth investigations into the exact mode of action of putative probionts. However, in order to investigate and identify the possible mechanism of action of a particular probiotic isolate for use in the aquaculture industry, it is important to understand the many functions that these microorganisms can perform within the aquatic environment.

Enteric bacteria, and therefore enteric probiotic bacterial strains, may perform many roles within the digestive tract of their aquatic invertebrate hosts, as reviewed in Harris (1993) and summarized in Figure 1.6. Enteric bacterial species enter the digestive tract of the host organism through associations with the host's food source, or they are present within the aquatic environment and enter the host through the water (Fig. 1.6). Ingested bacteria may be lysed and absorbed as an additional food source, or lysed and consequently release enzymes that contribute to the host's pool of digestive enzymes. Herry *et al.* (1989) showed that the clam, *Loripes lucinalis*, was capable of lysing and digesting bacterial cells. Proteins originating from the abalone probiont *Pseudoalteromonas* sp. strain C4 were found to be incorporated into the tissues (gills, foot muscle, adductor muscle, intestine and hepatopancreas) of *H. midae* fed a kelp-based diet supplemented with the probiotic strain C4 (ten Doeschate, 2005). Ingested bacteria may also transiently pass through the host's digestive tract, and in so doing may contribute to the digestion processes of the host animal through the secretion of hydrolytic enzymes. Studies have indicated that bacteria may be involved in shrimp nutrition through their ability to produce extracellular enzymes, such as proteases, carbohydrolases and lipases, as well as providing essential growth factors to the shrimp host (Arellano and Olmos, 2002; Ochoa and Olmos, 2006).

Table 1.2 Summary of some of the research conducted to date towards the development of probiotics for finfish, crustacean and molluscan aquaculture industries [Table adapted from Kesarcodi-Watson *et al.* (2008)].

Animals tested	**Probiotic strain(s)**	**Test method and effect**	***In vitro* or *in vivo***	**Reference**
Finfish				
Rainbow trout (*Onchorynchus mykiss* Walbaum)	*Pseudomonas fluorescens* AH2	Bath for 6 days; *V. anguillarum* challenge	*In vitro* and *in vivo*	Gram *et al.* (1999)
Rainbow trout (*O. mykiss* Walbaum)	*Vibrio fluvialis*	Dietary supplementation	*In vitro* and *in vivo*	Irianto and Austin (2002b)
Rainbow trout (*O. mykiss* Walbaum)	*Lactobacillus rhamnosus* (ATCC 53103)	Dietary supplementation; enhanced specific and innate immune parameters	*In vivo*	Nikoskelainen *et al.* (2003)
Atlantic salmon (*Salmo salar* L.)	*Vibrio alginolyticus*	Bath for 10 min; Improved survival when challenged with pathogens *Aeromonas salmonicida*, *V. anguillarum*, and *Vibrio ordalii*	*In vivo*	Austin *et al.* (1995)
Rainbow trout and Atlantic salmon	*Carnobacterium* sp. strain K1	Dietary supplementation; Improved disease resistance	*In vivo*	Robertson *et al.* (2000)
Turbot larvae (*Scophthalmus maximus*)	Three lactic acid bacterial strains	Added to culture of rotifer (*Brachionus plicatilis*) live feed	*In vivo*	Gatesoupe (1991)
Turbot larvae (*S. maximus*)	*Vibrio proteolyticus*	Dietary supplementation; Enhanced protein digestion	*In vivo*	De Schrijver and Ollevier (2000)
Sea bass larvae (*Dicentrarchus labrax*)	*Debaryomyces hansenii* HF1 and *Saccharomyces cerevisae*	Dietary supplementation; Improved digestion efficiency and survival	*In vivo*	Tovar *et al.* (2002)
Sea bream larvae (*Sparus aurata*)	*Cytophaga* sp., *Roseobacter* sp., *Ruergeria* sp., *Paracoccus* sp., *Aeromonas* sp., and *Shewanella* sp.	Natural larval survival study	*In vivo*	Makridis *et al.* (2005)
Common carp (*Cyprinus carpio*)	Photosynthetic bacterial strain and *Bacillus* sp.	Dietary supplementation; Improved growth, digestive enzyme activities and feed conversion	*In vivo*	Yanbo and Zirong (2006)

Table 1.2 (*Continued*)

Animals tested	Probiotic strain(s)	Test method and effect	***In vitro* or *in vivo***	Reference
Finfish (Continued)				
Common carp (*C. carpio*)	*Bacillus* sp.	Added to water; Reduction in pathogen (*Aeromonas hydrophila*) load, and improvement in water quality	*In vitro* and *in vivo*	Lalloo *et al*. (2007)
Poecilia reticulata (Peters), *P. sphenops* (Valenciennes), *Xiphophorus helleri* (Heckel) and *X. maculates* (Gunther)	*Bacillus subtilis*	Dietary supplementation; Enhanced disease resistance, digestive enzyme levels, growth and reproductive performance	*In vivo*	Ghosh *et al.* (2007a and b)
Nile tilapia (*Oreochromis niloticus*)	*Micrococcus luteus*	Dietary supplementation; Enhanced growth performance and fish-resistance against *A. hydrophila* infection.	*In vitro* and *in vivo*	Abd El-Rhman *et al.* (2009)
Crustacean				
Black tiger shrimp (*Penaeus monodon*)	*Bacillus* S11	Improvement in growth and survival when challenged with *Vibrio harveyi*	*In vivo*	Rengpipat *et al.* (1998) and (2003)
P. monodon	*Bacillus subtilis* BT23	Reduced mortality following challenge with *V. harveyi*	*In vitro* and *in vivo*	Vaseeharan and Ramasamy (2003)
Penaeus vannamei larvae		Growth improvement and decreased incidence and severity of disease	*In vivo*	Garriques and Arevalo (1995)
Penaeus vannamei juveniles	Commercial product (*S. cerevisiae*, *S. exiguous* and *Phaffia rhodozoma*)	Dietary supplementation; Improved vibriosis resistance	*In vivo*	Scholz *et al.* (1999)
Penaeus chinesis post-larvae	*Arthrobacter* sp. strain XE-7	Added to culture water; Improved water quality and survival following challenge with pathogenic vibrios (*Vibrio parahaemolyticus, V. nereis* and *Vibrio anguillarum*)	*In vivo*	Li *et al.* (2006)

Table 1.2 (*Continued*)

Animals tested	**Probiotic strain(s)**	**Test method and effect**	***In vitro* or *in vivo***	**Reference**
Crustacean (Continued)				
Indian white shrimp (*Fenneropenaeus indicus*)	Mixture of 5 commercial *Bacillus* probiotic strains	Improved digestion efficiency, survival and growth	*In vivo*	Ziaei-Nejad *et al.* (2006)
Pacific White shrimp (*Litopenaeus vannamei*)	*V. alginolyticus* UTM102, *B. subtilis* UTM126, *Roseobacter gallaeciensis* SLV03 and *Pseudomonas aestumarina* SLV22	Dietary supplementation; Improved feed conversion and disease resistance, following immersion challenge with *V. parahaemolyticus*	*In vitro* and *in vivo*	Balcázar *et al.* (2007)
L. vannamei	*Bacillus* OJ	Dietary supplementation; Enhanced survival and increased immune parameters	*In vivo*	Li *et al.* (2009)
Western king prawn juveniles (*Penaeus latisulcatus*)	*Pseudomonas synxantha* and *Pseudomonas aeruginosa*	Dietary supplementation; Enhanced survival	*In vivo*	Hai and Fotedar (2009)
Mollusc				
Pacific oyster larvae (*Crassostrea gigas*)	*Alteromonas* sp. strain CA2	Growth and natural survival experiment	*In vivo*	Douillet and Langdon (1994)
Pacific oyster larvae (*C. gigas*)	*Aeromonas media*	*Aeromonas* spp.; *Vibrio* spp.; *Photobacterium damsella*; *Yersinia rucheri*; and *Vibrio tubiashii*	*In vitro* and *in vivo*	Gibson *et al.* (1998)
Cortés oyster (*Crassostrea corteziensis*)	*Lactobacillus* strain NS6.1	Growth, survival and SOD measurement experiment; Improved growth	*In vivo*	Campa-Córdova *et al.* (2009)
Abalone (*Haliotis midae*)	*Pseudoalteromonas* sp. strain C4	Dietary supplementation; Enhanced growth rate	*In vivo* (field trials)	ten Doeschate and Coyne (2008)
Abalone (*H. midae*)	*V. midae* SY9, *D. hansenii* AY1 and *Cryptococcus* sp. strain SS1	Dietary supplementation; Improved growth rate and disease response	*In vivo* (field trials)	Macey and Coyne (2005)
Chilean scallop (*Argopecten purpuratus*)	*Vibrio* sp.	Enhanced survival following *V. anguillarum* challenge	*In vitro* and *in vivo*	Riquelme *et al.* (1997)

Abalone enteric bacteria have also been shown to secrete hydrolytic extracellular enzymes into the lumen of the digestive tract, which may in turn assist the abalone with digestion of complex food sources (Erasmus *et al.,* 1997; Sawabe *et al.,* 1998 and 2004a). In fact, almost all of the bacteria isolated from the digestive tract of *H. discus hannai* were shown to be capable of hydrolysing alginate (Sawabe *et al.,* 1995). The relative abundance of bacteria secreting extracellular hydrolytic enzymes may indicate a commensal or symbiotic relationship between these enteric microorganisms and their abalone host (Tanaka *et al.,* 2003). Therefore, supplementing the diet of cultured abalone species with probiotic bacteria able to secrete extracellular hydrolytic enzymes, may result in increased growth rates by improving the digestion efficiency of the abalone host through pre-digestion of the feed or enzymatic contribution to digestion (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008).

The proposed modes of action of probiotic microorganisms within an aquaculture environment can be summarized as one or more of the following: (i) production of inhibitory compounds; (ii) competitive exclusion of pathogenic microorganisms by competition for chemicals, available energy sources and/or adhesion sites; (iii) enhancing the immune response of the host; (iv) improvement of water quality; (v) interaction with phytoplankton; (vi) as a source of nutrients; and (vii) enzymatic contribution to digestion (Verschuere *et al.,* 2000; Balcázar *et al.,* 2006).

To date, the exact modes of action of probiotic microorganisms have not been completely elucidated (Junfeng *et al.,* 2008) and the ongoing development of probiotic technologies for aquaculture species in general, needs to be tempered with continued investigations into the precise mode of action of these probiotic microorganisms. A better understanding of how these microorganisms influence the host to induce improved health, disease resistance and growth will not only facilitate improved probiotic strain isolations, but will also assist with the development of strategies for the optimal use of probiotics within aquaculture facilities to improve production and revenue.

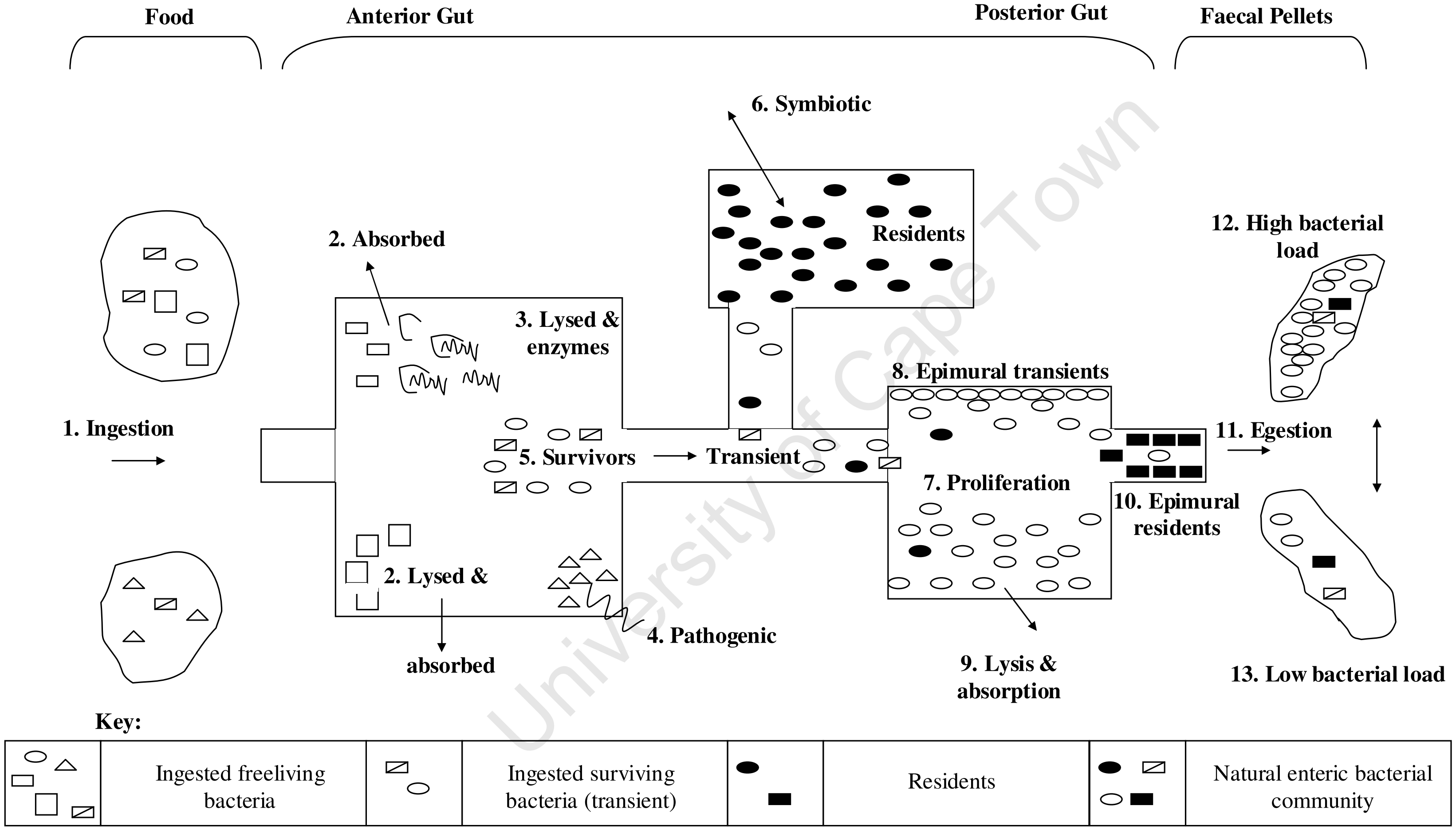


Figure 1. 6. The types of reported associations between aquatic invertebrates and their gut microflora (Adapted from Harris, 1993)

1.7.2 Probiotics used in the aquaculture industry

The probiotic microorganisms that have been investigated for use in the aquaculture industry have included Gram-negative and Gram-positive bacteria, bacteriophages, yeast and unicellular algae (Table 1.2) (Irianto and Austin, 2002). Gram-negative, facultative-anaerobic bacteria, such as *Vibrio* species, dominate the gut microbiota of finfish and shellfish (Prieur *et al.,* 1990; Sakata, 1990; Gatesoupe, 1999). Therefore, it is not surprising that some of the probiotic isolates that have been shown to be effective within aquaculture also belong to the *Vibrio* genus, including *V. alginolyticus* (Austin *et al.,* 1995) and *Vibrio midae* SY9 (Macey and Coyne, 2005). Gram-positive spore forming *Bacillus* ssp. have also been identified as effective probiotics for the aquaculture industry (Gullian *et al.,* 2004) and have been incorporated into commercial probiotic products that have been successful in improving shrimp production levels (Moriarty, 1998; Rengpipat *et al.,* 1998; Rengpipat *et al.,* 2003; Decamp and Moriaty, 2006; Balcázar *et al.,* 2007). Yeast strains, such as *Debaryomyces hansenii* HF1 (Tovar *et al.,* 2002) and *Debaryomyces hansenii* AY1 (Macey and Coyne, 2005) have also been proposed and utilized as probiotics for aquaculture species.

1.7.2.1 Probiotics for finfish aquaculture

Probiotics have been shown to be effective in all stages of finfish aquaculture, by improving growth and/or disease resistance of cultured species (Austin *et al.,* 1995; Gram *et al.,* 1999; Naik *et al.,* 1999; Robertson *et al.,* 2000; Spanggaard *et al.,* 2001; Hjelm *et al.,* 2004; Yanbo and Zirong, 2005; Ghosh *et al.,* 2007a) (summarized in Table 1.2). Gram *et al.* (1999) showed that *Pseudomonas fluorescens* AH2 inhibited the growth of a fish pathogenic strain of *Vibrio anguilarum* in an *in vitro* system. The survival of rainbow trout (*Oncorynchus mykiss* Walbaum) was significantly improved (46%) when challenged with a virulent *V. anguilarum* strain after pre-treatment of the culture water with *P. fluorescens* AH2 for five days. Dietary supplementation of rainbow trout with the probiotic *Kocuria* SM1 stimulated the immune parameters and improved disease protection in comparison to fish fed a basal diet (Sharifuzzaman and Austin, 2009). Probiotic dietary-supplementation of African Catfish

(*Clarias gariepinus*, Burchell 1822) with *Lactobacillus acidophilus* significantly improved growth, haemotology and immunological parameters in comparison with fish fed a control basal diet (Al-Dohail *et al.,* 2009). A previously isolated shrimp probiotic strain, *V. alginolyticus*, was also shown to reduce the mortalities of Atlantic salmon (Salmo salar L.) challenged with the pathogens *Aeromonas salmonicida*, *V. anguillarum*, and *Vibrio ordalii* (Austin *et al.,* 1995).

Probiotics may supply a direct nutritional benefit to the host through vitamin production, provision of essential minerals and trace elements, and secretion of additional digestive enzymes (Holzapfel *et al.,* 1998). Improved growth of probiotic finfish may be as a result of enhanced feed conversion efficiencies and better feed utilization (Ghosh *et al.,* 2007a). In so doing the probiotic may also improve the health and reproductive performance of the host fish (Ghosh *et al.,* 2007b). Dietary supplementation of *Bacillus subtilis* can lead to activation of the cellular and humoral immune responses of the host (Salinas *et al.,* 2005), resulting in enhanced disease resistance and survival when challenged with bacterial pathogens such as *Aeromonas hydrophila* (Ghosh *et al.,* 2007a). Similarly, dietary supplementation with the yeast *D. hansenii* HF1 enhanced digestive enzyme activity and survival of sea bass larvae, *Dicentrarchus labrax* (Tovar *et al.,* 2002). De Schrijver and Ollevier (2000) also showed that feeding the probiotic *Vibrio proteolyticus* to turbot larvae, *Scophthalmus maximus*, enhanced protein digestion.

1.7.2.2 Probiotics for shrimp aquaculture

Probiotic microorganisms, especially *Bacillus* spp., have been shown to be effective within the shrimp aquaculture industry in larviculture (Guo *et al.,* 2009), controlling disease (Rengpipat *et al.,* 1998; Rengpipat *et al.,* 2003; Balcázar *et al.,* 2007), increasing growth (Rengpipat *et al.,* 2003), enhancing the health of the shrimp (Gullian *et al.,* 2004; Tseng *et al.,* 2009), and improving the quality of water and sediments in shrimp ponds (Moriarty, 1998) (summarized in Table 1.2). Gullian *et al.* (2004) isolated two probiotic strains, *Bacillus* P64 and *Vibrio* P62, from the hepatopancreas of healthy wild *Penaeus vannamei* shrimp. The probiotic strains were able to colonise the shrimp's hepatopancreas and reduced colonisation of the hepatopancreas by pathogenic *Vibrio harveyi*. A significant growth advantage was also observed in the shrimp receiving the probiotic treatment. Although the authors acknowledge that probiotic microorganisms may increase a host's growth by improved digestion and synthesis of critical compounds, in this instance *Bacillus* P64 and *Vibrio* P62 are believed to have excluded microbial pathogens that hindered the growth of the shrimp in the control groups. In another study, the addition of selected *Bacillus* species to shrimp pond water displaced pathogenic luminous *Vibrio* species present in the water through competitive exclusion and improved the water quality and productivity of the culture ponds (Moriarty, 1998). Furthermore, probiotic treatment with the terrestrial lactic acid bacterium *Pediococcus acidilactici* (strain MA18/5M, CNCM) has been shown to effectively reduce the susceptibility of *Litopenaeus stylirostris* to the bacterial pathogen *Vibrio nigripulchritudo* (Castex *et al.,* 2008; Castex *et al.,* 2009).

1.7.2.3 Probiotics for molluscan aquaculture

While most of the initial aquaculture-related scientific research aimed at the isolation and development of probiotics has focused on the finfish and crustacean sectors of this industry, there have also been successful probiotics developed specifically for cultivated molluscan species (Douillet and Langdon, 1994; Riquelme *et al.,* 1997; Macey and Coyne, 2005; ten Doeschate and Coyne, 2008; Campa-Córdova *et al.,* 2009) (Summarized in Table 1.2). Most of the research has focused on probiotics for bivalve molluscs (Kesarcodi-Watson *et al.,* 2008), and has included work on the Pacific oyster, *Crassostrea gigas*; Cortés oyster, *Crassostrea corteziensis*; the scallop, *Pecten maximus*; Chilean scallop, *Argopecten purpuratus*; and the Manila clam, *Ruditapes philippinarum* (Table 1.2). A recent publication has also investigated the isolation of effective probiotics for use in the larviculture of Greenshell™ mussels, *Perna canaliculus* (Kesarcodi-Watson *et al.,* 2009).

There have been several recent publications on the development of probiotics specifically for the abalone aquaculture industry (Macey and Coyne, 2005; Macey and Coyne, 2006; ten Doeschate and Coyne, 2008; Iehata *et al.,* 2009). A significant focus within some of these publications has been the increased growth rate observed in abalone receiving probiotically supplemented diets (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). Maximising the growth rate of abalone is critical to the successful commercial abalone aquaculture industry (Capinpin *et al.,* 1999) and several other studies have also shown that bacterial isolates can improve the growth performance of numerous marine organisms (Douillet and Langdon, 1994; Ringø *et al.,* 1996; Riquelme *et al.,* 1997; Jory, 1998; Rengpipat *et al.,* 1998; Douillet, 2000a; Douillet, 2000b; Olafsen, 2001; Tovar *et al.,* 2002; Rengpipat *et al.,* 2003; Ziaei-Nejad *et al.,* 2006; Al-Dohail *et al.,* 2009; Merrifield *et al.,* 2009; Sáenz de Rodrigáñez *et al.,* 2009).

The marine bacterium *Pseudoalteromonas* sp. strain C4, isolated from the crop of the South African abalone *H. midae* (Erasmus, 1996; Erasmus *et al.,* 1997), secretes high levels of extracellular alginate lyase (ten Doeschate, 2005). Inclusion of strain C4 into an *H. midae* kelp-based diet increased the growth rate of the abalone by 38%, and resulted in significantly higher alginate lyase activity within the crop and stomach regions, compared to animals fed a basal kelp diet. The authors concluded that the increased growth rate observed in abalone receiving the *Pseudoalteromonas* sp. strain C4 supplemented diet was due to strain C4 being digested and utilized as an alternative protein source by the abalone, and the secreted alginate lyase enzyme improving the abalone host's ability to digest kelp. It is therefore thought that probiotic microorganisms can provide a source of nutrients for the host and contribute to the pool of digestive enzymes available to the host and thereby improve the digestion efficiency and growth of the host organism.

1.8 *Vibrio midae* SY9

Macey (2005) isolated the marine bacterium *Vibrio midae* SY9 from the digestive tract of *H. midae* (Fig. 1.7). This strain was isolated and selected for further investigation on the basis of high levels of extracellular protease activity against a broad range of protein substrates. In farm-based growth trials conducted on small (20 mm shell length) and large (67 mm shell length) abalone fed the high protein formulated feed ABFEED® S34 supplemented with *V. midae* SY9 and two yeast strains, the growth rate of the abalone was improved by 8% and 34% respectively, over an eight month growth trial (Macey and Coyne, 2005). Macey and Coyne (2006) also showed that *H. midae* animals fed ABFEED® S34 supplemented with *V. midae* SY9 had significantly increased levels of protease activity within the intestinal region of their digestive tract.

Figure 1.7 *Vibrio midae* SY9. An electron micrograph of a single *V. midae* SY9 cell (A), with a single polar flagellum (Macey, 2005). *V. midae* SY9 cultivated on a gelatine plate (B), which was stained with Amido black. The clear zones show regions of gelatine hydrolysis due to extracellular protease activity.

The increased intestinal protease activity of the animals fed *V. midae* SY9 was positively correlated with the presence of *V. midae* SY9. The increased intestinal protease activity was shown to result in improved protein digestion and absorption in abalone fed the *V. midae* SY9 supplemented diet. Therefore, supplementation of a high protein formulated feed such as ABFEED® S34 with *V. midae* SY9, could result in increased growth rates of farmed *H. midae* as a result of improved protein digestion due to the presence of the bacterial probiont and its extracellular protease(s).

1.9 Concluding remarks

Abalone are a highly sought after seafood delicacy, commanding very high prices, especially in the Far Eastern markets where they are sold as a canned, frozen or live product. A South African abalone species, *Haliotis midae*, has been the focus of the local commercial fishery since 1949. However, as a result of overfishing and illegal poaching the wild populations of *H. midae* have collapsed. The South African abalone aquaculture industry has emerged as the only viable solution to meet the high demand for *H. midae* products on the international market. Since its inception, the South African abalone aquaculture industry has developed rapidly and at present there are approximately 22 abalone aquaculture farms, with at least 14 farms at a commercial production level. One of the major challenges facing the South African abalone aquaculture industry is the relatively slow growth rate of *H. midae*, as it takes approximately five years to reach market size.

Probiotic microorganisms have been successfully used to improve the growth of several aquacultured organisms, including abalone (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). The observed increase in growth of probiotic-supplemented aquacultured organisms may be as a result of the probiotics being ingested and: (i) secreting extracellular enzymes, such as proteases, within the digestive tract of the host that improve the digestion efficiency of the host; (ii) digested by the host organism as an additional nutrient source; (iii) the probiotic may competitively exclude enteric microorganisms that negatively influence the host's growth, and in so doing improve the host's health and growth. Abalone gut associated bacteria have been shown to secrete hydrolytic extracellular enzymes within the abalone digestive tract and consequently, improve the digestion efficiency of the host (Erasmus *et al.,* 1997). An abalone enteric bacterial isolate, *V. midae* SY9, secretes high levels of extracellular protease, and has been shown to significantly increase protein digestion and the growth rate of cultured *H. midae* fed an artificial high protein diet (Macey and Coyne, 2005). However, molecular studies to further investigate and characterise the effect of nutritional probiotics, such as *V. midae* SY9, have not yet been conducted and the results that such a study may produce will enable a better understanding of the role played by probiotic microorganisms in conferring a growth advantage to their host.

1.10 Aims and objectives of this study

The current investigation should be viewed as an extension of the research previously conducted in the Marine Biotechnology Unit at the University of Cape Town, on the development of probiotics for the South African abalone aquaculture industry. The aim of this study was to characterise the detergent-stable extracellular protease produced and secreted by the probiotic strain *Vibrio midae* SY9. The detergent-stable protease gene was cloned, the protease protein purified and characterised at a molecular level. *V. midae* SY9 detergent-stable protease mutants were constructed and used to investigate the role that this protease plays in conferring the observed probiotic effect on the growth of cultured *Haliotis midae*. Polyclonal antibodies raised to the purified protease protein were employed to localize the *V. midae* SY9 detergent-stable protease protein within the digestive tract of probiotically supplemented abalone. A *V. midae* SY9 chromosomally tagged strain was constructed and used to localize *V. midae* SY9 within the digestive tract of probiotically supplemented *H. midae*. In so doing, we aim to better understand the role of the *V. midae* SY9 extracellular protease VmproA in the previously observed probiotic effect on cultured *H. midae*.

CHAPTER 2

CLONING AND CHARACTERISATION OF AN EXTRACELLULAR PROTEASE GENE FROM *VIBRIO MIDAE* SY9

CONTENTS

2.1 Summary

An *E. coli* clone displaying a proteolytic phenotype on skim milk Luria agar was isolated from a previously constructed and screened *Vibrio midae* SY9 genomic library. A restriction endonuclease map of the recombinant pBluescript SK cloning vector, pMR11, was assembled and a deletion sub-cloning strategy used to identify a region containing the protease gene(s) on the recombinant plasmids. A Southern blot confirmed the *V. midae* SY9 origin of the cloned DNA fragment in the recombinant plasmids. The protease gene was sequenced and analyzed. A 1605 bp ORF, a putative signal sequence and an energetically favorable transcriptional terminator were identified, as well as, three conserved domains within the deduced 534 amino acid protein. Primer extension identified the transcriptional start site of the *V. midae* SY9 extracellular protease mRNA transcript. A putative promoter region was identified upstream of the transcriptional start site. Northern blot hybridization demonstrated that the *V. midae* SY9 extracellular protease is encoded by a 1.7 kb mRNA transcript. Three active site amino acid residues were identified within the deduced amino acid sequence. These three active site residues were observed to be in the same order as those of other characterised subtilase serine proteases. A BLAST search of the GENBANK database showed very high levels of sequence identity between the deduced amino acid sequence of the *V. midae* SY9 extracellular protease and closely related protease proteins. The highest sequence identity, at an amino acid level, was 98% to a *Vibrio alginolyticus* alkaline serine exoprotease A precursor (Accession number: P16588). Phylogenetic analysis indicated that VmproA may be a member of the proteinase K-like sub-family of subtilases.

2.2 Introduction

Aquatic bacterial species of the genus *Vibrio* are commonly found in estuarine, coastal and oceanic waters and have been found associated with marine organisms, including fish, crustaceans and marine molluscs (as reviewed by Shieh *et al.*, 2003). *Vibrios* include non-pathogenic strains, as well as pathogenic strains with the ability to infect a broad range of host organisms. *Vibrio* species are the dominant bacterial species within the gut microbiota of marine invertebrates (Harris, 1993; Sawabe *et al.,* 1995; Tanaka *et al.,* 2004). They have been isolated from the digestive tract of abalone, and may play a role as probiotics for aquacultured abalone through contribution of extracellular hydrolytic enzymes to the pool of digestive enzymes available to the abalone host, thereby improving the digestion efficiency of the host (Sawabe *et al.,* 1998; Sawabe *et al.,* 2004b; Macey and Coyne, 2005).

Proteases, proteinases and peptidases are all terms for proteolytic enzymes that hydrolyze peptide bonds. Proteolytic enzymes are produced by a broad array of organisms, including both prokaryotes and eukaryotes (Deane, 1989). Microbial protease enzymes have important functions within the marine environment. Intracellular proteases assist in the regulation of metabolism and a range of other physiological functions (Kalisz, 1988). Secreted extracellular proteolytic enzymes are involved in nutrient acquisition for the bacterium through the breakdown of exogenous proteins to smaller peptides and amino acids that are more readily available to the bacterium (Kalisz, 1988; Albertson *et al.,* 1990), or they form part of a set of factors involved in virulence and pathogenicity (Farto *et al.,* 2002; Teo *et al.,* 2003). Proteolytic enzymes can be broadly classified into five distinct groups on the basis of their catalytic mechanism of action, namely serine, threonine, cysteine, aspartic and metallopeptidases.

Serine proteases are divided into approximately 50 families and nine clans, or superfamilies, of which the two largest are the (chymo)trypsin-like and subtilisin-like serine protease clans (Rawlings and Barrett, 1994). Proteolytic enzymes within both of these serine protease clans have a distinctive catalytic triad made up of histidine, aspartic acid and serine amino acid residues (Siezen and Leunissen, 1997). Over 200 members of the subtilisin-like serine protease clan, also known as subtilases

(Siezen *et al.,* 1991) are currently known and are all classified as endopeptidases or tripeptidylpeptidases (Siezen and Leunissen, 1997). All of the subtilases classified so far have a catalytic domain with structurally conserved regions that correspond to common secondary structure elements. The subtilases are generally secreted extracellular enzymes, and they have been further subdivided into six sub-families, A to F (Table 2.1), based on the sequence alignment of their respective catalytic domains. The majority of subtilases are initially synthesised as pre-pro-enzymes. The pre-peptide or signal sequence facilitates the secretion of the enzyme through the cell membrane, before being cleaved off. The cleavage of the pro-peptide then activates the enzyme and thereby, ensures that the enzyme is only catalytically active outside the cell.

Table 2.1 The sub-families within the subtilase superfamily of serine proteases.

Symbol	Subtilase sub-family
A	Subtilisin family
B	Thermitase family
C	Proteinase K family
D	Lantibiotic peptidase family
E	Kexin family
F	Pyrolysin family

Subtilases have been found to occur in Archaea (Kannan *et al.,* 2001; Palmieri *et al.,* 2005), bacteria (Suzuki *et al.,* 1997; Valbuzzi *et al.,* 1999; Arnórsdóttir *et al.,* 2002), fungi (Abraham and Breuil, 1995), plants (Meichtry *et al.,* 1999; Berger and Altmann, 2000; Popovič *et al.,* 2002) and other eukaryotes (Siezen *et al.,* 1994; Louie and Conrad, 1999). In most bacteria, subtilases are secreted, extracellular enzymes, with fairly non-specific substrate specificity, employed for growth on proteinaceous substrates. Serine proteases have previously been reported to be an important component in nutrient acquisition in fungi (Segers *et al.,* 1999) and, therefore, serine proteases such as subtilases may fulfil a similar role in bacteria.

Protease enzymes are one of the most abundant extracellular products of the *Vibrio* sp. (Marcello *et al.,* 1996). *Vibrio* strains, such as *Vibrio anguillarum*, *Vibrio cholerae*, *Vibrio pelagius, Vibrio metschnikovii*, *Vibrio mimicus, Vibrio harveyi* and

Vibrio alginolyticus, produce a range of extracellular proteases, of which several have been characterised (Young and Broadbent, 1982; Deane *et al.,* 1986; Kwon *et al.,* 1995; Farto *et al.,* 2002; Lee *et al.,* 2003; Teo *et al.,* 2003). Strains of *V. alginolyticus* and *V. metschnikovii* have been observed to secrete extracellular SDS-resistant serine proteases that have high homology to subtilisin-like serine proteases (Deane *et al.,* 1987a; Deane *et al.,* 1987b; Kwon *et al.,* 1995).

2.2.1 Aim of this chapter

The aim of this chapter is to investigate the extracellular proteolytic enzyme activity of the marine bacterium *Vibrio midae* SY9 and in particular the major extracellular protease produced by *V. midae* SY9. A proteolytic *Escherichia coli* clone harbouring a recombinant plasmid from a previously constructed and screened genomic library (Taylor, 2002) of *V. midae* SY9 will be characterised. The nucleotide sequence of the gene encoding a *V. midae* SY9 extracellular protease will permit further molecular and bioinformatic analysis in order to characterise the extracellular protease and compare it to closely related enzymes that have already been investigated. Cloning and characterizing the protease gene will enable genetic manipulation of the wild-type *V. midae* SY9 protease, through the construction of mutants. These mutants will allow a better understanding of the role of the protease gene in the previously observed probiotic effect in *H. midae* (Macey and Coyne, 2005).

2.3 Materials and methods

All media and solutions used in this study are listed in Appendix A.

2.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids that were used to clone and characterise the protease gene from *Vibrio midae* SY9 are listed in Table 2.2.

2.3.2 Media and culture conditions

The *Escherichia coli* strains were either grown in Luria-Bertani broth (LB) (Appendix A.1.5) or on Luria-Bertani agar (LA) (Appendix A.1.6) at 37 ºC. *E. coli* strains that harbored pBluescript SK and KS plasmids (Table 2.2), and recombinant pBluescript SK and KS plasmids (Table 2.2) were cultured in LB or on LA containing 100 µg ml^{-1} ampicillin (Appendix A.2.2). *V. midae* SY9 (Table 2.2) was maintained at 22 ºC on Marine agar (MA) (Appendix A.1.2) and cultured in Marine broth (MB) (Appendix A.1.1) or, the high-protein growth media, Peptone marine basal medium (P-MBM) (Appendix A.1.3) at 22 ºC, unless otherwise stated.

2.3.3 Identification of recombinant proteolytic *E. coli* clones

The genomic library of *V. midae* SY9 had previously been constructed and screened for proteolytic activity on LA supplemented with 2% (w/v) skim milk (Appendices A.1.8.2 and A.1.8.3) and 100 µg ml^{-1} ampicillin (Appendix A.2.2) (Taylor, 2002). Recombinant constructs displaying a proteolytic phenotype were identified by visual detection of clear zones of skim milk hydrolysis around the bacterial colony on the solid agar growth medium.

Table 2.2 Bacterial strains and plasmids used in this study

Strain/ plasmid	**Genotype/ relevant feature(s)**[a]	**Reference**
Strains:		
Escherichia coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F'(traD36 proAB' $lacI^q$ lacZΔM15)	Sambrook *et al.* (1989)
Escherichia coli DH5α	[(φ80*lac*ZΔM15) *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17($r_K^- m_K^+$) *sup*E44 (Nal^r) *rel*A1 Δ(*lac*ZYA-*arg*F)$_{U169}$ *pho*A	Hanahan (1983)
Vibrio midae SY9	Isolated from the digestive tract of *H. midae,* South Africa	Macey and Coyne (2005)
Plasmids:		
pBluescript SK/KS	Amp^r, β-galactosidase	Short *et al.* (1988)
pMR11	pBluescript SK containing ~7 kb *V. midae* SY9 genomic DNA; Amp^r	Taylor (2002)
pDel11	Derivative of pMR11 containing ~2.5 kb of *V. midae* SY9 genomic DNA; Amp^r	This study
pSub11	Derivative of pMR11 containing ~3.1 kb of *V. midae* SY9 genomic DNA; Amp^r	This study
pProH3	1.9 kb *Hin*dIII-*Hin*dIII fragment from pMR11 cloned into pBluescript SK; Amp^r	This study

[a] Amp^r, ampicillin resistant

2.3.4 DNA analytical methods

2.3.4.1 Restriction endonuclease analysis of pMR11

The recombinant plasmid pMR11 was isolated from an *E. coli* JM109 (Table 2.2) clone displaying a proteolytic phenotype on solid skim milk growth medium. Preliminary digestions with restriction endonucleases *Bam*HI, *Pvu*II and *Sma*I (Fermentas) were performed on pMR11 in order to calculate the size of the *V. midae* SY9 genomic DNA insert within the recombinant plasmid. Thereafter, the recombinant plasmid was subjected to further restriction endonuclease mapping in order to determine the approximate positions of the restriction endonuclease sites within the recombinant plasmid. The following restriction endonucleases were used: *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Not*I, *Pst*I, *Pvu*II, *Sac*I, *Sca*I, *Spe*I, and *Xba*I (Roche). Restriction enzyme digestions were conducted as outlined in Appendix B.5. The resulting restriction enzyme fragments were separated on 0.8% (w/v) TAE agarose gels (Appendix B.6). DNAFRAG version 3.03 was used to calculate the size of the fragments based on a least squares fit of fragment size to gel mobility (Schaffer and Sederoff, 1981).

2.3.4.2 Southern hybridization analysis of pMR11

Southern hybridization analysis was performed in order to confirm the *V. midae* SY9 origin of the cloned insert DNA within the recombinant plasmid pMR11. Chromosomal DNA was extracted (Appendix B.4.1) from *V. midae* SY9 and *E. coli* JM109 (Table 2.2). The recombinant plasmid pMR11 was double digested with the restriction enzymes *Eco*RI (Roche) and *Cla*I (Roche), as outlined in Appendix B.5. A 316 bp *Eco*RI - *Cla*I DNA fragment was resolved on a 2% (w/v) TAE agarose gel (Appendix B.6), before being gel purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. The purified DNA fragment was then radioactively labelled ([α^{32}P]-dCTP, Amersham) using a random-primed DNA labelling kit (Roche) according to the manufacturer's instructions, as outlined in Appendix B.8.2.

The labelled DNA was used as a probe against equally loaded amounts (10 µg) of *V. midae* SY9 and *E. coli* JM109 chromosomal DNA that had been digested with the restriction enzymes *Bgl*II, *Eco*RI and *Hin*dIII. The resulting fragments were separated on a 0.7% (w/v) TAE agarose gel at 35 V overnight (Appendix B.6). The Southern hybridization procedure that was followed is described in Appendix B.8.

2.3.4.3 Deletion analysis of pMR11

Deletion analysis of pMR11 was conducted in order to identify the regions within the cloned DNA that were responsible for the proteolytic activity in *E. coli* JM109 harbouring the recombinant plasmid pMR11. The recombinant construct pMR11 was digested with the restriction enzymes *Bgl*II and *Bam*HI (Appendix B.5). The resulting 3.0 and 5.6 kb DNA fragments were gel purified using the Qiagen Gel Purification kit. The 5.6 kb fragment was re-ligated (Appendix B.12.1) and the resulting construct designated pDel11. The 3.0 kb DNA fragment was subcloned (Appendix B.12.2) into pBluescript SK (Table 2.2) that had been digested with the restriction enzyme *Bam*HI, and the ensuing recombinant plasmid was designated pSub11.

The resulting constructs were transformed (Appendix B.2) into competent *E. coli* JM109 cells (Appendix B.1.2) and assayed for a proteolytic phenotype by plating onto LA, supplemented with 2% (w/v) skim milk and 100 µg ml^{-1} ampicillin, as described in section 2.3.3.

2.3.4.4 Sub-cloning of the proteolytic region into pBluescript SK

Further sub-cloning was conducted in order to identify and isolate the region within the cloned *V. midae* SY9 DNA fragment that was responsible for the observed extracellular proteolytic phenotype of *E. coli* JM109 harbouring pMR11. The 1.9 kb *Hin*dIII – *Hin*dIII restriction DNA fragment from pMR11 was sub-cloned (Appendix B.12.2) into pBluescript SK that had been linearised by restriction enzyme digestion with *Hin*dIII (Fermentas) (Appendix B.5) and the resulting recombinant plasmid designated pProH3 (Table 2.2). The recombinant construct pProH3 was transformed (Appendix B.2) into competent *E. coli* JM109 cells (Appendix B.1.2), and assayed for a proteolytic phenotype by plating onto LA containing 100 µg ml^{-1} ampicillin (Appendix A.2.2) and supplemented with 2% skim milk, as described in section 2.3.3.

2.3.4.5 DNA sequencing of the *Vibrio midae* SY9 extracellular protease gene

Synthetic oligonucleotide primers (M13Fwd and M13Rev, Table 2.3) were used to sequence the insert DNA by the dideoxy chain-termination method (Sanger *et al.,* 1977) (Appendix B.26.1). The recombinant pBluescript SK plasmids, pMR11, pDel11, pSub11 and pProH3 were isolated using an Eppendorf FastPlasmid Mini kit, according to the manufacturer's instructions and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems). The amplicons were separated on an Amersham Biosciences MegaBACE 500 Automated Capillary DNA Sequencing System (Amersham and Pharmacia Biotech Molecular Dynamics), according to the manufacturer's instructions, and analyzed using MegaBACE 500 Sequence Analyser version 2.4 software.

Table 2.3 Oligonucleotide primers used in this study

Primer	Sequence (5' – 3')	Reference
M13Fwd	CGCCAGGGTTTTCCCAGTCACGAC	Yanisch-Perron *et al.* (1985)
M13Rev	GAGCGGATAACAATTTCACACAGG	Yanisch-Perron *et al.* (1985)
PSeqFwd	CCAGCACGGACGTACGTTCGAG	This study
PSeqRev	CTCGAACGTACGTCCGTGCTGG	This study
ProFwd1	CTGTCGGCTCGACCAC	This study
ProRev1	CGCGGCTAACGATCAA	This study
PExt	GCACTGTCCGCAATCTCATTGG	This study
PExtCy5[a]	GCACTGTCCGCAATCTCATTGG	This study

[a] PExtCy5 was 5' - end labeled with Cy5-Far Red flouroscein

Sequence data was edited using CHROMAS version 2.01 software (Technelysium Pty Ltd., Australia) and analyzed using DNAMAN for windows version 4.13 (Lynnon

Biosoft, Canada) software. Homology and similarity searches of DNA sequences were performed using the basic local alignment search tool (BLAST) programs (Altschul *et al.,* 1989; Altschul *et al.,* 1997; Marchler-Bauer *et al.,* 2005), as provided by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Homology and similarity searches of translated DNA sequences were performed using the BLASTP program (Altschul *et al.,* 1997) as provided by NCBI. Conserved domains and conserved domain architecture searches were performed using the CD-search and conserved domains architecture retrieval tool (CDART), respectively, as provided by NCBI (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.,* 2009). Interproscan (www.ebi.ac.uk/interproscan/) together with a hydrophobicity analysis (DNAMAN) was used to identify a putative signal peptide. The DNAMAN software was also used to identify a putative terminator region.

2.3.5 RNA analytical methods

2.3.5.1 Isolation of total RNA

Total cellular RNA was isolated (Ausubel *et al.,* 1989) (Appendix B.7) from *V. midae* SY9 which had been cultured for 24 hours in P-MBM (Appendix A.1.3) at 22 °C and *E. coli* JM109 (pSK) cultured for 24 hours in LB broth (Appendix A.1.5) containing 100 µg ml^{-1} ampicillin (Appendix A.2.2) at 37 °C with shaking.

Ten millilitres of each culture was collected by centrifugation (13,000x g for 10 minutes at 4 ºC) and resuspended in 10 ml protoplast buffer (Appendix A.2.9). The resulting protoplasts were collected by centrifugation (7,000x g for 5 min at 4 ºC) and resuspended in 500 µl lysis buffer (Appendix A.2.9) containing 15 µl DEPC (Sigma). Samples were incubated at 37 ºC for 5 minutes before salt-saturated NaCl (Appendix A.2.9) was added to precipitate most of the SDS contained within the lysis buffer, and the resulting white precipitate was removed by centrifugation (13,000x g for 10 minutes at 4 ºC). Supernatant fractions were collected and the nucleic acid precipitated with ice-cold absolute ethanol (Merck) followed by centrifugation (13,000x g for 10 minutes at

4 ºC). The resulting nucleic acid pellets were washed in ice-cold 70% (v/v) ethanol (Appendix A.2.9) to remove any remaining salt, air-dried and resuspended in a final volume of 20 µl of DEPC treated water (Appendix A.2.9).

RNA samples were treated with DNAse I (Promega) in order to remove any chromosomal DNA contamination. Ten units of DNAse I was added to the RNA sample in a total volume of 50 µl, and incubated at 37 ºC for 3 hours. The RNA was recovered by adding an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1) (Appendix A.2.9), before centrifuging the samples at 13,000x g for 10 minutes at 22 ºC and transferring the aqueous phase to a clean sterile microfuge tube. In order to precipitate the RNA, 5 µl 3M sodium acetate (Appendix A.2.9) and 150 µl ice-cold absolute ethanol (Merck) was added to the RNA sample. The mixture was centrifuged at 13,000x g for 10 minutes at 4 ºC. The RNA sample was then resuspended in 20 µl DEPC water and quantified using the Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.). RNA integrity was assessed by electrophoresis of 1 µg of total RNA on a 1.5% formaldehyde agarose gel (Appendix B.13.1 and B.13.2).

2.3.5.2 Primer extension analysis of *vmproA*

The putative transcriptional start site of the *V. midae* SY9 protease gene, *vmproA*, was identified by a non-radioactive primer extension method (Appendix 26.2), adapted from Eikmanns *et al.* (1994) and Miroslav Pátek (Institute of Microbiology, Czech Republic, personal communication). A Cy5 end-labelled oligonucleotide primer (PExtCy5, Table 2.3) was designed complementary to the nucleotides +258 to +279 bp downstream of the *vmproA* ATG start codon. Total cellular RNA was extracted from a 16 hour culture of *V. midae* SY9 cultivated in P-MBM, as described in section 2.3.5.1 (Ausubel *et al.,* 1989). RNA integrity was assessed by electrophoresis of 1 µg of total RNA on a 1.5% formaldehyde agarose gel (Appendix B.13.1 and B.13.2).

Prior to the primer hybridization step, 40 µg of total RNA was denatured at 95 °C for 10 minutes and rapidly cooled on ice. A final concentration of 20 µM PExtCy5 and 20 units (U) of RNAse inhibitor (RNasin® Ribonuclease Inhibitor, Promega) were added (in a total volume of 15 µl) and the reaction mixture was incubated overnight at

45 °C in the dark to allow the primer to anneal. The reverse transcription (ImProm II™ Reverse Transcription System, Promega) reaction was set up, according to the manufacturer's instructions, and incubated at 42 ºC for 2-3 hours in the dark, before the addition of an extra 1 µl of reverse transcriptase enzyme and hybridization of the reaction mixture continued overnight at 42 °C in the dark.

The reaction was terminated by the addition of 10 µl 1 N NaOH (Appendix A.2.3) and 0.5 M EDTA (Appendix A.2.3), and incubated at 65 ºC for 15 minutes, before 10 µl 1 M HCl (Appendix A.2.3) was added. The cDNA was purified from the reaction mixture by passing the cDNA samples through RNeasy MinElute columns (RNeasy® MinElute™ Cleanup Kit, Qiagen). The cDNA was eluted from the RNeasy columns with two serial passages of 30 µl sterile water. The water was evapourated to reduce the volume of the cDNA sample using a Speed Vac Concentrator (Savant), and the cDNA pellet resuspended in 8 µl TE buffer (Appendix A.2.3). The samples were quantitated using a Nanodrop ND-1000 spectrophotometer and stored in the dark at -70 ºC.

The recombinant plasmid pMR11 was sequenced using the un-labelled oligonucleotide primer PExt (Table 2.3), according to the manufacturer's instructions on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems).

The primer extension products were defrosted on ice, before 3 µl of tracking dye was added. After a 5 minute denaturation at 96 ºC the primer extension products were rapidly cooled on ice and loaded on the ALFexpress™ automated DNA sequencer (Pharmacia Biotech). Analysis of the sequencing reaction products of pMR11, obtained using the oligonucleotide primer PExt, was performed in parallel in the appropriate sequencing lanes on the same sequencing gel as described in Appendix B.26.2. This enabled the primer extension product to be compared and analysed with respect to the nucleotide sequence upstream of the ATG start codon. The DNA sequence was determined, according to the manufacturer's instructions, on an ALFExpress™ DNA Sequencer (Pharmacia Biotech). Data was analysed using the AlfWin AM version 3.0 software. The sequence was edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft) and Chromas version 2.01 (Technelysium).

2.3.5.3 Northern hybridization analysis of *vmproA*

Northern hybridization analysis was performed in order to determine the size of the mRNA transcript encoding VmproA. The recombinant plasmid pMR11 (Table 2.2) was isolated using an Eppendorf FastPlasmid Mini kit according to the manufacturer's instructions. The resultant plasmid DNA was used as the template for PCR amplification of a 231 bp internal protease gene fragment. PCR amplification was performed with the oligonucleotide primers, ProFwd1 and ProRev1 (Table 2.3; Appendix B.25.8). The amplified PCR product was analyzed by agarose gel electrophoresis (Appendix B.6) to confirm reaction specificity and product size, before being purified using the Qiagen Gel Purification kit from a 2% (w/v) TAE agarose gel according to the manufacturer's instructions. The purified DNA fragment was then radioactively ([^{32}P]-dCTP, Amersham) labelled using a random-primed DNA labelling kit (Roche) according to the manufacturer's instructions (Appendices B8.2 and B.8.3).

Total cellular RNA was isolated (Appendix B.7) from *V. midae* SY9 cultured for 24 hours in P-MBM (Appendix A.1.3) at 22 °C and *E. coli* JM109 (pSK) cultivated for 24 hours in LB broth containing 100 µg ml^{-1} ampicillin (Appendix A.2.2) at 37 °C. Twelve micrograms of total RNA from each sample, and 5 µl RNA Molecular Weight Marker (Roche), were prepared and separated on a 1.5% formaldehyde gel (Appendix B.13.1 and B.13.2). The electrophoresed RNA samples were capillary transferred to Nylon membrane (Hybond N+ Nylon Membrane, Amersham) for 16 hours with 1x SSC (Appendix B.13.3), whereafter the membrane was UV cross linked (Ultraviolet Crosslinker, Amersham Life Science). The membrane was probed with the radioactively labelled internal gene fragment, as described in Appendix B.13.4.

2.3.6 Phylogenetic analysis of VmproA

For phylogenetic analysis of VmproA, reference strains were identified and selected for comparison from those identified by Siezen and Leunissen (1997). Sequences were downloaded from the GenBank database, aligned using CLUSTAL_X, version 1.81 (Thompson *et al*., 1997) and a portion of the protein sequences, including the catalytic domains, manually edited to a common length of 255 amino acids. Phylogenetic analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004) and a neighbour-joining (Saitou and Nei, 1987) tree constructed. Bootstrap values were based upon 1000 re-sampled data sets (Felsenstein, 1985) and only bootstrap values greater than 40% are indicated.

2.4 Results

2.4.1 Restriction enzyme mapping of the recombinant plasmid pMR11

An *E. coli* JM109 transformant displaying a proteolytic phenotype when cultured on skim milk solid media, was isolated from a previously constructed *V. midae* SY9 genomic library (Taylor, 2002). This protease clone was restriction mapped and designated pMR11 (Fig. 2.1).

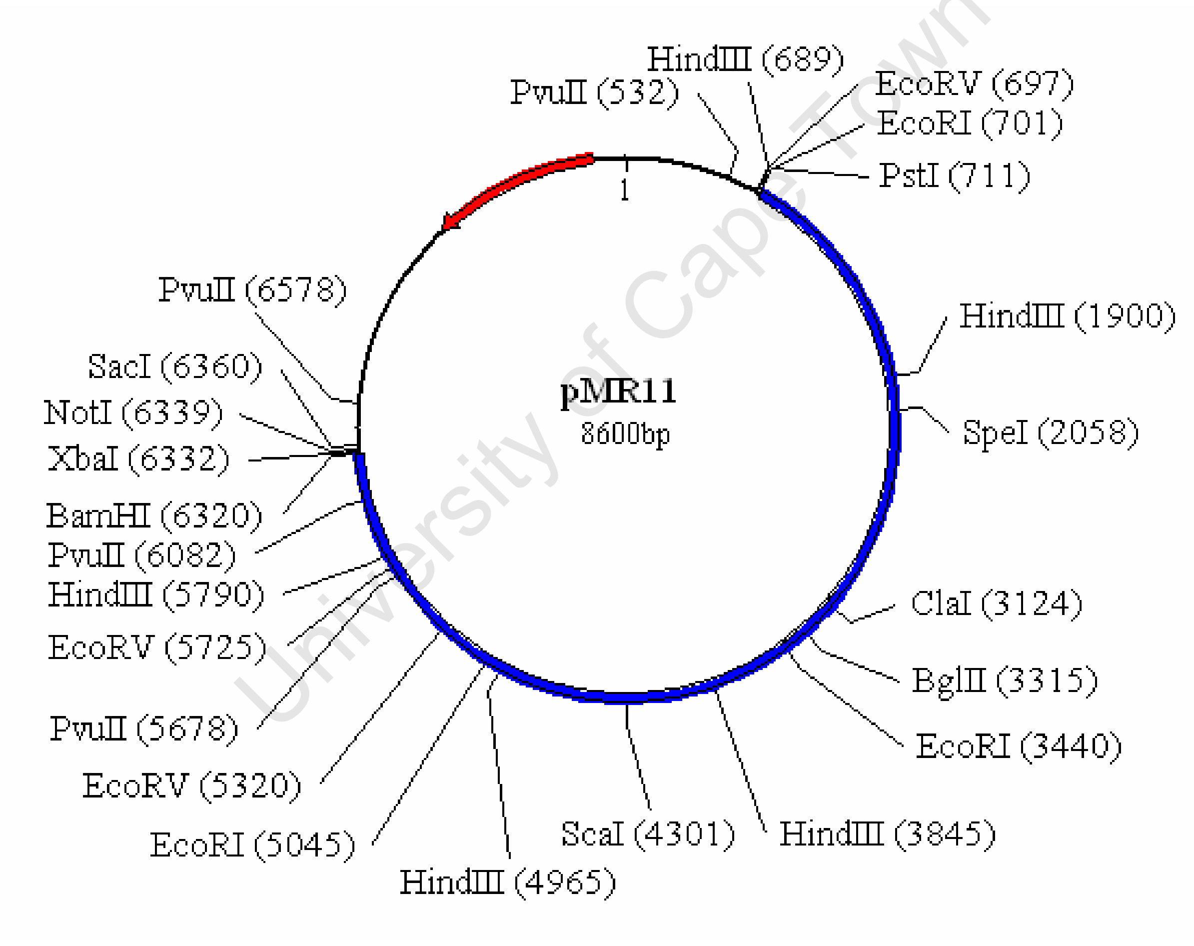


Figure 2.1 Restriction enzyme map of the recombinant plasmid pMR11. The thick blue line (■) represents cloned *V. midae* SY9 chromosomal DNA, whilst the thin black line represents the vector, pBluescript SK (+), DNA. The size of the recombinant plasmid pMR11 is shown in base pairs (bp). The positions of the restriction enzyme recognition sequences in bp are indicated in brackets. The position of the β-lactamase gene (→) is shown.

2.4.2 Southern hybridization analysis

The 316 bp *Cla*I- *Eco*RI fragment (at position 3124 and 3440, respectively) was excised from pMR11 (Fig. 2.1) and used as a probe against *V. midae* SY9 chromosomal DNA in order to confirm that the insert DNA fragment carried by the recombinant plasmid pMR11 was of *V. midae* SY9 origin (Fig. 2.2). *E. coli* JM109 chromosomal DNA was also assessed as a negative control.

For *V. midae* SY9, the *Cla*I-*Eco*RI fragment hybridized to two *Bgl*II fragments of approximately 16.6 and 6.4 kb in size (Fig. 2.2, lane 1), a 4.9 kb *Eco*RI fragment (Fig. 2.2, lane 2) and a 1.9 kb *Hin*dIII fragment (Fig. 2.2, lane 3). The *Cla*I - *Eco*RI fragment did not hybridize to any fragments generated by the *Bgl*II (Fig. 2.2, lane 4), *Eco*RI (Fig. 2.2, lane 5) and *Hin*dIII (Fig. 2.2, lane 6) digests of *E. coli* JM109 chromosomal DNA.

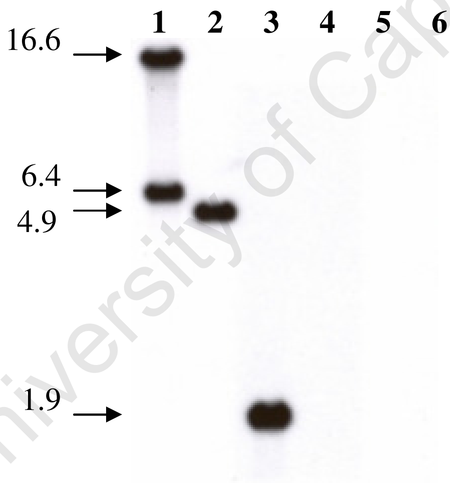


Figure 2.2 Southern hybridisation of the radiolabeled 316 bp *Eco*RI - *Cla*I internal gene fragment of *vmproA* from pMR11 to *V. midae* SY9 and *E. coli* JM109 chromosomal DNA. *V. midae* SY9 chromosomal DNA digested with *Bgl*II (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *E. coli* JM109 chromosomal DNA digested with *Bgl*II (lane 4), *Eco*RI (lane 5), *Hin*dIII (lane 6). The arrows indicate the approximate sizes of the bands in kilobase pairs (kb).

2.4.3 Deletion sub-cloning analysis of pMR11

Since the recombinant plasmid pMR11 (Fig. 2.1) contained a large insert of *V. midae* SY9 chromosomal DNA, it was necessary to determine which portion of this fragment was responsible for the observed extracellular proteolytic activity. This was achieved by making use of a deletion subcloning strategy whereby a portion of the insert from pMR11 was deleted, separately subcloned, and visually investigated (as in section 2.4.1) to determine the extracellular proteolytic phenotype of the resulting recombinant constructs when transformed into *E. coli* JM109. The deletion subcloning strategy that was followed is shown in Figure 2.3.

The 3005 bp *Bgl*II (3315)-*Bam*HI (6320) fragment was deleted from pMR11 in order to generate the plasmid pDel11 (Fig. 2.3). *E. coli* JM109 competent cells transformed with pDel11 did not display an extracellular proteolytic phenotype when cultured on skim milk solid media (Fig. 2.3). *E. coli* JM109 cells transformed with pSub11, which was constructed by subcloning the 3005 bp *Bgl*II (3315)-*Bam*HI (6320) fragment from pMR11 (Fig. 2.3) into pBluescript SK, also did not exhibit an extracellular proteolytic phenotype when cultured on skim milk solid media (Fig. 2.3). However, *E. coli* JM109 transformed with pProH3, generated by subcloning the 1945 bp *Hin*dIII (1900)-*Hin*dIII (3845) fragment from pMR11 into pBluescript SK, displayed an extracellular proteolytic phenotype (Fig. 2.3). Therefore, the cloned 1945 bp *Hin*dIII -*Hin*dIII pMR11 DNA fragment contained a functional *V. midae* SY9 extracellular protease gene(s).

2.4.4 DNA sequencing of the protease gene cloned from *V. midae* SY9

An open reading frame (ORF) of 1605 nucleotides was observed to begin at the putative ATG start codon at position 187 and continue to the TAG stop codon at position 1789 (Fig. 2.4). This ORF encodes a 534 amino acid protein, with a predicted size of 56.03 kDa. A putative signal peptide from amino acids $M^1 – A^{21}$ was identified (Fig. 2.4). A putative Shine Dalgarno sequence, AAGGAA, at positions 175 – 180 was identified upstream of the ATG translational start site (Fig. 2.4).

The 7 bp inverted repeat at positions 1819-1825 and 1836-1842 has a ΔG of formation of -5.6K cal/mol (DNAMAN) and may function as an energetically favorable transcriptional terminator of *vmproA* (Figs. 2.4 and 2.5). This region represents the

3'-end of the mRNA transcript and has the potential to form a stable mRNA hairpin structure with a stem and loop, followed by several uracil (U) residues (Fig. 2.5).

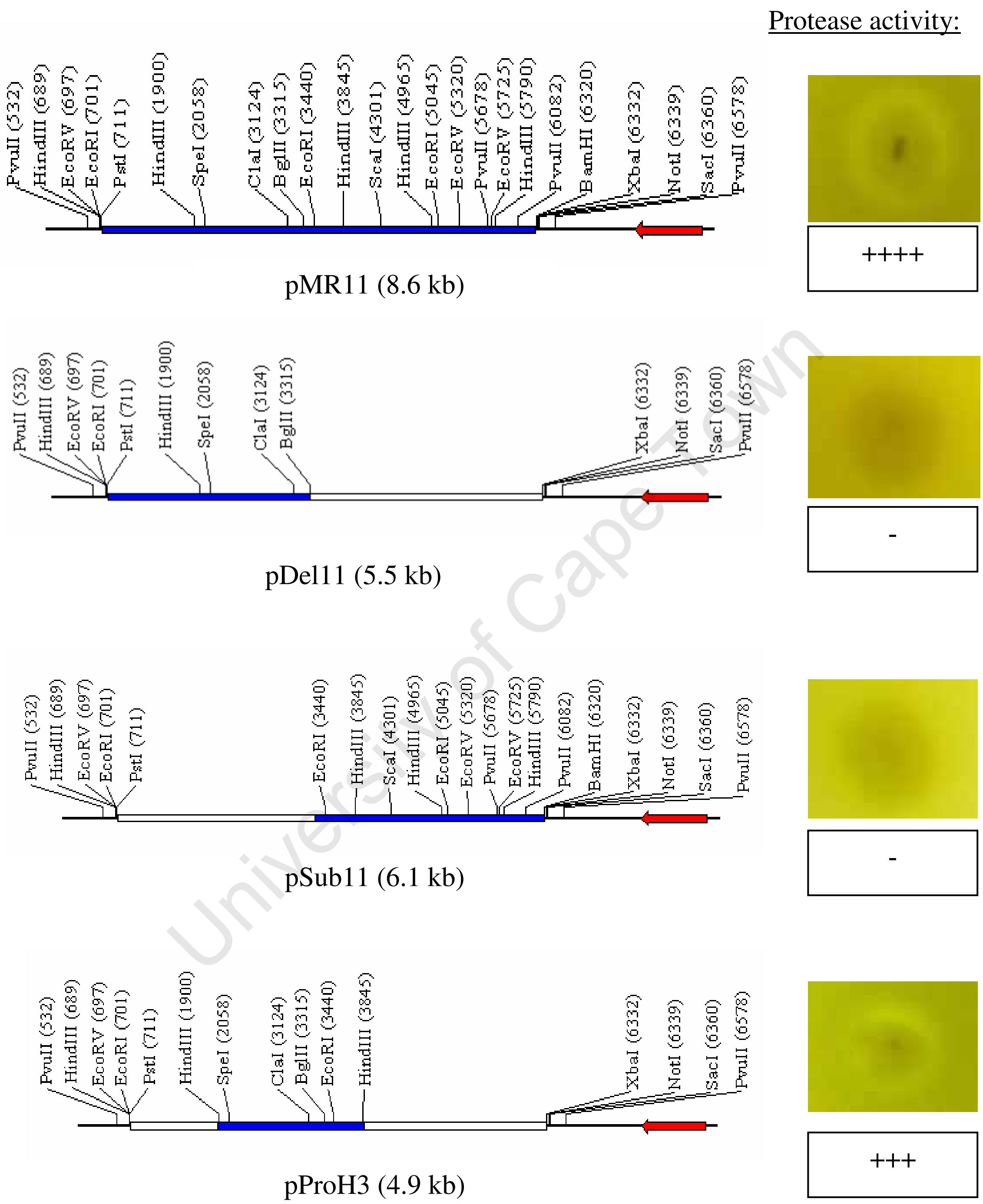


Figure 2.3 The deletion and subcloning strategy of the recombinant plasmid pMR11. DNA fragments were deleted (□) from pMR11 to create the plasmids pDel11, pSub11 and pProH3. The thick blue line (■) represents cloned *V. midae* SY9 chromosomal DNA, whilst the thin black line represents pBluescript SK (+) vector DNA. The position of the β-lactamase gene (→) is shown. **[Continued on following page]**.

[Continued from facing page] The size of the recombinant plasmids is shown in kilobase pairs (kb). The positions of the relevant restriction enzyme recognition sequences, in bp, are indicated in brackets. The extracellular proteolytic phenotypes of the *E. coli* JM109 strains harbouring the recombinant plasmids are shown. Extracellular protease activity was identified by visual detection of clear zones (designated and rated as: +++ or ++++) of skim milk hydrolysis around the bacterial colony growing on 2% skim milk LA. No hydrolysis of the skim milk surrounding the *E. coli* JM109 colony is considered as a lack of extracellular protease activity and is designated (-).

Primer extension analysis was performed in order to identify the putative transcriptional start site and approximate position of the promoter of *vmproA*. Primer extension indicated that the putative transcriptional start site is a thymine at position 83 and this is 104 bp upstream of the ATG start site (Figs. 2.4 and 2.6). A conserved promoter region based on the common -10 (TATAAT) and -35 (TTGACA) motifs generally observed in prokaryotic promoters could not be identified for *vmproA*. However, the nucleotides TCTAGT at positions -7 to -12 may form a possible -10 region and the nucleotides TTATGC at positions -30 to -35 may form a possible -35 region (Figs. 2.4 and 2.6).

2.4.5 Northern hybridization studies

In order to determine the size of the mRNA transcript of *vmproA*, total cellular RNA was isolated from *V. midae* SY9 cells cultivated for 24 hours in P-MBM at 22 ºC. A single 1.7 kb transcript of *vmproA* was detected in the 24 hour sample by northern hybridization analysis (Fig. 2.7, lane 1). The radiolabelled probe did not hybridize to *E. coli* JM109 (pSK) mRNA transcripts (Fig. 2.7, lane 2).

```
      HindIII                                               -35
   1  AAGCTTGGTTATTGATCATGTTTCTGAGTATGCGAATTATTATCAGTTATGCATATGATT
                 -10       +1
  61  GCTCATTGTCTAGTTTGACTTGTGTAACGTCACTTCAGCACGCACTACGCTGATTGTCTG
                                                              RBS
 121  TGTTTTCGTGTTGACAGTTTGGTACTAGGTAAAAATCAACAAGACTATGATTTCAAGGAA

 181  CATTTTATGTTAAAGAAACTACTAAGTTGTTGTATCACCTCCGCGCTCTGTTTTCATTCT
   1        M  L  K  K  L  L  S  C  C  I  T  S  A  L  C  F  H  S
            ----------------------------------------------------
 241  TCTCTCGCTTTTTCGCAACCCAATGAGATTGCGGACAGTGCAGAGTTACAACAAGCTCCC
  19   S  L  A  F  S  Q  P  N  E  I  A  D  S  A  E  L  Q  Q  A  P
       ---------
 301  GACACATTGCCTGCCACTTTGATGCTTGCACCGGACGACATCGCCATTGCAGATCGCTAT
  39   D  T  L  P  A  T  L  M  L  A  P  D  D  I  A  I  A  D  R  Y
 361  ATTGTGGTATTTCAACAGCCGCAAATGATGGTGAGCAATTCAACGGAATTCGAGCAATTC
  59   I  V  V  F  Q  Q  P  Q  M  M  V  S  N  S  T  E  F  E  Q  F
 421  ACGCAACAATCCGTAGACCGCATGTCTGGTTTGTATTCGATACAAGTGGAGTCGGTTTTT
  79   T  Q  Q  S  V  D  R  M  S  G  L  Y  S  I  Q  V  E  S  V  F
 481  GACCACTCGATAAGTGGATTTGTCGCTAACTTGAGTCCTGAGCAACTAAAAGATCTGCGT
  99   D  H  S  I  S  G  F  V  A  N  L  S  P  E  Q  L  K  D  L  R
 541  TCTGATCCTCGTGTGGACTACATTGAGCAAGACAGAATCCTATCGCTTGACCCAATAGTC
 119   S  D  P  R  V  D  Y  I  E  Q  D  R  I  L  S  L  D  P  I  V
 601  TCGGCAGACGCAAATCAAACCAATGCCATTTGGGGACTAGACCGAATCGACCAACGAAAC
 139   S  A  D  A  N  Q  T  N  A  I  W  G  L  D  R  I  D  Q  R  N
 661  TTGCCACTCGATAACAACTACAGTGCCAACTTTGATGGGACTGGGGTAACGGCTTATGTT
 159   L  P  L  D  N  N  Y  S  A  N  F  D  G  T  G  V  T  A  Y  V
 721  ATCGATACGGGTGTGAATAACGCGCATGTTGAGTTCGGTGGTCGCTCAGTTTCAGGGTAT
 179   I  D  T  G  V  N  N  A  H  V  E  F  G  G  R  S  V  S  G  Y
 781  GACTTTGTCGATAACGATGCAGATGCAAGTGACTGTAATGGACACGGCACACACGTAGCT
 199   D  F  V  D  N  D  A  D  A  S  D  C  N  G  H  G  T  H  V  A
 841  GGCACCATTGGCGGCAGCTTGTATGGTGTTGCAAAAAACGTCAACCTTGTCGGCGTGAGA
 219   G  T  I  G  G  S  L  Y  G  V  A  K  N  V  N  L  V  G  V  R
 901  GTATTGAGCTGTACCGGATCGGGTTCTACTTCTGGTGTTATTGCCGGTGTGGACTGGGTG
 239   V  L  S  C  T  G  S  G  S  T  S  G  V  I  A  G  V  D  W  V
 961  GCTGCGAACGCTTCCGGACCTTCAGTCGCCAACATGAGTTTAGGCGGCGGTCAATCCGTC
 259   A  A  N  A  S  G  P  S  V  A  N  M  S  L  G  G  G  Q  S  V
1021  GCTCTGGATAGTGCGGTGCAAAGTGCGGTTCAGTCAGGTGTCAGCTTTATGCTTGCAGCA
 279   A  L  D  S  A  V  Q  S  A  V  Q  S  G  V  S  F  M  L  A  A
1081  GGTAACTCCAACGCCGATGCATGTAACTATTCTCCAGCACGCGTTGCTTCCGGAGTAACT
 299   G  N  S  N  A  D  A  C  N  Y  S  P  A  R  V  A  S  G  V  T
1141  GTCGGCTCGACCACCAGCACGGACGTACGTTCGAGTTTTTCAAACTGGGGCAGTTGTGTG
 319   V  G  S  T  T  S  T  D  V  R  S  S  F  S  N  W  G  S  C  V
1201  GACGTGTTTGCGCCAGGTTCACAAATCAAATCCGCATGGTATGACGGTGGCTACAAAACC
 339   D  V  F  A  P  G  S  Q  I  K  S  A  W  Y  D  G  G  Y  K  T
1261  ATTAGTGGCACTTCGATGGCGACGCCACATGTGGCGGGTGTAGCAGCATTGTATCTTCAA
 359   I  S  G  T  S  M  A  T  P  H  V  A  G  V  A  A  L  Y  L  Q
1321  GAAAACCGTTCACTGTCTCCAAGCCAAGTAGAGGCCTTGATCGTTAGCCGCGCAACTAGC
 379   E  N  R  S  L  S  P  S  Q  V  E  A  L  I  V  S  R  A  T  S
1381  GGAAAAGTGACGGACACAAGAGGCAGCGTGAACAAGCTACTTTATAGCTTAACGGATACA
 399   G  K  V  T  D  T  R  G  S  V  N  K  L  L  Y  S  L  T  D  T
1441  GATTGTGGCCAAGACTGCGGTGGCCCAGATCCAACACCGGACCCAGAAGGCAAATTAACC
 419   D  C  G  Q  D  C  G  G  P  D  P  T  P  D  P  E  G  K  L  T
1501  TCGGGCGTGCCAGTAAGTGGTTTAAGTGGTTCAACTGGTCAAGTAGCGTATTACTATGTT
 439   S  G  V  P  V  S  G  L  S  G  S  T  G  Q  V  A  Y  Y  Y  V
1561  GATGTAGAAGCTGGGCAGCGCTTAACCGTACAAATGTATGGTGGCAGTGGCGATGCGGAT
 459   D  V  E  A  G  Q  R  L  T  V  Q  M  Y  G  G  S  G  D  A  D
1621  TTGTATCTCCGTTTTGGAGCAAAACCAGCACTGAATGCATGGGACTGCCGACCTTTCAAA
 479   L  Y  L  R  F  G  A  K  P  A  L  N  A  W  D  C  R  P  F  K
1681  TACGGTAACAATGAAACATGTACGGTCAGTGCGACGCAAAGTGGACGTTATCACGTCATG
 499   Y  G  N  N  E  T  C  T  V  S  A  T  Q  S  G  R  Y  H  V  M
```

```
1741    ATTCAAGGTTACTCAAACTATAGCGGTGTCAGCATTCAAGCGAGCTACTAGTTATTTTTC
 519     I  Q  G  Y  S  N  Y  S  G  V  S  I  Q  A  S  Y  *
1801    TCGTTATTAGAAGAGAACAAAGCCCGCTTTTCGGTGGGCTTTTCTTATGGGAATACTGTG

1861    CTTTAGGGAATGCAGTGTTAACGAGGAGACGCTATTCAAACGTATTGGCCTTTTGCTTCA
                                                       HindIII
1921    AAAACTCAACTCGAAGCGGCATCGAAGCTT
```

Figure 2.4 Nucleotide sequence of *vmproA* and the deduced amino acid sequence of the protease protein, VmproA. The -10 and -35 regions of a putative promoter region and a putative ribosome binding site (RBS) are underlined. The putative transcriptional start site is highlighted in bold. An asterisk (*) indicates the stop codon TAG. The amino acid active site residues are bolded and underlined. A pair of inverted repeats, representing the putative terminator, is indicated by a pair of inverted arrows. The putative signal sequence predicted using Interproscan (www.ebi.ac.uk/interproscan/) is underlined by a dotted line. *Hin*dIII restriction endonuclease sites are labeled and shown in bold.

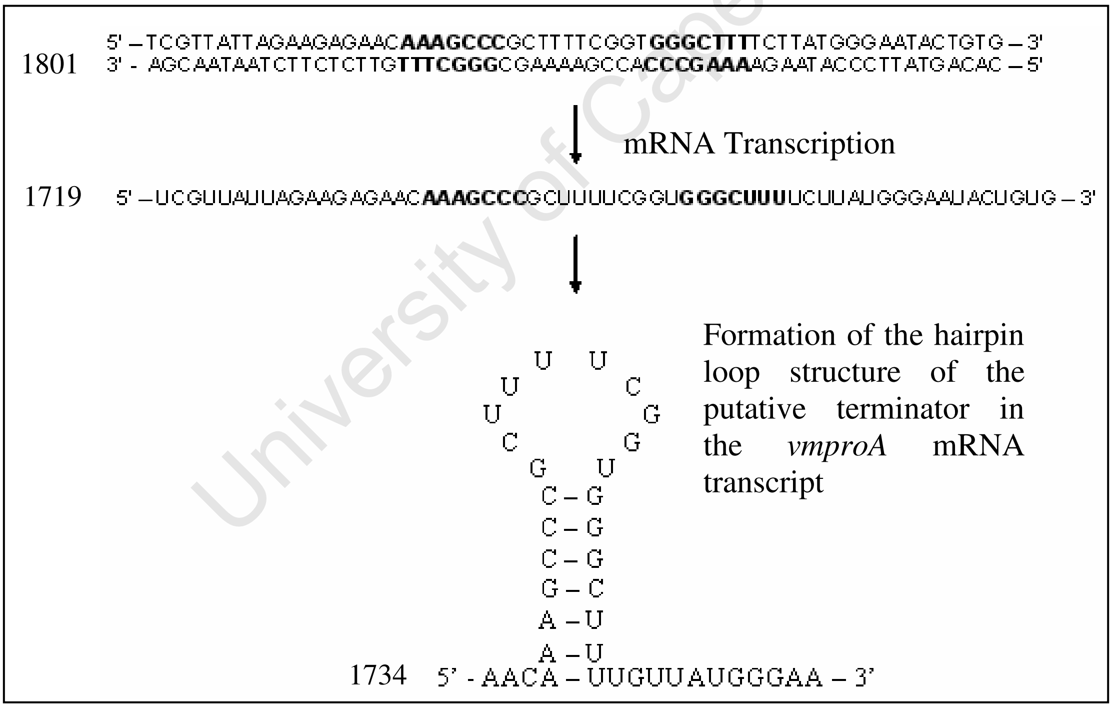
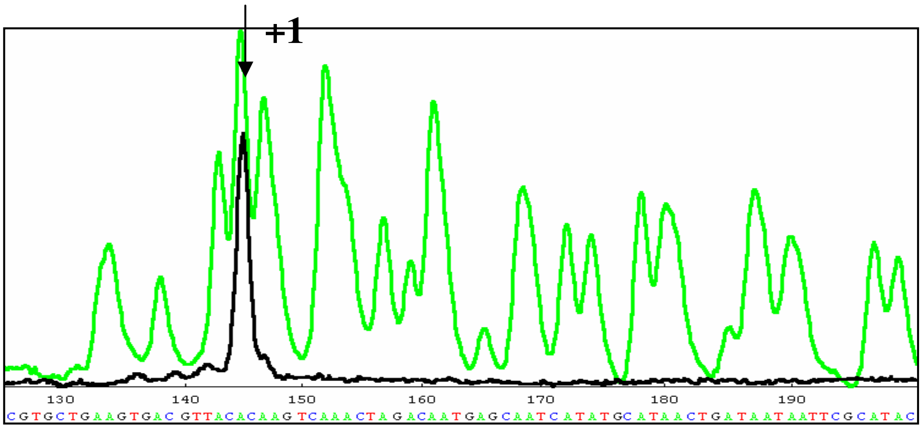
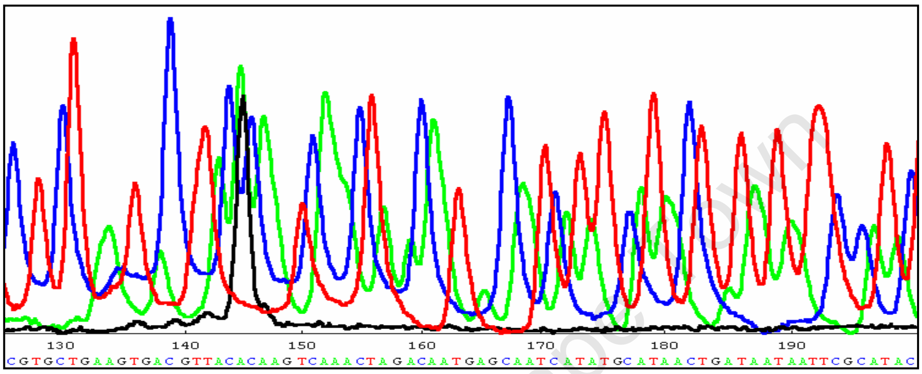


Figure 2.5 The formation of a hairpin loop structure of a potential transcriptional terminator in the transcribed mRNA of the *V. midae* SY9 *vmproA* protease gene. The inverted repeats are highlighted in bold. The formation of this inverted repeat is energetically favourable: ΔG= -5.6K cal/mol (DNAMAN version 4.13).

A)



B)



C)

*Hin*d**III** **-35**
1 **AAGCTT**GGTTATTGATCATGTTTCTGAGTATGCGAATTATTATCAG**TTATGC**ATATGATT
-10 +1
61 GCTCATTG**TCTAGT**TTGACTTGTGTAACGTCACTTCAGCACGCACTACGCTGATTGTCTG
RBS
121 TGTTTTCGTGTTGACAGTTTGGTACTAGGTAAAAATCAACAAGACTATGATTTC**AGGAA**
181 CATTTT**ATG**TTAAAGAAACTACTAAGTTGTTGTATCACCTCCGCGCTCTGTTTTCATTCT
241 TCTCTCGCTTTTTCGCAACCCAATGAGATTGCGGACAGTGCAGAGTTACAACAAGCTCCC

Figure 2.6 Identification of *vmproA* transcriptional start site by primer extension analysis. (A) Primer extension reaction product (black line) and adenines (green line). The transcriptional start (+1) site is indicated by the arrow. (B) Chromatogram of the corresponding DNA sequencing reaction using the same primer PExt (underlined in blue). (C) Nucleotide sequence of the putative promoter region of *vmproA*: ATG start codon (highlighted in green), transcriptional start site (red, +1), putative -10 and -35 motifs (underlined and bold), the ribosome binding site (RBS) and *Hin*dIII restriction enzyme recognition sequence (bold type). The Cy5 end-labelled reverse primer (PExtCy5) used to synthesise cDNA is underlined in blue. The numbering in (A) and (B) is based on the sequencing reaction and does not represent the numbering for the nucleotide sequence of *vmproA* as indicated in Figs. 2.4 and 2.6C.

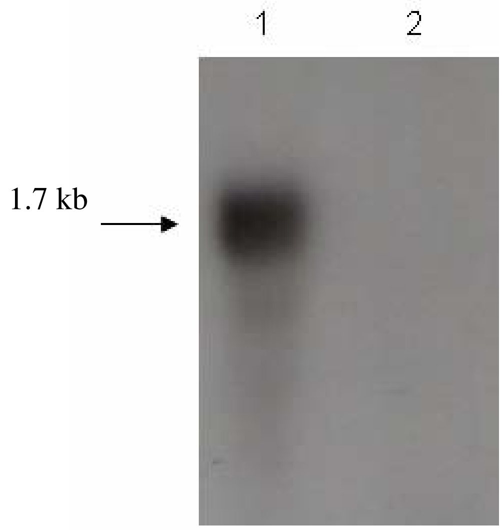


Figure 2.7 Northern hybridization of the 245 bp PCR product from *E. coli* JM109 (pMR11) to 10 µg of *V. midae* SY9 (lane 1) and *E. coli* JM109 (pSK) (lane 2) total cellular RNA isolated after 24 hours of growth in P-MBM at 22 ºC and LB containing 100 µg ml^{-1} Ampicillin at 37 ºC, respectively. The 231 bp PCR product representing an internal gene fragment (1793 - 2024) of *vmproA* was radiolabelled with [α-^{32}P]dCTP and used to probe the northern blot. The arrow indicates a single mRNA transcript of 1.7 kb in size.

2.4.6 Bioinformatic analyses of VmproA

Bioinformatic analysis of the deduced VmproA amino acid sequence indicated that it shared very high sequence identity at the amino acid level with other proteases produced and secreted by *Vibrio* species (Table 2.4). The deduced VmproA amino acid sequence had 98% (P16588), 97% (AAZ06359) and 96% (ZP_01259334) amino acid sequence identity with alkaline serine exoproteases of three *V. alginolyticus* species, and 86% amino acid sequence identity to a *V. harveyi* extracellular protease (ABL75266).

The active site amino acid residues, forming the catalytic triad (D^{180}/ H^{213}/ S^{363}), were identified by comparing the deduced VmproA amino acid sequence with known consensus sequences surrounding the active site residues of characterised subtilases (data not shown).

Table 2.4 Identities of the closest related protein sequences, obtained from a BLAST search of the GENBANK database (accessed 04/11/2009), to the deduced VmproA 534 amino acid sequence.

Organism	Accession no.	Description	% Identity	*E*-value
V. alginolyticus	P16588	Alkaline serine exoprotease A precursor	98%	2.00E-149
V. alginolyticus	AAZ06359	Alkaline serine exoprotease A	97%	2.00E-149
Vibrio sp. Ex25	ZP_01475709	Hypothetical protein VEx2w_02001655	97%	1.00E-148
V. alginolyticus 12G01	ZP_01259334	Alkaline serine protease	96%	3.00E-147
V. harveyi	ABL75266	Extracellular protease	86%	2.00E-129
Vibrio sp. PA-44	AAO16017	Extracellular subtilisin-like serine proteinase precursor	71%	4.00E-103
V. splendidus 12B01	ZP_00990201	Alkaline serine protease	70%	4.00E-103
V. cholerae MZO-3	EAY39642	Alkaline serine protease	56%	1.00E-85
V. cholerae O1 biovar eltor str. N16961	NP_229814	Alkaline serine protease	57%	2.00E-85
V. cholerae 1587	EAY32398	Alkaline serine protease	56%	2.00E-85

A neighbour-joining tree of the catalytic domain of VmproA and reference strains was constructed (Fig. 2.8). VmproA clustered within the sub-family of proteinase K-like subtilases. Furthermore, VmproA grouped together with the proteinase K-like subtilases produced and secreted by Gram-negative bacteria and not with those of fungal or yeast origin.

A conserved domain search revealed three putative conserved protein domains within the deduced amino acid sequence of the *V. midae* SY9 protease, VmproA (Fig. 2.9). A peptidase inhibitor domain was found at the N-terminus of VmproA (D^{56} to D^{135}) and is a match to protein family pfam05922. A peptidase S8 or subtilase domain, matching the protein family pfam00082, was found within VmproA (D^{155} to G^{399}). A bacterial pre-peptidase C-terminal (PPC) domain was observed within the VmproA deduced protein sequence (T^{450} to S^{533}) and is a match to protein family pfam04151. The conserved domain architecture within the VmproA amino acid sequence of sequentially ordered inhibitor, peptidase and PPC conserved domains is similar to 45 bacterial proteolytic enzymes, which includes extracellular alkaline serine proteases produced by several *Vibrio* species, including *V. alginolyticus*, *V. cholerae*, and *V. harveyi* (data not shown).

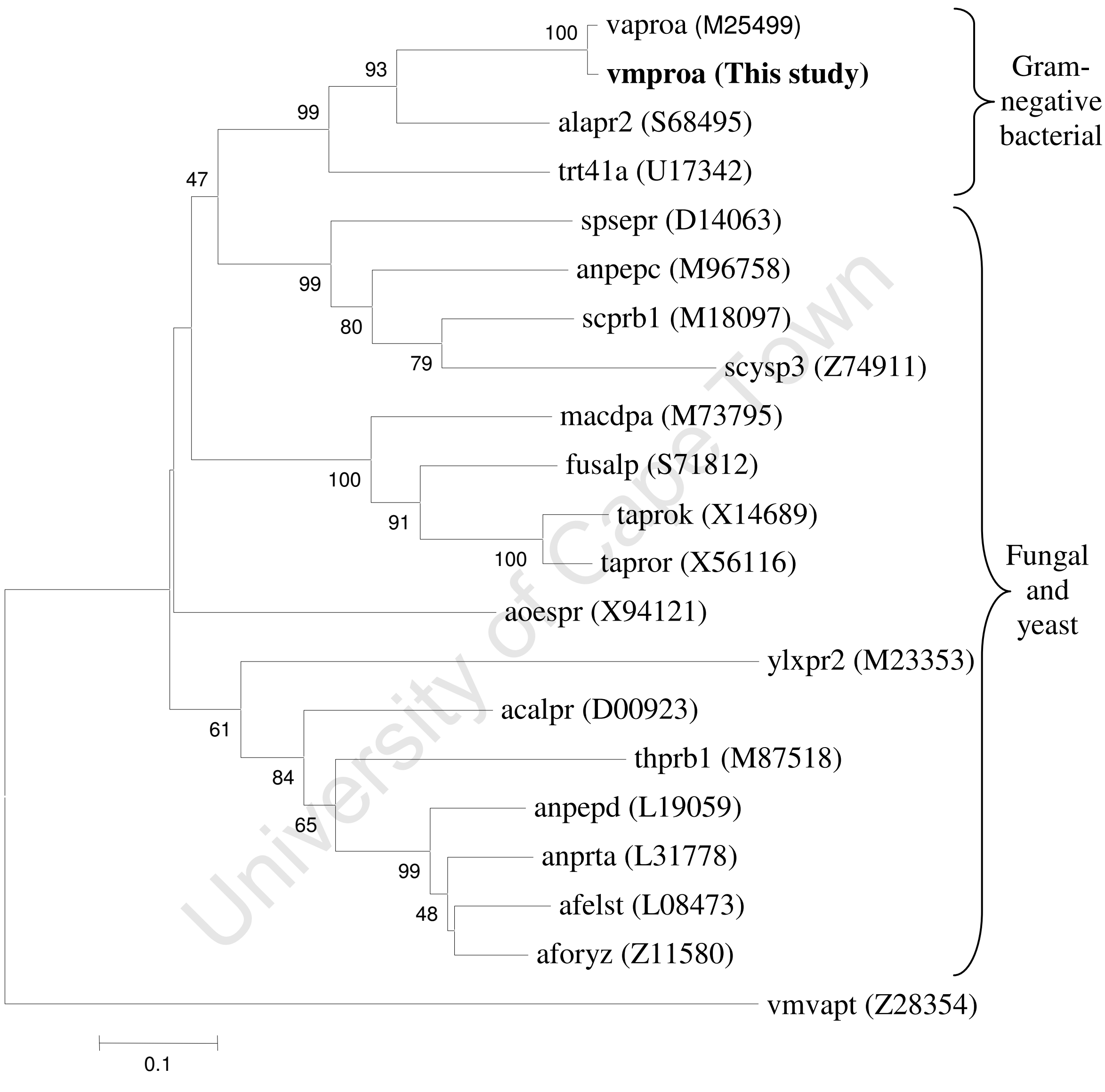


Figure 2.8 Unrooted phylogenetic tree of members of the Proteinase K sub-family, within the subtilase superfamily, based on the sequence alignment of a common length portion, including the catalytic domains (255 amino acids). The tree was constructed using the neighbour joining method. VmproA (vmproa) is highlighted in bold. Bootstrap values are based upon 1000 resampled data sets and only values of greater than 40% are indicated. A subtilisin sub-family member, vmvapt, was set as the out-group. Accession numbers (where available) are shown in brackets. The bar represents 0.1 amino acid substitutions per amino acid position.

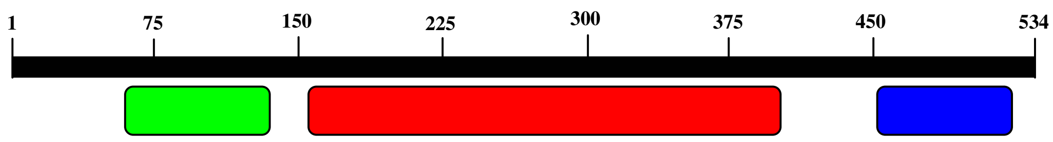


Figure 2.9 A representation of the conserved protein domain architecture within the deduced amino acid sequence of VmproA (number black bar). Amino acids 56-135 (green bar) are similar to N-terminal peptidase inhibitor domain; a subtilase (Peptidase S8 family) conserved domain was found from amino acid 155-399 (red bar) and amino acids 452-521 (blue bar) are similar to bacterial pre-peptidase C-terminal domains. (http://www.ncbi.nlm.nih.gov/Structure).

2.5 Discussion

An *E. coli* JM109 clone displaying an extracellular proteolytic phenotype on skim milk agar plates was isolated from a previously constructed genomic library of *V. midae* SY9 (Taylor, 2002). The proteolytic clone harboured the recombinant plasmid pMR11 that was mapped by restriction enzyme analysis. Southern hybridization analysis performed on pMR11 indicated that the cloned insert DNA originated from *V. midae* SY9. A deletion sub-cloning strategy employed to identify the proteolytically active portion of the plasmid indicated that the protease activity was located around the *Bgl*II (3315) restriction enzyme site of pMR11. The protease activity of pMR11 was localised to a 1.9 kb *Hin*dIII restriction fragment which was subsequently subcloned into the pBluescript SK cloning vector and designated pProH3. *E. coli* JM109 strains harbouring either pMR11 or pProH3 had an extracellular proteolytic phenotype when plated on skim milk agar plates, confirming that the *vmproA* protease gene was located within the 1.9 kb *Hin*dIII - *Hin*dIII *V. midae* SY9 chromosomal DNA fragment.

The recombinant plasmids pMR11 and pProH3, and the constructs pSub11 and pDel11, were sequenced. The nucleotide sequence obtained from these constructs was assembled and the full-length *vmproA* nucleotide sequence analysed. A BLAST search of the GENBANK database revealed that the putative ORF, contained within the *V. midae* SY9 cloned and sequenced chromosomal DNA fragment of pMR11, had high amino acid sequence identity to other *Vibrio* species' proteases. The majority of these proteases are alkaline serine proteases. The highest identity (98%) was to a *V. alginolyticus* calcium-dependent detergent-resistant alkaline serine exoprotease A (Deane *et al.,* 1989).

Deane (1989) visually identified a putative promoter region within the nucleotide sequence of the *V. alginolyticus* alkaline serine exoprotease A gene *proA*, that had homology to *E. coli* promoter sequences. A region upstream of the *vmproA* start codon corresponds to the putative *V. alginolyticus proA* promoter region. However, primer extension analysis performed on total RNA isolated from *V. midae* SY9 cultivated in a protease inducing growth medium, was used to determine the approximate position of a *vmproA* promoter region in relation to the transcriptional start site. This strategy for identification of putative promoter regions within *Vibrio* species gene sequences has been employed in several studies (Litwin *et al*., 1992; Swartzman *et al*., 1992; Okuda and Nishibuchi, 1998; Jeong *et al*., 2001; Stewart and McCarter, 2003). Primer extension analysis identified the transcriptional start site of *vmproA* as being 104 nucleotides upstream of the in-frame ATG start codon. Although a conserved promoter region was not identified, the sequences TAGTTT at nucleotide positions -12 to -7 and TATGCA at -35 to -30 could potentially function as the -10 and -35 regions of the *vmproA* putative promoter, respectively. Heinikoff shortening of the putative promoter region, coupled with promoter probe vector fusion constructs, could be employed in future studies to further investigate the promoter region and identify possible regulatory sequences within the DNA region upstream of the proposed *vmproA* promoter sequence.

The putative *vmproA* ORF of 1605 nucleotides is predicted to encode a 534 amino acid protein with a predicted M_r of 56.026 kDa. An energetically favourable palindromic sequence was identified downstream of the in-frame stop codon of *vmproA* that could function as a Rho-independent transcriptional terminator. Northern hybridization analysis indicated that a 1.7 kb mRNA transcript is encoded by the *vmproA* protease gene. The sequence data, transcriptional start site and transcriptional terminator of *vmproA* correlate with the predicted *vmproA* mRNA transcript size. A possible ribosome binding site (RBS), or Shine-Dalgarno sequence, was identified upstream of the putative in-frame start codon (Stormo *et al.,* 1982).

The first 21 amino acids (M^1 to A^{21}) of the VmproA deduced N-terminal amino acid sequence are hydrophobic residues and may function as a signal sequence to facilitate the secretion of the extracellular protease through the bacterial cell membrane. The putative cleavage site of the signal sequence may be between amino acid residues A^{21} and F^{22}. As with other bacterial proteolytic enzymes, including members of the subtilisin family (Deane *et al.,* 1989; Arnórsdóttir *et al.,* 2002), the overall architecture of VmproA can be divided into three conserved domains: an N-terminal pro-peptide domain, a catalytic subtilisin domain, and a pre-peptidase C-terminal domain. This suggests that the protease may be expressed and translated in a precursor form with pre- and pro-domains.

The subtilisin N-terminal domain, identified at the N-terminus of VmproA, may function as a pro-peptide. The N-terminal domain family is often found at the N-terminus of subtilisin proteases and is thought to function as an intramolecular chaperone (Shinde *et al.,* 1997) to promote correct folding before being auto-proteolytically cleaved off prior to activation of the enzyme (Lee *et al.,* 1991; Lee *et al.,* 1992; Eder *et al.,* 1993; Shinde and Inouye, 1993; Bryan *et al.,* 1995). The *Bacillus subtilis* extracellular proteolytic enzyme, Subtilisin E, is produced as a 352-residue pre-pro-subtilisin (Shinde and Inouye, 2000; reviewed in Takagi and Takahashi, 2003). The *B. subtilis* pre-pro-subtilisin is composed of a pre-domain that consists of a 29 amino acid signal sequence involved in protein secretion across the cell membrane, a 77 amino acid pro-domain and an active protease domain of 275 amino acid residues (Wong and Doi, 1986). Although the exact cleavage sites of the VmproA pro-peptidase domain were not determined, the approximate size of the pro-peptidase domain (75 amino acids) and that of the catalytically active domain (244 amino acids) are similar to that determined for Subtilisin E.

The VmproA catalytic subtilisin domain has homology to the subtilase superfamily of serine proteases, and contains the three active site amino acid residues (histidine, aspartic acid and serine) making up the catalytic triad (Siezen *et al.,* 1991; Siezen and Leunissen, 1997). Subtilases, like that of VmproA, have an Asp/Ser/His ordered catalytic triad and these three essential catalytic triad amino acid residues are conserved in all isolated subtilases (as reviewed by Siezen and Leunissen, 1997).

A bacterial Pre-peptidase C-terminal domain (PPC) was identified at the C-terminus of VmproA. PPC domains are generally 90 amino acid residues in length and are only found in bacteria and Archaea (Yeats *et al.,* 2003). These PPC domains have typically been found at the C-terminus of secreted bacterial peptidases, including three metalloprotease families and the subtilase superfamily of serine proteases (Rawlings *et al.,* 2002; Yeats *et al.,* 2003). PPC domains may assist the secretion of the extracellular proteolytic enzyme or function as an initial inhibitor, to prevent un-desired proteolytic activity, as post-secretion autocatalytic removal of this domain usually results in the enzyme being activated (Babé and Schmidt, 1998; Yeats *et al.,* 2003).

Sequencing and bioinformatic analyses of the predicted VmproA amino acid sequence indicated that the proteolytic enzyme is approximately 56 kDa in size. However, the presence of the conserved domains suggests that the mature VmproA is possibly N- and/or C-terminally processed and, therefore, the apparent size of the mature VmproA proteolytic enzyme may be smaller than the deduced and predicted size. Based on the high identity between VmproA and the *V. alginolyticus* detergent-resistant alkaline serine exoprotease, ProA, it is possible that VmproA may represent an alkaline detergent-stable extracellular serine protease.

The VmproA catalytic domain shares sequence homology with members of the proteinase K-like sub-family of the subtilase superfamily of serine proteases and specifically clusters with proteinase K-like enzymes produced by Gram-negative bacteria, including the *V. alginolyticus* serine exoprotease, ProA. The proteinase K-like enzymes represent a large family of secreted endopeptidases, that to date have only been found in fungi, yeast and Gram-negative bacteria (Siezen and Leunissen, 1997).

The putative active site of VmproA is comprised of three amino acids forming a catalytic triad. The catalytic triad and charge relay system between the three amino acids forming the catalytic triad are critical for serine protease activity (Kraut, 1977). The active site and the amino acid residues surrounding the active site of VmproA are highly conserved when compared to that of other related serine proteases and, as with other subtilases, the VmproA catalytic triad is ordered Aspartic acid/ Histidine/ Serine (Siezen and Leunissen, 1997).

This chapter describes an initial investigation into the extracellular proteolytic enzymes of *V. midae* SY9. The gene encoding an extracellular detergent resistant protease was cloned from a previously constructed and screened *V. midae* SY9 genomic library. Bioinformatic analysis of *vmproA* has enabled classification of VmproA as a member of the proteinase K-like subfamily of the subtilase superfamily of serine proteases on the basis of sequence identity, catalytic domain homology and the presence and order of three highly conserved active site residues within the catalytic domain. The cloned *V. midae* SY9 extracellular protease gene, *vmproA*, will enable further investigation into the specific role this gene and its secreted product, VmproA, potentially plays with regard to the previously observed (Macey and Coyne, 2005) increased growth rate of probiotically supplemented *Haliotis midae*.

CHAPTER 3

PURIFICATION AND PARTIAL CHARACTERISATION OF THE *VIBRIO MIDAE* SY9 EXTRACELLULAR PROTEASE PROTEIN AND RAISING OF POLYCLONAL ANTIBODIES TO THE PURIFIED PROTEIN

CONTENTS

3.1 Summary

The growth and extracellular protease activity of *V. midae* SY9 was investigated by growth curve and zymogram analysis. *V. midae* SY9 extracellular protease activity was shown to respond to incubation temperature, the growth medium and the growth phase of the *V. midae* SY9 culture. The *V. midae* SY9 extracellular protease(s) may also be regulated by catabolite and end-product repression, in response to the presence of glucose and casamino acids, respectively, within the culture medium. Casein and gelatine zymography showed that *V. midae* SY9 produced three extracellular proteases (designated proteases A, B and C, respectively). The predominant 40 kDa protease was shown to be SDS-stable on a gelatine-SDS-PAGE zymogram, while proteases B and C appeared to be minor proteases based on the zone of gelatine hydrolysis. The secreted proteolytic enzymes displayed maximal activity at a pH of 9.0. The presence of the protease inhibitors phenyl-methylsulfonyl (PMSF), EDTA and EGTA inhibited the activity of *V. midae* SY9 extracellular proteases, while the inhibitor, 1,10-phenanthroline, had no effect on *V. midae* SY9 extracellular protease activity. VmproA, the predominant, SDS-resistant, extracellular protease was purified 28.3-fold to homogeneity by ammonium sulphate bulk fractionation and precipitation, and successive ion-exchange and gel-filtration column chromatographies. The purified protein was observed to be a single band of approximately 40 kDa following SDS-PAGE and casein-zymogram analysis. The purified VmproA enzyme had maximal activity within the alkaline pH range of 9 - 10. Polyclonal antibodies raised to purified VmproA were shown to bind specifically to the purified VmproA protein.

3.2 Introduction

Active secretion of extracellular proteins by bacteria is an essential component of nutrient acquisition, adaptation to environmental change, quorum sensing, and virulence (Dautin and Bernstein, 2007). *Vibrio* species have been observed to produce and secrete a wide variety of extracellular proteins, including toxins, deoxyribonulease (Focareta and Manning, 1987), haemagglutinin (Oishi *et al.,* 1979; Finkelstein and Hanne, 1982), neuramidase and hydrolytic enzymes such as amylases (Oishi *et al.,* 1979), agarases (Farrell and Crosa, 1991; Sugano *et al.,* 1993; Araki *et al.,* 1998), alginate lyases (Sawabe *et al.,* 1998) and proteases (Sakurai *et al.,* 1973; Marcello *et al.,* 1996; Wang *et al.,* 2007).

Extracellular proteases are one of the most abundant proteins produced and secreted by *Vibrio* species (Marcello *et al.,* 1996). *Vibrio* extracellular proteases have been implicated as virulence factors involved in the degradation of host tissues (Farrell and Crosa, 1991). Therefore, research within this area has primarily focused on extracellular proteases produced by pathogenic *Vibrio* species such as *V. cholerae* (Häse and Finkelstein, 1991; Jobling and Holmes, 1997; Scott *et al.,* 2001; Stewart-Tull *et al.,* 2004), *Vibrio vulnificus* (Chuang *et al.,* 1997), *V. anguillarum* (Inamura *et al.,* 1985; Norqvist *et al.,* 1990), *Vibrio parahaemolyticus* (Sudheesh and Xu, 2001), *Vibrio pelagius* (Farto *et al.,* 2002), *V. harveyi* (Teo *et al.,* 2003) and *V. alginolyticus* (Lee *et al.,* 1997; Cai *et al.,* 2007).

The extracellular protease activity of several *Vibrio* species has been shown to be due to production and secretion of multiple proteases (Long *et al.,* 1981; Farrell and Crosa, 1991; Wang *et al.,* 2007). The variety of extracellular proteolytic enzymes produced and secreted may depend on the source of the isolate, the bacterial strain itself, the growth medium and culture conditions (Morita and Kusuda, 1994). A *V. alginolyticus* strain (Welton and Woods, 1973) has been observed to secrete a collagenase (Reid *et al.,* 1980), six extracellular alkaline serine proteases (Hare *et al.,* 1981; Hare *et al.,* 1983; Deane *et al.,* 1986) and an extracellular detergent resistant alkaline serine protease (Deane *et al.,* 1987b) during stationary phase growth (Hare *et al.,* 1983). *Vibrio metschnikovii* RH530 was observed to secrete six alkaline serine proteolytic enzymes, including a detergent-stable protease and a metalloprotease (Kwon *et al.,* 1992). *V. vulnificus* produces and secretes an extracellular metalloprotease (Miyoshi *et al.,* 1987), an elastolytic protease (Kothary and Kreger, 1985; Kothary and Kreger, 1987) and a collagenase (Smith and Merkel, 1982).

Serine extracellular proteases have been purified from *Vibrio* species, including *V. alginolyticus* strain Swy (Lee *et al.,* 1997), *V. anguillarum* strain PT-81049 (Inamura *et al.,* 1985), *V. parahaemolyticus* no. 93 (Lee *et al.,* 2002) and *Vibrio metschnikovii* strains (Kwon *et al.,* 1994; Mei and Jiang, 2005). Many *Vibrio* species' extracellular serine proteolytic enzymes have either been bulk purified from culture supernatant using ion-exchange and/or gel-filtration chromatography (Lee *et al.,* 1997; Lee *et al.,* 2002; Mei and Jiang, 2005). Alternatively, they have been heterologously expressed and purified from an *E. coli* host (Chung *et al.,* 2001; Arnórsdóttir *et al.,* 2002).

Serine proteases are broadly characterised as being sensitive to PMSF and extracellular serine proteolytic enzymes purified from *Vibrio* species are no exception (Lee *et al.,* 1997; Lee *et al.,* 2002). Many *Vibrio* species' extracellular serine proteolytic enzymes have also been found to be resistant to detergents and denaturants, such as SDS, and have high activity over a broad pH and temperature range (Kwon *et al.,* 1992; Chung *et al.,* 2001; Mei and Jiang, 2005).

3.2.1 Aim of this chapter

The aim of this study was to obtain additional information regarding the growth and extracellular protease activity of *V. midae* SY9. This will enable a better understanding of the production and secretion of extracellular proteolytic enzymes by *V. midae* SY9, thereby enhancing our understanding of their potential role in improving the digestion of *V. midae* SY9 supplemented protein-based artificial abalone diets. A further aim of this study will be to purify the predominant extracellular protease, VmproA, from *V. midae* SY9 and to raise antibodies to purified VmproA. These antibodies will in turn be employed (in Chapter 6) to attempt to visually localise VmproA within the digestive tract of probiotically supplemented juvenile abalone.

3.3 Materials and Methods

All media and solutions used in this study are listed in Appendix A.

3.3.1 Growth and extracellular protease analysis of *V. midae* SY9

The cell growth and extracellular protease activity of *V. midae* SY9 was investigated by conducting comparative growth curve analyses of *V. midae* SY9 in MB (Appendix A.1.1), P-MBM (Appendix A.1.3) and P-MBM supplemented with 0.2% (w/v) glucose (Appendix A.1.4). *V. midae* SY9 cultures were grown for 12 hours in a 5 ml starter culture of MB, P-MBM and P-MBM supplemented with 0.2% glucose, respectively, on an orbital shaker (100 rpm) at 22 °C. The whole volume of the starter cultures were inoculated into 100 ml MB, P-MBM and P-MBM supplemented with 0.2% glucose, respectively, and incubated at 22 °C for 16 hours on an orbital shaker (100 rpm). These cultures were inoculated into three 5 litre conical flasks containing 1000 ml of culture medium, so as to achieve an initial A_{600} (Beckman Du® 530 Life science UV/VIS Spectrophotometer) of 0.02, and incubated at 22 ºC on an orbital shaker at 100 rpm. Triplicate samples were removed aseptically from the cultures at regular intervals for measuring cell growth (A_{600}), and determination of extracellular protease enzyme activity and reducing sugar concentration. The samples were centrifuged (13,000x g for 5 min at 4 °C) to pellet the bacterial cells, and the supernatant fraction collected and stored at -20 °C for later analysis. The reducing sugar concentration and the protease activity in the growth medium was determined using standard DNS (Miller, 1959) (Appendix B.23) and Azo-casein protease assays (Appendix B.22), respectively.

3.3.2 Analysis of *V. midae* SY9 extracellular protease activity

V. midae SY9 was cultured for 24 hours to stationary phase in 100 ml P-MBM (Appendix A.1.3) at 22 ºC with shaking, as described in section 3.3.1 The cells were harvested by centrifugation (13,000x g for 10 minutes at 4 ºC), and the supernatant fraction (containing the protease) retained. Protease containing samples were filtered through a syringe filter (0.22 µm, Whatman) to remove any remaining bacterial cells. Thereafter, the cell-free supernatant was treated with 2.5% (w/v) SDS (Appendix A.2.18) and 2% (v/v) glycerol (Appendix A.2.18), before being analyzed using conventional SDS-PAGE (Appendix B.19) and denaturing casein- and gelatine-zymograms (Heussen and Dowdle, 1980; Saborowski *et al.,* 2004), as described in Appendix B.20. Electrophoresis was performed at 30 mA for 3 hours at 4 ºC in a Mighty Small II SE250 (Hoefer Scientific Instruments). Following electrophoresis, the SDS-PAGE gel was treated as described in Appendix B.19, while the gelatine- and casein-zymograms were performed as described in Appendix B.20.1 and B.20.2, respectively.

3.3.3 Characterisation of *V. midae* SY9 extracellular protease activity

The optimal pH for *V. midae* SY9 extracellular protease(s) activity was investigated. Determining the optimal pH conditions will facilitate purification of VmproA. The effect of protease inhibitors on *V. midae* SY9 extracellular protease activity was also investigated to better characterise the extracellular proteases of *V. midae* SY9.

3.3.3.1 Effect of pH on *V. midae* SY9 extracellular protease activity

In order to establish the effect of pH on *V. midae* SY9 extracellular protease(s), protease activity was assayed across a range of pH values. A *V. midae* SY9 culture was grown for approximately 24 hours in 5 ml of P-MBM (Appendix A.1.3) on an orbital shaker (100 rpm) at 22 °C. The cells were harvested by centrifugation (13,000x g for 5 minutes at 4 ºC). The cell-free supernatant was retained and stored on ice. The Azo-casein protease assay (as described in Appendix B.22) was performed on the cell-free culture supernatant using a synthetic Azo-casein substrate (Appendix A.2.20) that had been adjusted to pHs of 5.5, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. A negative control was performed using each Azo-casein solution at the appropriate pH and subtracted from the calculated enzyme activity at that pH value. Proteolytic activity was expressed as units of protease enzyme per ml of supernatant (U ml^{-1}) at 37 °C.

3.3.3.2 Effect of protease inhibitors on *V. midae* SY9 extracellular protease activity

The effect of protease inhibitors on *V. midae* SY9 extracellular protease(s) was investigated by determining the change in protease activity following pre-incubation of the proteases in the presence of chemical agents relative to an untreated control sample.

The effect of protease inhibitors on *V. midae* SY9 extracellular proteases was determined by performing the Azo-casein protease assay (as described in Appendix B.22) with protease containing supernatant (section 3.3.2) that had been pre-incubated with known concentrations of protease inhibitors. The inhibitors, 1,10–phenanthroline (Sigma) (Appendix A.2.20), PMSF (Roche) (Appendix A.2.20), EGTA (Calbiochem) (Appendix A.2.3) and EDTA (Saarchem) (Appendix A.2.3) were added to the following final concentrations: 0.1, 0.5, 1.0, 5 and 10 mM, and pre-incubated together with the extracellular proteases for 15 minutes at 22°C prior to being assayed for protease activity. Control experiments were conducted to show that the ethanol used to dissolve the 1,10-phenanthroline and PMSF stock solutions did not affect the protease activity. Protease activity was determined as a percentage of the untreated control with no inhibitors added.

3.3.4 Purification of VmproA from *V. midae* SY9 culture supernatant

All of the steps in the purification process were performed at 4 ºC, unless otherwise stated, in order to reduce the loss of protease protein during the purification process.

3.3.4.1 Ammonium sulphate precipitation of VmproA from *V. midae* SY9 culture supernatant

A preliminary investigation of the optimal ammonium sulphate concentration for fractionation and precipitation of VmproA from *V. midae* SY9 culture supernatant was performed as follows. A five milliliter starter culture of *V. midae* SY9 in P-MBM (Appendix A.1.3) was incubated at 22 ºC for 16 hours, before being inoculated into 1000 ml P-MBM in order to achieve an initial A_{600} of 0.02. The 1000 ml culture was subsequently incubated at 22 °C on an orbital shaker at 100 rpm for 24 hours. The cells were harvested by centrifugation (13,000x g for 15 minutes at 4 °C), and the supernatant was collected and maintained in sterile glass bottles at 4 ºC. The extracellular proteins in the supernatant were precipitated at 4 °C. This was done by adding increasing amounts of solid ammonium sulphate (Merck) in 100 g increments in a stepwise fashion, in order to achieve the desired ammonium sulphate concentration in the protease-containing supernatant, i.e. 30, 40, 50, 60, 70, 80, and 90% (Englard and Seifter, 1990). The solid ammonium sulphate was dissolved by gentle stirring at 4 °C.

The protein precipitate at each ammonium sulphate concentration was collected by centrifugation (13,000x g for 30 minutes at 4 °C) and resuspended in 1 ml 50 mM Tris-HCl, pH 9.0 (Appendix A.2.14). The resuspended proteins were dialysed against 50 mM Tris-HCl, pH 9.0 at 4 °C for a period of 16 hours. The proteolytic activity (Appendix B.22), A_{280} protein concentration (Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.)), and volume of the dialysed sample was determined for each ammonium sulphate concentration.

3.3.4.2 Purification of VmproA

For the large-scale purification of VmproA, twelve 1000 ml *V. midae* SY9 cultures were grown in P-MBM (Appendix A.1.3) for 24 hours at 22 °C on an orbital shaker at 100 rpm for 24 hours, as described in section 3.3.1. The cultures were centrifuged (13,000x g for

15 minutes at 4 °C) to pellet and remove the bacterial cells and any insoluble residues. The 12 liters of supernatant was collected in sterile glass bottles and maintained at 4 ºC.

The supernatant was adjusted to a final ammonium sulphate concentration of 30% (164 g l^{-1}) by adding solid ammonium sulphate in 100 g increments. The solid ammonium sulphate was dissolved by stirring at 4 ºC. The protein precipitate was collected by centrifugation (13,000x g for 20 minutes at 4 °C). The supernatant was collected and adjusted to a final ammonium sulphate concentration of 90% (402 g l^{-1}) by adding solid ammonium sulphate in 100 g increments. The solid ammonium sulphate was dissolved by stirring at 4 ºC. The protein precipitate was collected by centrifugation (13,000x g for 30 minutes at 4 °C) and resuspended in 160 ml 50 mM Tris-HCl, pH 9.0. The resuspended proteins were dialysed against 50 mM Tris-HCl, pH 9.0 for approximately 16 hours at 4 °C.

The following procedures were performed at 4 °C. A DE-52 (Pre-swollen microgranular anion exchanger, Whatman®) column (26 x 40 cm) was prepared according to the manufacturer's instructions. Thereafter, the column was equilibrated with 50 mM Tris-HCl, pH 9.0 (Appendix A.2.14). The dialysed ammonium sulphate protein concentrate (90% final concentration) was loaded onto the packed column and allowed to pass through the column by gravity flow. The loaded column was washed with 750 ml of 50 mM Tris-HCl, pH 9.0. The proteins were eluted from the column in a stepwise fashion with an increasing NaCl molarity gradient, i.e. 250 ml volumes of 0.05 M NaCl, 0.1 M NaCl, 0.25 M NaCl and 0.5 M NaCl (Appendix A.2.14) were passed through the column by gravity. The eluted fractions (2 ml each) were collected with a 7000 Ultrorac® fraction collector (LKB Bromma). The proteolytic activity (Appendix B.22) and A_{280} protein concentration (Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.)) were determined for every 5th fraction. The active fractions were pooled together and the volume reduced to 6 ml with an Amicon Centricon filter system (10,000 MW cut-off, Millipore).

A Sephadex G-100 (Sigma®) column (25 x 100 cm) was prepared according to the manufacturer's instructions. Thereafter, the column was equilibrated with 100 mM Tris-HCl (pH 9.0), 300 mM NaCl (Appendix A.2.14). The concentrated active fraction (6 ml) was loaded onto the gel-filtration column and 3 ml fractions of the eluate were collected with a 7000 Ultrorac® fraction collector (LKB Bromma). The proteolytic activity (Appendix B.20) and A_{280} protein concentration (Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.)) were determined for every 5th fraction. The active fractions were pooled together and the volume reduced with an Amicon® U-15 concentration spin column (10,000 MW cut-off, Millipore) to a final volume of 3 ml.

3.3.5 Characterisation of purified VmproA

3.3.5.1 Determination of the optimum pH for purified VmproA activity

The pH for optimal activity of purified VmproA was determined by performing the Azo-casein protease assay (Appendix B.20) with purified VmproA enzyme and pH-adjusted Azo-casein synthetic substrates (Appendix A.2.20), within the pH range of 7.0, 8.0, 9.0, 10.0 and 11.0. A negative control was performed using each Azo-casein solution at a different pH and subtracted from the calculated enzyme activity at that pH value. Extracellular protease activity was determined by the Azo-casein protease assay (Appendix B.22) and the protein concentrations determined by the Bradford method (Appendix B.24). Specific proteolytic activity was expressed as units of protease enzyme per mg of VmproA. The reaction data was analyzed by paired Student *t*-tests using SigmaStat 3.11.0 (Systat Software, Inc.). Statistically significant differences were established at critical levels of P<0.05.

3.3.5.2 Zymogram and SDS-polyacrylamide gel electrophoresis

Samples of *V. midae* SY9 culture supernatant, ammonium sulphate concentrated *V. midae* SY9 culture supernatant and purified VmproA protein were analyzed by conventional SDS-PAGE, on an 8% polyacrylamide gel according to the Laemmli method (Appendix B.19), and denaturing casein- and gelatine-zymograms (Heussen and Dowdle, 1980; Saborowski *et al.,* 2004) (Appendix B.20). Electrophoresis was performed at 30 mA for 3 hours at 4 ºC in a Mighty Small II SE250 (Hoefer Scientific Instruments). Following electrophoresis, the proteins were detected by a modified silver staining procedure (Appendix B.19.1), while the gelatine- and casein-zymograms were performed as described in Appendix B.20.1 and B.20.2, respectively.

3.3.6 Production of polyclonal antibodies to the purified *V. midae* SY9 extracellular detergent resistant protease, VmproA.

The production of polyclonal antibodies to the purified VmproA will provide another molecular tool to further investigate the *V. midae* SY9 protease.

3.3.6.1 Preparation of antigen

Purified VmproA was quantitated by the Bradford assay (Appendix B. 24) and diluted to a concentration of 0.5 mg ml^{-1}. Thereafter, the diluted protein sample was clarified by centrifugation (13,000x g for 30 minutes at 4 ºC). The protein sample was then mixed in a 1:1 ratio with Freud's incomplete adjuvant (Appendix A.2.21) in a total volume of 1 ml and emulsified, as described by Rybicki (1979).

3.3.6.2 Rabbit antiserum

A New Zealand White rabbit, older than ten weeks, was initially immunised (UCT Animal Ethics Committee approval - 04/034) with an intramuscular injection of 1 ml of antibody emulsion (section 3.3.6.1), as described by Rybicki (1979). The rabbit was boosted with additional 1 ml injections of prepared antigen (section 3.3.6.1) at weekly intervals for another two weeks. Thereafter, the rabbit was boosted with 1 ml injections of the antigen emulsion at 2 week intervals, so that the rabbit was immunised a total of five times. The rabbit was bled from the marginal vein in the ear, prior to the initial immunization and then at weekly intervals following the third injection. At each bleed, approximately 10 ml of blood was collected in a sterile beaker and allowed to clot. The clotted blood was centrifuged and the antibody containing serum fraction was collected and aliquoted into sterile bottles, labelled and stored at -20 ºC.

3.3.6.3 ELISA of anti-VmproA polyclonal antibodies

An ELISA was performed to confirm the presence of anti-VmproA polyclonal antibodies in the rabbit serum obtained from the various bleeds (section 3.3.6.2). Serum from each bleed (pre-bleed, bleed 1, bleed 2, bleed 3, bleed 4, pre-boost and post-boost) was tested for the presence of specific polyclonal antibodies to VmproA. Each well of the polysorb ELISA plate (Nunc™) was coated with 100 µg of purified VmproA antigen and the ELISA was performed as described in Appendix B.15.

3.3.6.4 Western hybridization analysis to confirm antibody specificity

A Western hybridization analysis was performed in order to confirm the specificity of the anti-VmproA polyclonal antibodies. Five millilitre cultures of *E. coli* JM109 (pMR11) in P-MBM (Appendix A.1.3) containing 100 µg ml^{-1} ampicillin (Appendix A.2.2) and *V. midae* SY9 in P-MBM were incubated at 22 ºC for approximately 16 hours on an orbital shaker (100 rpm), before being inoculated into 100 ml of the same growth medium.

These 100 ml cultures were incubated at 22 ºC for 24 hours on a rotary shaker at 100 rpm. The cells were harvested by centrifugation (13,000x g for 15 minutes at 4 ºC) and the supernatant fractions were collected. The supernatant fractions were adjusted to a final ammonium sulphate concentration of 90% with 60.3 g of solid ammonium sulphate at 4 ºC, as described in section 3.3.4.1. The protein precipitates were harvested by centrifugation (13,000x g for 40 minutes at 2 ºC) and resuspended in one ml of 50 mM Tris-HCl (pH 9.0) (Appendix A.2.14).

The harvested *E. coli* JM109 (pMR11) cells were resuspended in 50 mM Tris-HCl (pH 9.0) (Appendix A.2.14) and sonicated (four pulses of 20 seconds each, with 20 second pauses in-between; Virsonic Sonicator; as described in Appendix B.14) to obtain cell extracts. Insoluble cell debris was removed by centrifugation (13,000x g for 5 minutes at 4 ºC) and the supernatant containing soluble intracellular proteins was retained at 4 ºC. Soluble *E. coli* JM109 (pMR11) cell extracts and the ammonium sulphate precipitated supernatant fractions of *E. coli* JM109 (pMR11) and *V. midae* SY9 were dialysed against 10 litres of 50 mM Tris-HCl (pH 9.0) (Appendix A.2.14) for 16 hours at 4 ºC.

The soluble intracellular and precipitated extracellular protein fractions of *E. coli* JM109 (pMR11), the precipitated extracellular protein fraction of *V. midae* SY9, and purified VmproA were separated on a 8% denaturing polyacrylamide gel (Appendix B.19) and transferred to nitrocellulose membrane (Protran) using a Bio-Rad electro-transfer apparatus as described in Appendix B.21.1. The IgG fraction of anti-VmproA polyclonal antibodies was purified from the serum obtained from the final (post-boost) bleed (Appendix B.16) and pre-absorbed (Appendix B.17) against *E. coli* JM109 soluble intracellular protein extracts, prepared as described in Appendix B.14. Western blot analysis was performed using the IgG-purified, pre-absorbed anti-VmproA polyclonal antibodies, as described in Appendix B.21.

3.4 Results

3.4.1 The effect of culture conditions on *V. midae* SY9 growth and extracellular protease production

The effect of the growth medium and incubation temperature on the growth and extracellular protease activity of *V. midae* SY9 was assessed in a series of shake flask growth curve analyses (Figs. 3.1 to 3.4.).

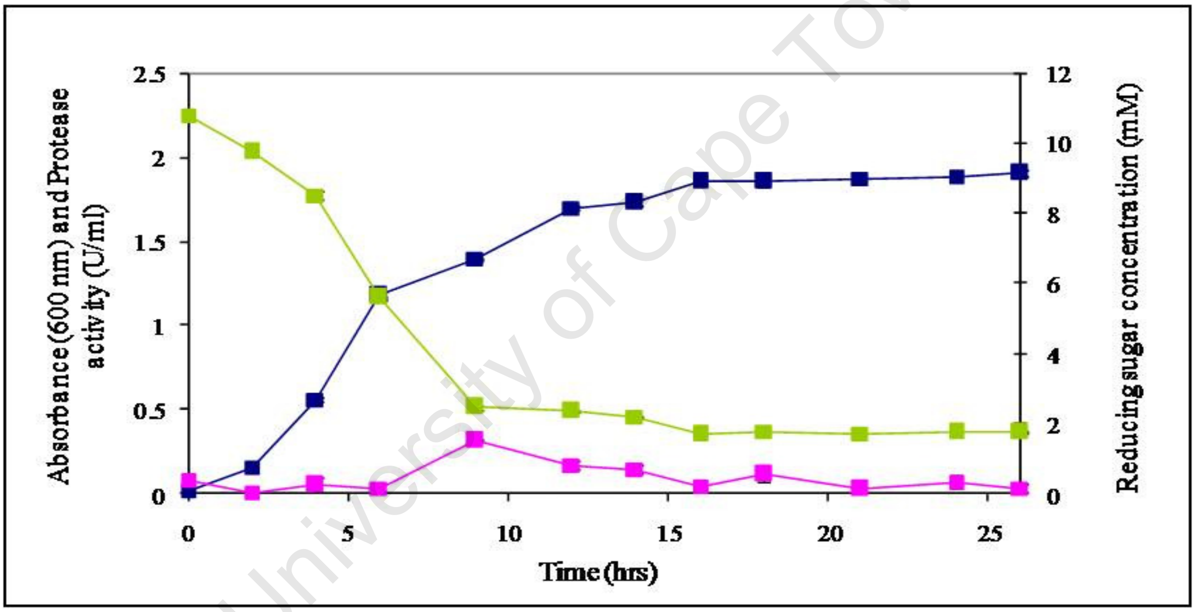


Figure 3.1 The growth profile of *V. midae* SY9 during shake flask cultivation in MB at 22 ºC. Cell growth was monitored by absorbance (600 nm) readings (■), while the alkaline protease activity (■) and reducing sugar concentration (■) within the culture supernatant were determined using standard assays. The vertical error bars represent the mean ± standard error.

When *V. midae* SY9 was cultured in MB at 22 ºC there was a brief lag phase, followed by a period of exponential growth (Fig. 3.1). During this period, there was a rapid decrease in the reducing sugar content of the growth medium, until the reducing sugar concentration reached a level of approximately 2 mM at approximately 9 hours of growth.

Thereafter, the culture began to enter stationary phase and the reducing sugar concentration within the culture supernatant remained around 2 mM throughout the remainder of the growth analysis. There was no significant increase in the levels of extracellular protease activity over the course of the growth curve analysis. The *V. midae* SY9 culture achieved a maximum biomass yield of approximately 1.9 A_{600} units after 30 hours of growth. When *V. midae* SY9 was cultured in P-MBM at 22 ºC (Fig. 3.2), there was a more gradual growth profile than was observed for *V. midae* SY9 cultivation in MB at 22 ºC (Fig. 3.1). *V. midae* SY9 cultured in P-MBM also achieved a higher cell biomass after 26 hours of growth (Fig. 3.2) than the culture grown in MB (Fig. 3.1). The reducing sugar concentration within the P-MBM culture medium was rapidly depleted during the initial growth phase to a level of approximately 0.9 mM, and remained at this level for the duration of the growth analysis (Fig. 3.2). The *V. midae* SY9 culture in P-MBM at 22 ºC did not enter stationary phase, as was observed when *V. midae* SY9 was cultivated in MB at 22 ºC (Fig. 3.1), but instead continued to grow at a slower growth rate for the remainder of the growth analysis (Fig. 3.2). Extracellular protease activity began to increase from 9 hours of growth towards the latter part of the exponential growth phase, with the highest level of approximately 10 U ml^{-1} detected at 26 hours of growth.

The growth rate was dramatically increased when *V. midae* SY9 was cultured in P-MBM at 37 ºC (Fig. 3.3). The *V. midae* SY9 culture achieved a maximum biomass yield of 2.5 A_{600} units after 9.5 hours. The available reducing sugars within the P-MBM culture medium was reduced within the first hour of growth to a level of approximately 0.75 mM, where it remained for the duration of the growth analysis. The extracellular protease activity levels began to increase between 2 and 4 hours of growth, reaching the highest level of approximately 2.5 U ml^{-1} after 9.5 hours.

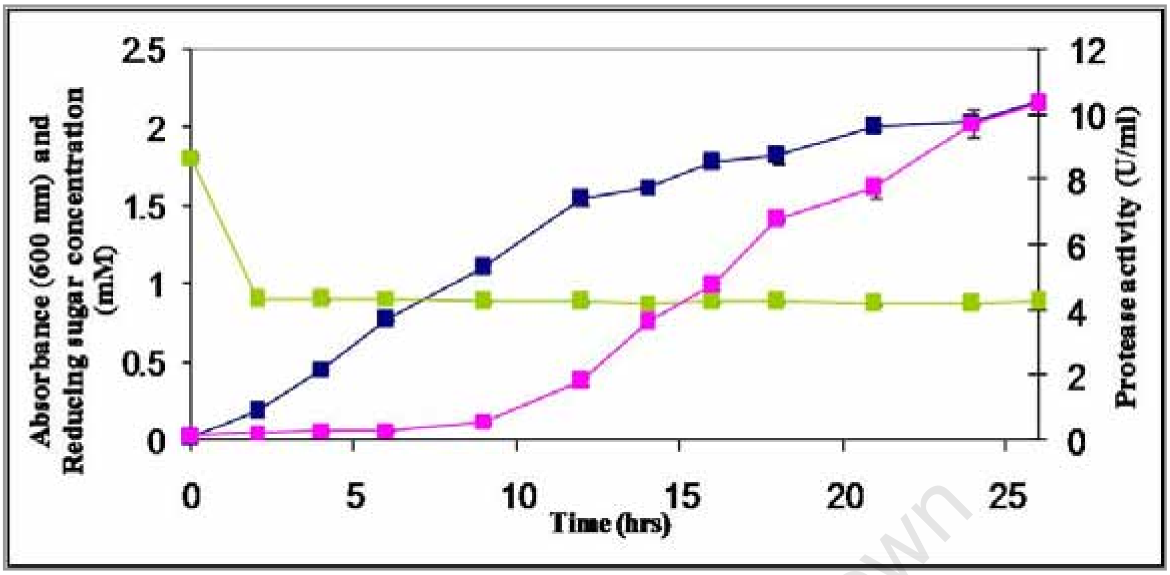


Figure 3.2 The growth and alkaline protease activity profiles of *V. midae* SY9 during shake flask cultivation in P-MBM at 22 ºC. The cell growth was monitored by absorbance (600 nm) readings (■), while the alkaline protease activity (■) and reducing sugar concentration (■) within the culture supernatant were determined using standard assays. The vertical error bars represent the mean ± standard error.

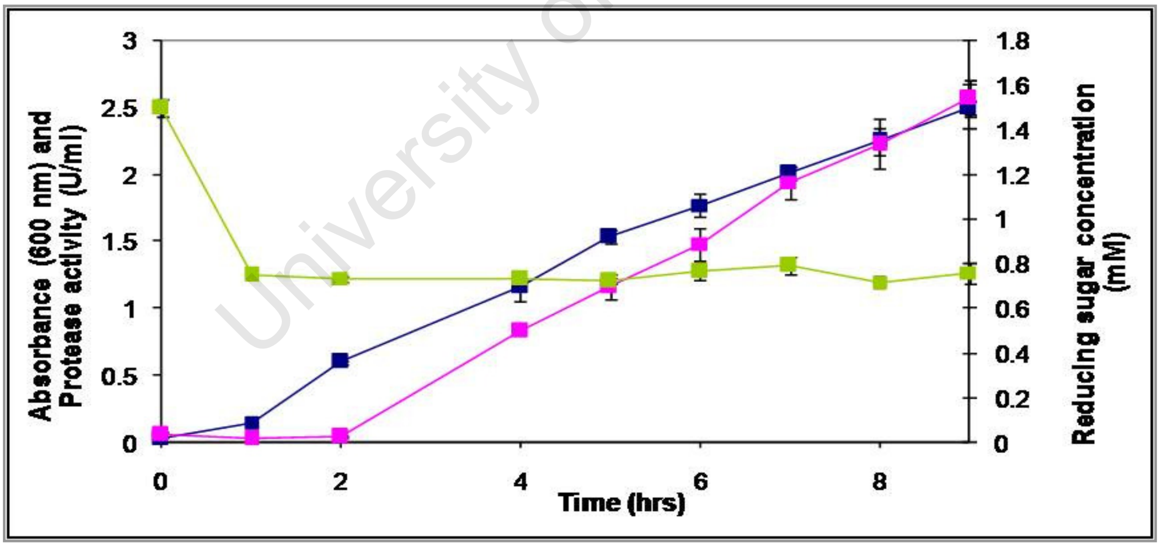


Figure 3.3 The growth and alkaline protease activity profiles of *V. midae* SY9 during shake flask cultivation in P-MBM at 37 ºC. The cell growth was monitored by absorbance (600 nm) readings (■), while the alkaline protease activity (■) and reducing sugar concentration (■) within the culture supernatant were determined using standard assays. The vertical error bars represent the mean ± standard error.

The highest growth rates and biomass yields were obtained when *V. midae* SY9 was shake flask cultured in P-MBM supplemented with 0.2% glucose (Appendix A.1.4) at 37 ºC (Fig. 3.4). The brief initial lag phase of approximately 1 hour was followed by an exponential growth phase of approximately 2 hours, during which the *V. midae* SY9 culture achieved a biomass of approximately 2.5 A_{600} units. The *V. midae* SY9 shake flask culture reached a maximum biomass yield of approximately 4.9 A_{600} units after 12 hours of growth. The available reducing sugar within the culture medium was utilized by the rapidly growing *V. midae* SY9 culture, decreasing from an initial concentration of 9 mM, to a concentration of approximately 1.2 mM after 4 hours of growth. The levels of extracellular protease activity began to increase after 5 hours of growth, with the highest activity of 4.4 U ml^{-1} being observed after 11 hours of growth.

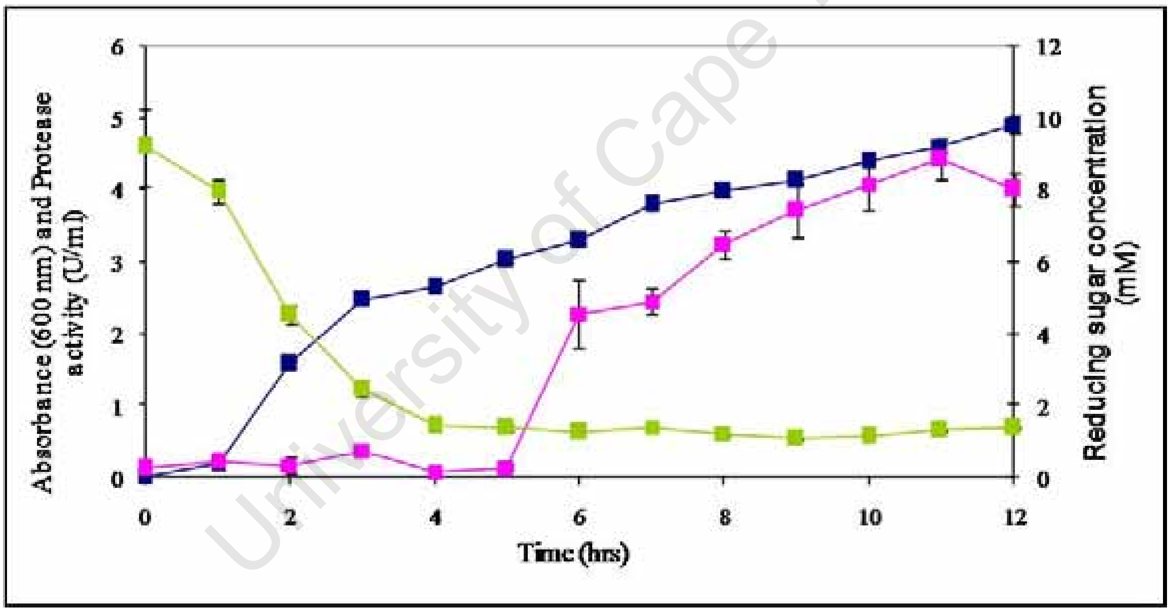


Figure 3.4 The growth and alkaline protease activity profiles of *V. midae* SY9 during shake flask cultivation in P-MBM containing 0.2% glucose at 37 ºC. The cell growth was monitored by absorbance (600 nm) readings (■), while the alkaline protease activity (■) and reducing sugar concentration (■) within the culture supernatant were determined using standard assays. The vertical error bars represent the mean ± standard error.

3.4.2 Zymogram of *V. midae* SY9 extracellular protease activity

The Azo-casein alkaline protease assay of *V. midae* SY9 culture supernatant samples from the growth analysis (Figs. 3.1 to 3.4) provides an indication of the protease activity of the enzyme(s) contained within the supernatant fraction. However, as previously discussed, *Vibrio* species have the ability to secrete a wide variety of extracellular proteolytic enzymes. In order to ascertain the relative contributions of the *V. midae* SY9 extracellular protease(s) to the total extracellular protease activity, the *V. midae* SY9 stationary phase culture supernatant was investigated by SDS-PAGE analysis, denaturing gelatine-SDS-PAGE (Fig. 3.5) and casein SDS-PAGE zymography (Fig. 3.6).

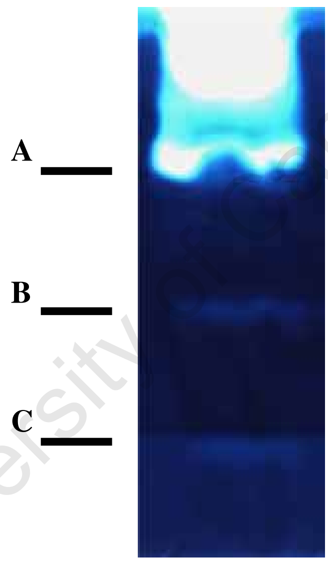


Figure 3.5 Gelatine-SDS-PAGE zymogram of culture supernatant from a *V. midae* SY9 24 hour culture in P-MBM. The relative positions of the three detected proteases A, B and C are indicated by the lines.

V. midae SY9 supernatant from a stationary phase culture had a distinctive extracellular protease activity profile, with three protease bands (A, B and C) detected in the *V. midae* SY9 culture supernatant after growth in P-MBM at 22 ºC (Fig. 3.5). A broad band of proteolytic activity was observed streaking downwards from the top of the gel (Fig. 3.5). This predominant protease (protease A) appears to be a detergent-stable extracellular protease. Two apparently minor proteases (proteases B and C) were also observed on the gelatine zymogram, resulting in fainter bands of protease activity.

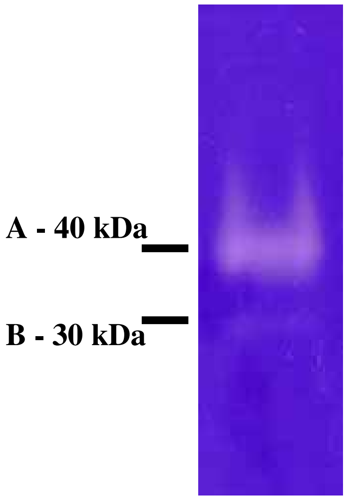


Figure 3.6 Casein-SDS-PAGE zymogram of culture supernatant from a *V. midae* SY9 24 hour culture in P-MBM. The relative positions of the two detected proteases A and B are indicated by the lines and the relative sizes are shown in kDa.

An accurate molecular weight for protease A could not be determined from the gelatine zymogram of *V. midae* SY9 culture supernatant due to the detergent-stable nature of the enzyme. Therefore, in order to obtain a more accurate measurement of the molecular weight of protease A, the culture supernatant was investigated on a casein zymogram (Fig. 3.6). The proteins within the culture supernatant were resolved by SDS-PAGE, before the gel was cut in half. One half was impregnated with casein substrate, and the protease(s) detected by negative staining. Silver staining of the other portion of the SDS-polyacrylamide gel revealed the molecular weight markers, but no protein bands were detected in the *V. midae* SY9 culture supernatant (data not shown). The bacterial supernatant fraction contains very low concentrations of protease protein, and these proteins can usually only be detected using sensitive zymography techniques (Deane, 1989; Kim *et al.,* 2007). Two proteases of approximately 40 and 30 kDa were observed on the casein zymogram (Fig. 3.6). The predominant 40 kDa protease observed on the casein zymogram may be protease A initially identified on the gelatine zymogram

(Fig. 3.5), while the minor 30 kDa protease band (Fig. 3.6) may correspond to protease B (Fig. 3.5).

3.4.3 Characterisation of *V. midae* SY9 extracellular protease activity

The supernatant from a 24 hour *V. midae* SY9 culture was investigated to determine the optimal pH of the *V. midae* SY9 extracellular protease(s). The optimal pH of the *V. midae* SY9 protease containing supernatant was approximately pH 9.0 (Fig. 3.7).

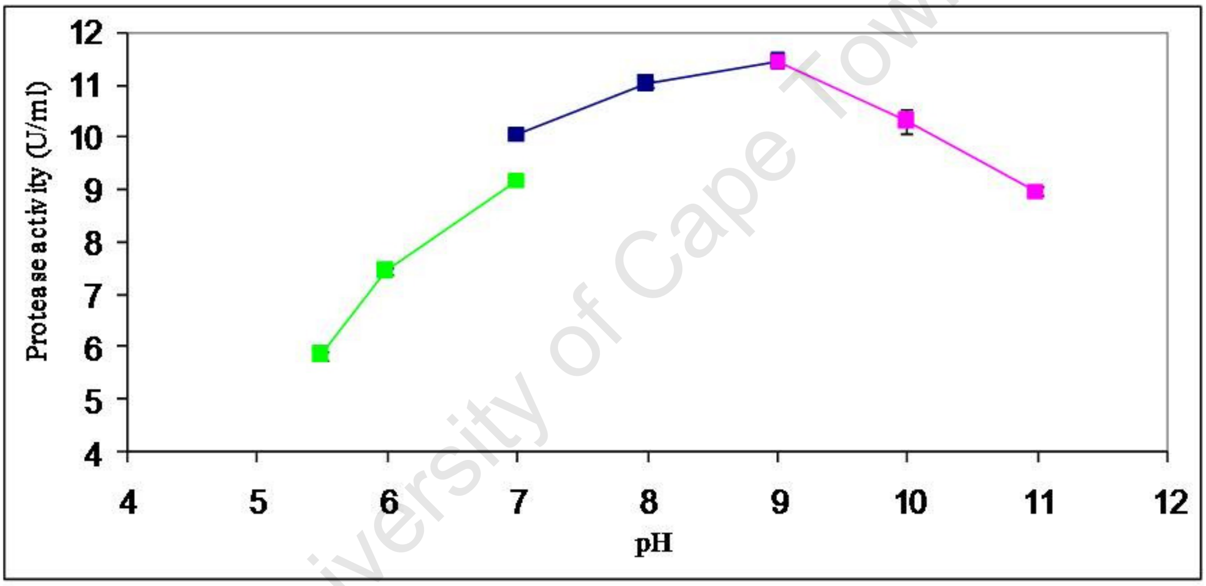


Figure 3.7 Effect of pH on *V. midae* SY9 extracellular protease(s) activity at 37 ºC. Buffers were 100 mM MES (pH 5.5 - 7.0) (■), 100 mM Tris (pH 7.0 - 9.0) (■) and CAPS (pH 9.0 - 11.0) (■). Data represents the mean ± standard error.

The effect of several protease inhibitors on protease activity of *V. midae* SY9 supernatant were investigated in order to further characterise the *V. midae* SY9 extracellular proteases (Fig. 3.8).

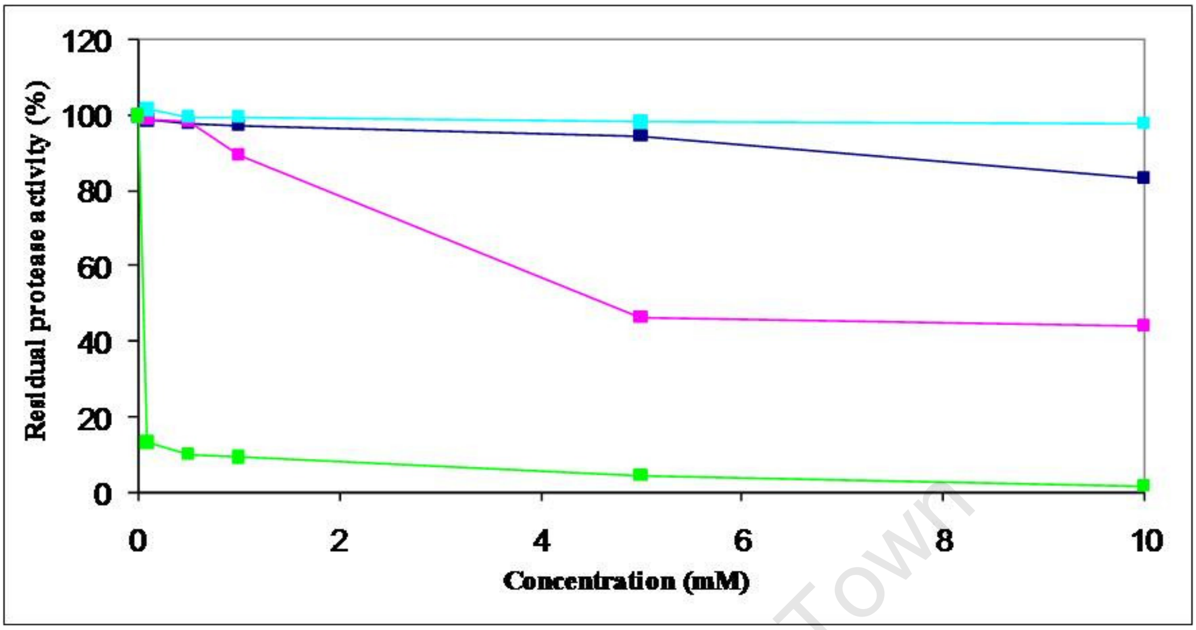


Figure 3.8 Effect of protease inhibitors on the protease activity of *V. midae* SY9 culture supernatant. *V. midae* SY9 supernatant was pre-incubated with increasing concentrations (mM) of 1,10-Phenanthroline (■), EDTA (■), EGTA (■) and PMSF (■). Residual alkaline protease activity was determined as a percentage of an untreated control sample.

Pre-treatment of *V. midae* SY9 enzyme containing supernatant with 1,10-phenanthroline did not affect the residual extracellular protease activity. The divalent metal cation-chelating agent EDTA, caused a slight reduction in the *V. midae* SY9 extracellular protease activity levels (Fig. 3.8). The most significant reduction in residual protease activity, of approximately 16.6%, was observed when *V. midae* SY9 culture supernatant was pre-incubated with 10 mM EDTA. Pre-treatment of *V. midae* SY9 supernatant with the Ca^{2+}-chelating agent EGTA, resulted in significant reductions in residual extracellular protease activity over the range of EGTA concentrations assessed in this study. A 55.7% inhibition of residual *V. midae* SY9 extracellular protease activity was detected when the culture supernatant was pre-treated with 10 mM EGTA. Pre-treatment with the serine protease inhibitor PMSF, resulted in 86.8% (0.1 mM PMSF) to 98.1% (10 mM PMSF) inhibition in the residual protease activity levels (Fig. 3.8).

3.4.4 Purification and characterisation of VmproA from *V. midae* SY9 culture supernatant

In order to determine the optimal ammonium sulphate concentration that would result in the precipitation of most of the protease proteins within the culture supernatant, a preliminary ammonium sulphate precipitation was conducted (Fig. 3.9). Most of the *V. midae* SY9 extracellular protease proteins within the culture supernatant were precipitated between 50 and 90% ammonium sulphate saturation. There was very little protease detected within the 30% ammonium sulphate precipitate.

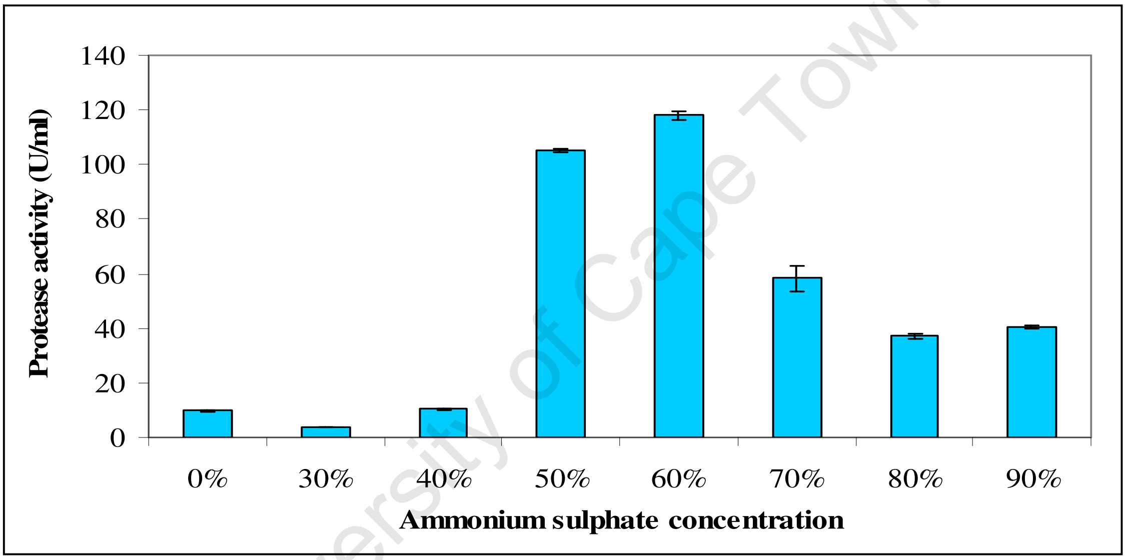


Figure 3.9 Protease activity of precipitated and dialysed ammonium sulphate protein fractions of *V. midae* SY9 culture supernatant. Data represents the mean ± standard error.

Therefore, to reduce particulate matter, contaminating large aggregates of proteins and denatured proteins that would be co-precipitated and carried through from the bulk harvest of *V. midae* SY9 extracellular proteins to the subsequent fine fractionation steps, a 30% ammonium sulphate saturation cut-off was employed as a first cut of the *V. midae* SY9 culture supernatant.

A total of 12 liters of culture supernatant obtained from a 24 hour *V. midae* SY9 P-MBM culture was initially cut with 30% ammonium sulphate. The protein precipitate was pelleted by centrifugation and discarded, while the supernatant fraction was retained. The supernatant fraction was saturated with solid ammonium sulphate to 90% ammonium sulphate and the *V. midae* SY9 protease protein containing precipitate harvested. The protein precipitate was resuspended in 50 mM Tris-HCl, pH 9.0, and dialysed against the same buffer to remove any contaminating salts, before being passed through a DE-52 ion-exchange column (Fig. 3.10).

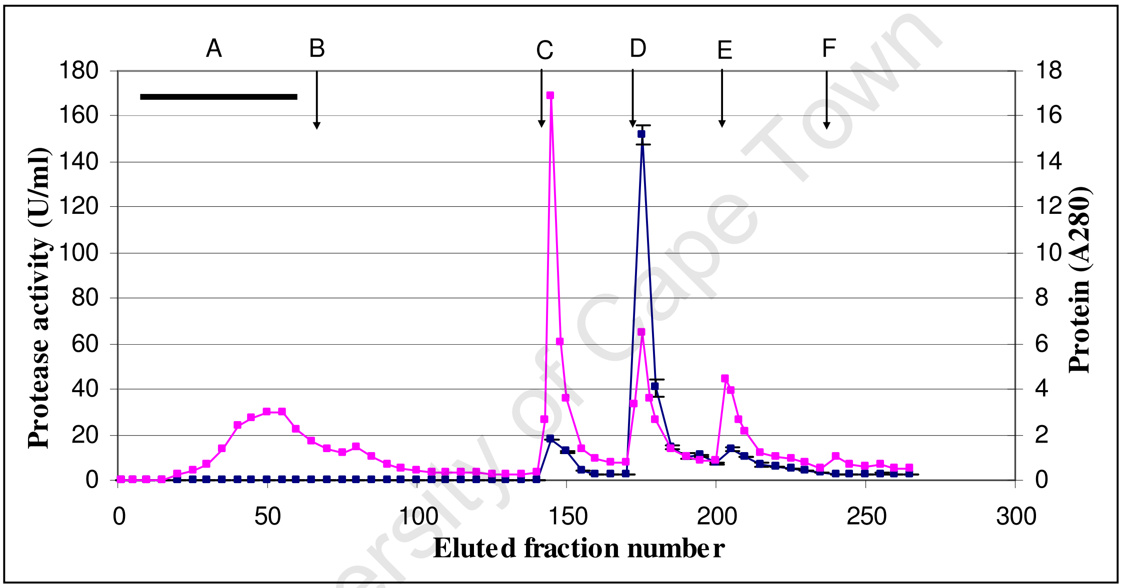


Figure 3.10 Elution profile of *V. midae* SY9 culture supernatant that had been bulk purified by ammonium sulphate precipitation and passed through a DE-52 ion-exchange column. A, Loading the column with the active concentrate; B, washing with 50 mM Tris-HCl, pH 9.0; C, 1st elution (50 mM NaCl); D, 2nd elution (100 mM NaCl); E, 3rd elution (250 mM NaCl); F, 4th elution (500 mM NaCl). Alkaline protease activity (■) is expressed as units of protease enzyme per ml (U ml^{-1}) at 37 °C and the protein concentration (■) was determined by measuring the absorbance at 280 nm (A_{280}). Data represents the mean ± standard error.

The active fractions (175 to 195) were collected off the DE-52 ion-exchange column (Fig. 3.10), concentrated by Amicon ultrafiltration and subjected to a Sephadex G-100 gel-filtration column (Fig. 3.11). Once again, the active fractions (35 to 55) were collected and concentrated by Amicon ultrafiltration.

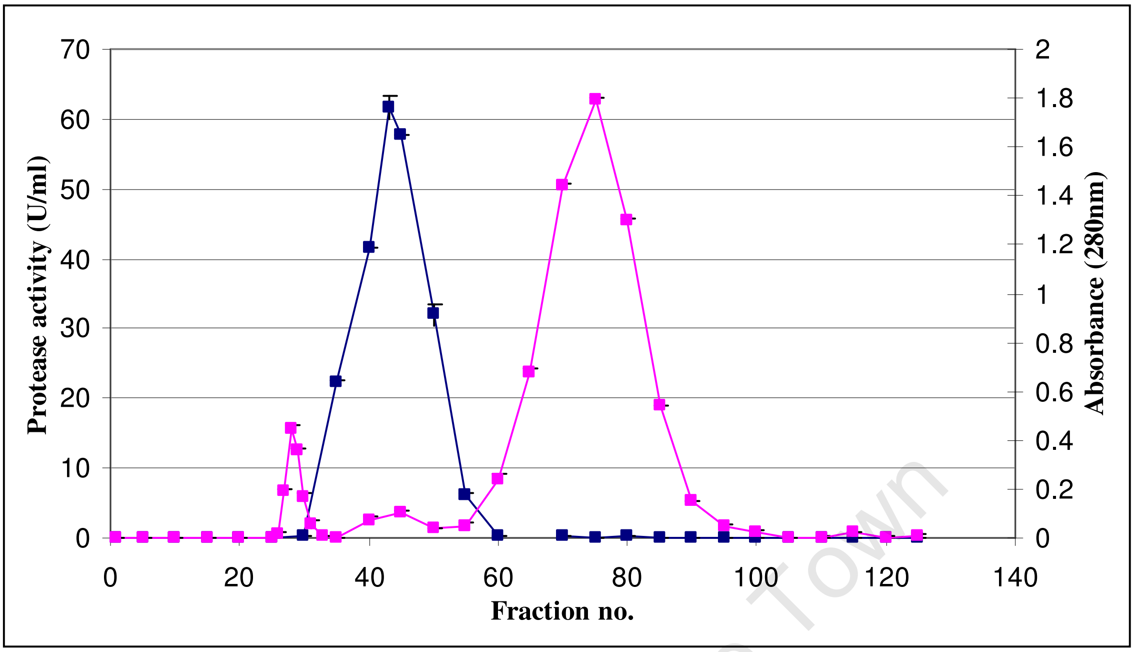


Figure 3.11 Elution profile of *V. midae* SY9 culture supernatant that has been bulk purified by ammonium sulphate precipitation, ion-exchange chromatography and passed through a Sephadex G-100 gel-filtration column. Alkaline protease activity (■) is expressed as units of protease enzyme per ml (U ml^{-1}) at 37 °C and the protein concentration (■) was determined by measuring the absorbance at 280 nm (A_{280}). Data represents the mean ± standard error.

The concentrated culture supernatant bulk purified using ammonium sulphate precipitation and the concentrated active fractions resolved by gel-filtration chromatography were analyzed by SDS-PAGE (Fig. 3.12). A single protein species of approximately 40 kDa was purified from the *V. midae* SY9 culture supernatant (Fig. 3.12 A, lane 2), and was shown to be a protease on a casein (Fig. 3.12 B, lane 2) and a gelatine zymogram (Fig. 3.12 C, lane 1). This corresponds to the size of the predominant, detergent-resistant extracellular protease detected in the supernatant of *V. midae* SY9 (Fig. 3.5 and 3.6). The purified protease protein represented a 4.2% recovery and 28.3-fold purification from the 12 liters of *V. midae* SY9 culture supernatant (Table 3.1).

Table 3.1 Summary of the purification of VmproA

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture supernatant	807.3	38400	47.6	100	1
Ammonium sulphate preparation	115.7	26800	231.6	69.8	4.9
DE-52	17.7	14190	803.1	37.0	16.9
Sephadex G-100	1.2	1600	1347.6	4.2	28.3

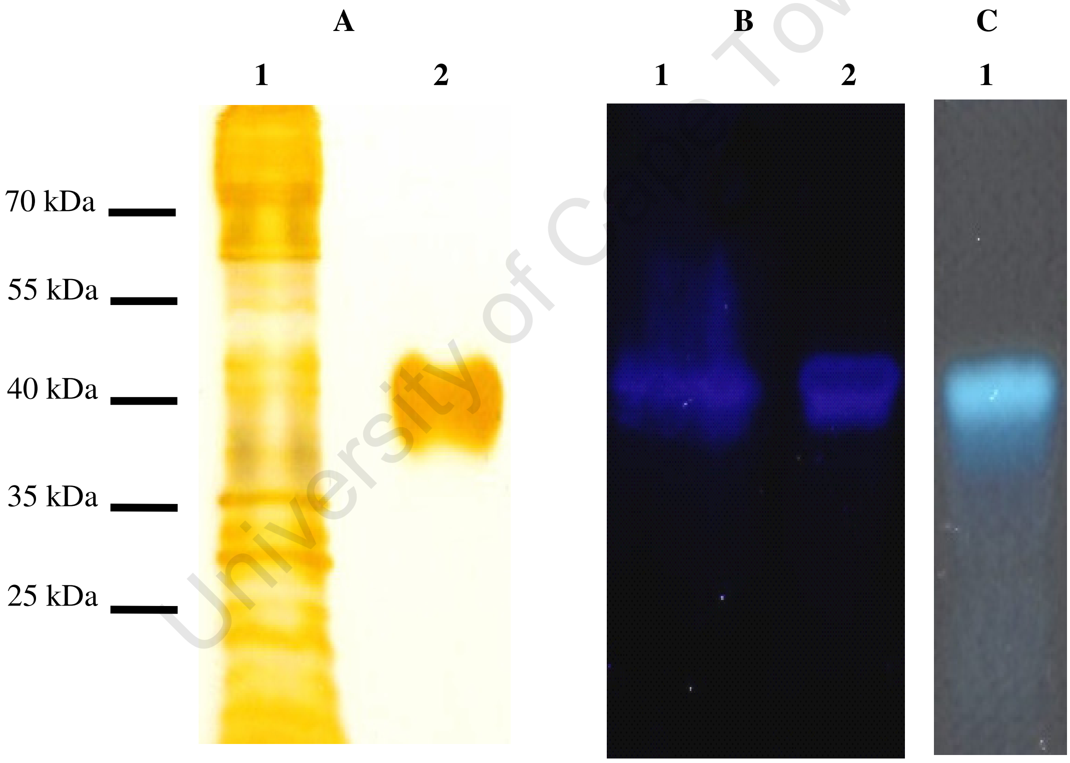


Figure 3.12 SDS-PAGE (A), casein zymogram (B) and gelatine zymogram (C) analysis of the ammonium sulphate concentrated *V. midae* SY9 culture supernatant (A and B, lane 1) and the final concentrated purified VmproA protease protein (A and B, lane 2; C lane 1). The protein bands were detected on the SDS-PAGE gel by silver staining (A), and the corresponding protease activity bands were detected by negative staining with coomassie stain (B) and Amido black (C). The lines represent the approximate sizes, in kDA, and positions of the molecular weight marker.

The protease activity of purified VmproA was significantly (P<0.05) higher between pH 9 and 10 (Fig. 3.13). Therefore, the optimal pH for the *V. midae* SY9 extracellular detergent resistant protease is in the alkaline range, between 9 and 10.

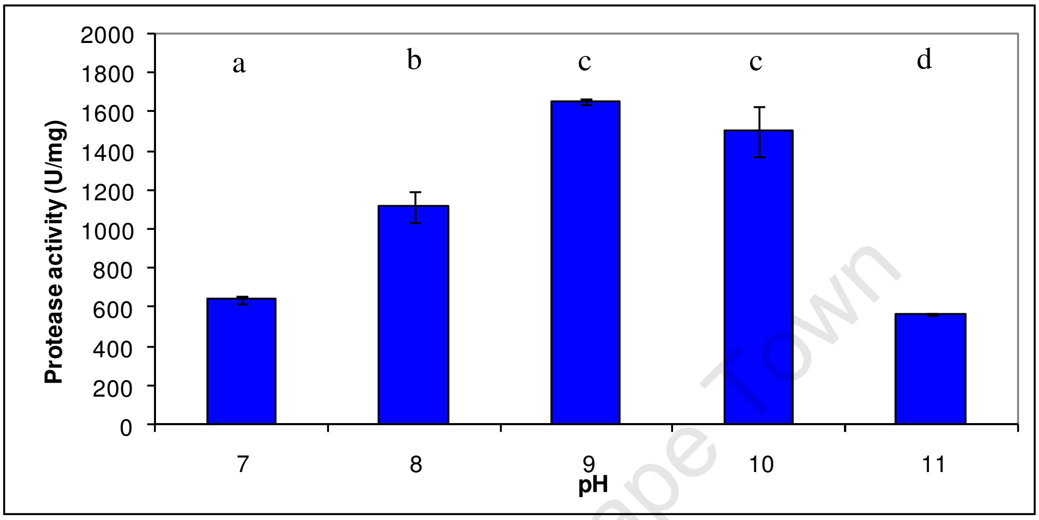


Figure 3.13 The effect of pH on the activity of purified VmproA. Protease activity is expressed as units of protease enzyme per mg of VmproA (U mg^{-1}). Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between sample means.

3.4.5 Polyclonal antibodies raised to purified VmproA

An ELISA was performed to determine which bleed contained the highest titre of anti-VmproA polyclonal antibodies. Serial dilutions of the anti-serum obtained from each bleed were used in conjunction with equal quantities (100 µg) of purified VmproA protein in the ELISA. No signal was detected for the pre-immunization bleed (Fig. 3.14 A), while increasing signal levels indicated increasing amounts of anti-VmproA antibodies were detected in the serum of the subsequent bleeds (Fig. 3.14 B-G). The highest titre of anti-VmproA polyclonal antibodies was detected in the serum obtained from the post-boost (final) bleed (Fig. 3.14 G).

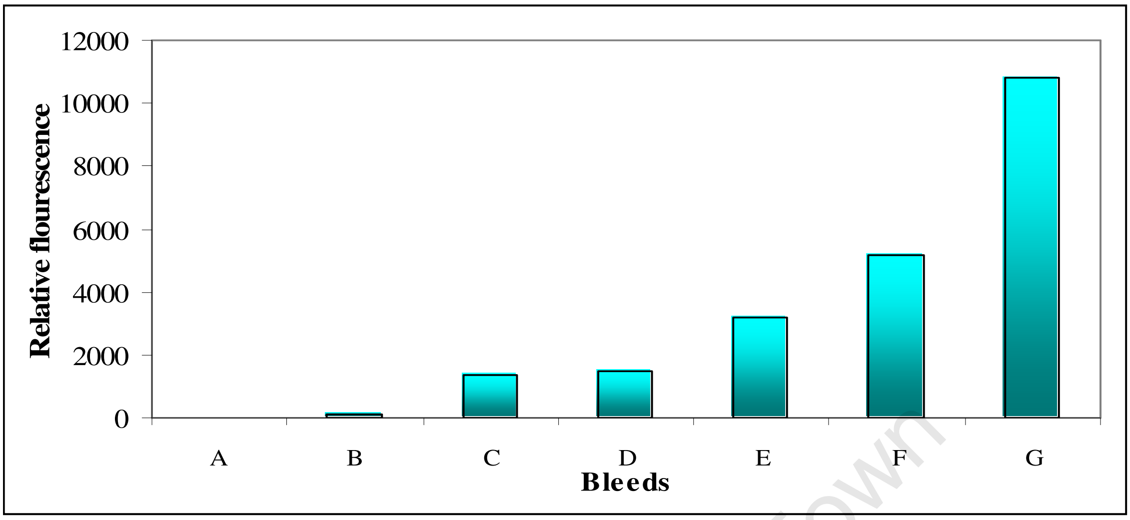


Figure 3.14 An ELISA performed to determine which bleed (A to G) had the highest titre of anti-VmproA polyclonal antibodies. A) Pre-immunization bleed; B) Post-immunization bleed 1; C) Post-immunization bleed 2; D) Post-immunization bleed 3; E) Post-immunization bleed 4; F) Pre-boost bleed; and G) Post-boost (final) bleed. An equal (100 µg) amount of purified VmproA was used to coat the wells of the ELISA microtitre plate. The ELISA was read on a TitreTek Multiscan Machine (405 nm) and after taking the dilution factor into account, converted to a relative fluorescence value.

The specificity of the anti-VmproA polyclonal antibodies was assessed by western blot analysis (Fig. 3.15). The soluble intracellular and extracellular protein fractions of *E. coli* JM109 (pMR11), the extracellular protein fraction of *V. midae* SY9 and the purified VmproA protein were separated on a 8% SDS-PAGE before being transferred to nitrocellulose membrane and subjected to western blot analysis using the post-boost (final) bleed anti-serum.

Western blot analysis showed that the anti-VmproA polyclonal antibodies recognized and bound to purified VmproA (Fig. 3.15, lane 5). The anti-VmproA antibodies also bound to VmproA within the culture supernatant (Fig. 3.15, lane 3) of *V. midae* SY9 cultivated in P-MBM. Anti-VmproA antibodies identified the *V. midae* SY9 detergent resistant protease protein within the intracellular fraction of *E. coli* JM109 harbouring the

V. midae SY9 protease clone, pMR11 (Chapter 2; Fig. 3.15, lane 2). However, anti-VmproA did not recognize or bind to proteins in the extracellular fraction of *E. coli* JM109 (pMR11) (Fig. 3.15, lane 1).

1 2 3 4 5

Figure 3.15 Western blot analysis of the specificity of polyclonal antibodies raised to VmproA. Lane 1, ammonium sulphate precipitated extracellular proteins of *E. coli* JM109 (pMR11); lane 2, soluble intracellular proteins of *E. coli* JM109 (pMR11); lane 3, ammonium sulphate precipitated extracellular proteins of *V. midae* SY9; lane 4, empty and lane 5, purified VmProA. A total of 10 µg of each of the ammonium sulphate and soluble cell extract protein samples, and 150 ng of purified VmproA, were loaded on the polyacrylamide gel.

3.5 Discussion

V. midae SY9 cell growth and regulation of extracellular protease production was investigated in different growth media and at two different culture temperatures. When *V. midae* SY9 was cultured in P-MBM the extracellular protease activity was evident towards the latter stages of exponential growth. This may indicate that expression of *V. midae* SY9 extracellular alkaline proteases may be growth phase dependent. Repression of extracellular enzyme production during the exponential growth phase is a feature of many extracellular enzymes (Pollock, 1962), of which, extracellular proteases are no exception (Watanabe *et al.,* 2004).

V. midae SY9 was observed to grow at increased growth rates under increasing culture incubation temperatures and when cultured in growth media containing casamino acids and glucose, as compared to the observed growth rate of *V. midae* SY9 at 22 ºC in P-MBM. Extracellular protease activity was detected in the supernatant of *V. midae* SY9 cultivated in an inducing growth medium at incubation temperatures of both 22 and 37 ºC. While the growth rate of *V. midae* SY9 was increased by increasing the incubation temperature, the observed extracellular protease activity was lower at 37 ºC than at 22 ºC. Similarly, expression of the extracellular proteolytic enzymes secreted by a *V. alginolyticus* strain were affected by increased incubation temperature (Deane *et al.*, 1987b). Synthesis of two minor extracellular proteases was lowered when the *V. alginolyticus* strain was cultured at 37 ºC.

The presence and concentrations of specific nutrients within the microbial extracellular environment play a key role in controlling the production of extracellular proteins in terms of both induction and repression of these proteins. Repression of extracellular protein production is often controlled by end-product inhibition and catabolite repression (Glenn, 1976). In particular, extracellular protease production has in many cases been shown to be influenced by various carbon sources, especially by high concentrations of glucose in the case of catabolite repression (Hanlon and Hodges, 1981) and readily metabolizable nitrogen sources in the case of end-product repression (Glenn, 1976; Hare *et al*., 1981). The lack of detectable extracellular protease activity when *V. midae* SY9 was cultivated in marine broth may be due to the presence of casamino acids and/or glucose within the culture medium, suggesting that synthesis of *V. midae* SY9 extracellular protease(s) may be regulated by catabolite and/or end-product repression. Gene expression studies using real-time RT-PCR *in vitro* and *in vivo* could be performed in order to further identify and confirm the *V. midae* SY9 extracellular protease regulatory mechanisms. In particular, *in vivo* investigation of the regulation and expression of *V. midae* SY9 extracellular protease(s) within the digestive tract of cultured *H. midae* would provide invaluable information to researchers investigating the role of *V. midae* SY9 extracellular protease(s) in the observed probiotic effect on cultured abalone (Macey and Coyne, 2005).

Zymography is a commonly employed method to detect and investigate proteolytic enzymes (Deane *et al.,* 1987a; Deane *et al.,* 1987b; Raser *et al.,* 1995; Shi *et al.,* 1997; Thangam and Rajkumar, 2002; Venugopal and Saramma, 2006; Kim *et al.,* 2007; Chougule *et al.,* 2008). Gelatine zymogram analysis of cell-free culture supernatant of *V. midae* SY9 grown in a protease inducing growth medium (P-MBM), resulted in the detection of three extracellular proteases. The predominant protease, designated protease A, produced a broad band of streaking when electrophoresed through the gelatine-SDS-PAGE zymogram. Similarly, *V. alginolyticus* (Deane *et al.,* 1987a; Deane *et al.,* 1987b) and *V. metschnikovii* (Kwon *et al.,* 1995) have been shown to produce proteases capable of hydrolysing gelatine in the presence of a denaturing surfactant while being electrophoresed through a polyacrylamide gel. Since a similar pattern was observed for *V. midae* SY9 culture supernatant it has been concluded that the broad band of streaking was due to proteolytic digestion of the co-polymerised gelatine substrate as the proteases were electrophoresed through the gelatine-SDS-polyacrylamide gels. Based on the high degree of identity between VmproA and the *V. alginolyticus* detergent-resistant alkaline serine exoprotease, ProA, it was previously hypothesized (Chapter 2) that *vmproA* encodes an alkaline detergent-stable extracellular serine protease. Hence, VmproA is likely to be the detergent-stable protease A.

The predominant 40 kDa protease detected on the casein zymogram of *V. midae* SY9 culture supernatant may represent the detergent-resistant protease A, and therefore VmproA, observed on the gelatine zymogram, while the 30 kDa protease detected on the casein zymogram may represent protease B. The minor protease, designated protease C, was only detected on the very sensitive gelatine-SDS-PAGE zymogram. Gelatine-zymography was utilised by Kim *et al.* (2007) to detect *V. vulnificus* extracellular protease VvpE more efficiently and sensitively than other methods, including skim milk zymography. Gelatine-zymography indicates that protease A is the predominant extracellular alkaline protease within the *V. midae* SY9 supernatant when cultured under these growth conditions, and that the two minor proteases contribute a significantly lower proportion of the observed extracellular protease activity. The production and secretion of multiple extracellular proteases by *V. midae* SY9 is not unexpected, as several *Vibrio* species have been observed to produce and secrete multiple extracellular proteases (Long *et al.,* 1981; Farrell and Crosa, 1991; Kwon *et al.,* 1992; Wang *et al.,* 2007). Of these *Vibrio* species, the predominant extracellular SDS-resistant alkaline proteases of *V. alginolyticus* (Deane *et al.,* 1987b) and *V. metschnikovii* (Kwon *et al.,* 1995) have been cloned and expressed in *E. coli* HB101.

The nucleotide sequence of the *V. midae* SY9 *vmproA* gene encodes a predicted 56 kDa protein (Chapter 2). However, the apparent molecular mass of the mature VmproA was shown to be approximately 40 kDa from a casein zymogram. Based on the deduced amino acid sequence of VmproA and the three conserved domains within the protease protein (Chapter 2), VmproA possibly represents a protein with the signal sequence cleaved off and processed N- and/or C-terminal domains. A subtilase gene isolated from *Vibrio* strain PA44 encoded a 530 amino acid precursor protein with a predicted molecular mass of 55.7 kDa (Arnórsdóttir *et al.,* 2002). However, the active enzyme was processed to remove a 139 amino acid N-terminal pro-sequence, and had a molecular weight of 40.6 kDa. The *Vibrio* strain PA44 subtilase could be further processed to produce a fully active 29.7 kDa proteolytic enzyme. Therefore, cleavage of the C-terminal domain and the N-terminal domain from the VmproA peptide could result in an active protease protein, as seen on the casein zymogram, with a predicted molecular mass of between 39.4 -

41.0 kDa, depending on the position of the cleavage site between the subtilisin N-terminal and catalytic domains. However, the position of the cleavage site would need to be confirmed by peptide sequencing of the *V. midae* SY9 pre-pro-peptide and the VmproA active protease protein.

Based on gelatine zymogram analysis of culture supernatant from *V. midae* SY9 cultivated in protease inducing growth media, it is apparent that *V. midae* SY9 secretes at least three extracellular proteases, of which VmproA is the major protease. In order to identify the genes responsible for the observed minor extracellular proteases, future work may involve screening the previously constructed *V. midae* SY9 genomic library (Taylor, 2002) for additional extracellular protease clones.

The proteolytic enzymes within the supernatant of *V. midae* SY9 cultured in a protease-inducing growth medium were investigated to gain a better understanding of the optimal reaction conditions and facilitate the purification of VmproA. The secreted *V. midae* SY9 extracellular proteases were active across a neutral to alkaline pH range, however, the optimal reaction pH was 9.0.

The loss of protease activity following EDTA and EGTA treatment indicates that the *V. midae* SY9 extracellular protease(s) require divalent metal cations as co-factors for activity. The enzyme activity of a purified *V. metschnikovii* DL 33-51 alkaline protease was similarly decreased by 40% following treatment with 2 mM EDTA (Mei and Jiang, 2005). In the same way, a purified *V. anguillarum* extracellular protease was substantially inhibited following pre-treatment with EDTA (Inamura *et al.,* 1985). Inhibition following treatment with metal chelating agents, such as EDTA and EGTA, is considered indicative of metalloproteases (Coleman and Vallee, 1960) which require metal ions as co-factors for enzyme activity. However, EDTA and EGTA treatment may result in inhibition of subtilisin-like proteases, as many of these enzymes bind calcium-ions for stability. *V. midae* SY9 extracellular protease activity was unaffected by treatment with *o*-phenanthroline which is a metalloprotease inhibitor (McConn *et al.,* 1964) and was significantly inhibited by the serine protease inhibitor PMSF. Lee *et al.* (1997) classified a partially purified *V. alginolyticus* extracellular proteolytic enzyme as an alkaline serine protease on the basis that it had optimal activity at pH 10 and was strongly inhibited by PMSF. Therefore, I propose that the extracellular protease VmproA responsible for the majority of the alkaline protease activity observed in the extracellular fraction of *V. midae* SY9 is a serine protease.

The *V. midae* SY9 secreted detergent resistant protease was purified 28.3-fold from culture supernatant to apparent homogeneity by a combination of ammonium sulphate precipitation, ion-exchange chromatography and gel-filtration chromatography. Inamura *et al.* (1985) purified a *V. anguillarum* extracellular protease 2.3-fold, with a purification yield of 18%, using a combination of ammonium sulphate precipitation and gel-filtration chromatography. The purification yield achieved by Inamura *et al.* (1985) was higher than that achieved in the current study. VmproA purification was performed at 4 ºC in an attempt to reduce and/or prevent autocatalytic degradation, particularly as the purification procedure involved an additional ion-exchange chromatographic step that was not employed by Inamura *et al.* (1985). However, despite performing the purification at 4 ºC, the lower purification yield achieved in this study may have been the result of loss of protease proteins during the precipitation and concentration steps and/or autocatalytic degradation of VmproA during the course of the purification procedure.

The molecular weight of the purified protease VmproA was approximately 40 kDa on a SDS-PAGE and a casein-zymogram which corresponds to the size of the predominant extracellular protease detected in the culture supernatant. The molecular mass is slightly above that of microbial alkaline proteases which are generally within the range of 15-36 kDa (Beg and Gupta, 2003). The reported molecular weights of Subtilisin Carlsberg from *B. licheniformis* (Jacobs *et al.,* 1985) and Subtilisin BPN' produced by *B. amyloliquifaciens* (Wells *et al.,* 1983) are 27.3 and 27.5 kDa, respectively. However, higher molecular weight microbial extracellular alkaline proteases have been identified, including a 45, a 54 and a 90 kDa protease produced and secreted by *V. metschnikovii* strain RH530 (Kwon *et al.,* 1994), *V. alginolyticus* (Deane *et al.,* 1987b) and *B. subtilis* (*natto*) (Kato *et al.,* 1992), respectively. Similar to VmproA, both the 45 kDa proteolytic enzyme VapT and the 54 kDa protease ProA produced and secreted by *V. metschnikovii* strain RH530 (Kwon *et al.,* 1994) and *V. alginolyticus* (Deane *et al.,* 1987b), respectively, are SDS-stable extracellular proteolytic enzymes.

Purified VmproA was observed to have an optimal pH profile of between 9 and 10. Therefore, VmproA is an alkaline serine protease as the optimal pH range of alkaline proteases is generally between 8.0 and 11.0 (Durham *et al.,* 1987; Kumar *et al.,* 1999; Beg and Gupta, 2003).

The polyclonal antibodies raised to the extracellular alkaline serine protease VmproA, purified from *V. midae* SY9, specifically cross-reacted with the 40 kDa purified VmproA and VmproA secreted by *V. midae* SY9. The 40 kDa band was the only protein detected within the ammonium sulphate concentrated fraction of *V. midae* SY9 culture supernatant. Therefore, the anti-VmproA polyclonal antibodies do not cross-react with the two other proteases that were observed to be produced and secreted by *V. midae* SY9 on a gelatine zymogram. The anti-VmproA polyclonal antibodies also cross-reacted with the intracellular fraction of *E. coli* JM109 harbouring the recombinant plasmid pMR11 which contains the cloned *vmproA* gene. However, anti-VmproA polyclonal antibodies did not recognize any proteins within the ammonium sulphate concentrated fraction of *E. coli* JM109 (pMR11) culture supernatant. Häse and Finkelstein (1991) observed that *E. coli* harbouring a recombinant plasmid containing a cloned *V. cholerae hap* gene did not recognize the *V. cholerae* transcription and/or translation signals as it did not display an extracellular proteolytic phenotype or display specific bands on western blots. Although many *Vibrio* genes encoding extracellular proteins have been cloned into *E. coli* strains, many of the proteins encoded by these cloned genes were not actively secreted and instead accumulated within the *E. coli* cells (Pearson and Mekalanos, 1982; Mercurio and Manning, 1985; Nishibuchi and Kaper, 1985; Taniguchi *et al.,* 1985; Howard and Buckley, 1986; Focareta and Manning, 1987). It has been suggested that *Vibrio* species may have a specific protein secretory mechanism that is not found in *E. coli* (Ichige *et al.,* 1988). As VmproA was only detected by western blot analysis within the *E. coli* (pMR11) intracellular protein fraction, the extracellular proteolytic phenotype observed for *E. coli* JM109 harbouring pMR11 and pProH3 (Chapter 2) may not have been the result of active secretion of the cloned *V. midae* SY9 protease. Instead, the observed extracellular phenotype of these *E. coli* JM109 transformants may be due to intracellular accumulation of VmproA. This may have resulted in the cell membrane of *E. coli* JM109 cells becoming compromised and 'leaky', leading to release of protease protein in an uncontrolled manner and hydrolysis of the skim milk within the solid growth medium surrounding the *E. coli* JM109 (pMR11) and *E. coli* JM109 (pProH3) colonies (Chapter 2).

Purification of VmproA from *V. midae* SY9 culture supernatant has enabled further biochemical characterisation of this proteolytic enzyme as an alkaline protease, and has complemented the data obtained from bioinformatic analysis of the sequenced *vmproA* gene (Chapter 2). The subsequent raising of specific polyclonal antibodies to the purified protein has provided a valuable molecular tool that will be employed (Chapter 6) to investigate the presence and location of VmproA within regions of the digestive tract of juvenile *H. midae* fed *V. midae* SY9 supplemented ABFEED® S34.

CHAPTER 4

CONSTRUCTION AND CHARACTERISATION OF THE *VMPROA* MUTANTS, *VIBRIO MIDAE* SY9PRO2 AND *VIBRIO MIDAE* SY9MUT2

CONTENTS

4.1 Summary

The *V. midae* SY9 detergent resistant extracellular protease, VmproA, may play a role in the previously observed growth advantage of cultured abalone following probiotic dietary supplementation, by improving the protein digestion efficiency of the abalone host. Therefore, the detergent-stable protease gene, *vmproA*, was targeted for gene mutagenesis, through *vmproA* gene duplication and disruption, using recombinant pGP704 suicide vectors. The *vmproA* gene was duplicated within the *V. midae* SY9 genome by recombination of the recombinant suicide vector pGPro.Cm into the *vmproA* gene. The *vmproA* gene was insertionally inactivated by integration of the recombinant suicide vector pGP13.Cm into the *vmproA* gene within the *V. midae* SY9 genome. The *V. midae* SY9 insertionally inactivated mutant was complemented by integration of the recombinant suicide vector pGPro.Km, containing a full-length copy of *vmproA*, into the chromosome of the *V. midae* SY9 mutant strain. The *vmproA* gene duplication, disruption, and complementation did not significantly influence the growth of the mutant strains in comparison to *V. midae* SY9. The *vmproA* gene duplication mutant had a slightly lower level of extracellular protease activity compared to the wild-type strain when cultivated in a minimal medium, an equivalent level of extracellular protease activity when cultured in P-MBM, and significantly increased protease activity when cultured in minimal medium supplemented with peptone as a protein source. The extracellular protease activity of the *vmproA* mutant was significantly reduced in comparison with that of the wild-type and *vmproA* gene duplication mutant strains, when cultured in all of the growth media assessed in this investigation. The extracellular protease activity of the complemented mutant strain was equivalent to that of the wild-type and *vmproA* duplication strains, depending on the growth medium. Gelatine and casein zymogram analysis of cell-free supernatant from *V. midae* SY9 and the *vmproA* gene duplication, disruption and complementation mutants showed that chromosomal duplication of *vmproA* did not affect the extracellular protease phenotype in comparison to the wild-type strain and that the predominant 40 kDa protease was still produced. Insertional inactivation of *vmproA* resulted in a loss in the production and secretion of the distinctive detergent-stable 40 kDa extracellular protease, VmproA, on both the gelatine

and casein zymograms. Casein zymogram analysis of the culture supernatant from the insertional inactivation mutant showed the presence of at least five minor extracellular proteases of approximately 34, 30, 28, 17, and 10 kDa. Complementation of the insertional inactivation mutant resulted in the production and secretion of the distinctive detergent-stable 40 kDa extracellular protease, VmproA, on both the gelatine and casein zymograms. A minor protease of approximately 30 kDa was also detected within the culture supernatant of the complemented mutant strain on the casein zymogram.

4.2 Introduction

Probiotics are believed to function in several ways within aquaculture environments, including through the secretion of extracellular enzymes that contribute to, and improve the digestion efficiency of the host organism (Verschuere *et al.*, 2000). It has been shown that *Haliotis midae* fed a high protein artificial diet supplemented with a mixture of probiotics, including *Vibrio midae* SY9, have increased growth rates when compared to abalone that were fed the same artificial diet but lacking the probiotic strain (Macey and Coyne, 2005). The abalone probiotic, *V. midae* SY9, produces and secretes high levels of extracellular proteases, with the predominant protease being identified as an extracellular detergent resistant alkaline serine protease.

The construction of mutations in bacteria, through reverse genetics, is a fundamental approach to understanding the function of prokaryotic genes at a molecular level (Hensel and Holden, 1996; Reyrat *et al.,* 1998). Mutation of prokaryotic genes, including protease genes, through insertional inactivation is a common strategy employed to genetically manipulate bacterial strains (Häse and Finkelstein, 1991). The construction of these mutant bacterial strains enables researchers to investigate the specific function of the mutated gene by direct comparisons of the mutant and wild-type strains. Suicide vectors are classically used for targeted gene disruption (Fig. 4. 1), and have been used for the construction of mutant strains in a wide variety of bacterial species (Miller and Mekalanos, 1988; Mintz *et al.,* 2002; Spory *et al.,* 2002; Tannock *et al.,* 2005; Walter *et al.,* 2005; Raychaudhuri *et al.,* 2006; Le Roux *et al.,* 2007; Schwab *et al.,* 2007). The main features of suicide vectors are that they are easily transferred to a variety of bacterial strains, and once transferred, are unable to replicate in the recipient strain. The transferred suicide vector either integrates (via homologous recombination; Fig. 4. 1A and B) a duplicate, or inactivated, copy of the target gene into the chromosome of the recipient strain under a selective pressure (Fig. 4. 1C) or the suicide vector does not integrate and is lost (Maloy *et al.,* 1996).

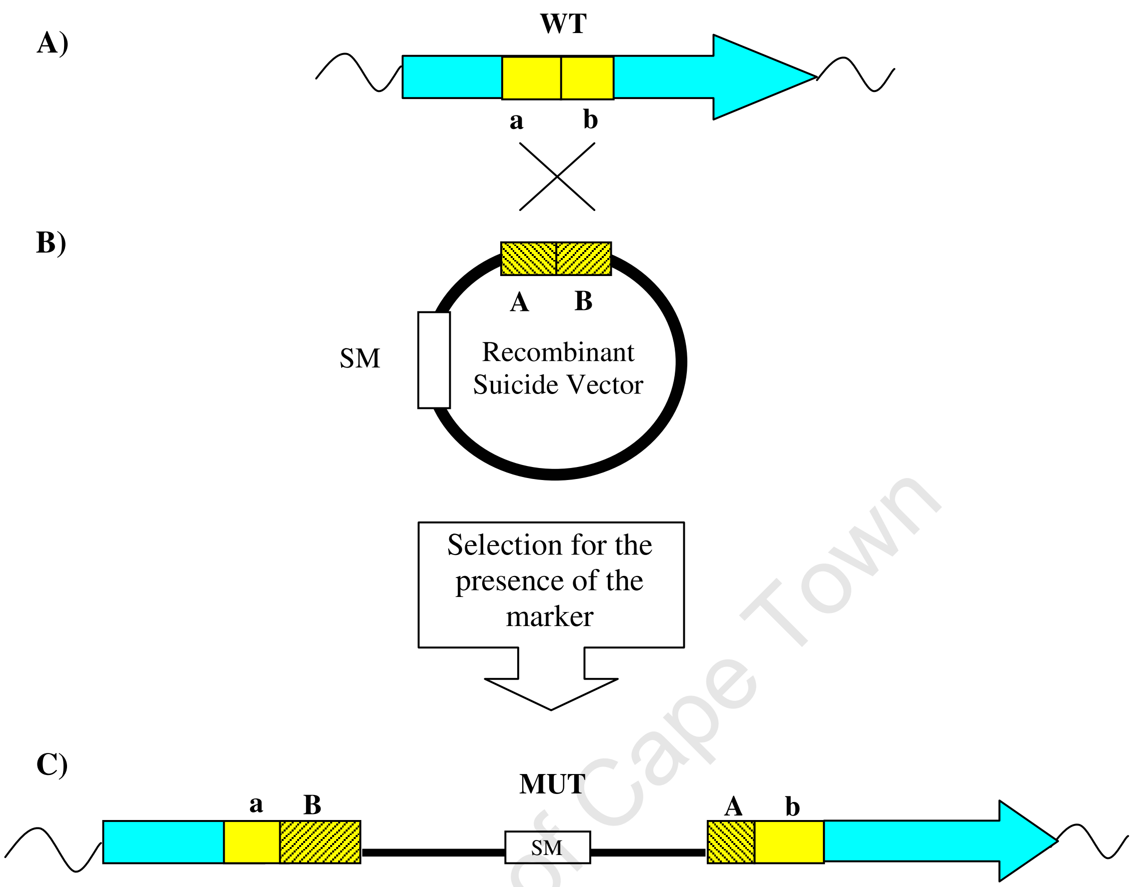


Figure 4.1 Schematic representation (not to scale) depicting homologous recombination of a recombinant suicide vector into the chromosome of a target prokaryotic microorganism. A chromosomal copy of the wild-type allele (WT) of the gene of interest (turquoise arrow) (A) is interrupted by homologous recombination (intermolecular) of a recombinant suicide vector (thick black line) harbouring an internal gene fragment (yellow hatched block) and a selectable marker (SM; white block) (B). The integration of the suicide vector into the homologous region of the gene fragment (yellow block) in the chromosome (wavy lines) interrupts the wild-type allele (WT) of the gene of interest, resulting in the formation of a mutated allele (MUT) of the gene (C). Figure adapted from Reyrat *et al.* (1998).

The pGP704 suicide vector system (Miller and Mekalanos, 1988) has been used successfully to study gene function in *Vibrio cholerae* strains (Paul, 2006; Raychaudhuri *et al.*, 2006), as well as, in other bacterial strains (Mintz *et al.*, 2002). The pGP704 suicide vector is a derivative of the pBR322 cloning vector, with the pBR322 origin of replication (oriE1) having been replaced by the R6K origin of replication (oriR6K). The oriR6K requires the π-protein that is encoded by the *pir* gene, and therefore, pGP704 is unable to replicate in the absence of the π-protein. Specific host *E. coli* strains, such as *E. coli* SM10λpir and *E. coli* SY327λpir, harbour a prophage (λpir) that carries and expresses a copy of the *pir* gene, allowing pGP704 to replicate and to be manipulated within these strains. The suicide vector pGP704 also carries an RP4 mobilization fragment. The RP4 mobilization fragment enables pGP704 to be mobilized by the host/donor *E. coli* SM10λpir strain, containing a chromosomal copy of a RP4 plasmid, and conjugated to the target/recipient bacterial strain (Maloy *et al.,* 1996). Once transferred to the recipient strain, the recombinant pGP704 suicide vector, harbouring an internal fragment of the gene of interest, is unable to replicate as the recipient lacks the *pir* gene and its gene product the π-protein. An appropriate antibiotic is then used in a single selection step to identify recipient strains that have undergone an integration event, resulting in the recombinant suicide vector stably integrating via the homologous internal gene fragment into the chromosome (Fig. 4.1) (Maloy *et al.,* 1996; Reyrat *et al.,* 1998).

Increased expression mutant strains can be constructed through exposure of the wild type organism to chemical and/or DNA-damaging mutagenic agents. A mutant strain, *V. metschnikovii* DL 33-51 (Accession no.: M204008), producing a 4.74-fold increase in protease enzyme activity was isolated following chemical and UV mutagenesis of the *V. metschnikovii* DL 33 wild type strain (Mei and Jiang, 2005). However, the major concern regarding this approach to construction of an increased expression mutant is that this method may introduce additional random mutations within the genome that one is not able to initially detect and characterise. The effect of random mutations, as may be expected following chemical and UV mutagenesis, may not be a significant consideration in a study investigating increased enzyme activity and possible industrial strain improvement. However, the objectives of this study are to investigate the role of *vmproA* and its gene product VmproA, in conferring the previously observed growth advantage on cultured abalone (Macey and Coyne, 2005) and any other mutations that may be introduced by chemical mutagenesis would not be ideal.

4.2.1 Aim of this chapter

The aim of this chapter is to construct a *Vibrio midae* SY9 strain capable of increased extracellular protease activity and a *V. midae* SY9 strain unable to produce the predominant detergent stable extracellular protease, VmproA, by means of recombinant suicide vector(s). A *V. midae* SY9 strain harbouring a chromosomal *vmproA* gene-duplication will be constructed and assessed as a mutant strain capable of increased extracellular protease activity. A *vmproA* insertionally inactivated *V. midae* SY9 mutant strain, unable to produce the distinctive detergent stable VmproA protease, will also be constructed. The insertionally inactivated mutant will be complemented through chromosomal integration of a full-length and functional copy of *vmproA*. The *V. midae* SY9 mutant strains and the complemented mutant strain will be used, in conjunction with *V. midae* SY9, to investigate the role of *vmproA* and its gene product (VmproA) on *V. midae* SY9 cell growth and extracellular protease activity. The *V. midae* SY9 mutant strains will be employed in an abalone growth trial (Chapter 5) in order to gain a better understanding of the role of the *V. midae* SY9 *vmproA* protease gene in the previously observed probiotic effect.

4.3 Materials and methods

All of the growth media and solutions used in this study are listed in Appendix A.

4.3.1 Bacterial strains and plasmids

All of the bacterial strains and plasmids used in this study are listed in Tables 4.1 and 4.2, respectively.

Table 4.1 Bacterial strains used in this study

Strain	Genotype/relevant characteristic(s)[a]	Reference
Vibrio midae SY9	Isolated from the digestive tract of *H. midae*, South Africa	Macey and Coyne (2005)
Vibrio midae SY9Sm^r	Sm^r strain of *V. midae* SY9	This study
Vibrio midae SY9Mut2	*V. midae* SY9 strain harbouring the integrated recombinant suicide vector, pGP22.Cm; Cm^r	This study
Vibrio midae SY9Pro2	*V. midae* SY9 strain harbouring the integrated recombinant suicide vector, pGPro.Cm; Cm^r	This study
Vibrio midae SY9Mut2.Comp	*Vibrio midae* SY9Mut2 strain harbouring the integrated recombinant suicide vector, pGPro.Km; Cm^r and Km^r	This study
Escherichia coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F'(traD36 proAB' lacI^q lacZΔM15)	Sambrook *et al*. (1989)
Escherichia coli SM10λpir	*thi thr leu ton*A *lac*Y *sup*E (λpir) *rec*A::RP4-2-Tc::Mu; Km^r	Simon *et al.* (1983)
Escherichia coli SY327λpir	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56* (λpir)	Miller and Mekalanos (1988)

[a] Cm^r, chloramphenicol resistant; Km^r, Kanamycin resistant; Sm^r, streptomycin resistant

Table 4.2 Plasmids used in this study

Plasmids	Genotype/relevant characteristic(s)[a]	Reference
pGP704	*ori* R6K, mob Rp4, MCS of M13tg131, Amp^r	Herrero *et al.* (1990)
pCAT19	Derivative of pBR325 and pUC19 containing the 1053 bp Tn9-CAT cassette; Amp^r, Cm^r	Fuqua (1992)
pGreenII	Vector for *Agrobacterium*-mediated transformation; Km^r	Hellens *et al.* (2000)
pMR11	pBluescript SK containing ~7 kb *V. midae* SY9 genomic DNA; Amp^r	Taylor (2002)
pProH3	1.9 kb *Hin*dIII - *Hin*dIII fragment from pMR11, containing the entire *vmproA* gene, cloned into pBluescript SK; Amp^r	This study (Chapter 2)
pGP13	Derivative of pGP704, containing the 0.256 kb internal *vmproA* gene fragment and in-frame translational stop codons; Amp^r.	This study
pGP13.Cm	Derivative of pGP13, containing the 986 bp *Xba*I-*Xba*I fragment from pCAT19; Amp^r, Cm^r	This study
pGPro.1	Derivative of pGP704, containing the 1.9 kb *Hin*dIII - *Hin*dIII fragment from pProH3, comprising the entire *vmproA* gene; Amp^r.	This study
pGPro.Cm	Derivative of pGPro.1, containing the 986 bp *Xba*I-*Xba*I fragment from pCAT19; Amp^r, Cm^r.	This study
pGPro.Km	Derivative of pGPro.1, containing a 1046 bp DNA fragment that includes the Km^r gene from pGreenII; Amp^r, Km^r.	This study

[a] Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, Kanamycin resistant; Sm^r, streptomycin resistant

4.3.2 Media and culture conditions

Escherichia coli SM10λpir, *E. coli* SY327λpir and *E. coli* JM109 (Table 4.1) were cultured in Luria-Bertani broth (LB) (Appendix A.1.5) and maintained on Luria-Bertani agar (LA) (Appendix A.1.6) at 37 ºC. *E. coli* SM10λpir harbouring the suicide vector pGP704 (Table 4.2) and the recombinant plasmids pGP13, pGPro.1 and pGPro.Km (Table 4.2) were cultured in LB-10 broth (Appendix A.1.9) or maintained on LA-20 agar (Appendix A.1.9) containing 100 µg ml^{-1} ampicillin (Appendix A.2.2) at 37 ºC, unless otherwise stated. *E. coli* SM10λpir harbouring the recombinant plasmids pGP13.Cm and pGPro.Cm were cultured in LB-10 broth or maintained on LA-20 agar containing 30 µg ml^{-1} chloramphenicol (Appendix A.2.2) at 37 °C, unless otherwise stated. *E. coli* SY327λpir harbouring the recombinant suicide vector pGPro.Km was cultured in LB-10 broth or maintained on LA-20 agar containing 100 µg ml^{-1} kanamycin (Appendix A.2.2) at 37 °C. *E. coli* JM109 harbouring the plasmid pGreenII was cultured in LB broth or on LA agar containing 100 µg ml^{-1} kanamycin at 37 °C. *E. coli* JM109 harbouring the plasmid pCAT19 was cultured in LB broth or on LA agar containing 30 µg ml^{-1} chloramphenicol at 37 °C. Wild-type *V. midae* SY9 was cultivated at 22 °C as described in section 2.3.2, unless otherwise stated. *V. midae* SY9Sm^r was either cultured in VNSS broth (Appendix A.1.9) or on VNSS agar (Appendix A.1.9) containing 120 µg ml^{-1} streptomycin (Appendix A.2.2) at 22 ºC, unless otherwise stated. The mutants, *V. midae* SY9Mut2 and *V. midae* SY9Pro2, were either grown in MB (Appendix A.1.1) or in P-MBM (Appendix A.1.3), or on MA (Appendix A.1.2), containing 30 µg ml^{-1} chloramphenicol at 22 °C. The complemented mutant *V. midae* SY9Mut2.Comp was either grown in MB, or P-MBM, or on MA, supplemented with 30 µg ml^{-1} chloramphenicol and 400 µg ml^{-1} kanamycin at 22°C.

4.3.3 Streptomycin resistant *V. midae* SY9

In order to mutate the *vmproA* protease gene using recombinant suicide vector constructs containing a second antibiotic resistance gene and a filter-mating protocol, a *V. midae* SY9 recipient strain capable of growth on streptomycin needed to be generated.

Table 4.3 Oligonucleotide primers used in this study

Primer	Sequence (5' – 3')[a]	Reference
KanFwd	CTCACGTTAAGCGGCCGCATTTTGG	This study
KanRev	CAAAGCCACGGAATTCGTGTCTC	This study
StopFwd	CGTC**TAG**CTTGTCGGCGTG	This study
StopRev	CTCC**CTA**AGCAACGCGTGC	This study
ProMutFwd	GCTTGGTTATTGATCATGTTTCTG	This study
ProMutRev	GAATAGCGTCTCCTCGTTAACAC	This study

[a] In frame translation stop codons, included in the oligonucleotide primer sequences StopFwd and StopRev are highlighted in bold text and underlined.

A streptomycin-resistant *V. midae* SY9 recipient strain will allow for the selection of recipients that have taken up and integrated a recombinant suicide vector following filter-mating with a streptomycin sensitive *E. coli* mating or donor strain harbouring the recombinant suicide vector. Therefore, a streptomycin resistant strain of *V. midae* SY9 was generated by inoculating a fresh culture of *V. midae* SY9 onto a MA/Sm gradient plate containing streptomycin (Appendix A.2.2) ranging in concentration from 0 to 120 µg ml^{-1}. The MA/Sm gradient plates were incubated at 22 ºC for approximately 24 hours. *V. midae* SY9 colonies capable of growing at the highest streptomycin concentration were repeatedly re-streaked onto fresh MA/Sm gradient plates until a *V. midae* SY9 strain capable of growing on streptomycin at a concentration of 120 µg ml^{-1} was isolated. The streptomycin-resistant *V. midae* SY9 strain was maintained on MA containing 120 µg ml^{-1} streptomycin at 22 °C and was designated *V. midae* SY9Sm^r (Table 4.1).

4.3.4 Construction and characterisation of *V. midae* SY9Pro2, harbouring two chromosomal copies of *vmproA*

A large-scale plasmid isolation was performed using the Qiagen Midi-prep kit (according to manufacturer's instructions), in order to isolate the plasmids pMR11, pCAT19 and pGP704 (Table 4.1). The recombinant construct pMR11 (Fig. 4.2) was digested with the restriction enzyme *Hin*dIII (Fermentas), as outlined in Appendix B.5. The resulting

1.9 kb *Hin*dIII - *Hin*dIII DNA fragment, containing the full-length *vmproA* gene (as described in Chapter 2), was blunt end-repaired using Klenow Fragment (Fermentas) (Appendix B.9), resolved on a 0.8% (w/v) TAE agarose gel, and finally purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions.

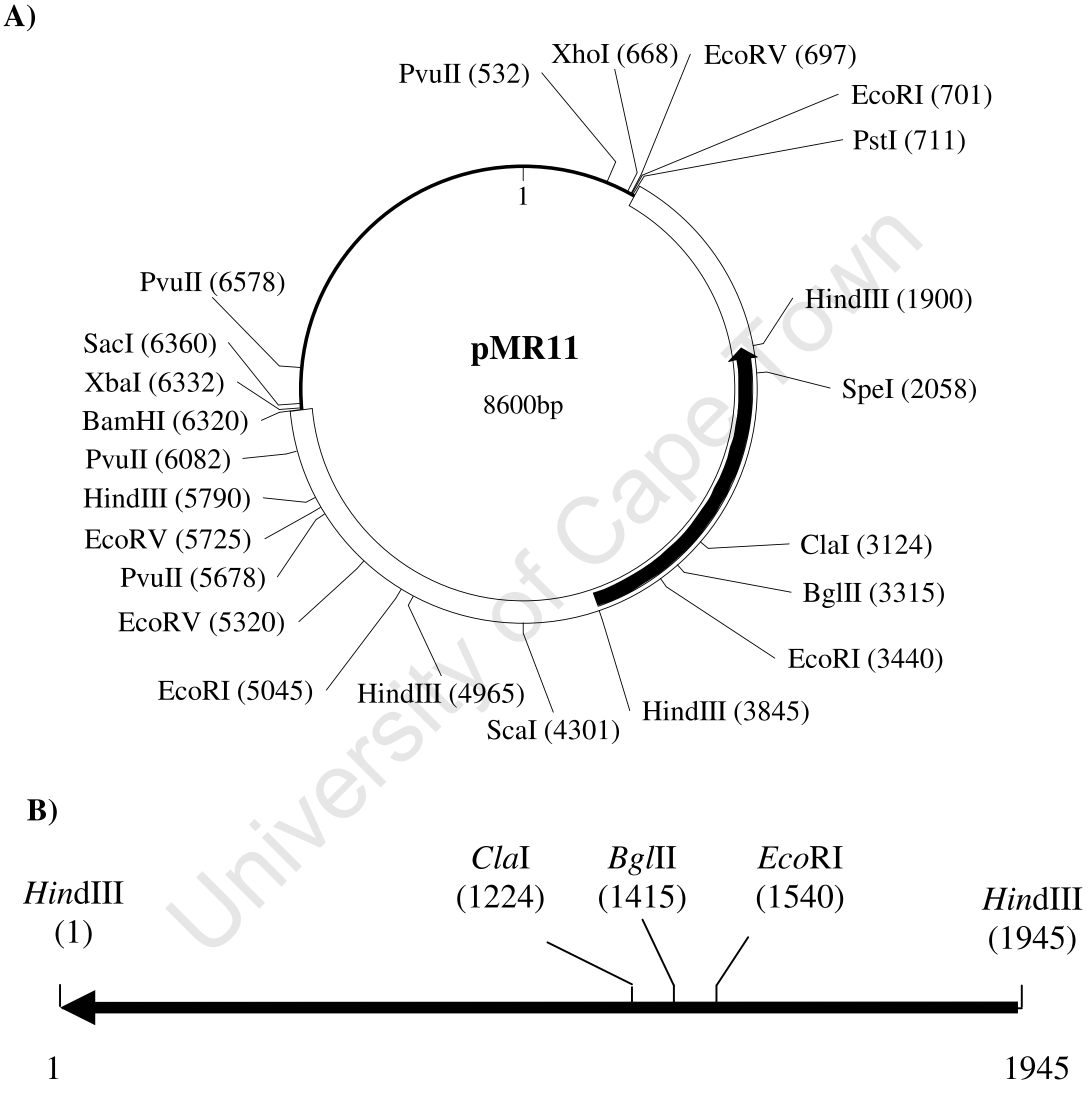


Figure 4.2 The recombinant plasmid pMR11 (A) was digested with *Hin*dIII to release a 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment (B) containing a full-length copy of *vmproA*. The thin line indicates pBluescript SK, the thick white line represents the 5.6 kb cloned fragment of *V. midae* SY9 chromosomal DNA and the black arrow represents the 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment containing *vmproA*. Relevant restriction sites are shown.

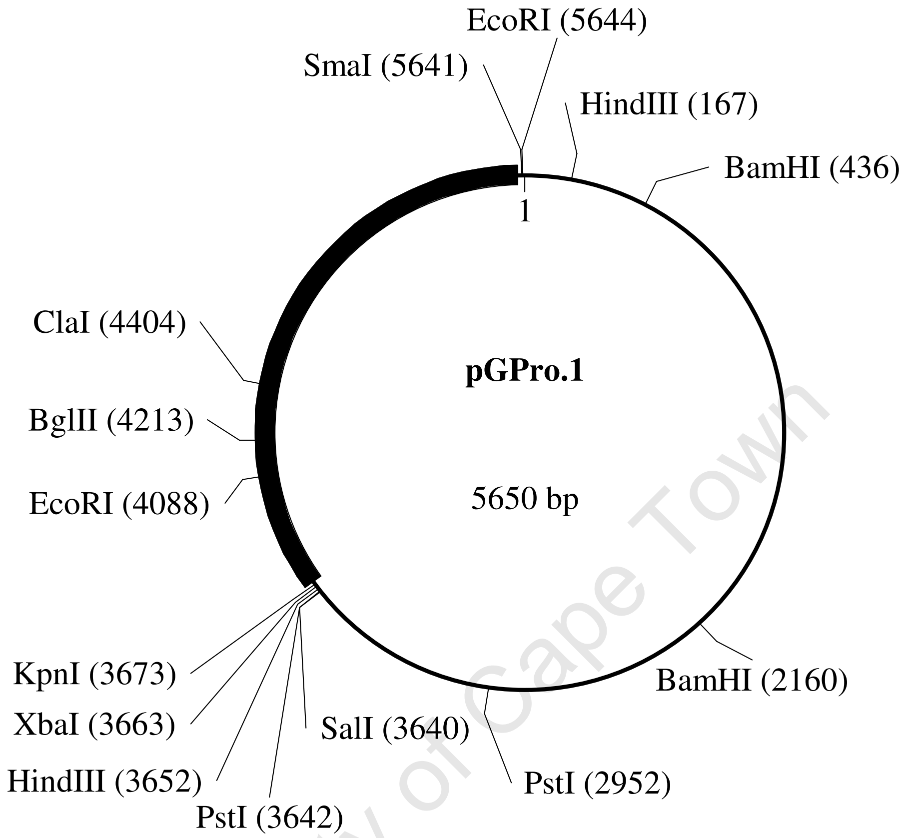


Figure 4.3 Restriction map of the recombinant suicide vector pGPro.1. The recombinant suicide vector pGPro.1 was constructed by subcloning the 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment, containing the *vmproA* gene, from pMR11 into the suicide vector pGP704. The thin line indicates pGP704 and the thick black line represents the cloned *V. midae* SY9 DNA fragment. Relevant restriction enzyme sites are indicated.

The suicide cloning vector pGP704 was linearised by digestion with the restriction enzyme *Eco*RV (Fermentas) (Appendix B.5), dephosphorylated (Appendix B.10) and resolved on a 0.8% (w/v) TAE agarose gel (Appendix B.6) before being purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions.

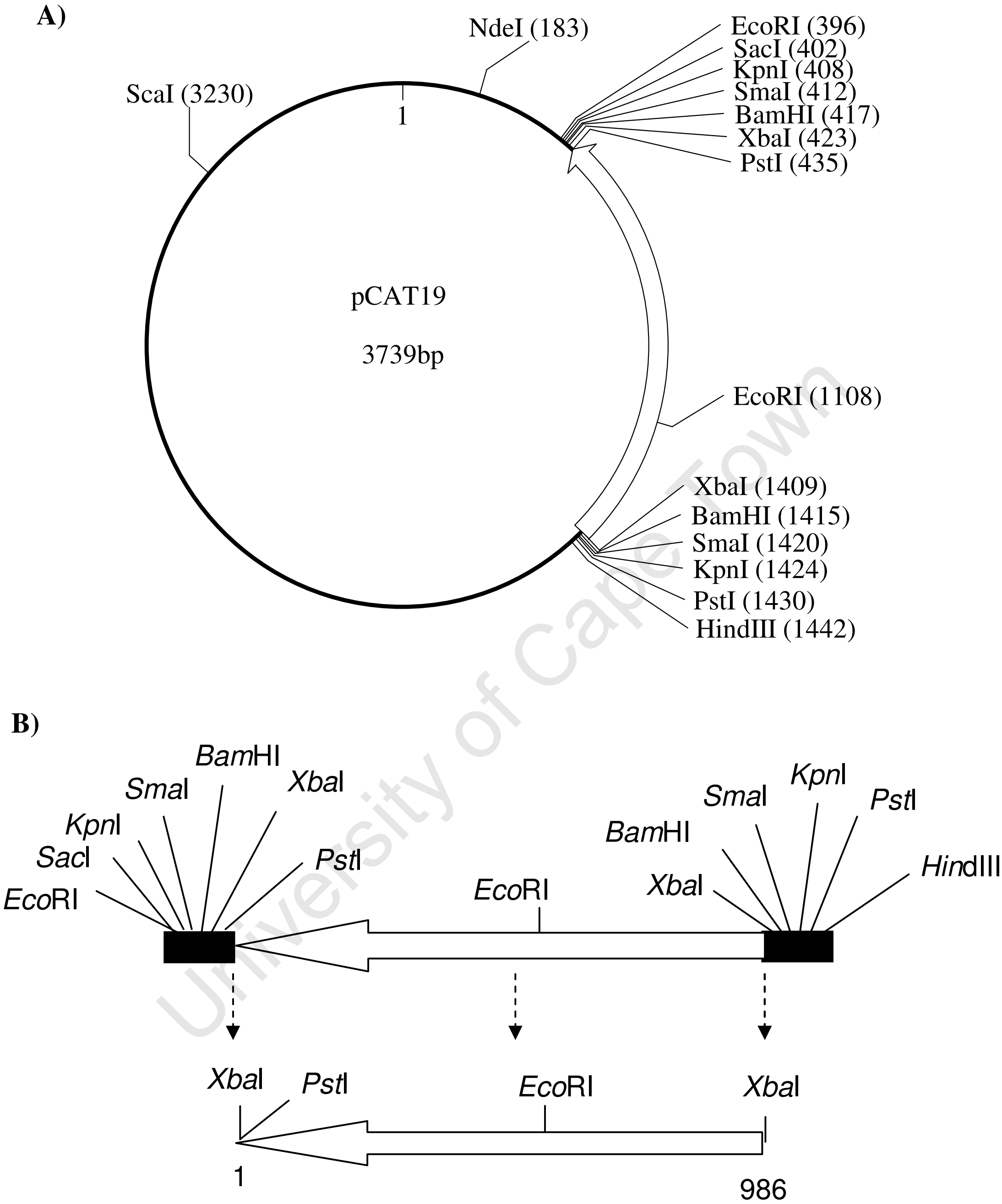


Figure 4.4 The plasmid pCAT19 (A) was digested with *Xba*I to release 986 bp *Xba*I - *Xba*I chloramphenicol resistance gene DNA fragment (B). The thin line indicates the pUC19 backbone and the thick white arrow represents the chloramphenicol resistance gene (CAT). Relevant restriction sites are shown.

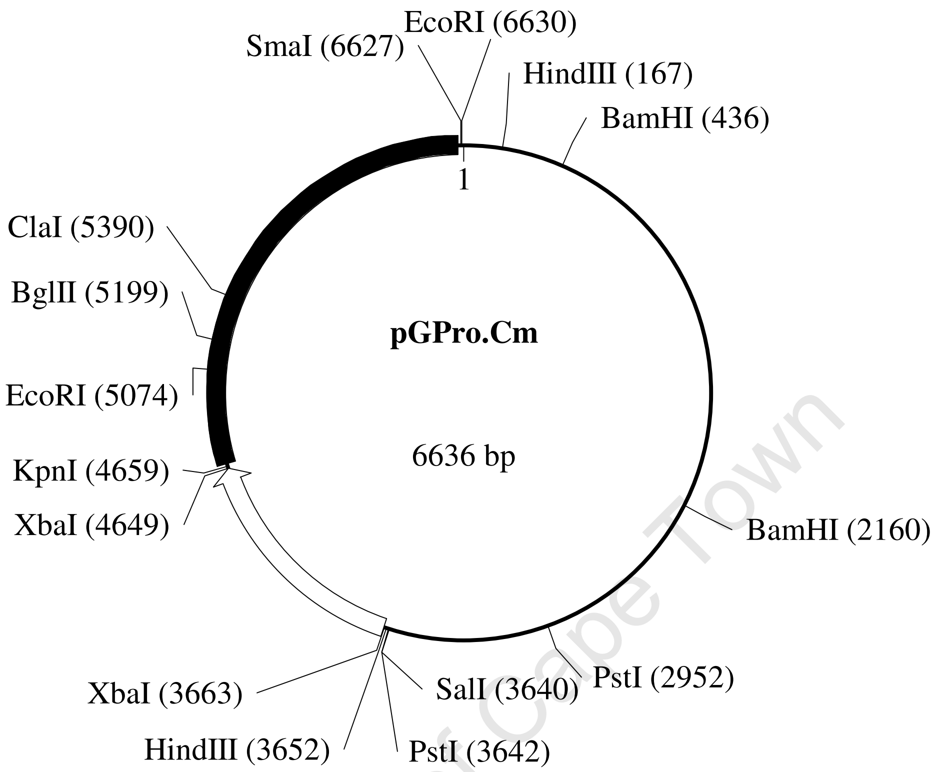


Figure 4.5 The recombinant suicide vector pGPro.Cm. The thin line indicates pGP704, and the thick black line represents the cloned 1.9 kb *Hin*dIII - *Hin*dIII *V. midae* SY9 chromosomal DNA fragment from pMR11. The white block arrow represents the 986 bp *Xba*I - *Xba*I DNA fragment containing the chloramphenicol resistance gene from pCAT19, cloned into pGPro.1. Relevant restriction enzyme sites are indicated.

The 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment from pMR11, containing the full-length *vmproA* gene (Chapter 2) (Fig. 4.2 A and B), was cloned into linearised and dephosphorylated pGP704 vector (Fig. 4.3; as described in Appendix B.12.2). The resulting constructs were transformed (Appendix B.2) into freshly prepared competent *E. coli* SM10λpir (Table 4.1), plated onto LA supplemented with 100 µg ml^{-1} ampicillin (Appendix A.2.2) and incubated at 37 ºC for approximately 16 hours. *E. coli* SM10λpir capable of growth on LA supplemented with 100 µg ml^{-1} ampicillin were inoculated into 5 ml LB supplemented with 100 µg ml^{-1} ampicillin and grown overnight with shaking at 37 ºC. Plasmid DNA was isolated from the overnight cultures using the alkaline lysis mini-prep method of Ish -Horowicz and Burke (1981) (Appendix B.3) and screened for the presence of the desired 1.9 kb insert by restriction enzyme analysis (Appendix B.5). A recombinant pGP704 construct containing the 1.9 kb protease gene fragment was identified and designated pGPro.1 (Fig. 4.3; Table 4.2).

The plasmid pCAT19 (Table 4.2; Fig. 4.4 A) was digested with the restriction enzyme *Xba*I (Fermentas) (Appendix B.5) to release the 986 bp *Xba*I - *Xba*I DNA fragment containing the Chloramphenicol acetyl-transferase (CAT) gene cassette (Fig. 4.4 B). The 986 bp *Xba*I - *Xba*I DNA fragment was gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions, and cloned (Appendix B.12.2) into the recombinant suicide vector pGPro.1 that had been linearised, by digestion with the restriction enzyme *Xba*I (Fermentas) (Appendix B.5) and gel purified (BioSpin Gel Extraction Kit (BioFlux)). The resulting constructs were transformed into competent *E. coli* SM10λpir (Appendices B.1.2 and B.2), plated onto LA containing 30 µg ml^{-1} chloramphenicol and 100 µg ml^{-1} ampicillin and incubated overnight at 37 °C. *E. coli* SM10λpir capable of growth on chloramphenicol and ampicillin were inoculated into 5 ml LB supplemented with 30 µg ml^{-1} chloramphenicol and 100 µg ml^{-1} ampicillin and grown overnight with shaking at 37 °C. Plasmid DNA was isolated from the overnight cultures using the alkaline lysis mini-prep method of Ish-Horowicz and Burke, 1981 (Appendix B.3) and screened for the presence of the 986 bp CAT gene by *Xba*I (Fermentas) restriction enzyme analysis (Appendix B.5). A recombinant pGPro.1 construct containing the 986 bp CAT gene DNA fragment was identified and designated pGPro.Cm (Fig. 4.5; Table 4.2).

The recombinant suicide vector pGPro.Cm was transferred from the donor *E. coli* SM10λpir strain to the recipient strain *V. midae* SY9Sm^r using a modified filter mating technique (Egan *et al.,* 2002). Briefly, 5 ml cultures of both *E. coli* SM10λpir harbouring the recombinant suicide vector pGPro.Cm and streptomycin resistant recipient *V. midae* SY9Sm^r were cultivated for approximately 16 hours. The overnight donor and recipient cultures were gently mixed at a volume ratio of 1:10 (100 µl donor and 1000 µl recipient) in 5 ml of wash solution (Appendix A.2.12). The mixture of donor and recipient cultures was passed through a 0.22 µm Whatman filter (∅ 2.5 cm) and washed with another 5 ml of wash solution. The filter discs were then placed cell-side up onto LA-20 agar (Appendix A.1.9) and incubated at 30 °C for 4 hours.

The filter discs were then placed inside sterile Sterilin tubes containing 2 ml of NSS (Appendix A.1.9) and vortexed vigorously to resuspend the bacteria on the filter discs. One hundred microlitre aliquots of the resuspended cells were spread plated onto VNSS agar (Appendix A.1.9) supplemented with 120 µg ml^{-1} streptomycin and 30 µg ml^{-1} chloramphenicol. The plates were subsequently incubated at 30 °C for 48 hours to select for recipient *V. midae* $SY9Sm^r$ strains harbouring the suicide vector pGPro.Cm. One hundred microlitre aliquots of each donor and recipient strain were spread plated onto both VNSS agar (Appendix A.1.9) and VNSS agar containing 120 µg ml^{-1} streptomycin and 30 µg ml^{-1} chloramphenicol as filter mating controls.

Putative mutants harbouring a second full-length copy of the detergent resistant protease gene were selected based on their ability to grow on VNSS agar containing 120 µg ml^{-1} streptomycin and 30 µg ml^{-1} chloramphenicol. One of these mutants, designated *V. midae* SY9Pro2, was analyzed further.

Southern hybridization analysis (Appendix B.8) was performed in order to determine whether the recombinant suicide vector pGPro.Cm had integrated into the chromosome of *V. midae* $SY9Sm^r$. A 256 bp internal DNA fragment of the detergent resistant protease gene was PCR amplified from pMR11 (Table 4.2) using the synthetic oligonucleotide primers, StopFwd and StopRev (Table 4.3). The 256 bp PCR amplicon was resolved on a 2% (w/v) TAE agarose gel (Appendix B.6) before being gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. The gel purified PCR product was DIG-labeled using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions (Appendix B.8.5 and B.8.6). The DIG-labeled DNA fragment was used as a probe against equal amounts (10 µg) of *V. midae* SY9 and *V. midae* SY9Pro2 chromosomal DNA that had been digested with the restriction enzymes *Eco*RI and *Cla*I (Fermentas) (Appendix B.5).

The resulting chromosomal DNA fragments were separated on a 0.7% (w/v) TAE agarose gel at 35 V overnight and transferred to Hybond N^+ nylon membrane (Amersham) according to Reed and Mann (1985). Southern hybridization was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions and as outlined in Appendices B.8.5 and B.8.6.

4.3.5 Construction and characterisation of an insertional inactivation mutant, *V. midae* SY9Mut2

Plasmid pGP704 (Table 4.2) was digested with the restriction enzyme *Eco*RV (Fermentas) (Appendix B.5) and resolved on a 0.8% (w/v) TAE agarose gel (Appendix B. 6), before being purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. Plasmid pMR11 (Table 4.2), containing the *V. midae* SY9 detergent resistant protease gene (Chapter 2), was isolated from an overnight culture using the alkaline lysis mini-prep method of Ish-Horowicz and Burke (1981) (Appendix B.3). A 256 bp internal DNA fragment (Fig. 4.6 A) of the detergent resistant protease gene was PCR amplified (Appendix B.25.4) using the high fidelity *Pfu* Polymerase (Fermentas) and the oligonucleotide primers StopFwd and StopRev (Table 4.3; Fig. 4.6 A). The PCR primers StopFwd and StopRev were specifically designed to include in-frame translation stop codons (as indicated in Table 4.3). This ensured that an active protease could not be produced once the recombinant suicide vector had integrated into the *V. midae* SY9 chromosome. The same strategy of primer design has been employed in several other cases (Milton *et al.,* 1992; Tannock *et al.,* 2005; Walter *et al.,* 2005; Schwab *et al.,* 2007).

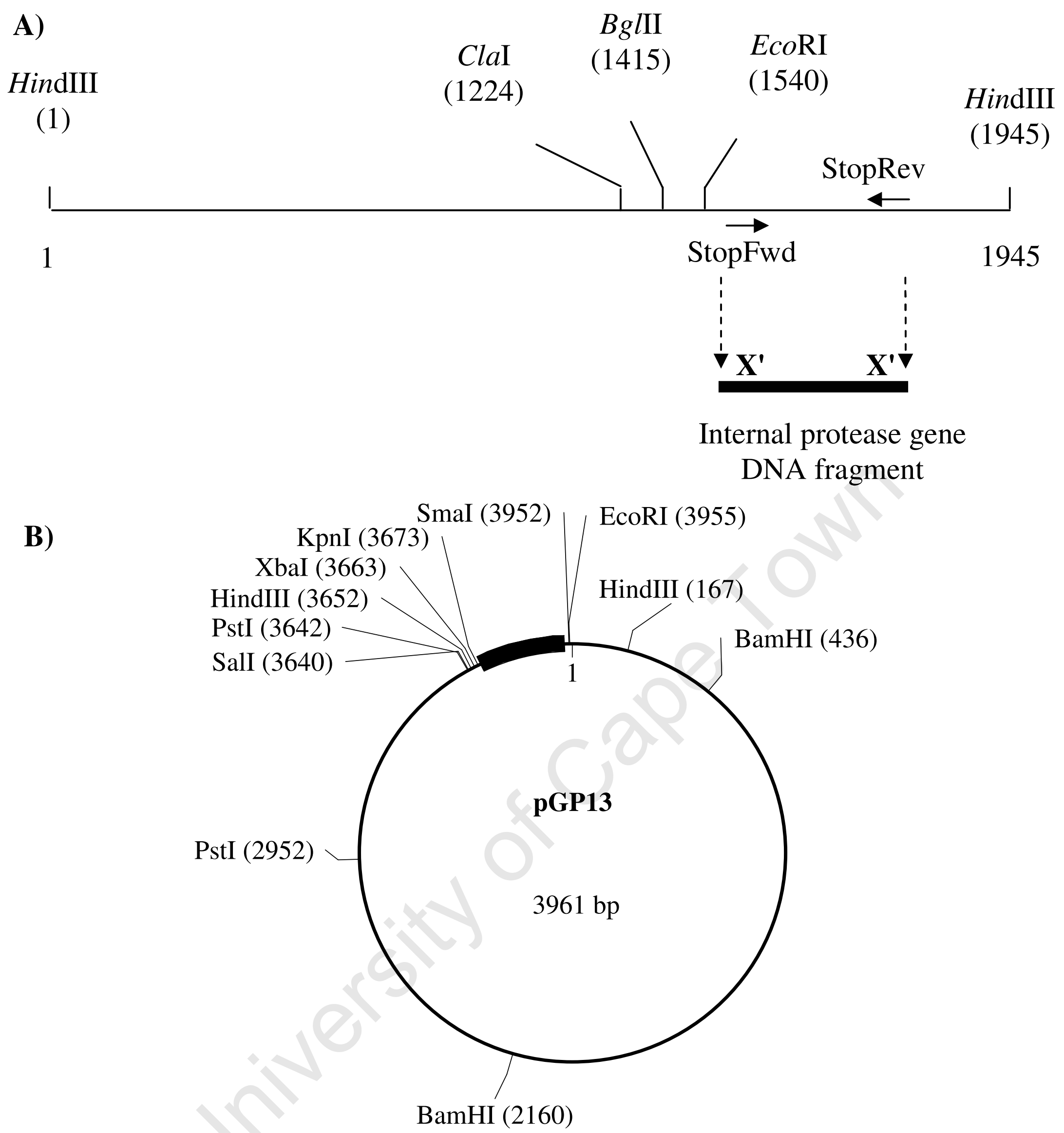


Figure 4.6 Construction of the recombinant suicide vector pGP13. An internal DNA fragment of the *V. midae* SY9 detergent resistant protease gene (A) contained within pMR11 was PCR amplified using the oligonucleotide primers StopFwd and StopRev and cloned into the suicide vector pGP704 to create recombinant construct pGP13 (B). A: Restriction map of the *V. midae* SY9 detergent resistant protease gene, showing the position of major restriction enzyme sites, the PCR primers and the in-frame stop codons (**X'**) included in the primers StopFwd and StopRev. B: Restriction map of the recombinant suicide vector pGP13. The thin line represents pGP704 and the thick black line represents the cloned *V. midae* SY9 256 bp internal DNA fragment. Relevant restriction sites are shown.

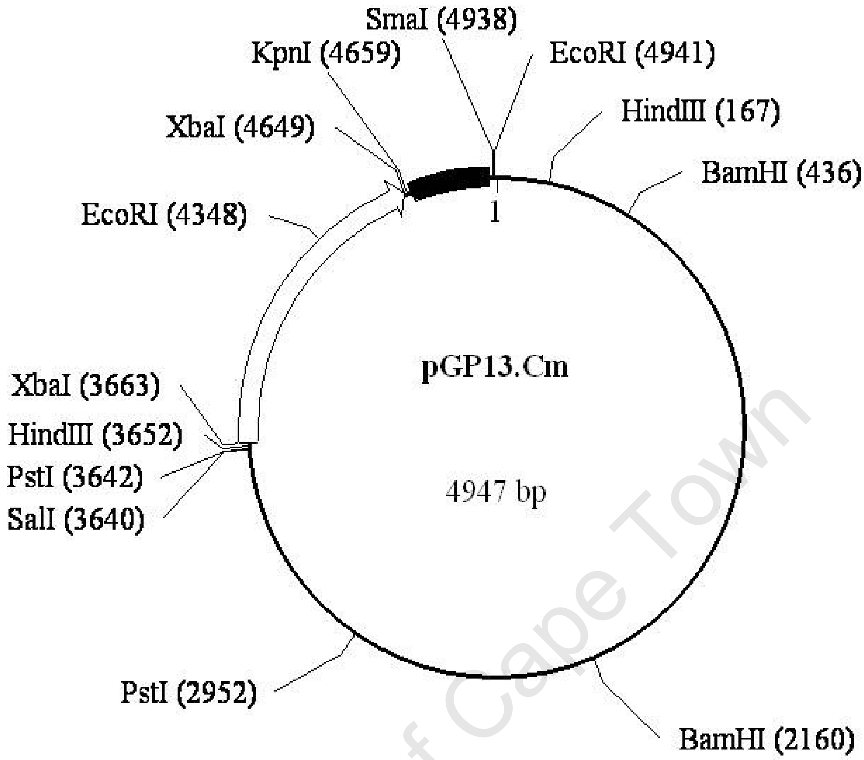


Figure 4.7 The recombinant suicide vector pGP13.Cm. The thin line represents pGP704 and the thick black line represents the cloned *V. midae* SY9 256 bp internal DNA fragment. The white block arrow represents the 986 bp *Xba*I - *Xba*I DNA fragment, containing the chloramphenicol resistance gene from pCAT19, cloned into pGP13. Relevant restriction sites are shown.

The 256 bp PCR amplicon was gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions, and cloned into pGP704 that had been linearised by digestion with the restriction enzyme *Eco*RV (Fermentas). The resulting construct was transformed (Appendix B.2) into freshly prepared competent *E. coli* SM10λpir (Table 4.1), plated onto LA supplemented with 100 µg ml^{-1} ampicillin and incubated at 37 °C overnight. *E. coli* SM10λpir strains capable of growth on LA supplemented with 100 µg ml^{-1} ampicillin (Appendix A.2.2) were screened for the presence of the 256 bp insert by colony PCR analysis using the oligonucleotide primers

StopFwd and StopRev (Table 4.3; Appendix B.23.5). A recombinant pGP704 construct containing the 256 bp internal protease gene fragment was identified and designated pGP13 (Table 4.2; Fig. 4.6 B).

The chloramphenicol resistance gene from plasmid pCAT19 (Table 4.2) was subsequently cloned into pGP13 as described in section 4.3.4. A recombinant pGP13 construct containing the 986 bp *Xba*I - *Xba*I DNA fragment was identified and designated pGP13.Cm (Table 4.2; Fig. 4.7). The recombinant suicide vector pGP13.Cm was transferred from the donor *E. coli* SM10λpir strain to the recipient strain *V. midae* SY9Sm^r using the modified filter mating technique (Egan *et al.,* 2002) described in section 4.3.4. Putative mutants containing an insertionally inactivated detergent resistant protease gene were selected based on their ability to grow on VNSS agar (Appendix A.1.9) containing 120 µg ml^{-1} streptomycin (Appendix A.2.2) and 30 µg ml^{-1} chloramphenicol (Appendix A.2.2). One of these mutants, designated *V. midae* SY9Mut2, was analyzed further.

Southern hybridization analysis was performed to determine whether the recombinant suicide vector pGP13.Cm had integrated into the chromosome of *V. midae* SY9. Genomic DNA was extracted (Appendix B.4.1) from *V. midae* SY9 and *V. midae* SY9Mut2 (Table 4.1). The recombinant plasmid pProH3 (Table 4.2), containing the full-length *vmproA* gene on a 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment, was digested with the restriction enzyme *Hin*dIII, as outlined in Appendix B.5. The resulting 1.9 kb *Hin*dIII - *Hin*dIII DNA fragment was resolved on a 0.8% (w/v) TAE agarose gel (Appendix B.6), before being gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to manufacturer's instructions. The purified DNA fragment was radioactively labeled ([α^{32}P]-dCTP, Amersham) using a random-primed DNA labeling kit (Roche) according to the manufacturer's instructions and as outlined in Appendix B.8.2. The radio-labeled DNA fragment was used as a probe against equal amounts (10 µg) of *V. midae* SY9 and *V. midae* SY9Mut2 genomic DNA that had been digested with the restriction enzymes *Hin*dIII and *Eco*RI (Appendix B.5). The resulting fragments were separated on a 0.7% (w/v) TAE agarose gel (Appendix B.6). The Southern hybridization procedure that was followed is described in Appendices B.8.3 and B.8.4.

4.3.6 Complementation of *V. midae* SY9Mut2

Integration of a fully functional full-length copy of *vmproA* into the chromosome of *V. midae* SY9Mut2 could complement the insertionally inactivated detergent resistant protease gene of *V. midae* SY9Mut2.

Plasmid pGreenII (Table 4.2; Fig. 4.8), containing a kanamycin resistance gene, was isolated from an overnight *E. coli* JM109 (Table 4.1) culture using the alkaline lysis mini-prep method of Ish-Horowicz and Burke, 1981 (Appendix B.3). The kanamycin resistance gene was PCR amplified using high-fidelity *Pfu* Polymerase (Fermentas) and the oligonucleotide primers KanFwd and KanRev (Table 4.3; Fig. 4.8 B) as described in Appendix B.25.2. The 1046 bp PCR amplicon was gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions, and cloned into pGPro.1 (Table 4.2; Fig. 4.3) that had been linearised by digestion with the restriction enzyme *Xba*I (Fermentas) (Appendix B.5), blunt end-repaired (Appendix B.9), resolved on a 0.8% (w/v) TAE agarose gel (Apprendix B.6) and purified (BioSpin Gel Extraction Kit (BioFlux), according to manufacturer's instructions). The resulting construct was transformed into competent *E. coli* SY327λpir (Table 4.1), plated onto LA (Appendix A1.6) containing 100 µg ml^{-1} kanamycin (Appendix A.2.2) and incubated overnight at 37 °C. *E. coli* SY327λpir colonies capable of growth on kanamycin were inoculated into 5 ml LB (Appendix A1.5) supplemented with 100 µg ml^{-1} kanamycin and grown overnight at 37 °C with shaking. Plasmid DNA was isolated from the overnight cultures using the alkaline lysis mini-prep method of Ish-Horowicz and Burke, 1981 (Appendix B.3) and screened for the presence of the kanamycin resistance gene by PCR (Appendix B.25.6). A recombinant pGPro.1 construct containing the 1046 bp kanamycin resistance gene was identified and designated pGPro.Km (Table 4.2; Fig. 4.9). The recombinant suicide vector pGPro.Km was transformed into freshly prepared

E. coli SM10λpir competent cells (Appendices B.1.1 and B.2), plated onto LA containing 100 µg ml^{-1} kanamycin (Appendix A.2.2) and incubated overnight at 37 °C.

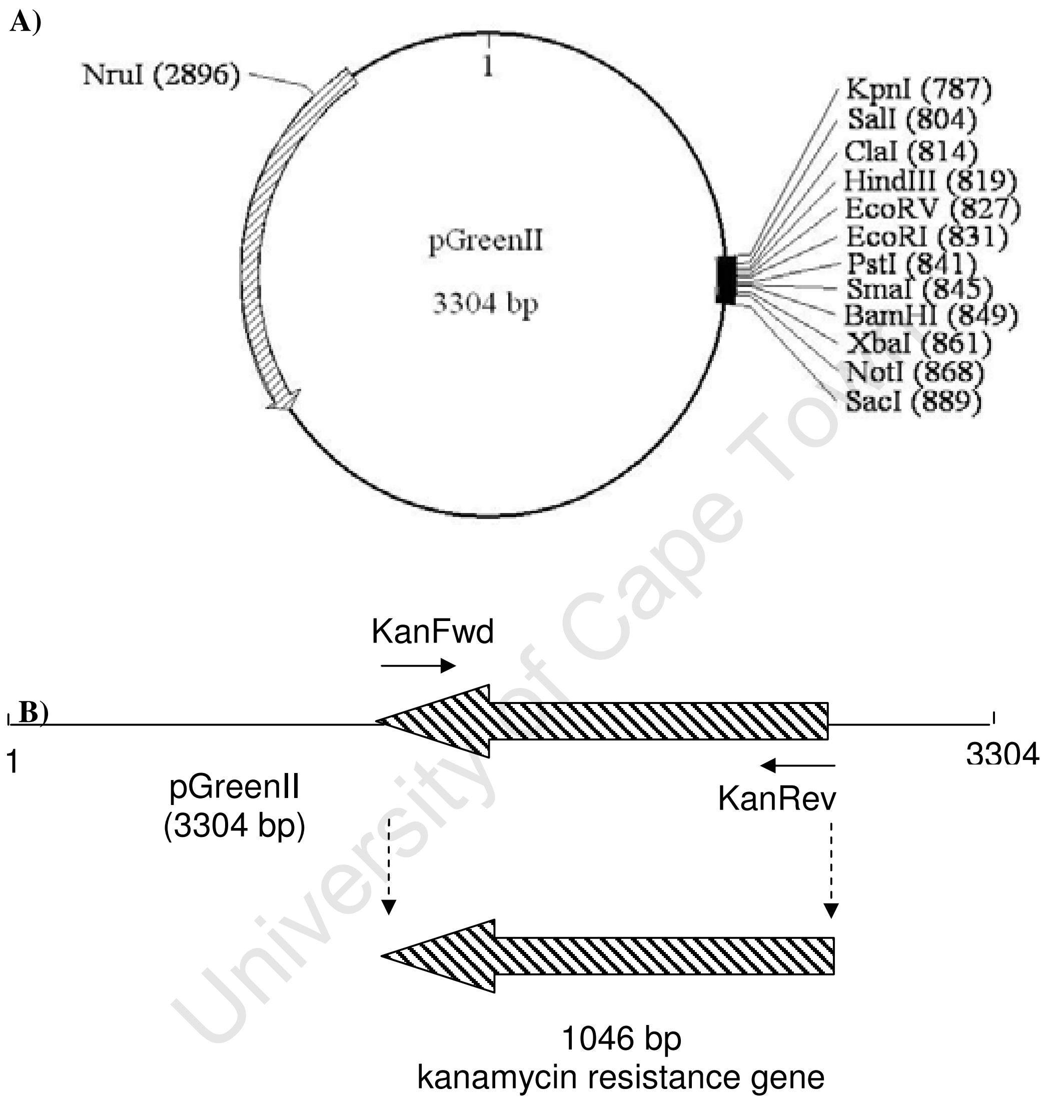


Figure 4.8 The pGreen II (A) 1046 bp kanamycin resistance gene was PCR amplified using the oligonucleotide primers, KanFwd and KanRev (B). The thin line indicates pGreenII and the thick black- and white-hatched arrow represents the kanamycin resistance gene. Relevant restriction sites are shown.

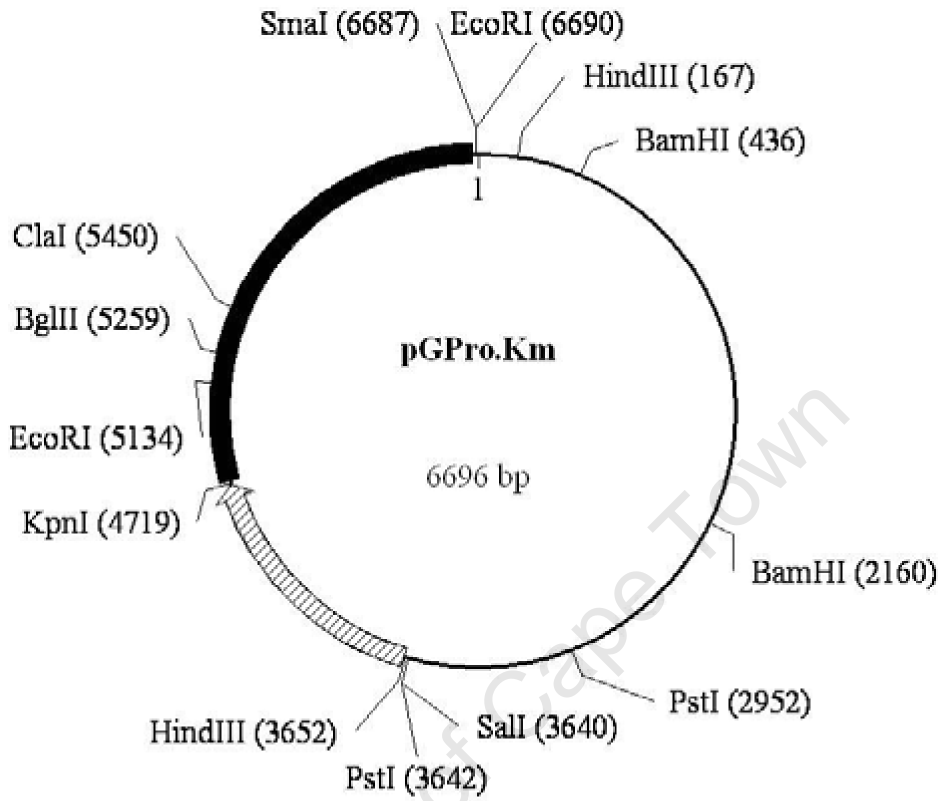


Figure 4.9 The recombinant suicide vector pGPro.Km. The thin line represents pGP704 and the thick black line represents the cloned *V. midae* SY9 1.9 kb *Hin*dIII - *Hin*dIII internal DNA fragment containing the full-length *vmproA* gene. The hatched-black arrow represents the 1046 bp kanamycin resistance gene, PCR amplified from pGreenII and cloned into pGPro.1. Relevant restriction sites are shown.

The recombinant suicide vector pGPro.Km was transferred from the donor *E. coli* SM10λpir strain to the recipient strain *V. midae* SY9Mut2 using the modified filter mating technique (Egan *et al.,* 2002) described in section 4.3.4. Putative *V. midae* SY9Mut2 mutants harbouring a full-length copy of the detergent resistant protease gene were selected based on their ability to grow on VNSS agar (Appendix A.1.9) containing 120 µg ml^{-1} streptomycin, 30 µg ml^{-1} chloramphenicol and 400 µg ml^{-1} kanamycin (Appendix A.2.2).

A total of 96 *V. midae* SY9Mut2 kanamycin-resistant mutant strains were isolated. Each strain was inoculated into 5 ml MB (Appendix A.1.1) supplemented with 30 µg ml^{-1} chloramphenicol and 400 µg ml^{-1} kanamycin, and grown overnight at 22 ºC on a rotary shaker (100 rpm). Chromosomal DNA was isolated from these overnight cultures using a chromosomal DNA extraction method adapted from Wang *et al.,* 1996 (Appendix B.4.2). The kanamycin-resistant *V. midae* SY9Mut2 strains were initially screened for the presence of the kanamycin resistance gene by PCR (Appendix B.25.2) using the oligonucleotide primers KanFwd and KanRev (Table 4.3). The presence of the kanamycin resistance gene would indicate that pGPro.Km had integrated into the chromosome of *V. midae* SY9Mut2. The recombinant *V. midae* SY9Mut2 strains that were shown to be harbouring a kanamycin resistance gene were further investigated to confirm complementation of *V. midae* SY9Mut2 by PCR for the presence of a full-length detergent resistant protease gene using the oligonucleotide primers ProMutFwd and ProMutRev (Table 4.3; Appendix B.25.7). One of these mutants, designated *V. midae* SY9Mut2.Comp, was analyzed further.

4.3.7 Growth and protease activity of V. midae SY9, V. midae SY9Pro2, V. midae SY9Mut2 and V. midae SY9Mut2.Comp

A growth curve was conducted to determine the effect of mutations to the *vmproA* gene on cell growth and extracellular protease activity of *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp. The growth and extracellular protease activity profiles of the mutant strains were compared to that of *V. midae* SY9 when cultured in Marine Minimal Medium (MMM) (Appendix A.1.10), Marine Minimal Medium supplemented with 1% (w/v) peptone (MMM-P) (Appendix A.1.10) and P-MBM (Appendix A.1.3). The wild-type and mutant strains were each grown for approximately 12 hours in a 5 ml starter culture of MMM, MMM-P and P-MBM at 22 ºC on an orbital shaker (100 rpm). The entire volume of each of the starter cultures was inoculated into 100 ml of MMM, MMM-P and P-MBM, respectively and incubated at 22 °C for 16 hours on an orbital shaker (100 rpm). The cultures were inoculated into two

5 liter conical flasks, each containing 500 ml of MMM, MMM-P and P-MBM, respectively, so as to achieve an initial A_{600} (Beckman Du® 530 Life science UV/VIS Spectrophotometer) of 0.02, and incubated at 22 °C on an orbital shaker at 100 rpm. Triplicate 2 ml aseptic samples were removed from the experimental flasks at regular intervals for A_{600} and extracellular protease enzyme activity determination. For the determination of extracellular protease activity, the samples were centrifuged (13,000x g for 5 minutes at 4 °C) to pellet the bacterial cells. The supernatant fraction was collected and stored at -20 °C for later extracellular protease enzyme analysis using the Azo-casein protease assay (Appendix B.22).

The maximum specific growth rate was calculated from the growth curve for *V. midae* SY9, the *vmproA* mutant strains, cultivated in MMM, MMM-P and P-MBM at 22 ºC. As a result of the exponential nature of bacterial growth, the logarithm of the relative population size is usually plotted against time (Zwietering *et al.,* 1990) and the maximum specific growth rate can then be determined by calculating the slope of the tangent drawn to the inflection of the curve (Baranyi and Pin, 1999).

4.3.8 Zymogram analysis of V. midae SY9, V. midae SY9Pro2, V. midae SY9Mut2 and V. midae SY9Mut2.Comp

Zymogram comparative analysis was conducted in order to investigate the extracellular protease phenotype of *V. midae* SY9 in comparison with that of the mutant strains *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp when cultured in P-MBM (Appendix A.1.3), a protease inducing growth medium.

4.3.8.1 Gelatine zymogram analysis of V. midae SY9, V. midae SY9Pro2, V. midae SY9Mut2 and V. midae SY9Mut2.Comp

The effect of mutating *vmproA* on the detergent resistant extracellular proteolytic phenotype of *V. midae* SY9Pro2, *V. midae* SY9Mut2 and the complemented mutant *V. midae* SY9Mut2.Comp was investigated by gelatine zymography in denaturing slab gels containing gelatine as a co-polymerised substrate (Heussen and Dowdle, 1980) (Appendix B.20.1).

V. midae SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp were cultivated, as described in section 4.3.4.4, for approximately 24 hours. The cells were harvested by centrifugation (13,000x g, for 10 minutes at 4 ºC) and the supernatant fraction retained. The supernatant from each of the bacterial strains was filtered (0.22 µm), treated with 2.5% (w/v) SDS (Appendix A.2.18) and 2% (v/v) glycerol (Appendix A.2.18), and analyzed on a gelatine zymogram as described in Appendix B.20.1. Electrophoresis was performed at 30 mA for 3 hours at 4 ºC in a Mighty Small II SE250 (Hoefer Scientific Instruments). Following electrophoresis, the gelatine zymogram gels were washed in Triton X-100 wash solution (Appendix A.2.18) for 60 minutes and then incubated in Glycine incubation buffer (Appendix A.2.18) for 3 hours at 37 ºC. Zones of proteolytic activity were visualized by staining the gel with Amido Black (Appendix A.2.18) as described in Appendix B.20.1.

4.3.8.2 Casein zymogram analysis of V. midae SY9, V. midae SY9Pro2, V. midae SY9Mut2 and V. midae SY9Mut2.Comp

The effect of mutating *vmproA* on the profile of extracellular proteases of *V. midae* SY9Pro2, *V. midae* SY9Mut2 and the complemented mutant *V. midae* SY9Mut2.Comp was investigated by casein zymography in denaturing polyacrylamide gels as described in appendix B.20.2.

V. midae SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp were cultivated, as described in section 4.3.4.4, for approximately 24 hours. The cells were harvested by centrifugation (13,000x g, for 10 minutes at 4 ºC) and the supernatant fractions retained. The supernatant from each of the bacterial strains was filtered (0.22 µm), treated with 2.5% (w/v) SDS (Appendix A.2.18) and 2% (v/v) glycerol (Appendix A.2.18), and analyzed on an 8% SDS-PAGE as described in Appendices B.19 and B.20.2. Electrophoresis was performed at 30 mA for 3 hours at 4 ºC in a Mighty Small II SE250 (Hoefer Scientific Instruments). Following electrophoresis the polyacrylamide gels were washed in Triton X-100 wash solution (Appendix A.2.19) and then incubated in the casein penetration solution (Appendix A.2.19) at 4 ºC for 30 minutes, as described in appendix B.20.2, before being incubated at 37 ºC for 2 hours. Zones of proteolytic activity were visualized by staining the gel with Coomassie stain (Appendix A.2.16) as described in Appendix B.19.

4.4 Results

4.4.1 Chromosomal integration of a second full-length copy of the *V. midae* SY9 detergent resistant protease gene

4.4.1.1 Isolation of *V. midae* SY9Pro2 mutants

A total of 44 *V. midae* SY9 colonies grew on VNSS agar containing 120 µg ml^{-1} streptomycin and 30 µg ml^{-1} chloramphenicol. All of these chloramphenicol-resistant *V. midae* SY9 isolates could possibly have undergone an integration event, where the recombinant suicide vector pGPro.Cm recombined into the detergent resistant protease gene resulting in two full-length detergent resistant protease genes in close proximity to each other on the *V. midae* SY9 chromosome.

4.4.1.2 Mapping the recombination of *vmproA* by pGPro.Cm

One of the 44 putative *V. midae* SY9 mutants was selected for further analysis and was designated *V. midae* SY9Pro2. Southern hybridisation analysis confirmed intergration of the recombinant suicide vector pGPro.Cm at a single site in the genome of *V. midae* SY9 (Fig. 4.10 and 4.11). Chromosomal DNA from *V. midae* SY9 and *V. midae* SY9Pro2 was digested with the restriction enzymes *Eco*RI and *Cla*I, and probed with a 256 bp DIG-labeled internal fragment of the detergent resistant protease gene. A single hybridization band of 4.9 kb (Fig.4.10; lane 1 and Fig. 4.11) was observed when *V. midae* SY9 chromosomal DNA was digested with *Eco*RI. The probe also hybridized to a single 1.7 kb fragment of *V. midae* SY9 chromososmal DNA digested with *Cla*I (Fig. 4.10; lane 2 and Fig. 4.11). Two hybridization fragments of approximately 1.55 and 4.9 kb in size (Fig. 4.10; lane 3 and Fig. 4.11) were observed when *V. midae* SY9Pro2 chromosomal DNA was digested with *Eco*RI. Digestion of *V. midae* SY9Pro2 chromosomal DNA with the restriction enzyme *Cla*I resulted in the probe hybridizing to two fragments of 1.7 and 6.6 kb (Fig. 4.10; lane 4 and Fig. 4.11). As a result of the integration of pGPro.Cm, an additional *Eco*RI and *Cla*I restriction site was introduced into the *V. midae* SY9 chromosome (Fig. 4.11).

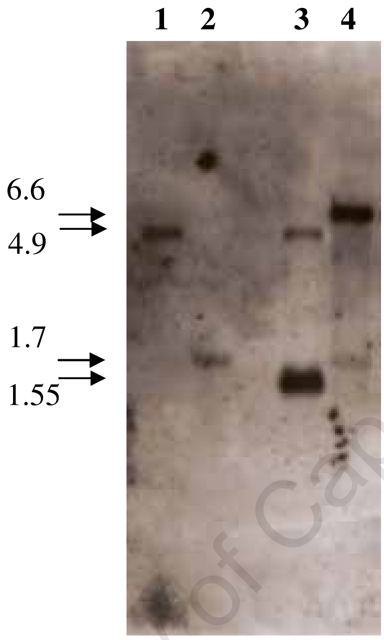


Figure 4.10 Southern hybridization of the 256 bp internal *vmproA* DIG-labelled DNA fragment PCR amplified from pProH3 to 10 µg of *V. midae* SY9 and *V. midae* SY9Pro2 chromosomal DNA. *V. midae* SY9 chromosomal DNA digested with *Eco*RI (lane 1), *Cla*I (lane 2), and *V. midae* SY9Pro2 chromosomal DNA digested with *Eco*RI (lane 3), *Cla*I (lane 4). The arrows indicate the approximate sizes of the bands in kilobase pairs (kb).

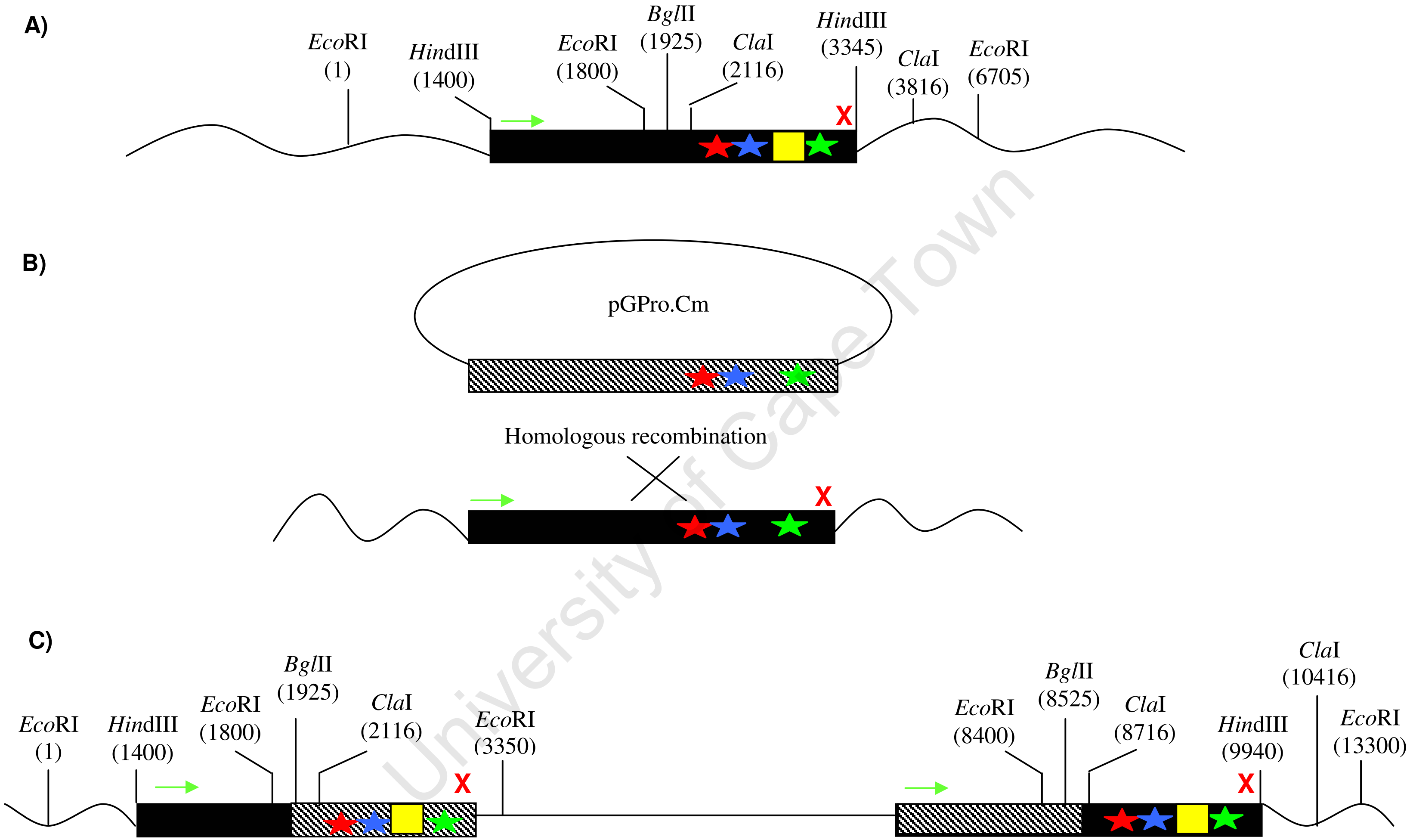

A)
EcoRI
(1)
HindIII
(1400)
EcoRI
(1800)
BglII
(1925)
ClaI
(2116)
HindIII
(3345)
ClaI
(3816)
EcoRI
(6705)
X
B)
pGPro.Cm
Homologous recombination
X
C)
EcoRI
(1)
HindIII
(1400)
EcoRI
(1800)
BglII
(1925)
ClaI
(2116)
X
EcoRI
(3350)
EcoRI
(8400)
BglII
(8525)
ClaI
(8716)
HindIII
(9940)
X
ClaI
(10416)
EcoRI
(13300)

Figure 4.11 **(Facing page)** Schematic representation (not to scale) depicting the homologous recombination event that interrupted the *V. midae* SY9 detergent resistant protease gene and the chromosomal arrangement of the resulting mutant *V. midae* SY9Pro2. A) The chromosomal arrangement of the wild type *V. midae* SY9 detergent resistant protease gene (black bar). The stars represent active site residues (red star - D^{180}, blue star - H^{213} and green star - S^{363}). The green arrow represents the promoter region and direction of transcription. The red cross (X) represents the stop codon. The yellow bar represents the 256 bp internal gene fragment used as a probe for the southern hybridization analysis. B) Recombinant suicide vector pGPro.Cm (circular black band represents plasmid pGP704) containing the detergent resistant protease gene on a 1.9 kb *Hind*III fragment (large white hatched band) and a chloramphenicol resistance gene (not shown). C) The resulting mutant, *V. midae* SY9Pro2, showing the intergrated pGPro.Cm recombinant suicide vector that resulted in the presence of two full-length detergent resistant protease genes in the chromosome of *V. midae* SY9Pro2. Relevant restriction enzyme recognition sites and their position relative to the *Eco*RI (1) restriction enzyme site are also shown.

4.4.2 Insertional inactivation of the *V. midae* SY9 detergent-stable protease gene

4.4.2.1 Isolation of *V. midae* SY9Mut2 mutants

Two *V. midae* SY9 colonies grew on VNSS agar containing 120 µg ml^{-1} streptomycin and 30 µg ml^{-1} chloramphenicol. These two chloramphenicol-resistant *V. midae* SY9 isolates could possibly have undergone a recombination event, resulting in integration of the recombinant suicide vector pGP13.Cm into the detergent resistant protease gene.

4.4.2.2 Mapping the interruption of *vmproA* by pGP13.Cm

One of the two putative *V. midae* SY9 mutants selected for further analysis was designated *V. midae* SY9Mut2. Southern hybridization analysis confirmed the chromosomal intergration of the recombinant suicide vector pGP13.Cm as well as the interruption of the *V. midae* SY9 detergent resistant protease gene (Fig. 4.12 and 4.13). Chromosomal DNA from the wild type strain, *V. midae* SY9, and the insertional inactivation mutant, *V. midae* SY9Mut2, was digested with the restriction enzymes *Hin*dIII and *Eco*RI, and probed with a radiolabelled 1.9 kb *Hin*dIII fragment from the recombinant plasmid pProH3. A single 1.9 kb hybridization band was observed when *V. midae* SY9 chromosomal DNA was digested with *Hin*dIII (Fig.4.12; lane 1). The single hybridization band is a result of the *Hin*dIII restriction enzyme not cutting within the *V. midae* SY9 detergent resistant protease gene. This hybridization fragment represents the 1.9 kb *Hin*dIII - *Hin*dIII fragment that was radiolabelled as the probe and was also previously shown to contain the full-length detergent resistant protease gene (section 2.4.3). The probe hybridized to two fragments of approximately 4.9 and 1.8 kb (Fig. 4.12; lane 2) of *V. midae* SY9 chromosomal DNA digested with *Eco*RI, indicating that *Eco*RI cuts once within the detergent resistant protease gene (Fig. 4.13). When the chromosomal DNA of *V. midae* SY9Mut2 was digested with *Hin*dIII the radiolabelled probe hybridized to two fragments of approximately 2.1 and 1.5 kb in size (Fig. 4.12; lane 3). As a result of the integration of pGP13.Cm into the *V. midae* SY9 chromosome, two additional *Hin*dIII restriction sites were introduced within the interrupted *vmproA* detergent resistant protease gene (Fig. 4.13). Digestion of *V. midae* SY9Mut2 genomic DNA with the restriction enzyme *Eco*RI resulted in the 1.9 kb *Hin*dIII fragment hybridizing to three fragments of 4.6, 1.8 and 1.4 kb (Fig. 4.12; lane 4). The 1.8 kb fragment is common to the *Eco*RI digest of both *V. midae* SY9 and *V. midae* SY9Mut2 chromosomal DNA (Fig. 4.12; lane 2 and 4). This is due to the probe hybridizing to a homologous portion of the detergent resistant protease gene that was not influenced by the integration of the recombinant suicide vector pGP13.Cm. As a result of the integration of pGP13.Cm into the *V. midae* SY9 chromosome, two additional *Eco*RI restriction sites were introduced within the *vmproA* detergent resistant protease gene (Fig. 4.13).

4.4.3 Complementation of *V. midae* SY9Mut2 by chromosomal integration of a full-length copy of *vmproA*

A total of 96 *V. midae* SY9Mut2 colonies grew on VNSS agar containing 120 µg ml^{-1} streptomycin, 30 µg ml^{-1} chloramphenicol and 400 µg ml^{-1} kanamycin. All of these kanamycin resistant *V. midae* SY9Mut2 isolates could have undergone a recombination event, resulting in integration of pGPro.Km into the *V. midae* SY9Mut2 chromosome and complementation of *V. midae* SY9Mut2. All of the kanamycin resistant *V. midae* SY9Mut2 isolates screened contained a kanamycin resistance gene and a full length detergent resistant protease gene (data not shown), indicating that the 1.9 kb *Hin*dIII fragment harboured on pGPro.Km integrated into the *V. midae* SY9Mut2 chromosome unaltered and was potentially capable of complementing the detergent resistant phenotype that was lost when *V. midae* SY9Mut2 was constructed.

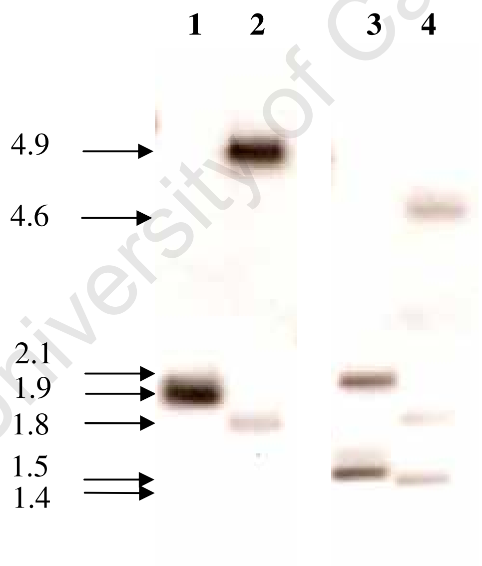


Figure 4.12 Southern hybridisation of *V. midae* SY9 and *V. midae* SY9Mut2 chromosomal DNA to show integration of pGP13.Cm into the genome of *V. midae* SY9 and interruption of *vmproA*. *V. midae* SY9 chromosomal DNA digested with *Hind*III (lane 1) and *Eco*RI (lane 2), and *V. midae* SY9Mut2 chromosomal DNA digested with *Hind*III (lane 3) and *Eco*RI (lane 4). The arrows indicate the approximate sizes of the bands in kilobase pairs (kb).

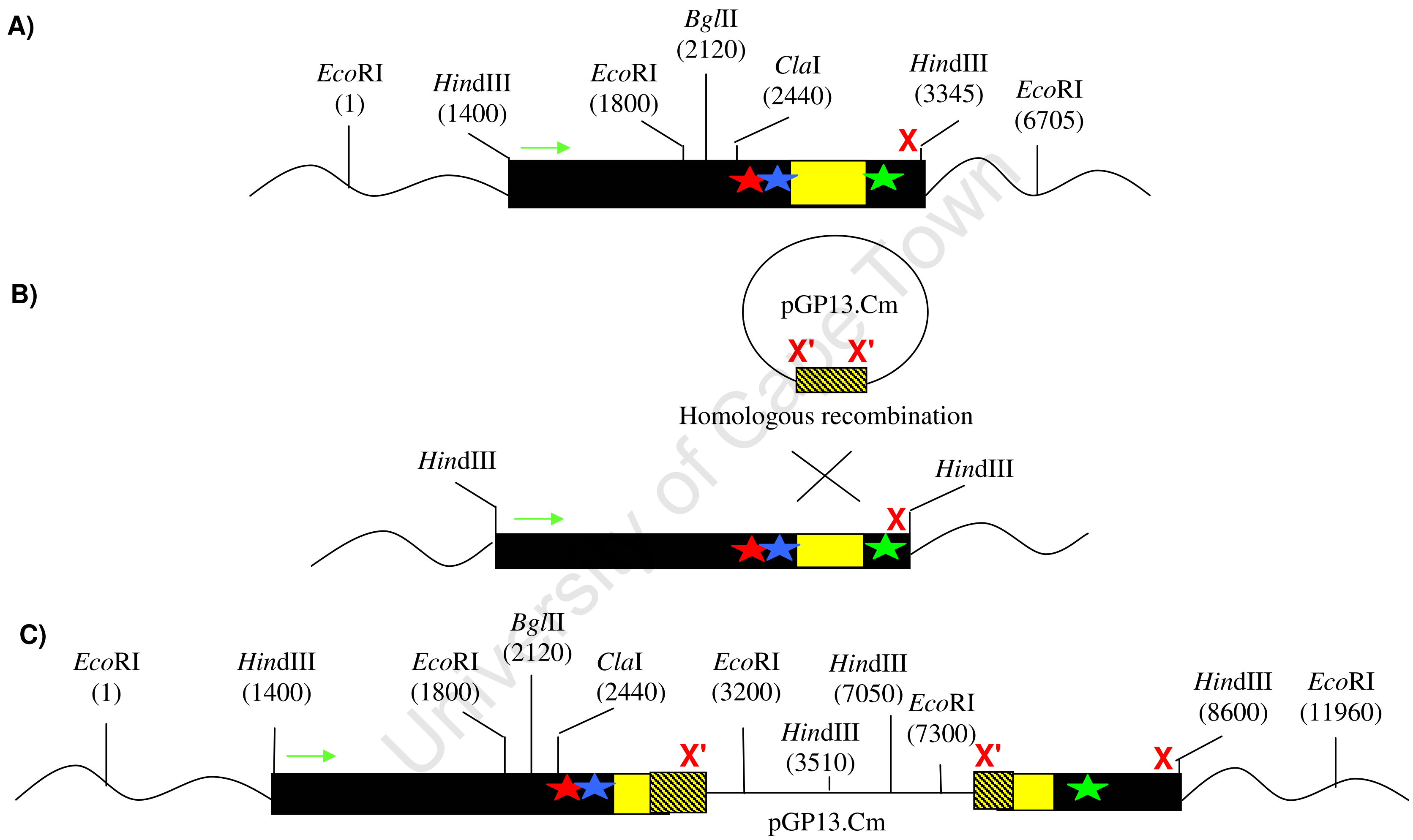

A)
*Eco*RI (1)
*Hin*dIII (1400)
*Eco*RI (1800)
*Bgl*II (2120)
*Cla*I (2440)
*Hin*dIII (3345)
*Eco*RI (6705)
X
B)
pGP13.Cm
X' X'
Homologous recombination
*Hin*dIII
*Hin*dIII
X
C)
*Eco*RI (1)
*Hin*dIII (1400)
*Eco*RI (1800)
*Bgl*II (2120)
*Cla*I (2440)
X'
*Eco*RI (3200)
*Hin*dIII (3510)
*Hin*dIII (7050)
*Eco*RI (7300)
pGP13.Cm
X'
X
*Hin*dIII (8600)
*Eco*RI (11960)

Figure 4.13 **(Facing page)** Schematic representation (not to scale) depicting the homologous recombination method used to interrupt the *V. midae* SY9 detergent resistant protease gene and the chromosomal arrangement of the resulting mutant, *V. midae* SY9Mut2. A) The chromosomal arrangement of the wild type *V. midae* SY9 detergent resistant protease gene (black bar). The stars represent the active site residues (red star - D^{180}, blue star - H^{213} and green star - S^{363}). The green arrow represents the promoter region and direction of transcription. The red cross (X) represents the wild type stop codon. The yellow bar represents the 256 bp internal *vmproA* gene fragment that was PCR amplified and cloned into pGP704. B) Recombinant suicide vector pGP13.Cm (circular black band represents the plasmid pGP704), containing an internal protease gene fragment (yellow hatched bar) and a chloramphenicol resistant gene (not shown). The cloned internal gene fragment (yellow hatched bar) has in-frame stop codons (X') that were included in the PCR primers StopMutFwd and StopMutRev (Table 4.3) used to amplify the 256 bp internal gene fragment. C) The resulting insertional inactivation mutant, *V. midae* SY9Mut2, with the inactivated detergent resistant protease gene containing the intergrated pGP13.Cm recombinant suicide vector in the chromosomal DNA. Relevant restriction enzyme recognition sites and their position relative to the *Eco*RI (1) restriction enzyme site are indicated.

One of the two putative complemented *V. midae* SY9Mut2 mutants selected for further analysis was designated *V. midae* SY9Mut2.Comp. A single 1924 bp DNA fragment was amplified from the recombinant construct pMR11 (Fig. 4.14, lane 2; Fig. 4.15) harbouring the full-length *V. midae* SY9 detergent resistant protease gene (Fig. 4.2) with the detergent resistant protease gene primers ProMutFwd and ProMutRev (Table 4.3). There was no amplification of a product for the no template control (Fig. 4.14, lane 1; Fig.4.15) or *V. midae* SY9Mut2 (Fig. 4.14, lane 5; Fig. 4.15). A PCR product of approximately 7.2 kb would have been expected for *V. midae* SY9Mut2. However, under the PCR conditions employed, amplification of such a large product was not likely. A single 1924 bp amplicon was amplified from *V. midae* SY9 (Fig. 4.14, lane 4; Fig. 4.15) and *V. midae* SY9Mut2.Comp (Fig. 4.14, lane 6; Fig. 4.15). Therefore, a full-length detergent resistant protease gene is present in pMR11, the wild type strain *V. midae* SY9 and the complemented mutant *V. midae* SY9Mut2.Comp.

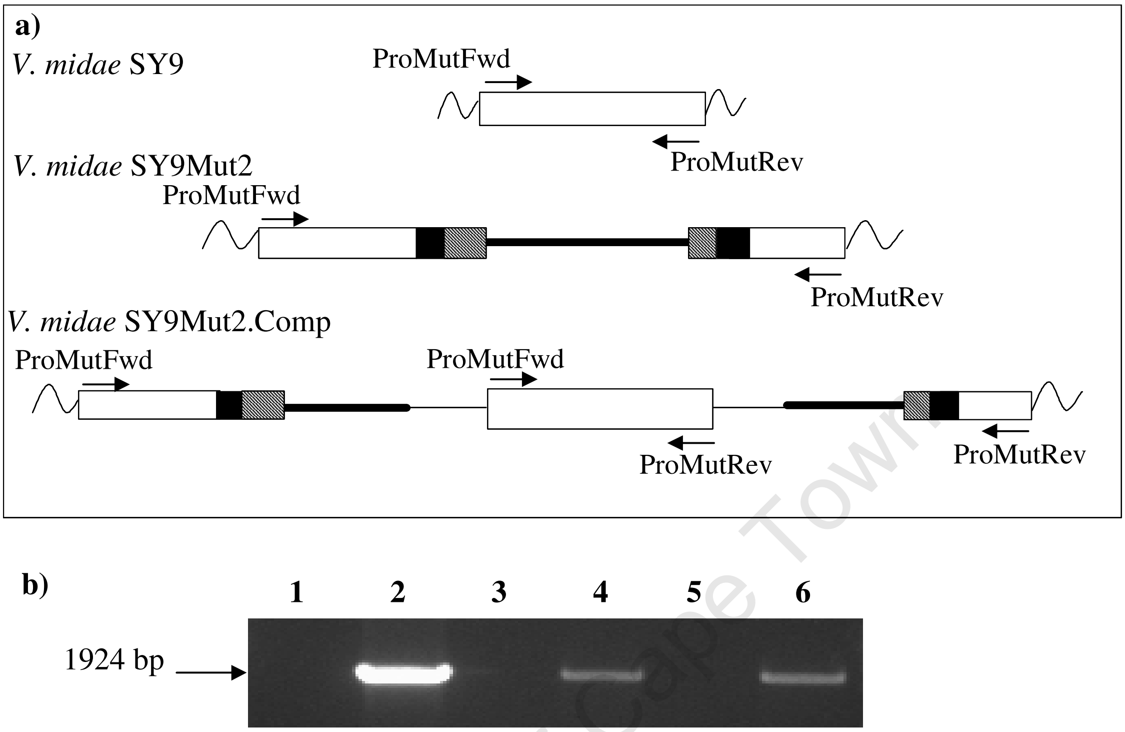


Figure 4.14 PCR confirmation of complementation of the insertionally inactivated mutant, *V. midae* SY9Mut2, by the recombinant suicide vector pGPro.Km. A schematic diagram (not to scale) of the chromosomal arrangement of *V. midae* SY9, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp (a), indicating the position of the primers ProMutFwd and ProMutRev. The black squares and the black- and white-hatched squares represent the 256 bp internal gene fragment cloned into pGP704 to construct pGP13. The thick black line represents the integrated pGP704 portion of pGP13, while the thin black line represents the pGP704 portion of pGPro.Km. Gel electrophoresis of amplified 1924 bp PCR products, using primers ProMutFwd and ProMutRev (b). Lane 1, No template control; lane 2, pMR11 (positive control); lane 3, empty; lane 4, *V. midae* SY9 chromosomal DNA; lane 5, *V. midae* SY9Mut2 chromosomal DNA; lane 6, *V. midae* SY9Mut2.Comp chromosomal DNA. The arrow indicates the approximate size of the amplified PCR product in base pairs (bp).

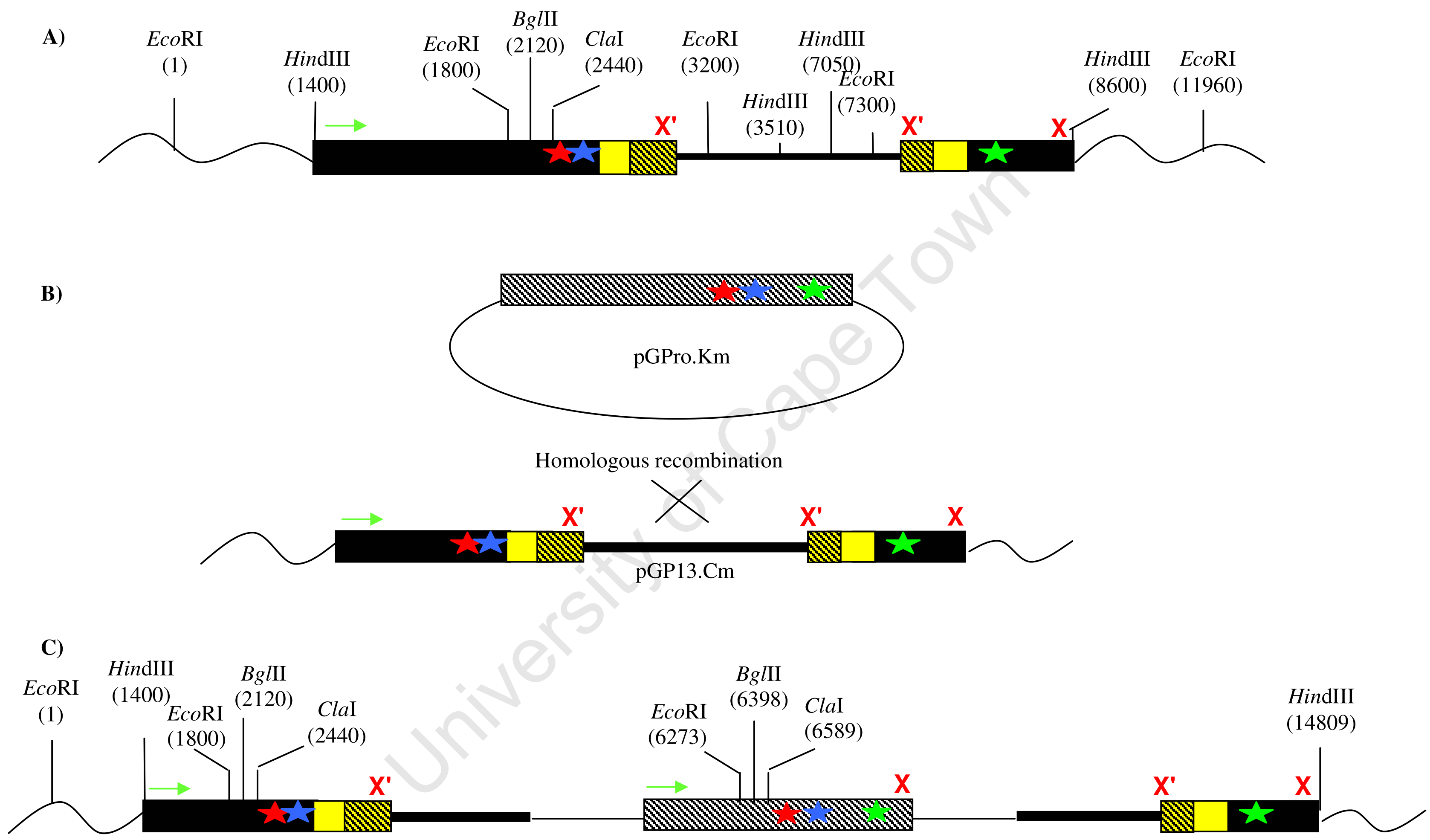

A)
EcoRI (1)
HindIII (1400)
EcoRI (1800)
BglII (2120)
ClaI (2440)
EcoRI (3200)
HindIII (3510)
HindIII (7050)
EcoRI (7300)
HindIII (8600)
EcoRI (11960)
X'
X'
X
B)
pGPro.Km
Homologous recombination
X'
X'
X
pGP13.Cm
C)
EcoRI (1)
HindIII (1400)
EcoRI (1800)
BglII (2120)
ClaI (2440)
X'
EcoRI (6273)
BglII (6398)
ClaI (6589)
X
HindIII (14809)
X'
X

Figure 4.15 **(Facing page)** Schematic representation (not to scale) depicting the homologous recombination event that complemented *V. midae* SY9Mut2 and the chromosomal arrangement of the resulting strain, *V. midae* SY9Mut2.Comp. A) The chromosomal arrangement of the mutant *V. midae* SY9Mut2. The stars represent the active site residues (red star - D^{180}, blue star - H^{213} and green star - S^{363}). The green arrow represents the promoter region and direction of transcription. The red crosses (X and X') represent the wild-type and inserted in-frame stop codons, respectively. The yellow bar and the yellow hatched bar represent the 256 bp internal *vmproA* gene fragment cloned into pGP704 to form the recombinant suicide vector pGP13. The thick black line represents the integrated pGP704 portion of the recombinant suicide vector pGP13.Cm. B) Recombinant suicide vector pGPro.Km (thin black line) containing the 1.9 kb *Hind*III - *Hind*III fragment (large white hatched band) and a kanamycin resistant gene (not shown). C) The chromosomal arrangement of the complemented insertional inactivation mutant, *V. midae* SY9Mut2.Comp, with the intergrated pGPro.Km recombinant suicide vector in the chromosomal DNA of *V. midae* SY9Mut2. Relevant restriction enzyme recognition sites and their position relative to the *Eco*RI (1) restriction enzyme site are indicated.

4.4.4 Growth and extracellular protease activity of *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp

A growth curve analysis was preformed to investigate the effect of mutating *vmproA* on cell growth and extracellular protease activity. *V. midae* SY9, the mutants *V. midae* SY9Pro2 and *V. midae* SY9Mut2, and the complemented mutant *V.midae* SY9Mut2.Comp, were cultivated in shake flasks containing MMM, P-MBM and MMM-P.

The growth profile and maximum specific growth rates of *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp are similar to that of *V. midae* SY9 when cultured in MMM at 22°C (Fig. 4.16 A to D; Table 4.4).

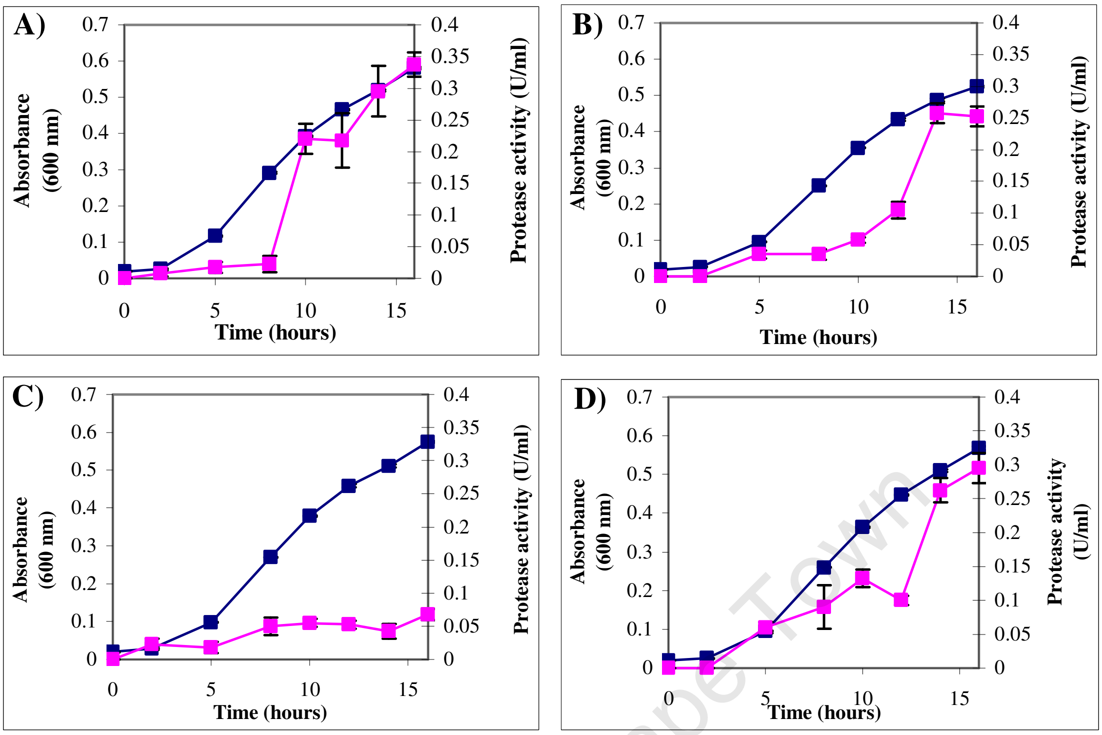


Figure 4.16 The growth and extracellular protease activity profiles of *V. midae* SY9 (A), *V. midae* SY9Pro2 (B), *V. midae* SY9Mut2 (C) and *V. midae* SY9Mut2.Comp (D) in MMM at 22°C on an orbital shaker (100 rpm) over the course of a 16 hour period. The cell growth was monitored by absorbance (600 nm) readings (■) and alkaline protease activities (■) within the culture supernatant were determined using standard assays. Data represents the mean ± standard error (n=3).

The extracellular protease activity of the *vmproA* gene duplication mutant, *V. midae* SY9Pro2, was 0.25 U ml^{-1} after 16 hours of growth in MMM at 22 °C (Fig. 4.16 B), which is a little lower than that of *V. midae* SY9 (0.34 U ml^{-1}) when cultivated under the same conditions (Fig. 4.16 A). While insertional inactivation of *vmproA* through the chromosomal integration of the recombinant suicide vector pGp13.Cm did not alter the bacterial growth profile, the extracellular protease activity is lower after the 16 hour analysis period for the mutant *V midae* SY9Mut2 in comparison with the wild-type strain when cultured in MMM (Fig. 4.16 C). The highest extracellular protease activity (0.07 U ml^{-1}) was recorded for *V midae* SY9Mut2 after 16 hours of growth in MMM at 22 °C (Fig. 4.16 C). Complementation of the insertional inactivation mutant by chromosomal integration of pGPro.Km, resulted in wild-type equivalent levels of extracellular protease activity (0.30 U ml^{-1}) for *V. midae* SY9Mut2.Comp when cultured in MMM at 22 ºC for 16 hours (Fig. 4.16 D).

The growth profiles and calculated maximum specific growth rates (Table 4.4) of the mutants *V. midae* SY9Pro2 (Fig. 4.17 B) and *V. midae* SY9Mut2 (Fig. 4.17 C), and complemented mutant *V. midae* SY9Mut2.Comp (Fig. 4.17 D), are similar to that of *V. midae* SY9 (Fig. 4.17 A) when batch cultivated in shake flasks containing P-MBM at 22 ºC.

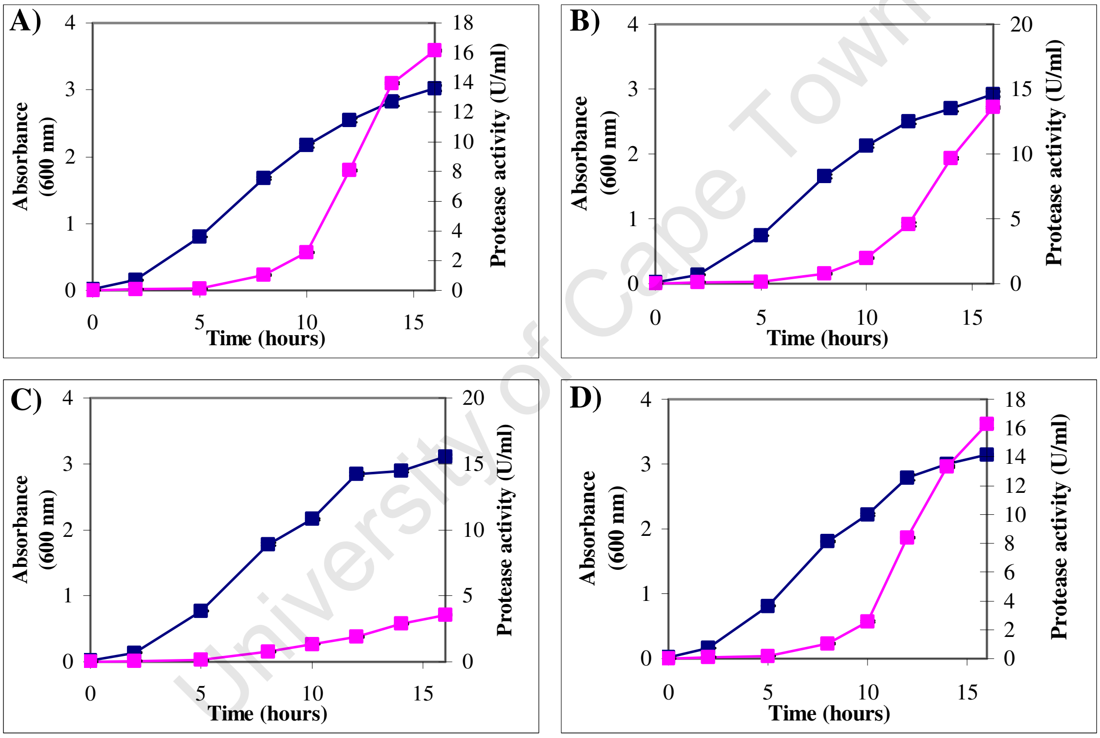


Figure 4.17 The growth and extracellular protease activity profiles of *V. midae* SY9 (A), *V. midae* SY9Pro2 (B), *V. midae* SY9Mut2 (C) and *V. midae* SY9Mut2.Comp (D) in P-MBM at 22 °C on an orbital shaker (100 rpm) over the course of a 16 hour period. The cell growth was monitored by absorbance (600 nm) readings (■) and alkaline protease activities (■) within the culture supernatant were determined using standard assays. Data represents the mean ± standard error (n=3).

The *vmproA* chromosomal duplication resulted in a slight decrease in the observed extracellular protease activity levels when *V. midae* SY9Pro2 was cultured in P-MBM at

22 ºC (Fig. 4.17B), when compared to *V. midae* SY9 (Fig. 4.17A). The profile of the *V. midae* SY9Pro2 extracellular protease activity profile is however similar to that of the wild-type strain (Fig. 4.17A). The extracellular protease activity levels of the insertional inactivation mutant *V. midae* SY9Mut2 (Fig. 4.17C) are significantly lower than that of *V. midae* SY9 (Fig. 4.17A) over the course of the 16 hour growth curve analysis. *V. midae* SY9 and *V. midae* SY9Mut2 extracellular protease activity levels are approximately 16.1 (Fig. 4.17A) and 3.6 U ml^{-1} (Fig. 4.17C), respectively, after 16 hours of growth in P-MBM at 22 ºC. The extracellular protease activity of the complemented mutant (Fig. 4.17D) is higher than that of *V. midae* SY9Mut2 (Fig. 4.17C), and the protease activity profile and activity levels over the course of the growth curve are similar to that of the wild type strain (Fig. 4.17 A) when cultured in P-MBM at 22 ºC.

The growth curve profiles of *V. midae* SY9 (Fig. 4.18A), *V. midae* SY9Pro2 (Fig. 4.18B), *V. midae* SY9Mut2 (Fig. 4.18C) and *V. midae* SY9Mut2.Comp (Fig. 4.18D) cultured in MMM-P at 22 ºC are comparable over the course of the 16 hour analysis period. Accordingly, the maximum specific growth rates of *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp are very similar when these strains are cultured in MMM-P at 22 ºC (Table 4.4).

The extracellular protease activity levels of the gene duplication mutant *V. midae* SY9Pro2 are higher than that of *V. midae* SY9, when both of the strains are cultivated in MMM-P at 22 ºC. After 16 hours of growth the extracellular protease activity of *V. midae* SY9 was approximately 6.62 U ml^{-1}, while *V. midae* SY9Pro2 culture supernatant contained approximately 11.80 U ml^{-1} of alkaline protease activity. The extracellular protease activity levels of the insertionally inactivated mutant *V. midae* SY9Mut2 were lower than that of *V. midae* SY9 (Fig. 4.18C) when cultured in MMM-P at 22 ºC. The mutant *V. midae* SY9Mut2 achieved a maximal level of protease activity of approximately 2.33 U ml^{-1} after 16 hours of growth in MMM-P (Fig. 4.18C). The complemented mutant strain *V. midae* SY9Mut2.Comp has a similar extracellular protease activity profile to *V. midae* SY9Pro2, achieving an extracellular protease activity level of approximately 10.2 U ml^{-1} after 16 hours of growth in MMM-P (Fig. 4.18D).

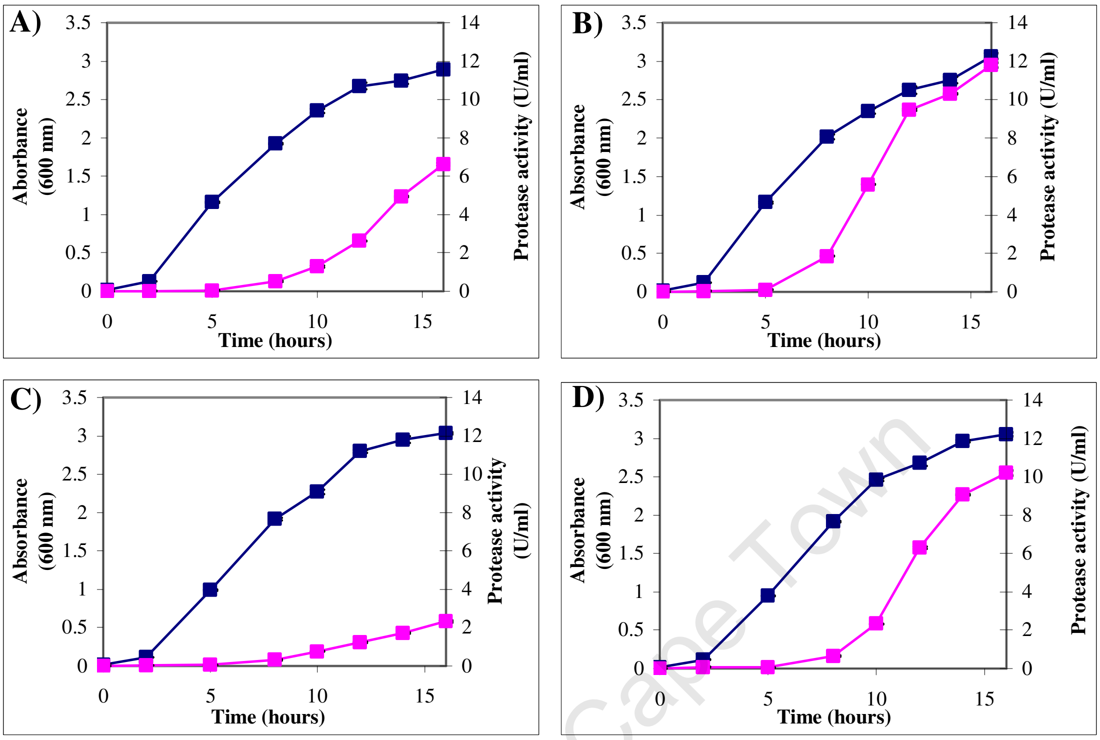


Figure 4.18 The growth and extracellular protease activity profiles of *V. midae* SY9 (A), *V. midae* SY9Pro2 (B), *V. midae* SY9Mut2 (C) and *V. midae* SY9Mut2.Comp (D) in MMM-P at 22 °C on an orbital shaker (100 rpm) over the course of a 16 hour period. The cell growth was monitored by absorbance (600 nm) readings (■) and alkaline protease activities (■) within the culture supernatant were determined using standard assays. Data represents the mean ± standard error (n=3).

Table 4.4 Maximum specific growth rate of *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp cultivated in shake flasks containing MMM, P-MBM and MMM-P at 22 °C.

Strains	**Maximum specific growth rate (h^{-1})**		
	MMM	**P-MBM**	**MMM-P**
***V. midae* SY9**	0.43	0.51	0.58
***V. midae* SY9Pro2**	0.40	0.53	0.60
***V. midae* SY9Mut2**	0.40	0.54	0.60
***V. midae* SY9Mut2.Comp**	0.41	0.52	0.59

4.4.5 Zymogram analysis of *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp

Gelatine and casein zymogram analyses were conducted in order to investigate the detergent resistant extracellular protease phenotype, and the molecular weight and number of extracellular alkaline proteases secreted by the *V. midae* SY9 mutant strains.

4.4.5.1 Gelatine zymogram analysis

A gelatine zymogram was performed on cell-free supernatant from *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp in order to investigate the extracellular proteolytic phenotype of these strains. The distinctive detergent resistant extracellular proteolytic enzyme is produced by *V. midae* SY9, *V. midae* SY9Pro2 and *V. midae* SY9Mut2.Comp (Fig. 4.19; lanes 1, 2 and 4), but was absent in the culture supernatant of *V. midae* SY9Mut2 (Fig. 4.19; lane 3). This confirms that the *V. midae* SY9 extracellular detergent resistant protease gene, *vmproA*, was inactivated leading to loss of the detergent resistant proteolytic phenotype of *V. midae* SY9Mut2.

4.4.5.2 Casein zymogram analysis

A casein zymogram was also performed on cell-free supernatant from *V. midae* SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 and *V. midae* SY9Mut2.Comp in order to further investigate the extracellular proteolytic phenotype of these strains. The 40 kDa extracellular protease is produced and secreted by *V. midae* SY9, *V. midae* SY9Pro2 and *V. midae* SY9Mut2.Comp (Fig. 4.20; lanes 1, 2 and 4), but not by *V. midae* SY9Mut2 (Fig. 4.20; lane 3). This confirms that *vmproA* was inactivated leading to the loss of the 40 kDa VmproA protease of *V. midae* SY9Mut2. A faint 30 kDa protease band was also detected on the casein zymogram in *V. midae* SY9Mut2.Comp culture supernatant (Fig. 4.20; lane 4). Five faint proteases, of approximately 34, 30, 28, 17 and 10 kDa in size, were detected in the cell-free culture supernatant of *V. midae* SY9Mut2 (Fig. 4.20; lane 5).

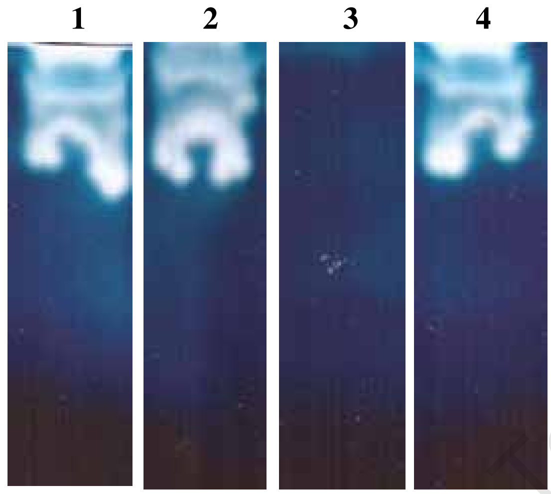


Figure 4.19 Gelatine zymogram investigating the detergent resistant proteolytic phenotype of *V. midae* SY9 (lane 1), *V. midae* SY9Pro2 (lane 2), *V. midae* SY9Mut2 (lane 3), and the complemented mutant *V. midae* SY9Mut2.Comp (lane 4) cultured in P-MBM for 24 hours at 22 °C. Each lane was loaded with an equal volume of cell-free culture supernatant from a 24 hour culture inoculated to the same initial A_{600}.

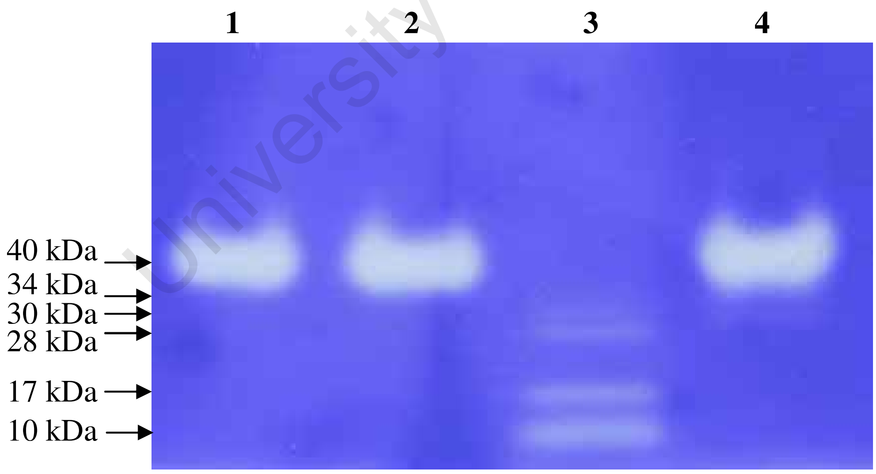


Figure 4.20 Casein zymogram of culture supernatant from *V. midae* SY9 (lane 1), *V. midae* SY9Pro2 (lane 2), *V. midae* SY9Mut2 (lane 3), and the complemented mutant *V. midae* SY9Mut2.Comp (lane 4) cultured in P-MBM for approximately 24 hours at 22 °C. Each lane was loaded with an equal volume of cell-free culture supernatant from a 24 hour culture inoculated to the same initial A_{600}. The arrows represent the approximate sizes, in kDa, of the protease bands.

4.5 Discussion

V. midae SY9 *vmproA* mutant strains were constructed and characterised in order to enable more advanced and specific investigations into the function of the detergent resistant protease gene, *vmproA*, and its gene product VmproA, in the increased growth advantage of abalone fed a probiotic-supplemented diet (Macey and Coyne, 2005).

The wild-type *V. midae* SY9 strain was altered by chromosomal *vmproA* gene duplication, in an attempt to produce an increased *vmproA* expression mutant, *V. midae* SY9Pro2. Both chromosomal copies of *vmproA* within *V. midae* SY9Pro2 would be under the control of their own promoter elements which are identical to the wild-type *vmproA* promoter. Therefore, the duplicate mutant *vmproA* genes would not be constitutively- or over-expressed as they might have been if the additional *vmproA* gene was contained on a non-integrating plasmid. Instead, the duplicated *vmproA* genes would presumably be expressed in response to the same stimuli as the wild-type, single copy *vmproA* gene; any increased expression and resulting extracellular protease activity would be as a result of mRNA transcripts being expressed from both *vmproA* genes within the chromosome of *V. midae* SY9Pro2.

Chromosomal integration of pGPro.Cm was confirmed by Southern hybridization and shown to have no significant influence on the growth profile and growth rate of *V. midae* SY9Pro2 when cultured in a minimal medium, a high-protein growth medium, or a minimal medium supplemented with peptone as a protein source. There was no observed increase in the activity profile or the levels of *V. midae* SY9Pro2 extracellular protease activity, compared to *V. midae* SY9, when cultured in either the minimal medium or P-MBM, despite the presence of a second chromosomal copy of *vmproA* within close proximity to the original *vmproA*. The low levels of extracellular protease activity of *V. midae* SY9 and *V. midae* SY9Pro2 when cultured in the minimal medium may be the result of the bacteria secreting hydrolytic enzymes into the surrounding growth medium for nutrient acquisition (Kalisz, 1988; Albertson *et al.,* 1990). The lack of a suitable protease-inducing complex protein source within the minimal medium may have prevented expression of *vmproA* to the levels observed for cultures grown in an inducing medium such as P-MBM. As a result, the alkaline protease activity remained relatively low over the course of the analysis period in comparison to the higher extracellular protease activity levels detected when the same strains were cultured in a high-protein growth medium.

When *V. midae* SY9Pro2 was cultured in a minimal medium supplemented with peptone, the levels of extracellular protease activity were higher than that of *V. midae* SY9 cultivated under the same conditions. The difference between protease activity levels of *V. midae* SY9Pro2 when cultivated in P-MBM and MMM-P may be as a result of a growth medium component, such as yeast extract, within P-MBM. Depending on the quality, properties and concentration of the reducing sugar, peptides, and amino acids present within a media component, such as yeast extract, the growth media may either induce or repress protease production and secretion (Singh *et al.,* 2004). The increased activity levels observed when *V. midae* SY9Pro2 was cultivated in MMM-P indicates that *vmproA* gene duplication has the potential to result in increased protease production under the appropriate culture conditions. Casein and gelatine zymography showed that *V. midae* SY9Pro2 produced and secreted the detergent-stable 40 kDa extracellular alkaline protease.

In this study, the *V. midae* SY9 detergent-stable extracellular protease gene was insertionally inactivated using the recombinant suicide vector pGP13.Cm. Similar insertional inactivation strategies, using recombinant suicide vectors targeting specific proteolytic genes, have been used to construct knockout mutant strains of several *Vibrio* species in order to evaluate the functions of these proteases (Häse and Finkelstein, 1991; Milton *et al.,* 1992; Jeong *et al.,* 2000). Chromosomal integration of pGP13.Cm into the *vmproA* gene resulted in a mutant strain displaying significantly reduced levels of extracellular protease activity in comparison with *V. midae* SY9. Despite insertional inactivation of the *vmproA* gene, *V. midae* SY9Mut2 produced a low level of extracellular protease activity in all of the growth media investigated in this study. This is however not unexpected since *Vibrio* species are known to produce a number of extracellular proteases (Long *et al.,* 1981; Farrell and Crosa, 1991; Wang *et al.,* 2007). Milton *et al.* (1992) constructed a mutant strain of *V. anguillarum* NB10 via the integration of a recombinant suicide vector into the 36 kDa metalloprotease gene. The mutant strain, *V. anguillarum* NB12, displayed significantly reduced levels of extracellular proteolytic activity in comparison with the wild-type strain. Despite inactivation of the metalloprotease gene, *V. anguillarum* NB12 produced a zone of clearing when cultured on gelatine solid media, although the zone was significantly smaller than that produced by the wild-type strain. Häse and Finkelstein (1991) constructed a Haemagglutinin/Protease (HA/Protease) -negative strain of *V. cholerae* by mutating the HA/Protease (*hap*) gene through chromosomal integration of a plasmid harbouring a kanamycin-interrupted copy of the wild-type *hap* gene. Despite the inactivation of the *hap* gene, *V. cholerae* HAP-1 displayed a proteolytic phenotype, although this was significantly diminished. Häse and Finkelstein (1991) explain the extracellular proteolytic phenotype of the HA/Protease-negative strain to be the result of several additional un-mutated extracellular proteases produced and secreted by *V. cholerae* (Young and Broadbent, 1982). The growth of the mutant strain, *V. cholerae* HAP-1, did not appear to be affected by insertional inactivation of the *hap* gene (Häse and Finkelstein, 1991). Similarly in this study, despite inactivation of the *vmproA* gene and the loss of the predominant detergent resistant extracellular protease, *V. midae* SY9Mut2 had similar growth profiles and growth rates to that of the wild type strain when cultivated in the marine growth media P-MBM and MMM-P at 22 ºC. The extracellular protease enzyme activity of *V. midae* SY9Mut2 was significantly reduced in all the culture media investigated in this study, in comparison with *V. midae* SY9. The low level of extracellular proteolytic activity observed for *V. midae* SY9Mut2 over the course of the 16 hour growth curve analysis was most likely the activity of several additional extracellular proteases. Five extracellular proteases, of approximately 34, 30, 28, 17, and 10 kDa in size, were detected in the culture supernatant of *V. midae* SY9Mut2. The 30 kDa protease detected within *V. midae* SY9Mut2 supernatant is approximately the same size as protease B (Chapter 3), which was previously observed on gelatine and casein zymograms of *V. midae* SY9 culture supernatant. Similarly, either the 17 or the

10 kDa protease band may be the smaller protease C (Chapter 3) that was previously detected on a gelatine zymogram. Milton *et al.* (1992) observed two additional protease bands on a gelatine-SDS zymogram within the culture supernatant of *V. anguillarum* ND12 that were not detected in the supernatant of the wild-type and/or complemented mutant strains. The two additional proteolytic enzymes were thought to either be the result of two extra protease genes that were up regulated in response to inactivation of the metalloprotease or two further proteolytic enzymes degraded in the presence of the 36 kDa metalloprotease (Milton *et al.,* 1992). Similarly, the remaining extracellular proteases that have to date only been detected on the *V. midae* SY9Mut2 casein zymogram may be the result of up-regulation of genes encoding these protease proteins as a result of the inactivation of *vmproA*.

In this study, the insertional inactivation mutant *V. midae* SY9Mut2 was complemented using the recombinant suicide vector pGPro.Km which integrated into the *V. midae* SY9Mut2 chromosome. Complementation of inactivated genes is often used to confirm that the phenotype of a constructed mutant strain is a direct effect of the inactivation of a particular gene of interest. *V. cholerae* HAP-1, a HA/Protease-negative strain of *V. cholerae*, was complemented with a non-integrating plasmid harbouring a full-length copy of the wild-type *hap* gene (Häse and Finkelstein, 1991). Similarly, Jeong *et al.* (2000) complemented the *V. vulnificus vvpE* knock-out mutant strain with a plasmid containing a full-length and fully functional copy of the wild-type *vvpE* gene. The *V. vulnificus vvpE* mutant was complemented in order to confirm that the reduced proteolytic activity observed for the mutant strain was the result of the insertional inactivation of the functional *vvpE* gene and not due to a downstream polar effect of chromosomal integration of the recombinant suicide vector. The *V. midae* SY9Mut2 complemented mutant regained the extracellular proteolytic phenotype of the wild-type strain. Indeed, *V. midae* SY9Mut2.Comp produced a larger zone of hydrolysis, in comparison with the wild-type strain, when cultured on solid skim-milk growth media (data not shown). A similar effect was observed for the *V. cholerae* HAP-1 complemented mutant (Häse and Finkelstein, 1991). Chromosomal integration of pGPro.Km containing a full-length copy of the *vmproA* gene resulted in the complemented mutant having the ability to produce and secrete VmproA. *V. midae* SY9Mut2.Comp produced and secreted the 40 kDa protease and displays the distinctive detergent-stable extracellular proteolytic phenotype of the wild-type strain.

The growth profile of *V. midae* SY9Mut2.Comp was comparable to that of *V. midae* SY9, *V. midae* SY9Pro2 and *V. midae* SY9Mut2 in all of the culture media investigated in this study. However, the protease activity of the complemented mutant strain was significantly higher than that of *V. midae* SY9Mut2 and was equivalent to that of the wild-type strain when the strains were cultivated in minimal medium or P-MBM. When *V. midae* SY9Mut2.Comp was cultivated in MMM-P for approximately 16 hours, there was a 1.54-fold increase in extracellular alkaline protease activity in comparison with *V. midae* SY9 and a 4.38-fold increase in comparison with *V. midae* SY9Mut2. The increased protease activity of the complemented mutant, in comparison with the wild-type strain, may either be due to the presence of two promoter elements for *vmproA* within close proximity on the chromosome, or that the intact *vmproA* gene may have been separated from a transcriptional repressor. Further investigation using northern blot hybridization analyses or RT-PCR to study *vmproA* expression in the complemented mutant, *V. midae* SY9 and *V. midae* SY9Mut2 may ascertain whether transcriptional up-regulation is indeed responsible for the increased extracellular protease activity observed for *V. midae* SY9Mut2.Comp when cultivated in MMM-P.

The *V. midae* SY9Pro2 and *V. midae* SY9Mut2 mutant strains of the probiont *V. midae* SY9 will be employed in an aquarium-based abalone growth trial (Chapter 5) to determine the role of *vmproA*, and its protease gene product VmproA, in the previously observed growth advantage of *H. midae* fed a probiotic-supplemented formulated diet (Macey and Coyne, 2005).

CHAPTER 5

THE EFFECT OF DIETARY SUPPLEMENTATION WITH *VIBRIO MIDAE* SY9, *VIBRIO MIDAE* SY9PRO2 AND *VIBRIO MIDAE* SY9MUT2 ON THE GROWTH OF *HALIOTIS MIDAE*.

CONTENTS

5.1 Summary

V. midae SY9, *V. midae* SY9Pro2 and *V. midae* SY9Mut2 were investigated in an abalone growth trial in order to elucidate the role of *vmproA* in the previously observed growth advantage of probiotically supplemented *H. midae* (Macey and Coyne, 2005). The feasibility of using vacuum impregnation technology to incorporate the probiotic strains into a formulated feed was assessed to ensure that the feed contained more than 1.0 x 10^6 culturable cells g^{-1} ABFEED® S34. The optimal storage condition for vacuum impregnated feed was found to be 4 ºC. Although there was a significant (P<0.05) decline in the number of culturable probiotic cells within the impregnated ABFEED® S34 stored at 4ºC, the viable number of probiotic cells remained above a critical level of 1.0 x 10^6 culturable cells g^{-1} ABFEED® S34 over a 10 day period for all three probiotic strains. *V. midae* SY9 supplementation of ABFEED® S34 weaning chips resulted in a 6.5% improvement in the growth rate of *H. midae*, in comparison with abalone fed a basal diet over the course of a 180 day growth trial. Supplementation of ABFEED® S34 with *V. midae* SY9Pro2 and *V. midae* SY9Mut2 increased abalone growth rates by 15.0 and 14.5%, respectively. *In situ* protease assays showed that the alkaline protease activity within the crop/stomach and intestinal regions of the abalone digestive tract was significantly (P<0.05) increased in animals fed the *V. midae* SY9 supplemented diet. Abalone fed ABFEED® S34 containing the mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2 did not have enhanced protease activity within the crop/stomach region when compared to the basal fed animals. The crop/stomach and intestinal *in situ* alkaline protease activity of animals fed *V. midae* SY9Pro2 supplemented feed was significantly lower (P<0.05) than in abalone that received the *V. midae* SY9 supplemented ABFEED® S34 diet. However, the *in situ* protease activity within the intestinal regions of animals fed *V. midae* SY9Pro2 supplemented feed was significantly higher (P<0.05) than in abalone being fed either the basal feed or a *V. midae* SY9Mut2 supplemented diet. The *in situ* protease activity within the intestinal regions of abalone fed either the basal diet or the basal diet supplemented with *V. midae* SY9Mut2 was not significantly (P>0.05) different.

5.2 Introduction

The success of any agricultural practice involving animal production relies on optimized growth of the cultured species so that the animals reach a marketable size in an economically viable timeframe (Fleming, 1995). One of the main challenges facing the abalone aquaculture industry is to improve the relatively slow growth rate of abalone. While the growth rate of wild populations of abalone is very slow, initial research into abalone aquaculture in South Africa showed that the growth rate of cultured animals was faster than that of natural abalone stocks (Cook, 1990). However, it still takes in the region of five years for cultured animals to reach a marketable size of approximately 100 grams. Research on improving the growth rate of cultured abalone has focused on the development of artificial diets (Simpson, 1994; Knauer *et al.,* 1996; Britz and Hecht, 1997), optimizing feed conversion ratios (Britz, 1996; Bautista-Teruel *et al.,* 2003) and culture conditions (Cook, 1990; Britz and Hecht, 1997; Britz *et al.,* 1997; Harris *et al.,* 1998a; Harris *et al.,* 1999).

The ongoing research into the development of artificial formulation diets for *H. midae* culture is considered critical to the long-term growth and success of the local abalone aquaculture industry (Britz, 1994). The most important nutritional parameters are the protein, essential amino acids, lipids, vitamins and mineral content of the artificial feed (Hahn, 1989a). Of these, protein is the most expensive dietary component of abalone artificial feeds (Fleming *et al.,* 1996). Abalone growth rates have been positively correlated to the dietary protein content of the artificial feed (Britz, 1996). Therefore, the protein content of artificial feed is essential for growth, and by either using easily digested protein sources or improving the ability of the animal to digest the available protein within the artificial feed, the growth rate can be improved, resulting in increased production and profits for the abalone aquaculture industry.

Enteric bacteria have been implicated in the nutrition of various marine organisms (Erasmus *et al.,* 1997; Simon and McQuaid, 1999; Bairagi *et al.,* 2002; Kar and Ghosh, 2008). Erasmus *et al.* (1997) showed that enteric bacteria are present throughout the digestive tract of the South African abalone, *H. midae* and that these enteric bacteria may have the potential to improve the digestion efficiency of the host through secretion of hydrolytic enzymes that contribute to the pool of digestive enzymes available for the digestion of complex polysaccharides by the abalone host. Therefore, Erasmus *et al.* (1997) suggested that dietary supplementation with selected bacteria might improve the growth rate of cultured abalone as a consequence of improved digestion efficiency and feed utilization.

In the first reported study of this nature on the South African abalone, the growth rate of farm-based *H. midae* was increased by dietary supplementation with a mixture of three probiotic strains, *Vibrio midae* SY9, *Cryptococcus* sp. SS1 and *Debaryomyces hansenii* AY1 (Macey and Coyne, 2005). The three-probiotic strains were mixed with the ingedients of ABFEED® S34, extruded and dried at the Marifeed (Pty.) Ltd. factory in Hermanus, South Africa. The probiotic strains were added to the formulation feed to achieve a final concentration of approximately 10^7 viable cells g^{-1} of dried ABFEED® S34. The probiotic-supplemented ABFEED® S34 pellets were fed to *H. midae* in two large, long-term, farm-based growth trials that were conducted on two size classes of abalone with initial shell lengths of 20 and 67 mm in order to assess the effect of dietary probiotic supplementation on both small and larger abalone. The monthly growth rate in shell length of the smaller animals increased by up to 8%, while the larger animals grew up to 33% faster following probiotic supplementation for 252 and 186 days, respectively. The improvement in the monthly growth rate in weight of the smaller animals was 7%, while the larger animals grew 35% faster following probiotic supplementation for 252 and 186 days, respectively.

Macey and Coyne (2005) also showed that abalone receiving the probiotic supplemented diet had significantly increased levels of intestinal protease activity and an improved ability to digest and absorb proteins in comparison to animals receiving the basal ABFEED® S34 diet. A significant correlation was detected between the presence of *V. midae* SY9 and enhanced alkaline protease activity within the intestinal regions of probiotically supplemented ABFEED® S34 (Macey, 2005). Therefore, Macey and Coyne (2005) concluded that the increased protein digestion efficiency may be due to *V. midae* SY9 secreted extracellular proteases within the abalone intestinal regions.

In the investigation conducted by Macey and Coyne (2005), the probiotically supplemented ABFEED® S34 was prepared at Marifeed (Pty.) Ltd. However, for the purposes of this study we were unable to make use of the mixing, extrusion and drying facilities of the commercial Marifeed (Pty.) Ltd. factory and investigated the feasibility of several alternative methods of incorporating the *V. midae* SY9 strains into ABFEED® S34. The only practical solution at our disposal at the time of this investigation was to supplement ABFEED® S34 weaning chips with *V. midae* SY9 and the mutant strains (Chapter 4) using a vacuum infusion system provided by Atlantic Sea Farm, St. Helena Bay.

Vacuum impregnation technologies have been employed to introduce liquid micro ingredient additives and physiologically active compounds such as probiotic microorganisms into porous products (Betoret *et al.,* 2003; reviewed by Alzamora *et al.,* 2005). Indeed, vacuum impregnation systems have been successfully used within the pet food and aquaculture feed manufacturing industries to incorporate heat labile additives such as vitamins, lipids, oils, minerals, digestive enzymes and probiotic bacteria into extruded and dried feed, in order to preserve the biological value of the infused micro ingredient (http://www.firstmate.com/default.aspx and http://www.uas.bc.ca). Investigations regarding the feasibility of incorporating viable microorganisms into porous foods have shown that *Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, *Phoma glomerata* and *Bifidobacterium* spp. Bb12 could be incorporated into apple pieces by vacuum impregnation (Rodríguez, 1998 and Maguiña *et al.,* 2002, as reviewed by Alzamora *et al.,* 2005). During vacuum impregnation, the products to be impregnated are placed in an airtight chamber and a vacuum is drawn within the chamber using a vacuum pump. During the vacuum cycle, air is extracted from both the air-filled spaces within the porous feed product and out of the airtight chamber (Fito, 1994 as reviewed in Alzamora *et al.,* 2005). Thereafter, the impregnation liquid containing the additive is sprayed into the chamber, thoroughly coating the feed and air is then allowed to re-enter the chamber in a slow and controlled manner. As the pressure within the chamber returns to atmospheric pressure, the impregnation liquid containing the micro ingredient additives is forced into the spaces within the feed pellets or chips by capillary action (Fito, 1994 as reviewed in Alzamora *et al.,* 2005). The additive-containing feed can then be air dried, packaged and stored at an appropriate temperature in order to preserve the additive (http://www.firstmate.com/default.aspx and http://www.uas.bc.ca).

5.2.1 Aim of this chapter

The aim of this study will be to determine the feasibility of vacuum infusion as a method to supplement ABFEED® S34 weaning chips with probiotic bacterial strains for use in an aquarium based growth trial. The ABFEED® S34 weaning chips will be vacuum infused with the probiotic strain *V. midae* SY9 and the mutant strains *V. midae* SY9Mut2 and *V. midae* SY9Pro2 (Chapter 4), and fed to juvenile *H. midae*. *In situ* protease activity and growth parameters of animals receiving either a control basal ABFEED® S34 weaning chip diet or an ABFEED® S34 diet supplemented with either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 will be compared. In so doing, we aim to better understand the role of *vmproA* in the previously observed probiotic effect of *V. midae* SY9 on cultured *H. midae*.

5.3 Materials and Methods

All the growth media and solutions used in this study are listed in Appendix A.

5.3.1 Bacterial strains and plasmids

All the bacterial strains used in this study are listed in Table 5.1.

Table 5.1 Bacterial strains employed in this study

Strains	**Genotype/relevant characteristic(s)**[a]	**Reference**
Vibrio midae SY9	Isolated from the digestive tract of *H. midae*, South Africa	Macey and Coyne, 2005
Vibrio midae SY9Mut2	*V. midae* SY9 strain harbouring the integrated recombinant suicide vector, pGP22.Cm; Cm^r	This study (Chapter 4)
Vibrio midae SY9Pro2	*V. midae* SY9 strain harbouring the integrated recombinant suicide vector, pGPro.Cm; Cm^r	This study (Chapter 4)

[a] Cm^r, chloramphenicol resistant.

5.3.2 Preparation of experimental ABFEED® S34 diets

The basal diet used in this study was ABFEED® S34 Weaning Chips (Marifeed (Pty.) Ltd., Hermanus, South Africa). The three probiotic-supplemented diets were prepared by incorporating the three bacterial strains, *V. midae* SY9, *V. midae* SY9Mut2 and *V. midae* SY9Pro2 (Table 5.1), separately into ABFEED® S34 weaning chips (section 5.3.2.2).

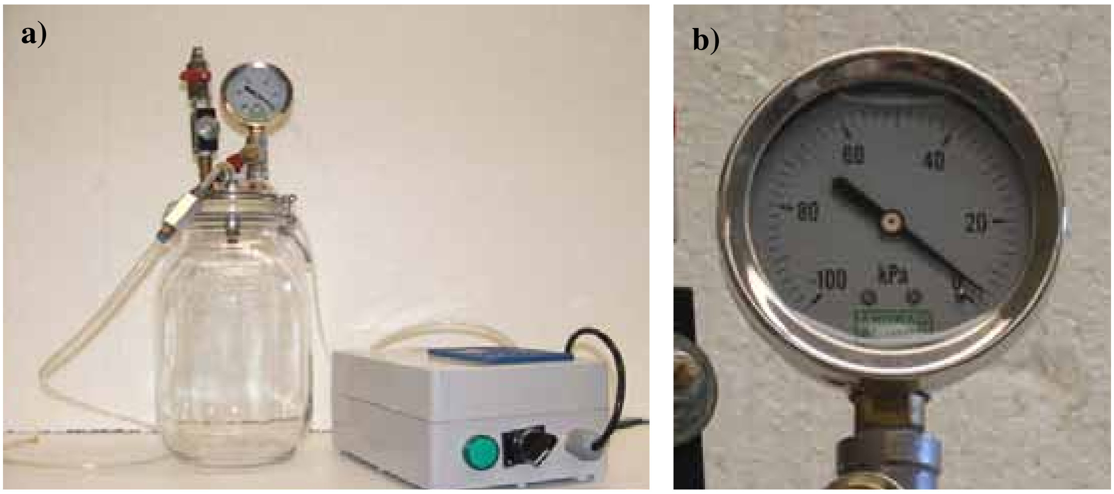


Figure 5.1 The vacuum impregnation apparatus (a) used for incorporation of the probiotic strains into ABFEED® S34 weaning chips. The vacuum pump is attached to a vacuum jar with a plastic hose and a valve. The vacuum jar has a lid with an efficient air-tight seal and a pressure gauge (b) on top to maintain and monitor the pressure within the vacuum jar during the vacuum impregnation process.

5.3.2.1 Cultivation of *V. midae* SY9, *V. midae* SY9Mut2 and *V. midae* SY9Pro2 for incorporation into the experimental diets

All three bacterial strains were grown for 8 hours in 5 ml starter cultures of P-MBM (Appendix A.1.3) for *V. midae* SY9 and P-MBM supplemented with chloramphenicol (Appendix A.2.2) to a concentration of 30 µg ml^{-1} for *V. midae* SY9Mut2 and *V. midae* SY9Pro2 on an orbital shaker (100 rpm) at 22 °C. Three flasks, each containing 100 ml P-MBM, were inoculated with the entire volume of each starter culture and incubated on an orbital shaker (100 rpm) for 12 hours at 22 ºC. The 100 ml cultures were inoculated into five litre flasks (in triplicate), containing 1000 ml of P-MBM, to achieve an initial A_{600} of 0.02 and incubated for 24 hours at 22 °C with shaking (100 rpm). Thereafter, the bacterial cells were harvested by centrifugation (8,000x g for 15 minutes at 4 °C), washed with one volume of ASW (Appendix A.2.1) and resuspended in 100 ml of ASW.

5.3.2.2 Incorporation of the probiotic bacterial strains into the ABFEED® S34 experimental diet by vacuum impregnation

The bacterial strains were incorporated into ABFEED® S34 weaning chips using vacuum impregnation technology (Fig. 5.1), to a final concentration of at least 1.0 x 10^8 culturable cells g^{-1} feed.

Approximately 200 g of the ABFEED® S34 weaning chips were placed inside the glass vacuum jar (Fig. 5.1a) which was then sealed with a rubber seal and a tight clamp. A vacuum pump was used to draw a vacuum within the glass jar to 80 kPa, and 20 ml of the resuspended bacteria (section 5.3.2.1) was then drawn into the vacuum jar and thoroughly mixed with the feed by shaking the vessel. During the process of drawing the bacterial suspension into the chamber, the vacuum within the vacuum jar decreased to 50 kPa and was subsequently maintained at 50 kPa for 5 minutes. Thereafter, the vacuum was slowly released to atmospheric pressure and the impregnated ABFEED® S34 weaning chips were removed and placed on clean Whatman 3MM paper to dry (section 5.3.2.2.1).

5.3.2.2.1 Optimal post-vacuum impregnation drying conditions

Post-vacuum impregnation drying conditions were assessed in order to determine the optimal conditions for cell survival and viability. A batch of *V. midae* SY9Mut2, cultivated as described in section 5.3.2.1, was vacuum impregnated as described in section 5.3.2.2 and then dried at either 37 ºC for 2 hours or 22 ºC for 16 hours. The cell viability and survival following the drying process was assessed by homogenizing 1 g quantities (in triplicate) of undried, freshly infused and dried (22 and 37 ºC) infused ABFEED® S34 weaning chips in 4 ml of ASW (Appendix A.2.1). Viable cell numbers were determined by spread-plating serial dilutions, in ASW, of the homogenized ABFEED® onto MA (Appendix A.1.2) containing 30 µg ml^{-1} chloramphenicol (Appendix A.2.2). Cell numbers were recorded after incubating the plates for 48 hrs at 22 ºC and viability recorded as the number of cfu g^{-1} of ABFEED®. The viable cell count data was natural log transformed and analyzed by paired Student *t*-tests using SigmaStat 3.11.0 (Systat Software, Inc.). Statistically significant differences were established at critical levels of $P<0.05$.

5.3.2.2.2 Optimal storage conditions of probiotic supplemented ABFEED® S34

In order to determine the optimal storage temperature for the probiotic impregnated ABFEED®, a batch of each of the three probiotic strains (cultivated as described in section 5.3.2.1) was impregnated, as described in section 5.3.2.2, dried at 22 ºC for 16 hours and then stored at 4 and 22 ºC for up to 10 days. Viable bacterial cell counts were determined by homogenizing 1 g quantities (in triplicate) of infused ABFEED® in 4 ml of ASW (Appendix A.2.1). Serial dilutions, in ASW, of the homogenized ABFEED® S34 weaning chips supplemented with the probiotic strains were spread-plated onto MA (Appendix A.1.2) to culture *V. midae* SY9 and MA supplemented with chloramphenicol (Appendix A.2.2) at a concentration of 30 µg ml^{-1} to culture the mutant strains *V. midae* SY9Mut2 and *V. midae* SY9Pro2. Culturable bacterial cell numbers were recorded after incubating the *V. midae* SY9 strains for 48 hours at 22 ºC. The viability was recorded as the number of cfu g^{-1} of ABFEED®. The viable cell count data was natural log transformed and analyzed by paired Student *t*-tests using SigmaStat 3.11.0 (Systat Software, Inc.). Statistically significant differences were established at critical levels of $P<0.05$.

5.3.2.2.3 Stability of *V. midae* SY9Mut2 vacuum impregnated ABFEED® S34

The stability of the probiotic bacteria in the vacuum impregnated ABFEED® S34 weaning chips incubated in the flow-through growth trial tanks was investigated. This was done in order to determine how often the impregnated ABFEED® S34 would need to be removed from the abalone tanks and replaced with fresh impregnated ABFEED® S34 during the course of the growth trial.

A batch of *V. midae* SY9Mut2 vacuum impregnated ABFEED® S34 weaning chips was prepared as described in section 5.3.2.2. Approximately 50 g of impregnated ABFEED® weaning chips were placed in an otherwise empty abalone basket (30 x 24 x 26 cm) suspended in a large plastic tank (68 x 40 x 38 cm). Seawater, pumped directly from the sea, passed through the tank at a rate of approximately 330 litres hour^{-1}. The tank was aerated by means of a large air stone suspended inside the tank.

A sample was immediately removed (T = 0) and after this, samples were removed at 30 minutes, 24 and 72 hours. Viable bacterial cell counts were determined by homogenizing 1 g quantities (in triplicate) of infused ABFEED® S34 weaning chip samples in 4 ml of ASW (Appendix A.2.1). Serial dilutions, in ASW, of the homogenized ABFEED® samples were spread-plated onto MA (Appendix A.1.2) supplemented with chloramphenicol (Appendix A.2.2) at a concentration of 30 µg ml^{-1} to culture the mutant strain, *V. midae* SY9Mut2. Culturable bacterial cell numbers were recorded after incubating the *V. midae* SY9Mut2 for 48 hours at 22 ºC. Viability was recorded as the number of cfu g^{-1} of ABFEED®. The viable cell count data was analyzed using SigmaStat 3.11.0 (Systat Software, Inc.) by one-way ANOVA. When the results of the one-way ANOVA were significant, the Tukey test was used to test for significant differences between the sample means. Statistically significant differences were established at critical levels of P <0.05.

5.3.3 *Haliotis midae* growth trial

The effect of the three probiotic strains, *V. midae* SY9, *V. midae* SY9Mut2 and *V. midae* SY9Pro2 included in ABFEED® S34 weaning chips, on *H. midae* growth was investigated in a laboratory based growth trial. This trial was conducted at the Marine and Coastal Management Research Aquarium, Sea Point, South Africa. Abalone were obtained from Atlantic Sea Farm, St. Helena Bay, South Africa. Abalone were housed in baskets (30 x 24 x 26 cm) suspended in large plastic tanks (68 x 40 x 38 cm) (Fig. 5.2 and 5.3). Seawater passed through the tanks at a rate of approximately 330 litres hour^{-1} and was aerated by means of a large air stone suspended in the tanks. The abalone were an average initial size of 28 mm (shell length) and were stocked at a density of 33 animals/basket. Animals were acclimatized to the conditions at the aquarium for 4 weeks prior to the beginning of the growth trial. During this acclimatization period, all the abalone were fed the control basal diet of ABFEED® S34 weaning chips. For the growth trial, the abalone were divided into four separate tanks, each containing two baskets. Abalone in each tank were fed a different diet (Fig. 5.2). Abalone in the first tank were fed the basal diet of ABFEED® S34 weaning chips, while the animals in the second tank were fed the basal diet supplemented with *V. midae* SY9. *H. midae* in the third tank were fed the basal diet supplemented with *V. midae* SY9Mut2, and the abalone in the fourth tank were fed the basal diet supplemented with *V. midae* SY9Pro2 (Fig. 5.2).

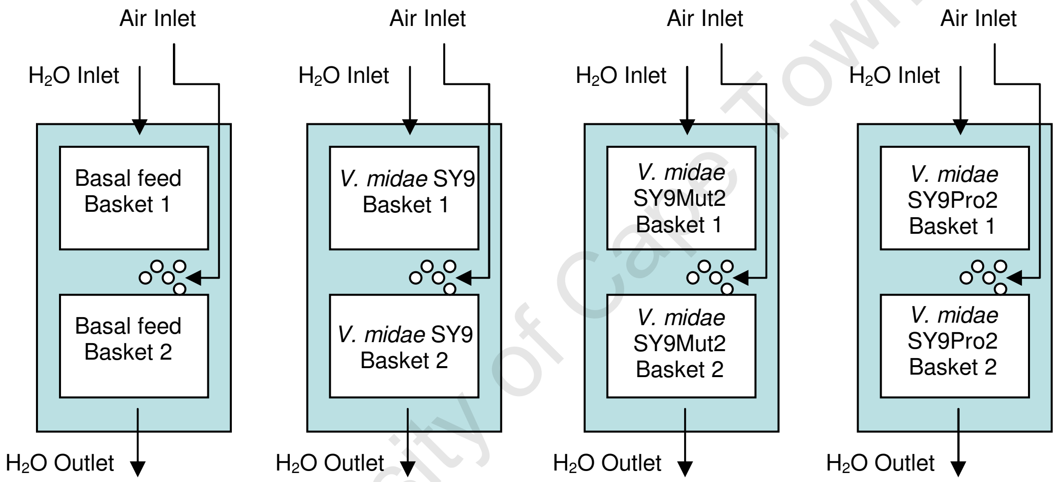


Figure 5.2 Schematic diagram of the experimental set-up of the *H. midae* growth trial.

Prior to the start of the growth trial, each animal was individually labeled with a numbered tag (Fig. 5.3b). This was done in order to enable monitoring of the growth of individual animals over the course of the growth trial, as the growth of individual abalone is inherently variable (Leighton and Boolootian, 1963). The numbered tags were made by laminating a small piece of paper with a unique number printed on it between two sheets of latex rubber (Rubber Dam) using cyanoacrylate glue (Super Glue, Alcolin). Once dry, these individual latex identification tags were stuck onto the dried shell of the animals using cyanoacrylate glue.

Vacuum impregnated feed was prepared once every 10 days for the duration of the growth trial. Random batches of feed were analyzed by determining the total number of culturable bacterial cells, to ensure that each batch of feed contained at least 1 x 10^6 cfu g^{-1} ABFEED® S34 weaning chips and that the bacterial count was approximately the same for each of the *V. midae* SY9 strains for the duration of the growth trial.

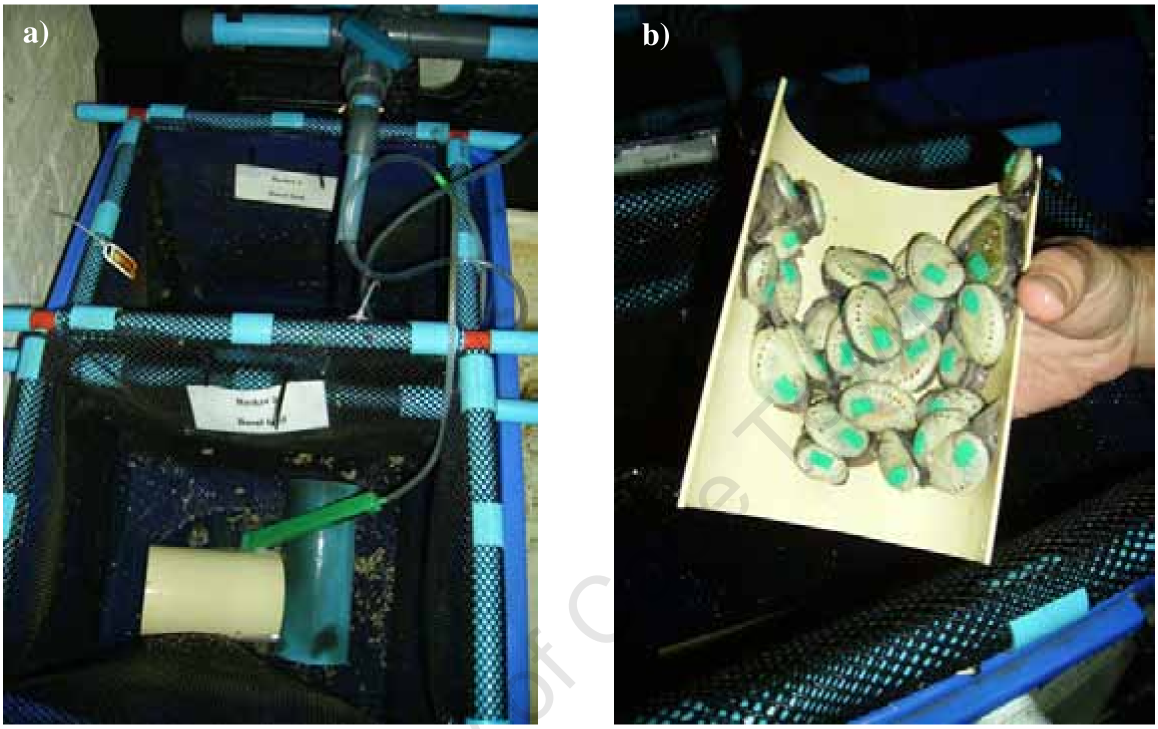


Figure 5.3 A tank (a) and juvenile abalone (b) used in the *Haliotis midae* growth trial. The tank receives freshly filtered seawater from the tap visible at the top of the picture, and contains two labeled baskets (a). Each basket contains half-round PVC pipes used as a refuge for the animals in the growth trial, and an air stone, as seen in one of the baskets (a). The individually numbered green latex tags are visible on the posterior part of the shells of the abalone clustered within a piece of half-round PVC pipe (b).

The abalone were fed the respective ABFEED® S34-based diets three times a week, with each basket receiving an equal amount of feed. The feed was evenly distributed to maximize the feeding opportunities of the abalone within each basket. All uneaten food was removed from the baskets, and the tanks and baskets were thoroughly cleaned three times a week before the addition of fresh feed. The duration of the growth trial experiment was six months. The weight, shell length, and shell width measurements were taken approximately every eight weeks throughout the course of the six month period. Shell length and width of the individual abalone was measured along the longest axis to the nearest 0.01 mm using electronic vernier calipers. Individual abalone were gently dried with paper toweling (Kimberly-Clarke) before their weight was measured to the nearest 0.01 g using an electronic balance (Mettler PE3600).

The total increase in length, width and mass at each sampling time was calculated by subtracting the final weight, width or length from the initial weight, width or length for that individual animal.

Monthly growth rate in shell length (mm/month) was calculated using the formula:

$$\text{Monthly growth rate (mm month}^{-1}\text{)} = \left(\frac{L_t - L_0}{t}\right)(30)$$

where L_o is the initial abalone shell length and L_t is the abalone shell length at time t (days).

Monthly growth rate in shell width (mm/month) was calculated using the formula:

$$\text{Monthly growth rate (mm month}^{-1}\text{)} = \left(\frac{Wd_t - Wd_0}{t}\right)(30)$$

where Wd_o is the initial abalone shell width and Wd_t is the abalone shell width at time t (days).

Monthly growth rate in weight was calculated according to the formula:

$$\text{Monthly growth rate (g month}^{-1}\text{)} = \left(\frac{W_t - W_0}{t}\right)(30)$$

where W_0 is the initial abalone weight and W_t is the abalone weight at time t (days).

The condition factor is a measure of the mass of an abalone per unit shell length (Britz, 1995).

$$\text{CF (g mm}^{-1}\text{)} = \left(\frac{\text{W}}{\text{L}^{2.99}}\right) \times 5575$$

where CF is the condition factor, W is the mean abalone weight (in grams) and L is the mean shell length (in mm).

The growth parameters of all the abalone were initially measured at the start of the growth trial (T = 0) and at each sampling point thereafter (56, 111 and 180 days). The shell length, shell width and weight were measured for each animal in order to obtain an average length, width and weight of the animals receiving each of the four dietary treatments at each sampling point. The monthly growth rate, in terms of shell length, shell width, and weight, and condition factor were calculated for each animal in order to obtain an average for the abalone receiving each of the four diets. The data obtained from the duplicate baskets within each treatment (diet) was grouped at each sampling point for statistical purposes.

5.3.3.1 Statistical analyses

The effects of probiotic dietary supplementation on the growth and growth rate parameters (shell length, shell width and weight) and CF of juvenile *H. midae* were analysed using repeated measures (RM) two-way analyses of variance (ANOVA). SigmaStat 3.11.0 (Systat Software, Inc.) was used to perform *post hoc* pairwise multiple comparisons on significant effects and interactions using the Tukey Test method. Statistically significant differences were established at critical levels of $P<0.05$.

5.3.4 In situ enzyme assays

At the end of the growth trial (180 days), three abalone from each basket (41.35 mm ± 2.12 mm shell length) were removed and immediately sacrificed. The entire digestive tract was aseptically removed from each animal. The animals were maintained on ice to prevent degradation of digestive tract proteins during the dissection. The crop/stomach and intestinal regions (Fig. 5.4) were carefully dissected away from each other. The crop/stomach and intestinal regions were placed into separate sterile microfuge tubes on ice. One milliliter of sterile ice-cold 1x PBS (Appendix A.2.3) was added to each microfuge tube and the samples were gently mixed with a sterile glass rod to extrude the contents of the crop/stomach and intestinal digestive tract regions. Thereafter, the digestive tract samples were centrifuged (13,000x g for 10 minutes at 4 ºC) to pellet any digestive tract debris, cellular material, bacterial cells and undigested food.

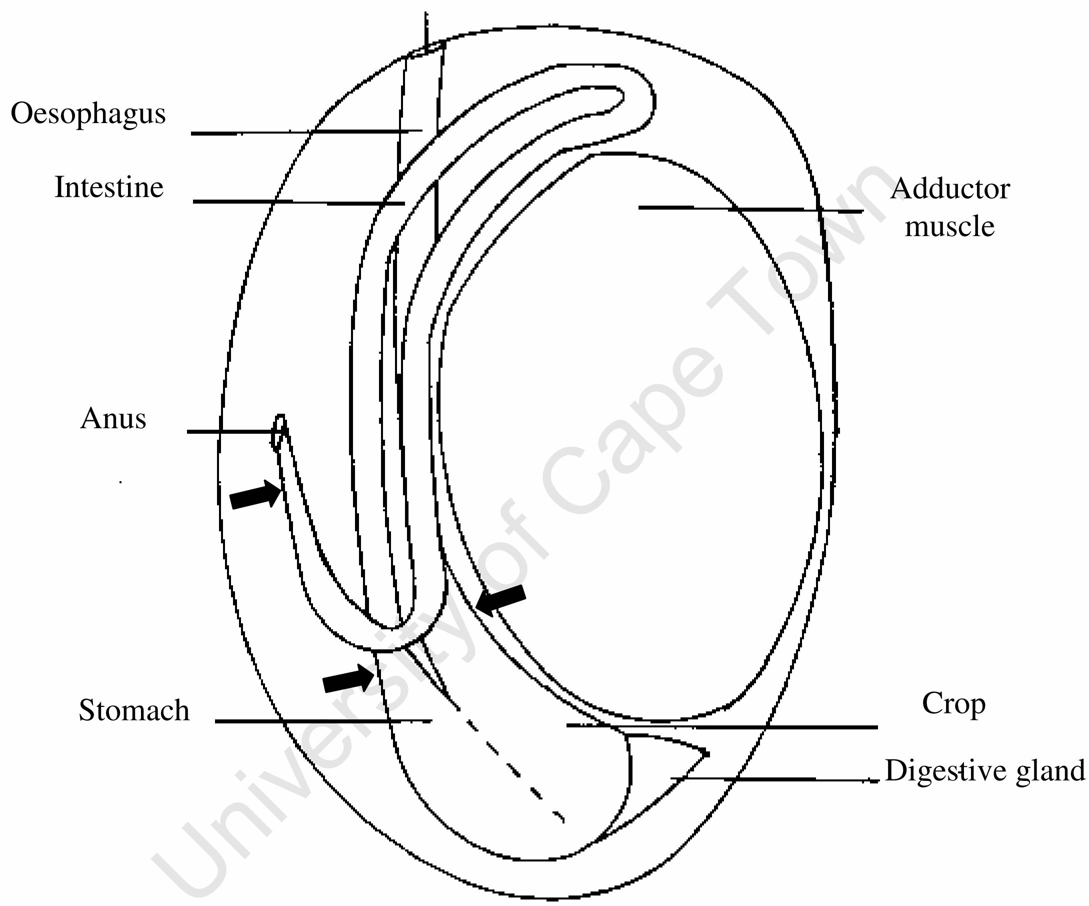


Figure 5.4 Schematic diagram of the *Haliotis midae* digestive tract regions, showing the portions that were excised for the *in situ* alkaline protease activity assay. The black arrows indicate where the crop/stomach and intestinal regions were dissected from the remainder of the digestive tract. Figure adapted from Erasmus *et al.* (1997).

As the volume of the individual digestive tract samples was insufficient for analysis, the supernatant from the digestive tract samples of the six-abalone samples within each dietary treatment were combined in a sterile standard container. The extracellular protease activity (section 5.3.4.1) and protein concentration (section 5.3.4.2) of the pooled samples was determined immediately.

5.3.4.1 Extracellular protease enzyme assay

The protease activity of the crop/stomach and intestinal samples was determined as described in Appendix B.22.

5.3.4.2 Protein quantitation

The extracellular protein concentration of the crop/stomach and intestinal samples was determined using the Bradford protein assay as described in Appendix B.24.

5.3.4.3 Statistical analyses

The *in situ* protease activity data for the intestinal samples was analysed by the Kruskal-Wallis one-way ANOVA on ranks, using SigmaStat 3.11.0. When the effects were significant, the Student *t*-test was used to test for significant differences between the sample means. Significant differences were established at $P<0.05$.

The *in situ* protease activity data for the crop/stomach samples was analysed by one-way ANOVA. When the results of the ANOVA were significant, the Holm-Sidak multiple comparison method was used in order to determine the significant differences in protease activity due to the type of feed, using SigmaStat 3.11.0. Significant differences were established at $P<0.05$.

5.4 Results

5.4.1 Optimal post- vacuum impregnation drying conditions for *V. midae* SY9Mut2 viability

In order to determine the optimal drying conditions for vacuum impregnated ABFEED® S34 weaning chips, the number of culturable cells was determined for impregnated ABFEED® S34 weaning chips dried at 22 ºC for 16 hours and 37 ºC for 2 hours. The viable number of *V. midae* SY9Mut2 cells decreased from a post-impregnated viable cell count of approximately 3.53 x 10^9 cfu g^{-1} ABFEED® S34 to approximately 2.83 x 10^7 cfu g^{-1} ABFEED® S34 following the 16-hour drying period at 22 ºC. The number of viable probiotic cells within the vacuum impregnated ABFEED® S34 weaning chips decreased from the level following vacuum impregnation to 4.22 x 10^7 cfu g^{-1} ABFEED® S34 following two hours drying at 37 ºC. The decline in viable *V. midae* SY9Mut2 cells during post-vacuum impregnation drying at both 22 and 37 ºC was a significant reduction (P<0.05) from the number of viable *V. midae* SY9Mut2 cells within the ABFEED® S34 immediately following vacuum impregnation (Fig. 5.5). However, there was no significant (P>0.05) difference between the viable cell counts following either method of drying the *V. midae* SY9Mut2 vacuum impregnated ABFEED® S34 weaning chips (Fig. 5.5). Thus, either temperature could be used to dry the vacuum impregnated ABFEED® S34 weaning chips prior to feeding the abalone in the growth trial.

5.4.2 Optimal storage temperature for *V. midae* SY9 vacuum impregnated ABFEED®S34

In order to determine the optimal storage temperature for the probiotic supplemented ABFEED® S34, the number of culturable cells was determined for impregnated ABFEED® S34 stored at constant temperatures of 4 and 22 ºC. When the feed was stored at 22 ºC (data not shown), there was a dramatic decline in the number of culturable cells for all three strains in comparison to the viability of the probiotic strains stored at 4 ºC (Fig. 5.6). After 7 days there were no culturable cells detected for any of the three strains impregnated and stored at 22 ºC (data not shown).

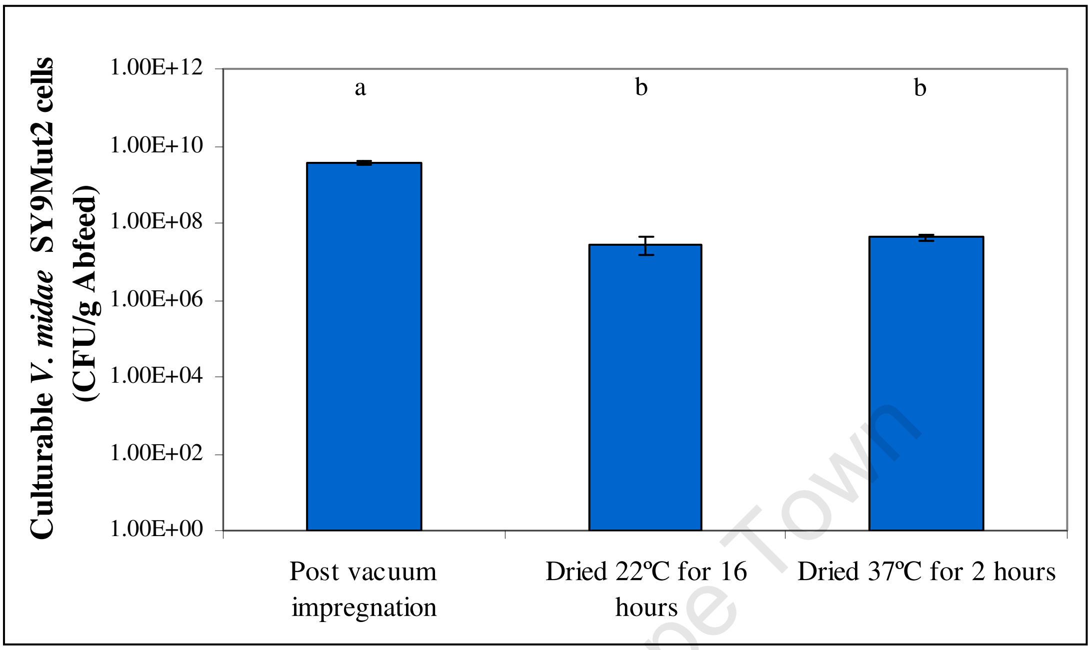


Figure 5.5 Viability of the mutant probiotic strain, *Vibrio midae* SY9Mut2, in vacuum impregnated ABFEED® (cfu gram^{-1} Abfeed® S34) after vacuum impregnation, and then after being dried at 22 ºC for 16 hours and 37 ºC for 2 hours. Data represents the mean ± standard error of three batches of impregnated ABFEED® S34 weaning chips. Different postscripts indicate a significant difference (P<0.05) between sample means.

There was a steady and significant (P<0.05; paired t-test) decline in the level of culturable cells between virtually all measurement points over the course of the 29 day period for all three *V. midae* SY9 strains when the impregnated feed was stored at 4 ºC (Fig. 5.6 and Appendix C Table 1). The only exception was that there was not a significant (P>0.05) decrease in the number of viable *V. midae* SY9 cells within the vacuum impregnated ABFEED® S34 weaning chips stored at 4 ºC between days 14 and 22 (Fig. 5.6 and Appendix C Table 1). Although the viability of the probiotic impregnated feed was improved by storage at 4 ºC, fresh feed would need to be prepared every 7 days in order to ensure that the animals in the growth trial received feed containing more than 1.0 x 10^6 cfu g^{-1} ABFEED® S34.

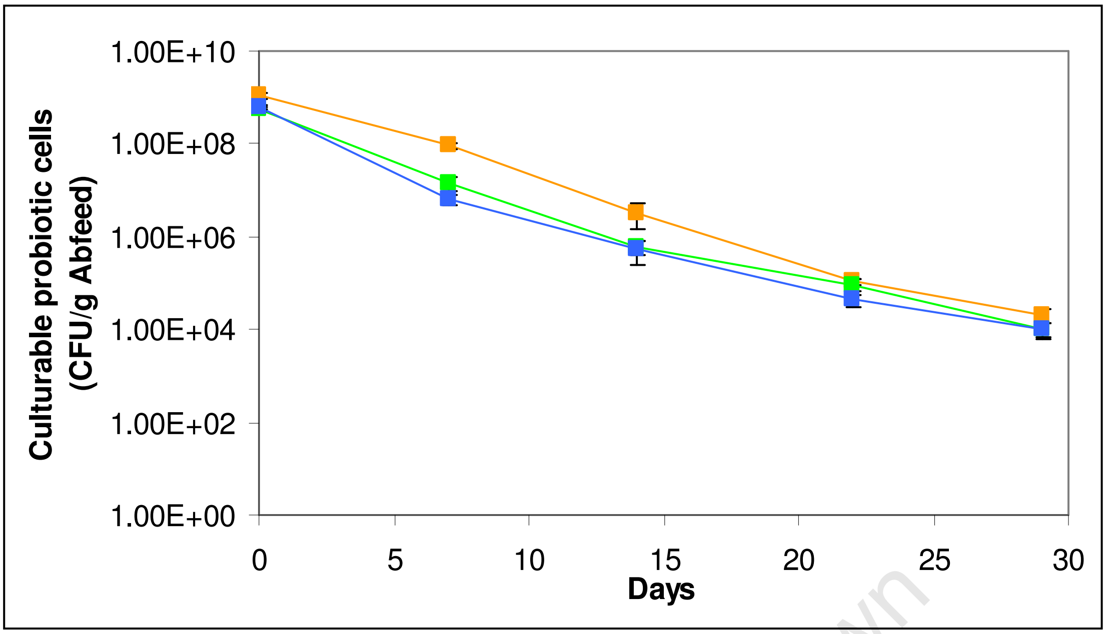


Figure 5.6 Viability of *Vibrio midae* SY9 (■), *Vibrio midae* SY9Mut2 (■), and *Vibrio midae* SY9Pro2 (■) probiotic strains in vacuum impregnated ABFEED® S34 (cfu g^{-1} ABFEED® S34) stored at a constant temperature of 4 ºC over the course of 29 days. Data represents the mean ± standard error of three batches of each strain of vacuum infused ABFEED® S34 weaning chips.

5.4.3 Leaching of the probiotic *V. midae* SY9Mut2 from vacuum impregnated ABFEED® S34

In order to investigate probiotic bacterial leaching from supplemented ABFEED® S34 weaning chips, the number of viable *V. midae* SY9Mut2 cells within the supplemented feed was determined over the course of three days in a flow-through abalone tank. The number of culturable *V. midae* SY9Mut2 cells within the probiotically supplemented ABFEED® S34 decreased from 3.29 x 10^8 cfu g^{-1} ABFEED® to 2.78 x 10^7 cfu g^{-1} ABFEED® after the feed had been immersed in the flowing seawater for only 30 minutes (Fig. 5.7). However, after a further 24 hours the number of culturable *V. midae* SY9Mut2 within the impregnated ABFEED® had significantly (P<0.05) increased from the viability level observed after 30 minutes in the seawater to 2.01 x 10^{10} cfu g^{-1} ABFEED®. After another 48 hours, the viable counts of *V. midae* SY9Mut2 associated with the feed had significantly (P<0.05) declined to 1.8 x 10^6 cfu g^{-1} ABFEED®. Therefore, probiotic supplemented ABFEED® S34 weaning chips would need to be replaced at least every three days for the duration of the growth trial to ensure the abalone had access to a minimum of 1.0×10^6 cfu g^{-1} ABFEED®.

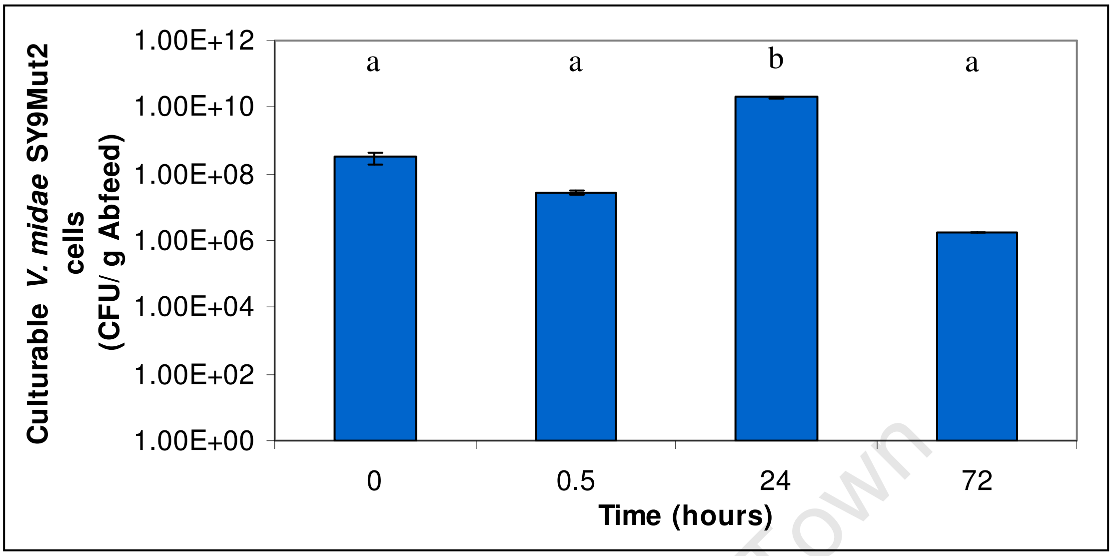


Figure 5.7 Viability of the probiotic mutant strain, *Vibrio midae* SY9Mut2 in vacuum impregnated ABFEED® (cfu $gram^{-1}$ ABFEED®), incubated in a flow-through abalone tank over the course of 72 hours. Data represents the mean ± standard error of three batches of vacuum impregnated ABFEED® S34 weaning chips. Different postscripts indicate a significant difference (P<0.05) between sample means.

5.4.4 Growth Trial

A growth trial was conducted in order to determine the effect of VmproA on the growth of *H. midae*. The abalone were fed either a basal control diet of ABFEED® S34 weaning chips or the basal diet supplemented with either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2. The abalone growth parameters of shell length, shell width and weight were monitored over a 180 day period.

There was no significant difference (P>0.05) between the initial shell length (Fig. 5.8 and Appendix C Table 2), shell width (Fig. 5.9 and Appendix C Table 3) or weight (Fig. 5.10 and Appendix C Table 4) of the individual abalone within each treatment group.

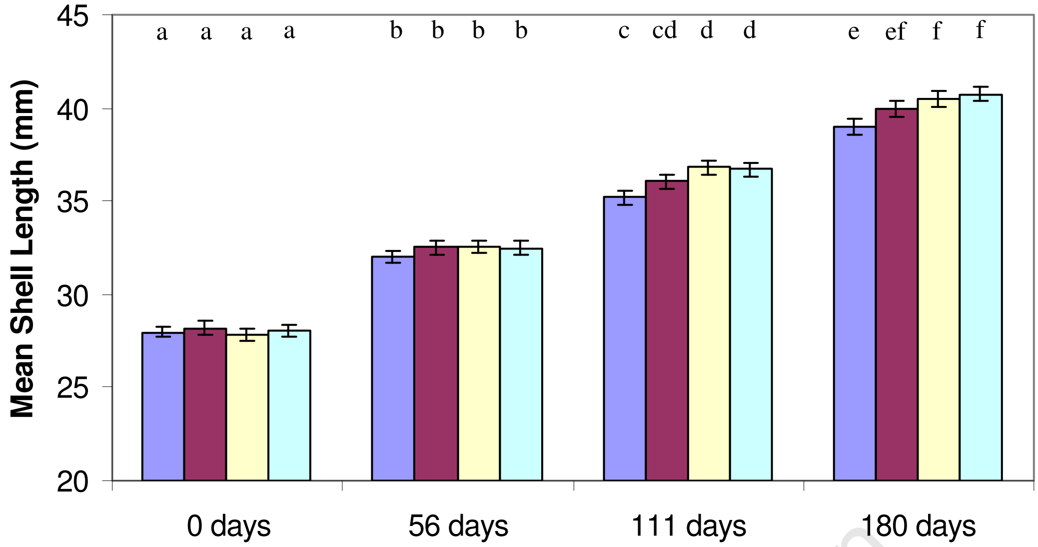


Figure 5.8 Mean length (mm) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), or ABFEED® S34 supplemented with *Vibrio midae* SY9 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean shell length values.

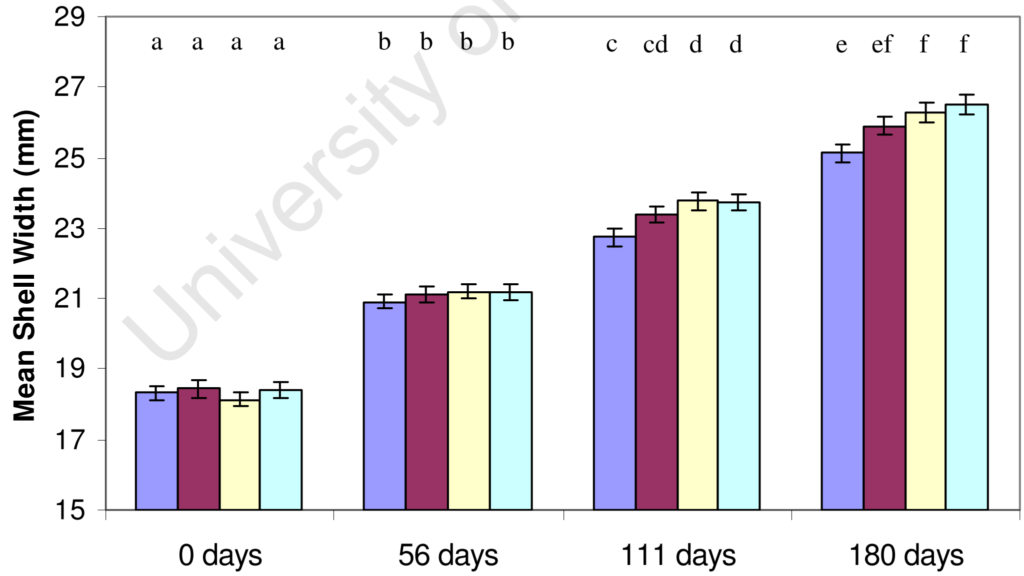


Figure 5.9 Mean width (mm) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), ABFEED® S34 supplemented with *Vibrio midae* SY9 (■) ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean shell width values.

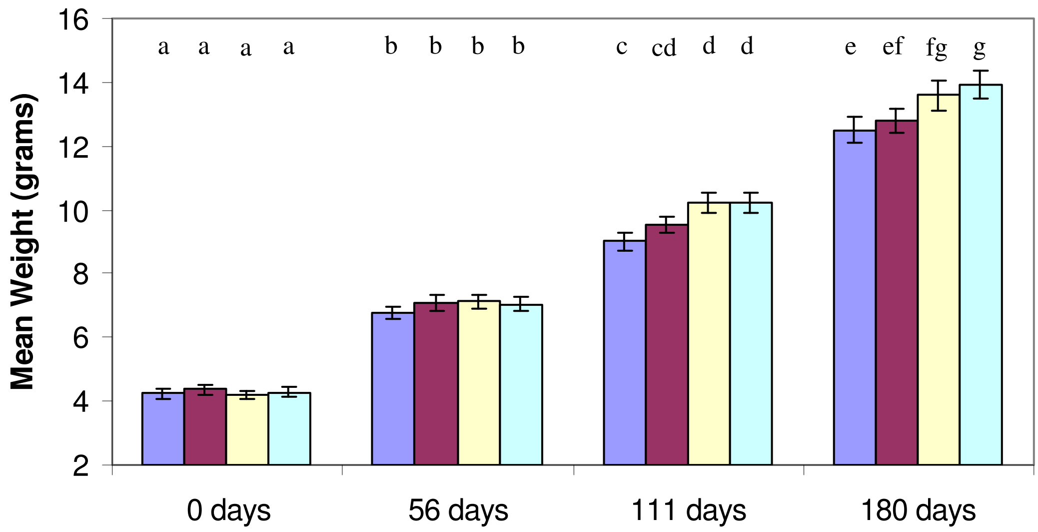


Figure 5.10 Mean weight (g) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), ABFEED® S34 supplemented with *Vibrio midae* SY9 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean weight values.

The animals within all of the treatment groups grew significantly (P<0.05) over the course of the 180 day experimental period in mean shell length (mm) (Fig. 5.8), width (mm) (Fig. 5.9) and weight (g) (Fig. 5.10).

The mean shell length (Fig. 5.8 and Appendix C Table 2) of the abalone receiving the basal and the three probiotically supplemented artificial diets were not significantly different (P>0.05) after 56 days of the growth experiment. After 111 and 180 days of treatment there was a slight, although not statistically significant (P>0.05), increase in the mean shell length (Fig. 5.8 Appendix C Table 2) of the animals receiving the *V. midae* SY9 supplemented artificial feed compared to the animals being fed the basal ABFEED® S34 weaning chips diet. The mean shell length (Fig. 5.8 and Appendix C Table 2) of the abalone fed ABFEED® S34 supplemented with *V. midae* SY9Pro2 and *V. midae* SY9Mut2 increased significantly (P<0.05) in comparison to the control animals after 111 and 180 days of the growth trial. There was no significant difference (P>0.05) between the shell length (Fig. 5.8 and Appendix C Table 2) of the abalone fed the three probiotic supplemented diets over the 180 day duration of the growth trial. Probiotic supplementation with

V. midae SY9, *V. midae* SY9Pro2, *V. midae* SY9Mut2 resulted in a 2.5, 4.6 and 3.9% improvement, respectively, in abalone shell length (mm) over the 180 day growth trial in comparison to the growth in shell length of the control animals (Table 5.2).

Table 5.2 The percentage increase in shell length and growth rate (in shell length) of *Haliotis midae* fed ABFEED® S34 weaning chips supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2 over the course of a 180 day growth trial.

Probiotic Treatment	Length (mm)[a]			Length (mm month^{-1})[b]		
	Days					
	56	**111**	**180**	**56**	**111**	**180**
***V. midae* SY9**	1.5	2.4	2.5	9.9	10.4	6.5
***V. midae* SY9Pro2**	1.4	4.2	4.6	10.5	21.3	15.0
***V. midae* SY9Mut2**	1.8	4.6	3.9	18.2	25.7	14.5

[a] - Percentage increase was calculated as a percentage relative to the shell length of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.
[b] - Percentage increase was calculated as a percentage relative to the growth rate of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.

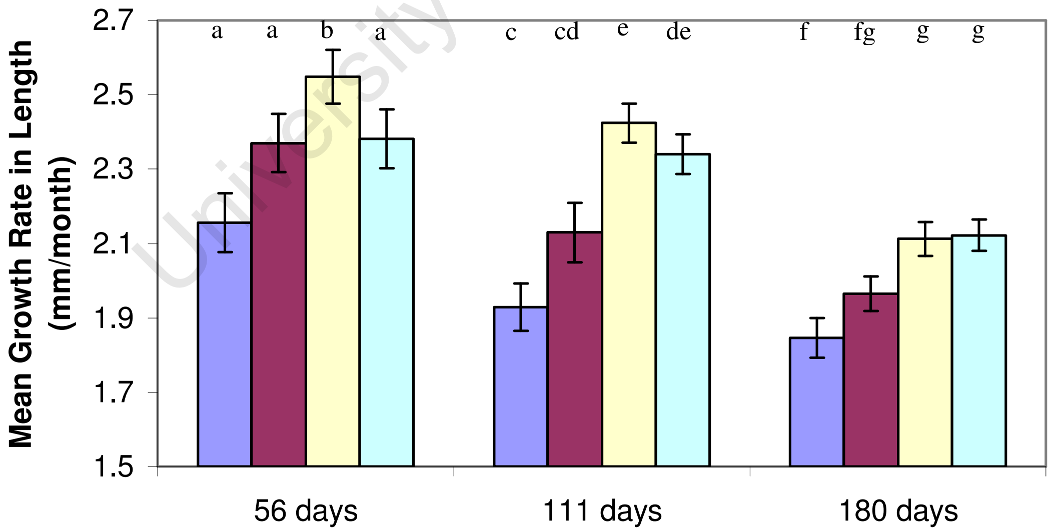


Figure 5.11 Mean growth rate in length (mm month^{-1}) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), ABFEED® S34 supplemented with *Vibrio midae* SY9 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean growth rate length values.

The mean abalone growth rate in shell length (mm month^{-1}) (Fig. 5.11 and Appendix C Table 5) was significantly improved (P<0.05) following dietary supplementation of the basal diet with *V. midae* SY9Mut2 in comparison to abalone fed the control diet over the course of the first 56 days of the growth trial. There was no significant difference (P>0.05) in the mean growth rate, in terms of shell length, between the animals receiving the ABFEED® S34 basal diet supplemented with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 at 56 and 180 days of the growth trial (Fig. 5.11 and Appendix C Table 5). There was however, a significant difference (P<0.05) between the growth rate of the animals fed the wild type strain and the animals fed the mutant strain *V. midae* SY9Mut2 at 111 days. Despite the growth rate of the abalone receiving the *V. midae* SY9 supplemented diet being improved in comparison to abalone fed the control diet, the differences in the growth rates of the control animals and the *V. midae* SY9 fed animals at 56, 111 and 180 days was not statistically significant (P>0.05) (Fig. 5.11 and Appendix C Table 5). However, abalone fed the basal diet supplemented with either *V. midae* SY9Pro2 or *V. midae* SY9Mut2 were significantly improved (P<0.05) in comparison to animals receiving the basal diet after 111 and 180 days (Fig. 5.11 and Appendix C Table 5).

On the whole, there was no significant (P>0.05) increase in the growth rate, in terms of shell length, between abalone fed the basal control diet and those fed the control diet supplemented with *V. midae* SY9 over the duration of the growth trial (Appendix C Table 6). However, supplementation of ABFEED® S34 weaning chips with the mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2, resulted in a significant (P<0.05) increase in growth rate, in terms of shell length, in comparison to basal ABFEED® S34 fed animals (Appendix C Table 6). Supplementation of ABFEED® S34 weaning chips with *V. midae* SY9Mut2, resulted in a significant (P<0.05) increase in growth rate, in terms of shell length, in comparison to abalone fed *V. midae* SY9 supplemented feed (Appendix C Table 6). Probiotic supplementation of the *H. midae* formulation diet resulted in a 6.5, 15.0 and 14.5% improvement in the growth rate in terms of shell length (mm month^{-1}) of abalone fed either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2, respectively, over the 180 day growth trial period (Table 5.2).

The initial mean shell width (mm) of the individual abalone was not significantly different (*P*>0.05) within each treatment group (Fig. 5.9 and Appendix C Table 3). There were no significant differences (*P*>0.05) between the mean shell widths (Fig. 5.9 and Appendix C Table 3) of the abalone receiving the four different experimental ABFEED® S34-based diets after the initial 56 days of the growth trial experiment. However, after 111 and 180 days, the animals receiving the two mutant probiotically-supplemented diets were observed to have increased shell widths in comparison to that of the basal diet fed abalone. Although the difference in mean shell widths between the basal fed animals and the *V. midae* SY9 supplemented animals was not statistically significant (*P*>0.05) at 111 and 180 days, the difference between the basal fed abalone and those receiving the two mutant strains of *V. midae* SY9 was significant (*P*<0.05) after 111 and 180 days of the growth trial. There was also no significant difference (*P*>0.05) between the mean abalone shell width (Fig. 5.9 and Appendix C Table 3) of the animals fed the three probiotic supplemented diets, at each measurement point, over the duration of the growth trial experiment. Probiotic supplementation with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 resulted in a 3.1, 5.6 and 4.7% improvement, respectively, in abalone shell width (mm) over the duration of the growth trial in comparison to the growth in width of the control fed abalone (Table 5.3).

The mean growth rate, in terms of shell width (mm month^{-1}), of the abalone receiving the *V. midae* SY9, *V. midae* SY9Pro2 and *V. midae* SY9Mut2 supplemented diets was higher than that of the animals being fed the basal control diet (Fig. 5.12). However, after 56 days of growth, only the growth rate in shell width of the animals fed the *V. midae* SY9Mut2 supplemented diet was significantly increased (*P*<0.05), in comparison to the growth rate of the basal, *V. midae* SY9 and *V. midae* SY9Pro2 fed animals (Appendix C Table 7). After a growth trial period of 111 days, the growth rate of the abalone fed the *V. midae* SY9 supplemented diet was not significantly increased (*P*>0.05), in comparison to the growth rate of the basal fed animals. The growth rate of the animals receiving the *V. midae* SY9Pro2 and *V. midae* SY9Mut2 supplemented diets was significantly improved (*P*<0.05) in comparison with the growth rate of the basal ABFEED® S34 weaning chips fed abalone (Fig. 5.12 and Appendix C Table 7). Furthermore, the mean growth rate, in terms of shell width, of abalone receiving the ABFEED® S34 basal diet supplemented with

V. midae SY9Pro2 and *V. midae* SY9Mut2 was significantly improved ($P<0.05$) in comparison to the control animals (Fig. 5.12 and Appendix C Table 7) over the duration of the 180 day growth trial period. Overall, the growth rate in terms of shell width of the animals receiving the basal ABFEED® S34 diet was not significantly different ($P>0.05$) to that of the animals fed the basal diet supplemented with *V. midae* SY9 (Appendix C Table 8). There was however a significant improvement ($P<0.05$) in the growth rate of the abalone fed the control diet supplemented with the two mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2 (Appendix C Table 8). The abalone fed the ABFEED® S34 basal diet supplemented with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 were observed to have a 11.2, 20.5 and 27.0%, respectively, improvement in the growth rate (shell width) after 111 days of the growth trial (Table 5.3). The growth rate of *H. midae* fed a formulation diet supplemented with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 was enhanced by 9.9, 19.6 and 19.9%, respectively, over the course of the 180 day growth trial (Table 5.3).

Table 5.3 The percentage increase in shell width and growth rate (in shell width) of *Haliotis midae* fed ABFEED® S34 weaning chips supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2 over the course of a 180 day growth trial.

Probiotic Treatment	**Width (mm)**[a]			**Width (mm month^{-1})**[b]		
	Days					
	56	**111**	**180**	**56**	**111**	**180**
***V. midae* SY9**	1.1	2.7	3.1	5.7	11.2	9.9
***V. midae* SY9Pro2**	1.2	4.3	5.6	7.0	20.5	19.6
***V. midae* SY9Mut2**	1.3	4.5	4.7	17.8	27.0	19.9

[a] - Percentage increase was calculated as a percentage relative to the shell width of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.

[b] - Percentage increase was calculated as a percentage relative to the growth rate in width of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.

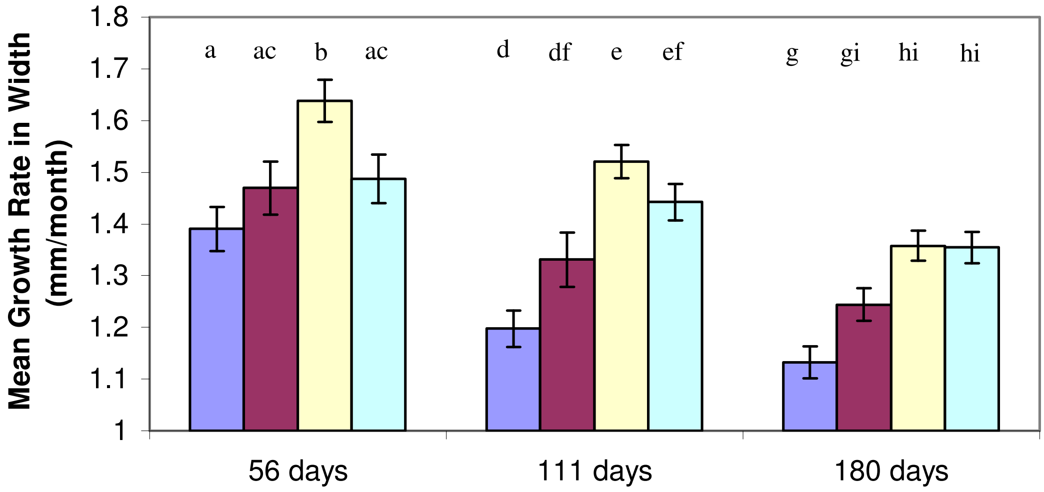


Figure 5.12 Mean growth rate in shell width (mm month^{-1}) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), ABFEED® S34 supplemented with *Vibrio midae* SY9 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean growth rate width values.

There was no significant difference (P>0.05) between the initial mean (0 days) weight of the individual abalone within each treatment group (Fig. 5.10 and Appendix C Table 4). The mean mass of the abalone receiving the basal diet and those being fed the three probiotically-supplemented diets were not statistically significantly (P>0.05) different after 56 days of the growth trial. After a period of 111 days there was no significant difference (P>0.05) between the mean weight of the animals fed the basal control diet and the animals fed the basal diet supplemented with *V. midae* SY9 (Fig. 5.10 and Appendix C Table 4). After 111 days there was also no significant difference (P>0.05) between the mean mass of the animals receiving the three different probiotically supplemented diets. However, the mean weight of the animals fed the basal diets supplemented with the two mutant strains, *V. midae* SY9Pro2 and *V. midae* SY9Mut2, were significantly improved (P<0.05) in comparison to the mean weight of the control animals after 111 days of the growth trial. After 180 days, the mean masses of the abalone fed the three probiotically supplemented ABFEED®-based diets were improved in comparison to that of the animals fed the basal ABFEED® S34 diet. However, there was no significant difference (P>0.05) between the mean weight of the abalone fed the basal control diet and the animals fed the basal diet supplemented with *V. midae* SY9 (Fig. 5.10 and Appendix C Table 4). The mean mass of the animals fed the basal diet supplemented with *V. midae* SY9Pro2 was significantly improved ($P<0.05$) in comparison to that of the animals fed either the basal control diet or the *V. midae* SY9 supplemented diet. There was no significant difference ($P>0.05$) between the mean mass of *H. midae* fed either the *V. midae* SY9Mut2 or the *V. midae* SY9Pro2 supplemented ABFEED® S34 diets. Probiotic supplementation with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 resulted in a 2.4, 11.5, and 8.8% improvement, respectively, in abalone mass (g) over the 180 day growth trial period (Table 5.4).

Table 5.4 The percentage increase in weight and growth rate (in weight) of *Haliotis midae* fed ABFEED® S34 weaning chips supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2 over the course of a 180 day growth trial.

Probiotic Treatment	**Weight (g)[a]**			**Weight (g month^{-1})[b]**		
	Days					
	56	**111**	**180**	**56**	**111**	**180**
***V. midae* SY9**	4.6	5.6	2.4	8.4	7.7	2.0
***V. midae* SY9Pro2**	4.0	13.5	11.5	8.5	24.3	16.7
***V. midae* SY9Mut2**	5.5	13.4	8.8	16.1	26.0	13.7

[a] - Percentage increase was calculated as a percentage relative to the weight of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.

[b] - Percentage increase was calculated as a percentage relative to the growth rate in weight of the abalone receiving the basal ABFEED® S34 weaning chips diet at 56, 111 and 180 days.

The growth rate in weight of the abalone receiving the three probiotically-supplemented diets was improved in comparison to that of the animals fed the basal ABFEED® S34 weaning chips diet over the course of the 180 day growth trial period. However, there was no significant difference ($P>0.05$) between the growth rate in weight of the animals fed the basal control diet and those receiving the *V. midae* SY9 supplemented diet at each measurement point over the duration of the growth trial (Fig. 5.13 and Appendix C Table 9). There was no significant difference ($P>0.05$)

between the growth rates in weight of the animals fed ABFEED® S34 weaning chips supplemented with the mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2 after 111 and 180 days of the growth trial (Fig. 5.13 and Appendix C Table 9). Over the same period, the growth rates in weight of abalone fed the control diet supplemented with *V. midae* SY9Pro2 were significantly (P<0.05) improved in comparison to animals fed the basal feed (Fig. 5.13 and Appendix C Table 9).

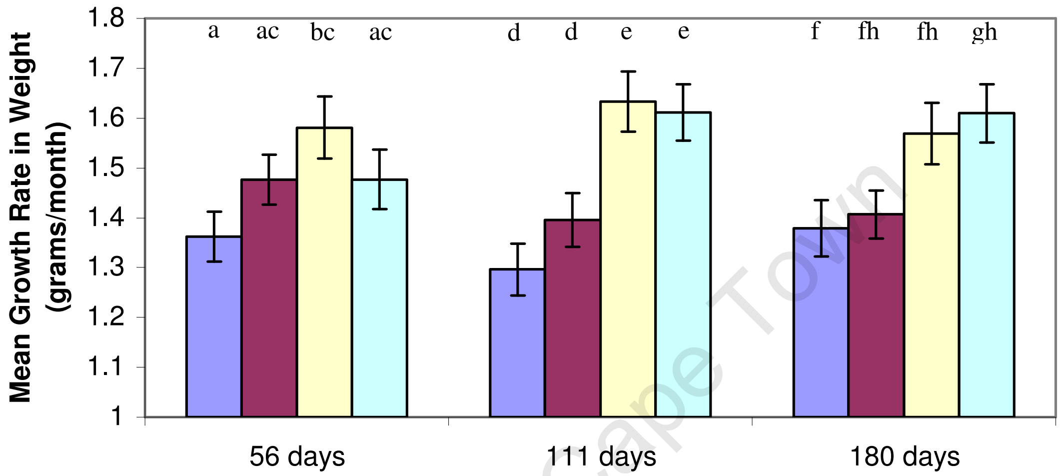


Figure 5.13 Mean growth rate in weight (grams month^{-1}) of *Haliotis midae* fed the ABFEED® S34 basal diet (■), ABFEED® S34 supplemented with *Vibrio midae* SY9 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Mut2 (■), ABFEED® S34 supplemented with *Vibrio midae* SY9Pro2 (■) during the course of the growth trial. Data represents the mean ± standard error. Different postscripts indicate a significant difference (P<0.05) between mean growth rate weight values.

Overall, the growth rate in weight of abalone receiving the basal ABFEED® S34 diet was not significantly different (P>0.05) to that of the animals fed the basal diet supplemented with *V. midae* SY9 or *V. midae* SY9Pro2 (Appendix C Table 10). However, there was a significant improvement (P<0.05) in the growth rate of abalone fed the control diet supplemented with the mutant strain *V. midae* SY9Mut2 in comparison to the growth rate of abalone fed either the control diet or the control diet supplemented with *V. midae* SY9 (Appendix C Table 10). The abalone fed the basal diet supplemented with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 had a 2.0, 16.7 and 13.7% improvement in the growth rate in terms of weight, respectively, over the course of the 180-day growth trial period in comparison to the growth rate of the basal fed animals (Table 5.4).

The CF of *H. midae* within all the treatment groups increased over the initial 56 day period (Table 5.5). However, statistical analysis revealed that there was no significant influence (P>0.05) on the CF of abalone fed the four experimental ABFEED® S34 weaning chip diets throughout the 180 day experimental period (Appendix C Table 11).

Table 5.5 The condition factor (mean ± standard error) of *Haliotis midae* fed ABFEED® S34 weaning chips, ABFEED® S34 weaning chips supplemented with *Vibrio midae* SY9, ABFEED® S34 weaning chips supplemented with *Vibrio midae* SY9Pro2 or ABFEED® S34 weaning chips supplemented with *Vibrio midae* SY9Mut2 over the course of a 180 day growth trial.

Probiotic Treatment	**Condition factor (g mm^{-1})**			
	Days			
	0	**56**	**111**	**180**
Basal	1.096±0.010	1.179±0.011	1.186±0.012	1.190±0.013
V. midae SY9	1.105±0.012	1.171±0.012	1.160±0.009	1.138±0.011
V. midae SY9Pro2	1.106±0.014	1.170±0.013	1.180±0.011	1.170±0.011
V. midae SY9Mut2	1.103±0.013	1.172±0.012	1.165±0.012	1.159±0.011

5.4.5 *In situ* protease activity

An *in situ* protease assay was conducted to determine the presence and levels of extracellular alkaline protease activity within the digestive tract of *H. midae* fed the different diets investigated in the growth trial (section 5.4.4). In the process, we hoped to be able to elucidate the possible extracellular protease enzyme contribution of the probiotic strain *V. midae* SY9, and the mutant strains *V. midae* SY9Pro2 and *V. midae* SY9Mut2, to the pool of proteolytic digestive enzymes within the crop/stomach and intestinal regions of *H. midae*. Thus, the soluble protein fraction of the contents of the crop/stomach and intestinal regions of *H. midae* fed either the basal ABFEED® S34 diet or the ABFEED® diets either supplemented with *V. midae*

SY9, *V. midae* SY9Mut2 or *V. midae* SY9Pro2 were compared in terms of the specific alkaline protease activity within the digestive tract samples.

There was no significant difference (*P*>0.05) between the protease activity levels within the crop/stomach (Fig. 5.14 A and Appendix C Table 12) and intestinal (Fig. 5.14 B and Appendix C Table 13) regions of abalone fed the basal ABFEED® S34 and the *V. midae* SY9Mut2 supplemented ABFEED® S34 diets. The lowest protease activity within the crop/stomach region was observed in the animals fed the ABFEED® diet supplemented with *V. midae* SY9Pro2 (Fig. 5.14 A).

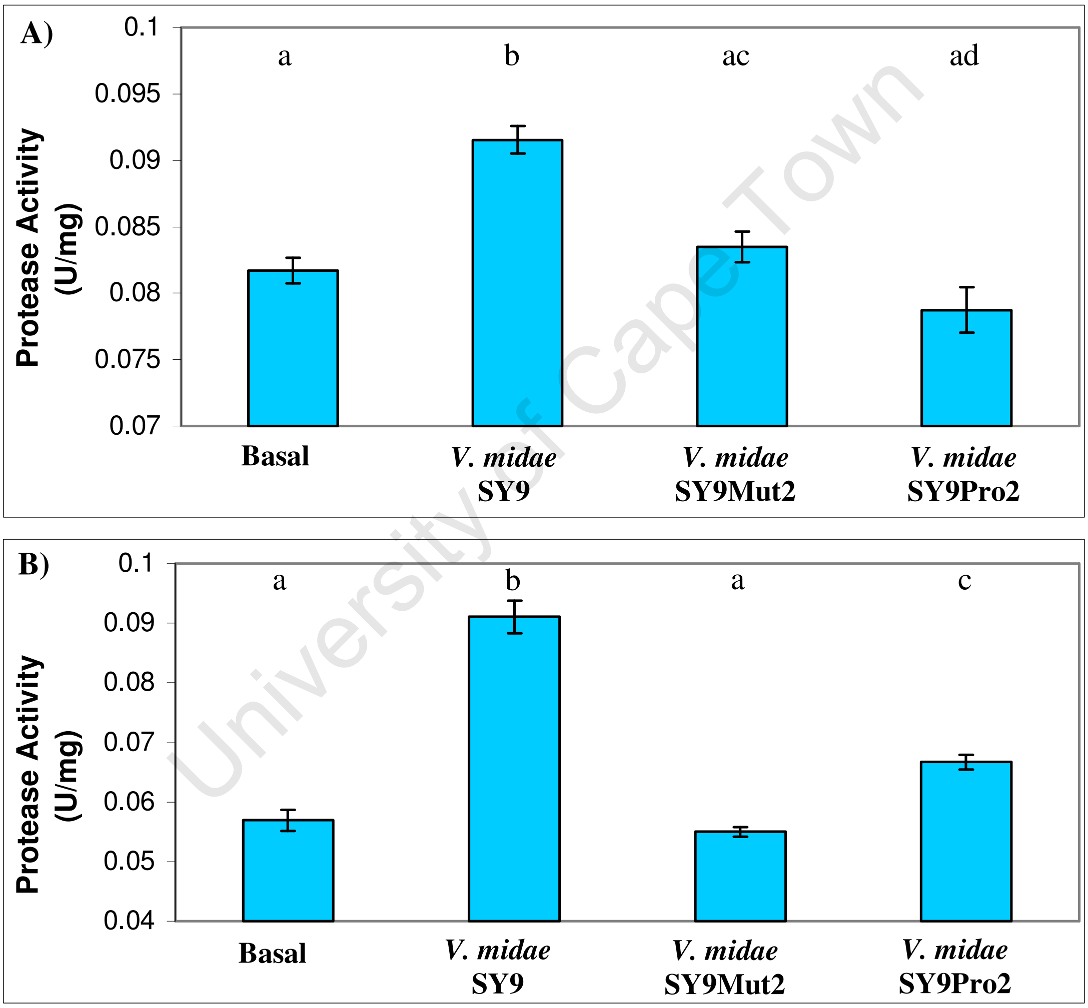


Figure 5.14 *In situ* protease activity in the crop/stomach (A) and the intestinal (B) regions of the digestive tract of *Haliotis midae* fed either the basal ABFEED® S34 weaning chips diet, or ABFEED® S34 diets supplemented with *Vibrio midae* SY9, *Vibrio midae* SY9Mut2 or *Vibrio midae* SY9Pro2. Data represents the mean ± standard error (n=6). Different postscripts represent a significant difference (*P*<0.05) between the *in situ* protease activity (U mg^{-1}) values for the different feeds.

In situ protease activity within the intestinal region of abalone fed *V. midae* SY9Pro2 supplemented ABFEED® S34 was significantly (*P*<0.05) higher than that in animals fed either the ABFEED® S34 control diet or the *V. midae* SY9Mut2 supplemented ABFEED® S34. However, the *in situ* protease activity within the intestinal region of abalone fed *V. midae* SY9Pro2 supplemented ABFEED® S34 was significantly (*P*<0.05) lower than that in animals fed the *V. midae* SY9 supplemented ABFEED® S34 weaning chips diet (Fig. 5.14 B and Appendix C Table 13). Indeed, the abalone fed the ABFEED® S34 weaning chips diet supplemented with *V. midae* SY9 had the highest specific protease activity within the crop/stomach (Fig. 5.14 A and Appendix C Table 12) and intestinal regions (Fig. 5.14 B and Appendix C Table 13) of the digestive tract.

5.5 Discussion

Abalone are relatively slow growing marine gastropods and even when cultured in an intensive aquaculture environment they take four to five years to reach a marketable size. Farm-based growth trials have however shown that the growth rate of the South African abalone species *Haliotis midae* can be improved by up to 30% through dietary probiotic supplementation (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). An increased growth rate following probiotic dietary supplementation may be the result of several possible modes of probiotic action, including enzymatic contribution to digestion through the secretion of extracellular enzymes. Secretion of extracellular enzymes by probiotic strains may increase the pool of digestive enzymes available to the host (De Schrijver and Ollevier, 2000; Lara-Flores *et al.,* 2003; Ziaei-Nejad *et al.,* 2006; Wang, 2007), thereby improving the digestion efficiency of the host (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). In so doing, the level of nutrient absorption within the digestive tract may increase (Macey and Coyne, 2005), and in addition the host may be able to use the probiotic bacteria as an additional source of protein and nutrients (ten Doeschate, 2005).

The aim of this study was to demonstrate the specific mode of action of *V. midae* SY9 and the *vmproA* protease gene thought to be responsible for improving the growth rate of cultured abalone. In this study we constructed (Chapter 4) and employed *vmproA* gene duplication and insertionally inactivated *vmproA* mutants *V. midae* SY9Pro2 and *V. midae* SY9Mut2, together with *V. midae* SY9, in a *H. midae* growth trial to investigate the effect of VmproA on abalone growth.

An important consideration is that the probiotic microorganisms are viable and consistently incorporated into the experimental feed. While the levels of probiotic organisms incorporated into supplemented feeds vary, all seem to constantly supply a minimum probiotic titre of greater than 10^6 cfu g^{-1} (Al-Dohail *et al.,* 2009; Castex *et al.,* 2008; Castex *et al.,* 2009; Iehata *et al.,* 2009; Li *et al.,* 2009; Macey and Coyne, 2005; Merrifield *et al.,* 2009; Rengpipat *et al.,* 1998; Saenz DE Rodrigáñez *et al.,* 2009; Sharifuzzaman and Austin, 2009; ten Doeschate and Coyne, 2008). Macey and

Coyne (2006) suggested that viable probiotic microorganisms need to be present within the gut of the host organism at a level of 10^6 cfu g^{-1} in order to make a significant contribution to the host's digestive enzyme levels. However, the levels of probiotic cells supplied to the host may be subject to the method of supplementation, bacterial species, and target organism. Finfish studies involving dietary supplementation with probiotic strains at doses within the range of 10^4 - 10^9 cfu g^{-1} have resulted in enhanced immune parameters and response to bacterial challenge (Gildberg *et al.,* 1997; Gildberg and Mikkelsen, 1998; Robertson *et al.,* 2000; Nikoskelainen *et al.,* 2003). Macey and Coyne (2005) incorporated probiotic strains into the formulation feed ABFEED® S34 prior to extrusion and drying, to achieve a viable bacterial concentration of approximately 10^7 cfu g^{-1} of dried feed. Rengpipat *et al.* (1998) employed a similar method of co-extrusion to incorporate the probiotic strain *Bacillus* S11 into a shrimp formulation diet to a final concentration of approximately 10^{10} cfu g^{-1}. The probiotic strains *Lactobacillus* sp. strain a3 and *Enterococcus* sp. strain s6 were added to a commercial abalone feed to achieve a final concentration of approximately 1.0 x 10^{10} cfu g^{-1} for use in a study of colonisation and probiotic effects on cultured *Haliotis gigantean* (Iehata *et al.,* 2009). In another investigation into the influence on *H. midae* growth by dietary supplementation with a probiotic strain *Pseudoalteromonas* sp. strain C4, abalone were fed kelp cakes containing the probiotic strain at a final concentration of approximately 10^{10} cfu g^{-1} (ten Doeschate and Coyne, 2008).

In this study, ABFEED® S34 weaning chips were supplemented with the three *V. midae* SY9 probiotic strains (Chapter 4) by means of vacuum impregnation. Vacuum impregnation technology has previously been used for probiotic supplementation of porous foods (Betoret *et al*., 2003). Betoret *et al.* (2003) combined vacuum impregnation and air-drying to enrich apples with 10^6 - 10^7 cfu g^{-1} of the human probiotic strains *S. cerevisiae* and *Lactobacillus casei* (spp. rhamnosus). A cryo-SEM technique was used to verify that the impregnating liquid, containing viable *S. cerevisiae* and *L. casei* cells, had replaced the gas within apple intercellular spaces. Dehydration of live probiotic cultures is a common method of viably maintaining probiotics for functional applications (reviewed in Meng *et al.,* 2008). Betoret *et al.* (2003) found that post-impregnation drying at 40 ºC for 48 hours resulted in a significant reduction in the viable cell concentrations of both probiotic strains within supplemented apple samples. *S. cerevisiae* viable cell counts declined from the post-infusion level of 2.8 x 10^9 cfu g^{-1} to 4.3 x 10^6 cfu g^{-1} following drying, while the *L. casei* viable cell numbers dropped from 1.8 x 10^8 after vacuum impregnation to 6.3 x 10^5 cfu g^{-1} after being air dried. However, the authors concluded that despite the loss in probiotic viability during the preparation process, vacuum impregnation was a satisfactory method of enriching porous foods with probiotic microorganisms (Betoret *et al.,* 2003).

Optimization of the manufacturing, drying and storage conditions could help to ensure that live probiotic cultures remain viable and retain their functionality through the abalone feed preparation process. Therefore, preparation and storage of ABFEED® S34 probiotically supplemented experimental diets was optimized in order to ensure that the probiotic strains could be viably, stably and consistently incorporated into ABFEED® S34 weaning chips for use in the growth trial. Similarly to Betoret *et al.* (2003), we observed a significant decline in the number of viable *V. midae* SY9Mut2 cells during the course of the post-impregnation drying processes at both 22 and 37 ºC. There was no difference in the observed survival of the probiotic strain under the two different post-impregnation drying conditions investigated in this study and so either temperature could be employed for drying the feed.

The optimal storage temperature was investigated to ensure that ABFEED® S34 weaning chips supplemented with the three different *V. midae* SY9 strains (Chapter 4) could be stored for a reasonable period, without a significant loss in the viability of the incorporated probiotic strains. Despite a gradual loss in viability of the three different *V. midae* SY9 strains within vacuum impregnated ABFEED®, the optimal storage temperature was determined to be 4 ºC. The feed could also be stored at 4 °C for approximately 10 days without the number of viable cells decreasing below the pre-determined threshold of approximately 10^6 cfu g^{-1}ABFEED®. Macey (2005) also observed an optimal storage temperature of 4 ºC for *V. midae* SY9 incorporated into ABFEED® S34, however, there was no significant decrease in culturable *V. midae* SY9 over a period of six weeks at the constant storage temperature of 4 ºC. The difference in the storage periods of *V. midae* SY9 supplemented ABFEED® S34 between the previous investigation by Macey (2005) and the present study could possibly be attributed to the different methods of supplementing the ABFEED® S34 formulation feed with the probiotic strain. As mentioned in the introduction to this chapter, Macey and Coyne (2005) mixed the probiotic strains with the formulation feed prior to extrusion and drying of ABFEED® at the Marifeed™ (Pty) Ltd. factory. Although ABFEED® S34 weaning chips can be stably supplemented with *V. midae* SY9 strains by vacuum impregnation technology, this method of supplementing ABFEED® may not be as effective as the co-extrusion method employed by Macey and Coyne (2005). This may be especially the case for long-term storage of viable probiotic strains within abalone formulation feeds, as there was a significant difference in the period of time that feeds could be viably stored at 4 ºC between this study and that of Macey and Coyne (2005).

The efficiency of vacuum impregnation for supplementing ABFEED® with probiotic strains was initially of concern during this study, especially the stability of the probiotic strains within the impregnated feed in the flow-through tanks. Therefore, the number of viable *V. midae* SY9Mut2 probiotic cells in supplemented ABFEED® S34 immersed in the abalone tanks was monitored over a three day period. Viable *V. midae* SY9Mut2 cells remained at levels of greater than 10^6 cfu g^{-1} vacuum impregnated ABFEED® S34 weaning chips after the three-day trial period. Therefore, fresh *V. midae* SY9 supplemented feed was supplied every three days over the duration of the *H. midae* growth trial. This would ensure that the abalone always had access to ABFEED® containing probiotics at a level of at least 10^6 cfu g^{-1}.

Previous investigations by Macey and Coyne (2005) and ten Doeschate and Coyne (2008) into the growth of abalone in response to dietary probiotic supplementation only measured shell length and animal wet weight as growth parameters. During the course of this study, in addition to measuring these growth parameters, we also measured and calculated abalone growth rates in terms of shell width, which in fact showed the most significant improvement in abalone growth rate. Abalone shells grow in spirals (Hahn, 1989a), forming rings that are similar to the growth rings on a tree (Lin *et al.,* 2008). New nacre is laid down along the growing edge towards the anterior of the shell, with less being added to the sides and posterior portions of the shell (Hahn, 1989a). The anterior edge of the abalone shell is very fragile and prone to damage and erosion. Extreme care was taken to ensure that the growing edges were not damaged during handling and measurements. However, there are differences between the calculated growth rates based on shell length and width. Therefore, due to the less-fragile nature of the sides of abalone shells, the width measurements may provide a more accurate assessment of shell growth and growth rate of abalone in growth trials of this nature.

Growth and growth rate, in terms of shell length, shell width and animal weight, of *H. midae* fed an ABFEED® diet supplemented with either *V. midae* SY9 or the two *V. midae* SY9 mutant strains were enhanced in comparison to the abalone fed the control diet of ABFEED® S34 weaning chips. This is in agreement with previous investigations showing that *H. midae* fed probiotically supplemented diets have increased growth rates when compared to abalone fed basal control diets (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008).

In this study, abalone fed the basal diet supplemented with *V. midae* SY9 grew faster and had increased growth rates compared to the animals fed the control diet, although these effects were not significant. The improvement in the growth parameters of *H. midae* fed the *V. midae* SY9 supplemented diet was not as considerable as previously observed by Macey and Coyne (2005). The growth trial conducted by Macey and Coyne (2005) was however carried out using three probiotic strains, including *V. midae* SY9, over a period of eight months and therefore a direct comparison can not be drawn. The enhanced growth observed for *H. midae* fed the *V. midae* SY9 supplemented diet in this study was not significantly ($P>0.05$) improved in comparison to the animals fed the basal control diet. This was especially noticeable between 111 and 180 days of the growth trial. The relative percentage growth rate improvement of animals fed the *V. midae* SY9 supplemented diet, in comparison to those animals fed the control diet, decreased substantially from 111 to 180 days for all of the growth parameters measured. Macey and Coyne (2005) did observe a slight decline in the growth rate of *H. midae* across the two dietary treatments over the course of their growth trial. However, the growth rates of the animals receiving the probiotically-supplemented diet were improved significantly in comparison to the animals fed the basal diet. For example, the growth rate in terms of shell length of small and large animals fed ABFEED® S34 supplemented with a mixture of *V. midae* SY9 and two yeast strains improved by 8 and 33%, respectively

(Macey and Coyne, 2005). To date, the effect of dietary supplementation with *V. midae* SY9 alone on the growth of cultured *H. midae* has not been investigated and, therefore, the probiotic effect of *V. midae* SY9 alone is not presently known.

In the present study, abalone fed *V. midae* SY9Pro2 or *V. midae* SY9Mut2 supplemented basal diets had significantly (P<0.05) enhanced growth and growth rates in terms of shell length and shell width when compared to *H. midae* fed the control diet over the course of the 180 day growth trial. Growth rates in shell length, shell width and mean weight were improved by 15.0, 19.6 and 16.7%, respectively, by *V. midae* SY9Pro2 supplementation. Similarly, *V. midae* SY9Mut2 dietary supplementation resulted in a 14.5, 19.9, and 13.7% improvement in growth rates of shell length, shell width and mean weight, respectively. These improvements in growth parameters represent a substantial increase in the growth rate of relatively small cultured *H. midae*.

V. midae SY9Mut2 supplementation also resulted in significant (P<0.05) enhancement of abalone growth and growth rate in comparison to *H. midae* fed the control diet supplemented with *V. midae* SY9 after 180 days of the growth trial. There was no significant difference (P>0.05) in the growth and growth rate of abalone fed the basal feed supplemented with *V. midae* SY9 in comparison to animals fed *V. midae* SY9Pro2 supplemented feed. Unexpectedly, the growth of the animals receiving the *V. midae* SY9 supplemented diet appeared to decline more substantially than that of the abalone fed either the basal ABFEED® S34 diet or the basal diet supplemented with *V. midae* SY9Mut2 or *V. midae* SY9Pro2 between 111 and 180 days of the growth trial. This may have resulted in the significant differences detected in the three growth parameters measured in abalone fed either the *V. midae* SY9 or the *V. midae* SY9Pro2 and *V. midae* SY9Mut2 supplemented diets at 180 days of the growth trial. It is unlikely that the differences detected in abalone growth rates after 180 days was due to a tank effect. The abalone in the growth trial were stocked at a similar density and received fresh sea water from the same source at approximately the same flow rate. The growth trial feeds supplemented with probiotic bacteria were prepared and stored in the same manner. In addition, the numbers of culturable probiotic cells within the experimental feeds were monitored routinely to ensure that each batch of vacuum impregnated ABFEED® S34 weaning chips contained the respective probiotic strains at levels of greater than 10^6 cfu g^{-1} and were similar to each other.

The condition factor (CF) relates the association between the mass of an abalone per unit shell length and describes the amount of ingested feed devoted to the development of both the body mass and shell length (Britz, 1995; Britz, 1996; Dlaza, 2006). Therefore, a higher CF value implies that more nutrients were invested in development of body mass rather than shell length. Dlaza (2006) recorded a significantly improved CF of 1.312 g mm^{-1} for juvenile *H. midae* (initial shell length 12.717±0.0151 mm) fed an ABFEED® S34 diet for 11 months. The mean CF of the abalone fed the four experimental diets increased over the 180 day growth trial period. Interestingly, a decline in the mean CF values at 111 to 180 days was observed for all the abalone fed the probiotically supplemented diets, while the mean CF of the animals fed the basal diet increased at each sampling point over the 180 day period. There were no statistically significant differences observed between the mean CF values of abalone fed the ABFEED® basal diet or the probiotically supplemented diets over the course of the 180 day trial. Therefore, none of the experimental diets investigated in this study significantly altered the growth ratio between weight and shell length.

The alkaline protease activity within the crop/stomach and intestinal regions of the *H. midae* digestive tract was compared in abalone fed an ABFEED® S34 basal diet and animals fed the basal diet supplemented with either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2. This was done in order to determine whether there was a difference in the levels of alkaline protease activity within the digestive tract of *H. midae* fed the three different probiotically supplemented diets and whether these differences could be correlated to the presence or absence of VmproA.

In situ alkaline protease activity was higher within the crop/stomach than the intestinal region of the *H. midae* digestive tract, regardless of whether the animals were fed the basal diet or the basal diet supplemented with any of the three *V. midae* SY9 strains. Similarly, Macey and Coyne (2008) observed higher levels of both *in situ* protease and amylase activity within the crop/stomach region in comparison to the intestinal region of the *H. midae* digestive tract, regardless of the animals' diet. The abalone hepatopancreas is the primary organ involved in the storage of digestive enzymes (Campbell, 1965; Bevelander, 1988; Picos-García *et al.,* 2000; Garcia-Esquivel and Felbeck, 2006), including proteolytic enzymes, which it secretes directly into the crop/stomach via the caecum (Bevelander, 1988). Therefore, proteolytic enzymes produced and secreted by the abalone into the crop/stomach are most likely responsible for the higher *in situ* alkaline protease activity observed in the crop/stomach compared to the intestine. Despite the many digestive enzymes secreted from the digestive gland into the crop/stomach region, this region is not considered the site of amino acid and peptide absorption in *H. midae* (Macey and Coyne, 2005). Macey and Coyne (2005) detected lower levels of protein digestion and absorption within the crop/stomach than in the intestinal regions of the *H. midae* digestive tract. They concluded that the intestine is the main site of protein digestion and absorption. Therefore, production and secretion of protease enzymes by *V. midae* SY9 within the abalone intestine in particular, may provide the most significant contribution to enhancing the digestion efficiency of *H. midae*.

Total protease activity within the crop/stomach and intestine of *H. midae* fed ABFEED® S34 supplemented with *V. midae* SY9 was significantly (P<0.05) increased when compared to the abalone receiving the basal ABFEED® diet and for this particular feed, increased protease activity did correlate with increased growth parameters. Similar results have been observed in related investigations into improved growth rates and increased digestive enzyme activity levels of aquacultured species through dietary supplementation with selected bacterial isolates (Macey and Coyne, 2005; Yanbo and Zirong, 2006; Ziaei-Nejad *et al.,* 2006; Wang, 2007; Suzer *et al.,* 2008; ten Doeschate and Coyne, 2008). Macey and Coyne (2005) reported increased protease activity (75%) as well as increased protein digestion and absorption within the intestinal digestive tract region of *H. midae* fed a probiotic-supplemented diet, compared to animals receiving the basal diet. Of the three-probiotic strains included in the formulation diet tested by Macey and Coyne (2005), *V. midae* SY9 was thought to be primarily responsible for the increased intestinal protease activity levels as it had been shown to produce and secrete high levels of extracellular protease activity. Since protein type and content of feed have been shown to influence abalone growth (Britz, 1996; Sales *et al.,* 2003), increased intestinal proteolytic enzyme activity and improved digestion of this protein may have contributed to the reported growth enhancement of cultured *H. midae* (Macey and Coyne, 2005). In a related study, supplementation of a kelp-based diet with *Pseudoalteromonas* species strain C4 resulted in a significant increase in alginate lyase activity within the crop/stomach region of the *H. midae* digestive tract in comparison to abalone fed a basal kelp-based diet (ten Doeschate and Coyne, 2008). In both of these investigations, increased levels of digestive enzymes within the digestive tract of *H. midae* fed probiotically supplemented diets were thought to have contributed to or resulted in enhanced *H. midae* growth rates (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008). These studies support the idea that increased levels of digestive enzymes could improve the nutritional status of the abalone host through increased digestion efficiency due to the synergistic action of bacterial and abalone hydrolytic digestive enzymes.

The *in situ* alkaline protease activity levels within the crop/stomach and intestine were equivalent in abalone fed either the basal diet or ABFEED® S34 supplemented with *V. midae* SY9Mut2. Therefore, dietary supplementation of ABFEED® S34 weaning chips with *V. midae* SY9Mut2 had no significant impact on the *in situ* alkaline proteolytic enzyme levels. Supplementation of ABFEED® S34 with *V. midae* SY9 resulted in significantly enhanced *in situ* alkaline proteolytic enzyme activity levels throughout the digestive tract in comparison to animals fed *V. midae* SY9Mut2 supplemented basal feed. This may be due to the production and secretion of VmproA by *V. midae* SY9 within the gastrointestinal tract of *H. midae* fed an ABFEED® diet supplemented with *V. midae* SY9.

Supplementation of the basal ABFEED® diet with *V. midae* SY9Pro2 resulted in a significant improvement in the *in situ* proteolytic activity levels within the abalone intestine compared to abalone fed either the control diet or the *V. midae* SY9Mut2 supplemented diet. These levels were however significantly lower than the *in situ* proteolytic activity levels within the intestine of animals fed the basal ABFEED® diet supplemented with *V. midae* SY9. This was surprising as studies of *V. midae* SY9Pro2 cultivation in a high-protein growth medium (Chapter 4) would lead us to expect that *in situ* protease activity levels in *H. midae* fed a *V. midae* SY9Pro2 supplemented artificial diet would be either slightly higher or similar to the protease activity levels in abalone fed the *V. midae* SY9 supplemented artificial diet. Interestingly, *in situ* alkaline protease activity levels within the crop/stomach region of *H. midae* fed *V. midae* SY9Pro2 supplemented ABFEED® was significantly lower than in animals fed either the control feed or the basal diet supplemented with *V. midae* SY9Mut2. This may indicate that regulation of *vmproA* expression within the abalone gut, especially in the case of *V. midae* SY9Pro2, may differ to *vmproA* expression in batch culture.

A comparison of the data obtained from the *H. midae* growth trial and the *in situ* protease assay shows that there is no direct role for VmproA in the growth advantage previously observed by Macey and Coyne (2005). However, the significant improvement in *H. midae* growth rates and the altered *in situ* protease activity levels of abalone fed ABFEED® supplemented with either of the two mutant *V. midae* SY9 strains suggests that the mutant strains are contributing in some manner to the observed abalone growth advantage. Recombination of suicide vectors into *vmproA* within the *V. midae* SY9 chromosome could have resulted in additional effects on the abalone and this warrants further investigation. A better understanding of the reason for the increased growth rate of cultured abalone fed these two mutant strains may assist in the isolation of additional growth enhancing probiotic strains. Successful isolation, identification and implementation of growth enhancing probiotic microorganisms for *H. midae* cultivation could dramatically improve the economics of abalone aquaculture in South Africa.

CHAPTER 6

IN SITU LOCALISATION OF *VIBRIO MIDAE* SY9 AND IMMUNOHISTOCHEMICAL LOCALISATION OF VMPROA WITHIN THE DIGESTIVE TRACT OF *HALIOTIS MIDAE*

CONTENTS

6.1 Summary

In order to localise *Vibrio midae* SY9 and its extracellular protease VmproA within the *Haliotis midae* digestive tract, *V. midae* SY9 was transformed with the mini-Tn*10*-*gfp*-*kan* transposon from pLOFKm*gfp* to chromosomally tag the abalone probiotic with a single chromosomal copy of a promotorless *gfp* gene. The resulting chromosomally-tagged strain, *V. midae* SY9::Tn10.52, was isolated and characterised in terms of growth and extracellular protease activity. *V. midae* SY9::Tn10.52 was stably and viably incorporated into ABFEED® S34 weaning chips using vacuum impregnation technology. Furthermore, the vacuum impregnated feed could be viably stored at 4 ºC for at least 7 days and was stable for a period of 3 days in the experimental abalone tanks. An *in situ* histological comparison of *H midae* fed a basal ABFEED® S34 diet and abalone fed the basal diet supplemented with *V. midae* SY9::Tn10.52, using three digoxigenin-labeled oligonucleotide DNA probes to the *gfp* gene, demonstrated that the probiotic cells incorporated into the feed could be localised to the crop/stomach and intestine of *H. midae*. The ingested probiotic microorganisms were visualized in association with the walls of the crop/stomach and adhered to feed and/or other particulate matter within the crop/stomach and intestinal regions. Similarly, polyclonal anti-VmproA antibodies localised VmproA to the crop/stomach and intestine of *H. midae*. The VmproA detected within these regions, by immunohistochemistry, appeared to be associated with feed and/or other particulate matter within the abalone gut.

6.2 Introduction

The use of probiotic microorganisms is becoming increasingly accepted as a viable method of improving the health and growth of aquacultured species (Gomez-Gil *et al.,* 2000). Several possible modes of probiotic action have been proposed for probiotic effects observed within aquaculture environments. These include competitive exclusion of pathogenic bacteria, production of inhibitory compounds that prevent the growth of pathogens, secretion of extracellular enzymes that contribute to and improve the host's digestion efficiency, provision of essential nutrients; and stimulation of the host's immune system (Vershuere *et al.,* 2000; Balcázar *et al.,* 2006). It is also necessary for putative probiotic microorganisms to possess certain features in order to successfully function as probiotics within an aquaculture environment. The putative probiotic should be readily and easily delivered to the host (e.g. through ingestion), they should be able to reach the location where the effect is expected to take place (e.g. intestinal region of the digestive tract), and they should colonise and replicate within the host (Vershuere *et al.,* 2000; Kesarcodi-Watson *et al.,* 2008). Therefore, it would be valuable to utilize molecular tools to visually localise probiotic microorganisms and any gene products believed to be involved in the observed probiotic effect, within the host organism.

In order to better understand the role that probiotic microorganisms, such as *V. midae* SY9, play in improving the growth rate of cultured abalone it is necessary to investigate the relationship between the probiotic and the rest of the enteric microbiota. Studies have shown that abalone have a unique microbiota capable of producing extracellular enzymes to degrade the major constituents of abalone feeds (Sawabe *et al.,* 1995; Erasmus *et al.,* 1997). However, less than 10% of the abalone gut associated microorganisms can be cultured using currently available techniques (Tanaka *et al.,* 2003). As over 90% of the enteric microbiota is presently unculturable, culture-independent techniques are invaluable to further investigate gut associated microorganisms within their natural habitat (Tanaka *et al.,* 2004).

In situ hybridization using specific 16S rDNA oligonucleotide probes is a culture-independent method of investigating bacterial population diversity (Tanaka *et al.,* 2004), and it is the ideal method to investigate microorganisms within their natural habitat or microenvironment (Moter and Gobel, 2000). *In situ* hybridization using oligonucleotide probes for use in bacteriology was first employed by Giovannoni *et al.* (1988). *In situ* hybridization-based approaches provide a distinct advantage over culture-based approaches for investigations into probiotic colonisation and localisation within a host organism's digestive tract, by enabling detection of the entire probiotic population, including any non-culturable probiotic cells, present within the host's gut. *In situ* hybridization techniques have been successfully used to investigate the microbiota of goldfish (Asfie *et al.,* 2003), abalone (Macey and Coyne, 2006), *Artemia* nauplii (Høj *et al.,* 2009) and salmon (Holben *et al.,* 2002), and to detect and specifically localise intracellular prokaryotes within abalone tissue sections (Antonio *et al.,* 2000; Horwitz, 2008).

The main principle behind *in situ* hybridization is the specific annealing of complementary nucleic acid molecules from the oligonucleotide probe and the target sequence. As a result, any nucleic acid sequence can be detected with a labelled complementary anti-sense probe and can provide direct visualization of the spatial location of the specific target sequence in question (Wilkinson, 1999). Therefore, *in situ* hybridization allows the visualization of a specific oligonucleotide probe hybridized to the complementary portion of DNA within the target microorganism within its natural microenvironment (Moter and Gobel, 2000).

The small size of oligonucleotide probes (typically 20 - 40 bp; Wilkinson, 1999) used for *in situ* hybridization, enables easy penetration of tissue sections and access to the specific nucleic acid target sequence (Polak and McGee, 1998). The oligonucleotide probes are labelled to facilitate the localisation of the target in the tissue sections (Rattray and Michael, 1999). Oligonucleotide probes can either be radioactively labelled and then detected by autoradiography, or labelled with a hapten and then visualized directly by fluorescence or indirectly by using an enzyme-conjugated antibody. A common and very sensitive hapten labelling method is to end-label the oligonucleotide probe with the steroid digoxigenin (DIG). Anti-DIG antibodies conjugated with alkaline phosphatase (AP), and a chromogenic substrate that yields an insoluble coloured product at the site of the probe-target hybridization, are then used to localise the probe (Wilkinson, 1999).

Relatively low signal intensity is one of the major problems associated with *in situ* hybridization experiments. Even if the oligonucleotide probes are designed correctly, efficiently labelled and specific for their complementary target sequence, a low hybridization signal may be due to either a low-abundance target or the probe not being able to access the target (Amann *et al.,* 1995). However, several studies have shown that the use of a cocktail of several labelled oligonucleotide probes that hybridise to different target sites of the same sequence can increase the strength of the probe-target hybridization signal (Lee *et al.,* 1993; Antonio *et al.,* 2000).

Ever since it was first described by Coons *et al.* (1941), immunohistochemical techniques for the localisation of cellular antigens within tissue sections have become a valuable diagnostic and research tool (Ramos-Vara, 2005). The fundamental concept behind immunohistichemical localisation of a tissue antigen, is the binding of specific antibodies to the specific antigen within the tissue sections, followed by a coloured histochemical reaction that can be visualized by light microscopy or flourochromed with ultraviolet light (Ramos-Vara, 2005). Electron microscopy, in conjunction with gold-labelled antibodies, has also been used successfully to identify and localise antigens within tissue sections (Schroeder *et al.,* 2003).

The detection of the antigen-antibody reaction by an indirect approach, specifically making use of enzyme conjugated secondary antibodies, is a very sensitive method of immunohistochemical localisation of tissue antigens. Alkaline phosphatase as an antibody conjugated reporter molecule, is being increasingly utilized for immunostaining as it avoids most of the issues related to endogenous enzyme activity when reporter molecules such as peroxidases are used for immunohistochemistry (Ramos-Vara, 2005).

Albrecht *et al.* (2001) employed immunohistochemistry using hemocyanin-specific antibodies, together with *in situ* hybridization using a 2.2 kb DIG-labelled cDNA probe, to investigate rhogocytes (pore cells) within *Haliotis tuberculata* connective tissues as the site of hemocyanin biosynthesis. The process of biomineralization in the formation of molluscan shells was investigated by Jolly *et al.* (2004) using a combination of histology and immunohistochemistry to study shell matrix proteins within the mantle epithelium of *H. tuberculata.* Rengpipat *et al.* (2008) employed monoclonal antibodies to detected *Vibrio vulnificus* within the tissues of infected *Penaeus monodon* using immunohistochemistry. Therefore, the combined use of immunohistochemistry, *in situ* hybridization and standard histological staining techniques may be useful for investigating the spatial distribution of bacterial isolates and their gene products, such as *V. midae* SY9 and VmproA, within the digestive tract of *H. midae.*

In the first reported study to investigate probiotic colonisation of the digestive tract of *H. midae*, Macey and Coyne (2006) employed a *gfp* chromosomally-tagged strain *V. midae* SY9.8 that was constructed using pLOFKm*gfp*, to monitor the colonisation potential of *V. midae* SY9 in the digestive tract of *H. midae*.

Green fluorescent protein (GFP) is a stable, non-toxic fluorescent protein obtained from the Pacific jellyfish, *Aequoria victoria* (Prasher, 1995). Variants of the *gfp* gene are available that display improved expression, folding-efficiency and detection (Heim *et al.,* 1995; Cormack *et al.,* 1996). Chromosomal tagging of bacterial strains with *gfp* has enabled visualization of bacteria as single cells and as cell populations (Eberl *et al.,* 1997). The *gfp* gene has also been successfully used as a marker for investigating the localisation and colonisation potential of bacteria (Scott *et al.,* 2000; Macey and Coyne, 2006; Rengpipat *et al.,* 2009).

Transposon vectors have been constructed to facilitate stable chromosomal integration of foreign marker genes, such as *gfp,* into a host genome (De Lorenzo *et al.,* 1990; Herrero *et al.,* 1990; Sohaskey *et al.,*1992; Albertson *et al.,* 1996; Matthysse *et al.,* 1996). The transposon vector pLOFKm*gfp* (Stretton *et al.,* 1998), containing a promotorless *gfp* gene, was constructed in order to investigate gene expression in marine bacteria through the construction of transcriptional fusions. The vector pLOFKm (Herrero *et al.,* 1990), which was used to create pLOFKm*gfp,* was constructed on a backbone of the suicide vector pGP704 (Miller and Mekalanos, 1988) and is unable to replicate in the absence of the π-protein encoded by the *pir* gene (See Chapter 4). The mini-Tn*10*-*gfp*-*kan* reporter transposon, contained within pLOFKm*gfp,* includes an $IS10_R$ transposase gene. This transposase gene, located outside of the 70 bp Tn*10* inverted repeat sequences, is under the control of the p*tac* promoter and is therefore induced in the presence of IPTG. Once transferred to the recipient strain, the vector pLOFKm*gfp* is unable to replicate as the recipient lacks the *pir* gene. Induced by the addition of IPTG the transposase gene enables the mobile transposable element to randomly integrate at a single site into the chromosome of the recipient strain. Kanamycin is then used in a single selection step to identify recipient strains that have undergone an integration event resulting in chromosomal integration of the mini-Tn*10*-*gfp*-*kan* reporter transposon.

As *V. midae* SY9 was initially isolated from the digestive tract of cultured *H. midae* (Macey, 2005), it is likely that this bacterial strain may be a common enteric bacterium of cultured *H. midae*. Therefore, a mini-Tn10 transposon containing a promotorless *gfp* gene was used to chromosomally-tag *V. midae* SY9. A chromosomally-tagged *V. midae* SY9 strain will allow localisation of *V. midae* SY9 ingested from supplemented ABFEED® S34 weaning chips within the digestive tract of *H. midae*, and not *V. midae* SY9 cells naturally present within the enteric microbiota of cultured *H. midae*.

Macey and Coyne (2005) observed significantly increased growth rates, and increased protease activity, protein digestion and protein absorption within the intestinal region of abalone fed a probiotic supplemented feed compared to animals not fed probiotics. This supports the argument that feeding aquacultured animal species with microorganism(s) with enhanced extracellular enzyme activity may lead to increased growth rates of the host, as a result of improved digestion efficiency of the host animal (Erasmus *et al.,* 1997; Verschuere *et al.*, 2000). Therefore, in addition to localizing *V. midae* SY9 within the digestive tract of *H. midae* fed a probiotic supplemented feed, it would also be advantageous to be able to detect the presence of the predominant *V. midae* SY9 extracellular protease, VmproA, within the gut of abalone fed probiotically supplemented ABFEED® S34. This requires that *in situ* hybridization of abalone tissue sections for detection of *V. midae* SY9 be conducted in conjunction with immunohistochemical localisation of the VmproA antigen.

6.2.1 Aim of this chapter

The aim of this chapter is to construct a chromosomally tagged strain of *V. midae*SY9 using a *gfp* transposon mutagenesis system. This *gfp* tagged *V. midae* SY9 strain will be used to further investigate the role of the probiotic *V. midae* SY9 and its extracellular protease VmproA in the observed probiotic effect on the growth of *H. midae*. Colonisation of the digestive tract of juvenile *H. midae* fed an ABFEED® S34 diet supplemented with *gfp*-tagged *V. midae* SY9 will be investigated by means of *in situ* hybridization. The role of the *V. midae* SY9 predominant extracellular protease, VmproA, in the observed increased *in situ* protease activity (Chapter 5) and improved protein digestion of abalone fed probiotically supplemented feed (Macey and Coyne, 2005) will be investigated further by means of immunohistochemical localisation. Therefore, we aim to employ these molecular techniques to further evaluate the role of *V. midae* SY9 and VmproA, the predominant extracellular protease secreted by *V. midae* SY9, in the previously observed growth advantage (Macey and Coyne, 2005) of abalone fed a probiotically supplemented diet.

6.3 Materials and Methods

The media and solutions used in this study are listed in Appendix A.

6.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 6.1.

6.3.2 Media and culture conditions

Wild-type *V. midae* SY9 was maintained at approximately 22 °C on MA (Appendix A.1.2) and cultured in MB (Appendix A.1.1) or P-MBM (Appendix A.1.3) at 22 °C as described in Chapters 2 and 3 unless otherwise stated. *Pseudoalteromonas* sp. strain C4 (Erasmus, 1996) was maintained on MA, and cultured in MB, at 22 °C. *E. coli* SM10λpir harbouring the pLOFKm*gfp* plasmid (Table 6.1 and Fig. 6.1), obtained from Molecular Biology Vectors (ATCC 87711), was either grown in LB10 (Appendix A.1.9) or on LA10 (Appendix A.1.9), supplemented with 100 µg ml^{-1} kanamycin (Appendix A.2.2) and 100 µg ml^{-1} ampicillin (Appendix A.2.2) at 37 °C. *V. midae* SY9Sm^{r} (Table 6.1) was maintained on either MA, or VNSS agar (Appendix A.1.9), or grown in VNSS broth (Appendix A.1.9), containing streptomycin (Appendix A.2.2) at a concentration of 120 µg ml^{-1} at 22 °C, unless otherwise stated. *Gfp* chromosomally-tagged strains, *V. midae* SY9::Tn10.11, *V. midae* SY9::Tn10.12 and *V. midae* SY9::Tn10.52, were either grown on MA or VNSS agar, or cultivated in P-MBM or MB supplemented with 120 µg ml^{-1} streptomycin and 400 µg ml^{-1} kanamycin at 22 °C unless otherwise stated.

6.3.3 Construction of *gfp* chromosomally-tagged *V. midae* SY9 strains

As *V. midae* SY9 was initially isolated from the digestive tract of cultured *H. midae* (Macey, 2005), it is likely that this bacterial strain may be a common enteric bacterium of cultured *H. midae*. Therefore, a mini-Tn10 transposon (Fig. 6.1) containing a promotorless *gfp* gene (Mut2 variant) was used to chromosomally-tag *V. midae* SY9. A chromosomally-tagged *V. midae* SY9 strain will allow localisation of *V. midae* SY9 ingested from supplemented ABFEED® S34 within the digestive tract of *H. midae*, and not *V. midae* SY9 cells present within the natural enteric microbiota of cultured *H. midae*.

A strain of *V. midae* SY9 capable of growth on streptomycin was generated, as described in section 4.3.3, as a recipient strain for the construction of a *V. midae* SY9 *gfp* chromosomally-tagged strain. The streptomycin-resistant *V. midae* SY9 strain was maintained on MA containing 120 µg ml^{-1} streptomycin at 22 °C and was designated *V. midae* SY9Sm^r (Table 6.1).

The vector pLofKm*gfp* (Table 6.1 and Fig. 6.1) was transferred from the donor *E. coli* SM10λpir (Table 6.1) strain to the recipient strain *V. midae* SY9Sm^r using a modified filter mating technique (Egan *et al.,* 2002). Briefly, 5 ml cultures of both *E. coli* SM10λpir harbouring pLofKm*gfp* and streptomycin resistant recipient *V. midae* SY9Sm^r (Table 6.1) were cultivated for 16 hours. The overnight donor and recipient cultures were gently mixed at a volume ratio of 1:10 (100 µl donor and 1000 µl recipient) in 5 ml of wash solution (Appendix A.2.12). The mixture of donor and recipient cultures was passed through a 0.22 µm Whatman filter (∅ 2.5 cm) and washed with another 5 ml of wash solution. The filter discs were then placed cell-side up onto LA-20 agar (Appendix A.1.9) and incubated at 30 °C for 4 hours.

The filter discs were then placed inside sterile 50 ml plastic tubes (Sterilin) containing 2 ml of NSS (Appendix A.1.9), and vortexed vigorously to resuspend the bacteria on the filter discs. One hundered microlitre aliquots of the resuspended cells were spread plated onto VNSS agar (Appendix A.1.9) supplemented with 120 µg ml^{-1} streptomycin and 400 µg ml^{-1} kanamycin. The plates were subsequently incubated at 30 °C for 48 hours to select for recipient *V. midae* SY9Sm^r strains harbouring the integrated mini-Tn10-*gfp-kan* transposable element. One hundered microlitre aliquots of each donor and recipient strain were spread plated (100 µl) onto both VNSS agar and VNSS agar containing 120 µg ml^{-1} streptomycin and 400 µg ml^{-1} kanamycin as filter mating controls.

Table 6.1 Strains and plasmid used in this study.

Strains / Plasmid	Genotype/Relevant characteristic (s)[a]	Reference
Strains:		
V. midae SY9	Isolated from the digestive tract of *H. midae*, South Africa	Macey (2005)
V. midae SY9Sm^r	Streptomycin resistant strain of *V. midae* SY9; Sm^r	This study
V. midae SY9::Tn10.11	*V. midae* SY9Sm^r::mini-Tn10-*gfp-kan*; Sm^r, Km^r	This study
V. midae SY9::Tn10.12	*V. midae* SY9Sm^r::mini-Tn10-*gfp-kan*; Sm^r, Km^r	This study
V. midae SY9::Tn10.52	*V. midae* SY9Sm^r::mini-Tn10-*gfp-kan*, Sm^r, Km^r	This study
E. coli SM10λpir	*thi thr leu ton*A *lac*Y *sup*E (λpir) *rec*A::RP4-2-Tc::Mu Km^r	Simon *et al.* (1983)
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F'(traD36 proAB' lacI^q lacZΔM15)	Sambrook *et al*. (1989)
Pseudoalteromonas sp. C4	*H. midae*, South Africa	Erasmus (1996)
Plasmid:		
pLOFKm*gfp*	pLOFKm with cloned promoterless *gfp* (GFPmut2), Km^r, Amp^r	Stretton *et al.* (1998)

[a] Sm^r, streptomycin resistant; Km^r, kanamycin resistant; Amp^r, ampicillin resistant.

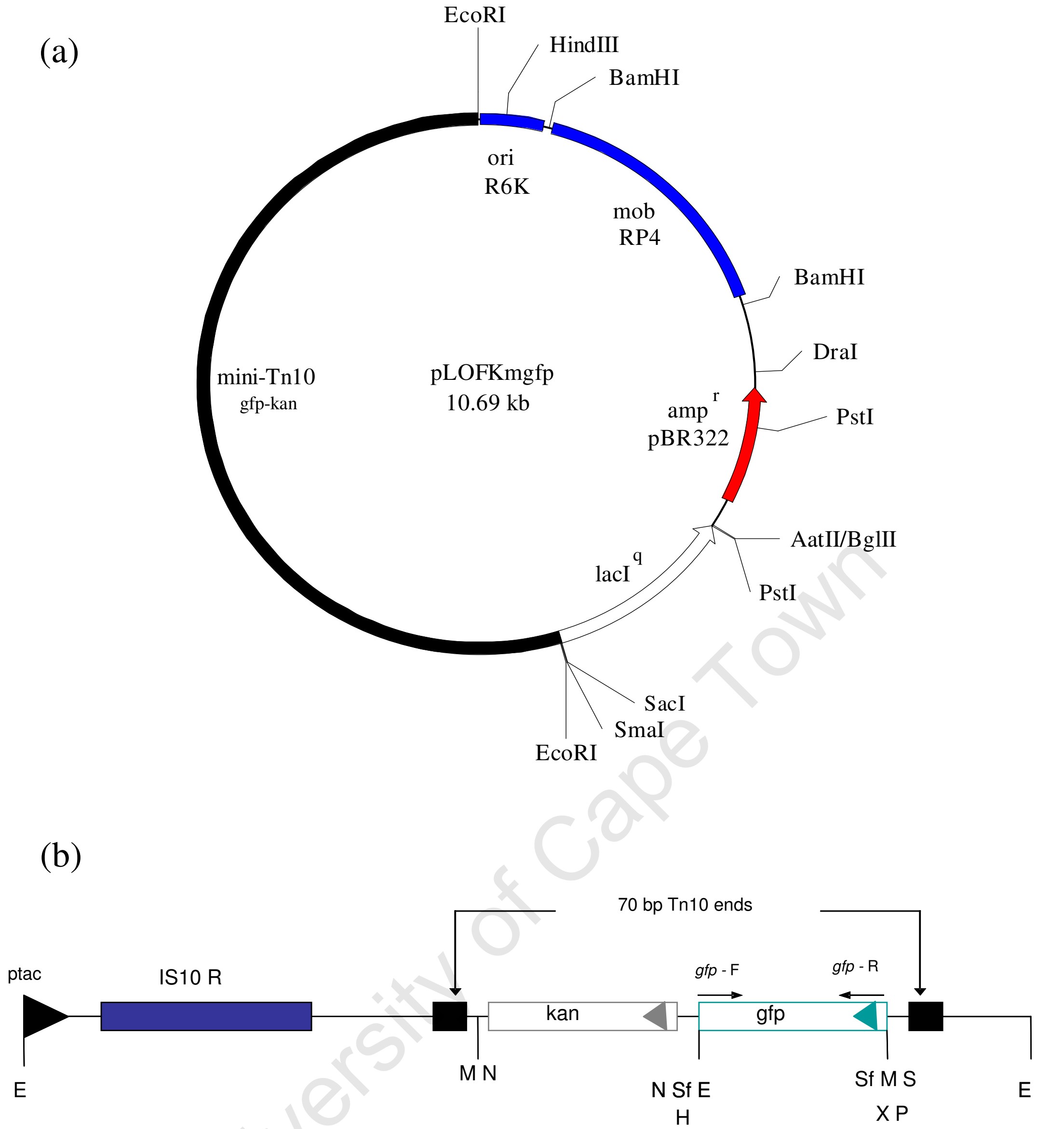


Figure 6.1 Diagramatic representation of the recombinant transposon vector pLOFKm*gfp* (a) and the mini-Tn10-*gfp-kan* transposable element (b) used to chromosomally tag *V. midae* SY9Sm^r. Plasmid pLOFKm*gfp* is a derivative of the suicide vector pGP704 and contains the mini-Tn10-*gfp-kan* transposable element. In mini-Tn10-*gfp-kan,* the transposase gene of IS10_R is located outside of the inverted repeats (black-boxes) of the mobile element and downstream of the p*tac* promoter (black triangle). The PCR primers (*gfp*-F and *gfp*-R; Table 6.2) are indicated above the open boxes depicting the kanamycin and *gfp* genes in mini-Tn10-*gfp-kan* (b). Relevant restriction enzyme sites are indicated. Abbreviations: E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sf, *Sfi*I; X, *Xba*I. The diagram (not to scale) of pLOFKm*gfp* was adapted from Herrero *et al*. (1990) and mini-Tn10-*gfp-kan* (not to scale) from Stretton *et al*. (1998).

6.3.4 Screening of recombinant strains

Fifty eight *V. midae* SY9 strains putatively harbouring the chromosomally integrated mini-Tn10-*gfp*-*kan* transposable element were selected on the basis of their ability to grow on VNSS agar containing 120 µg ml^{-1} streptomycin and 400 µg ml^{-1} kanamycin. Each of the strains was inoculated into 5 ml MB, containing 120 µg ml^{-1} streptomycin and 400 µg ml^{-1} kanamycin, and grown for approximately 16 hours at 22 ºC on a rotary shaker (100 rpm). Chromosomal DNA was isolated from the overnight cultures, as described in Appendix B.4.2. The 58 kanamycin-resistant *V. midae* SY9 strains were screened by PCR (Appendix B.25.1) using the oligonucleotide primers *gfp*-F and *gfp*-R (Table 6.2) for the presence of the *gfp* gene. PCR amplification of the *gfp* gene would indicate integration of the mini-Tn10-*gfp*-*kan* transposable element into the *V. midae* SY9 chromosome. Three *gfp* chromosomally-tagged strains were identified, designated *V. midae* SY9::Tn10.11, *V. midae* SY9::Tn10.12 and *V. midae* SY9::Tn10.52, and analyzed further.

Table 6.2 Oligonucleotide primers used in this study.

Primer	Sequence (5' - 3')	Reference
gfp-F	GAT TTC TAG ATT TAA GAA GC	This study
gfp-R	TCA TAT TTG TAT AGT TCA TCC	This study
fD1	AGA GTT TGA TCC TGG CTC AG	Weisburg *et al.* (1991)
Rp2	ACG GCT ACC TTG TTA CGA CTT	Weisburg *et al.* (1991)

6.3.5 Growth and extracellular protease activity of *V. midae* SY9, *V. midae* SY9::Tn10.11, *V. midae* SY9::Tn10.12 and *V. midae* SY9::Tn10.52

A growth curve was conducted to determine which *gfp* chromosomally-tagged *V. midae* SY9 strain would be best suited for further characterisation and use in *H. midae*-based experiments. The effect of the integrated mini-Tn10-*gfp*-*kan* transposable element on the cell growth and extracellular protease activity was assessed by comparative growth curve analysis of *V. midae* SY9::Tn10.11, *V. midae* SY9::Tn10.12, *V. midae* SY9::Tn10.52 and the wild-type strain in P-MBM (Appendix A.1.3) as described in section 4.3.7.

6.3.6 Southern hybridization

Southern hybridization analysis was performed to confirm whether the mini-Tn10-*gfp*-*kan* cassette had integrated into the chromosome of *V. midae* SY9Sm^r. Chromosomal DNA was extracted from *V. midae* SY9 and *V. midae* SY9::Tn10.52, as described in Appendix B.4.1. *E. coli* SM10λpir harbouring pLOFKm*gfp* was inoculated into 5 ml LB supplemented with 100 µg ml^{-1} ampicillin and grown overnight with shaking at 37 ºC. A small scale isolation of the plasmid pLOFKm*gfp* was performed, as described in Appendix B.3, from the overnight culture using the alkaline lysis mini-prep method of Ish-Horowicz and Burke (1981). A 717 bp DNA fragment of the *gfp* gene was PCR amplified (Appendix B.25.1) from pLOFKm*gfp* (Fig. 6.1a and b) using the synthetic oligonucleotide PCR primers, *gfp*-F and *gfp*-R (Table 6.2). The 717 bp PCR amplicon was resolved on a 1% (w/v) TAE agarose gel (Appendix B.6), before being gel purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. The purified 717 bp DNA fragment was then radioactively labelled (Appendix B.8.2) and used as a probe against equal amounts (10 µg) of *V. midae* SY9 and *V. midae* SY9::Tn10.52 chromosomal DNA that had been digested with the restriction enzymes *Hin*dIII, *Eco*RI and *Pst*I (Fermentas) (Appendix B.5). The resulting chromosomal DNA fragments were separated on a 0.7% (w/v) TAE agarose gel at 35 V overnight and transferred to Hybond N$^+$ nylon membrane (Amersham) according to Reed and Mann (1985). Southern hybridization was performed as described in Appendix B.8.

6.3.7 Differentiation of chromosomally-tagged *V. midae* SY9 strains from unlabelled bacterial strains

A PCR-based method was employed to investigate whether or not *V. midae* SY9::Tn10.52 could be differentiated from other bacterial strains on the basis of the presence of a chromosomally integrated copy of the *gfp* gene within the mini-Tn10-*gfp-kan* cassette. Chromosomal DNA was extracted from *V. midae* SY9, *V. midae* SY9::Tn10.52, *E. coli* JM109 and *Pseudoalteromonas* species strain C4, as described in Appendix B.4.1. Plasmid DNA was isolated from an overnight culture of *E. coli* SM10λpir harbouring pLOFKm*gfp*, using the alkaline lysis mini-prep method of Ish-Horowicz and Burke (1981) (Appendix B.3). The chromosomal DNA samples were screened for the presence of the *gfp* gene by PCR (Appendix B.25.1) using the oligonucleotide primers *gfp*-F and *gfp*-R (Table 6.2). The amplified PCR products were analyzed by agarose gel electrophoresis (Appendix B.6) to verify reaction specificity and fragment sizes. Amplification of the *gfp* gene from pLOFKm*gfp* and *V. midae* SY9::Tn10.52, and not from *V. midae* SY9, *E. coli* JM109 and *Pseudoalteromonas* sp. strain C4, would indicate that the *gfp* gene could be used as a selective marker for identification of *V. midae* SY9::Tn10.52.

6.3.8 Probe design and labeling

Three oligonucleotide probes (Table 6.3) were designed to the variant of the *gfp* gene, harboured by pLOFKm*gfp*, in order to enable *in situ* localisation of *gfp* chromosomally-tagged *V. midae* SY9 strains. It is critical that positive and negative controls be included in order to validate the results obtained in any *in situ* hybridization procedure (Polak and McGee, 1998). Therefore, the eubacterial probe EUB338 (Stahl *et al*., 1989) (Table 6.3) was employed as a positive control, while ECJ109 (Table 6.3) was included as a negative control in *in situ* hybridization experiments (section 6.3.12). The probe ECJ109 was designed by comparing the 16S rRNA gene sequences of *E. coli* JM109 and *V. midae* SY9, and identifying a region of low sequence homology (Fig. 6.2).

All of the oligonucleotide probes employed in this investigation (Table 6.3) were labelled with Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxy-succinimide ester (Roche) by 5' labelling. The 5'-amino substituted oligonucleotide (X, Table 6.3) was labelled with the digoxigenin moiety under weak alkaline conditions in a sodium borate buffer/dimethyl formamide mixture at 22 ºC overnight (Zischler *et al*., 1989).

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361   GATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGG
      ||||||||||||||||||||.|||||||||||||||||||||||||.||||||||..|.|
328   GATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTG

421   AGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGC
      |||||||..||..||||||||.||....|..|||||||||.|..||||||||||||||||
388   AGGAAGGTGGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGC
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Figure 6.2 A portion of the alignment of the 16S rRNA gene sequences of *E. coli* JM109 (Accession no.: AB305017) and *V. midae* SY9. The portion representing the oligonucleotide DNA probe EC109 is highlighted in bold. The upper line represents the *E. coli* JM109 16S rRNA gene sequence, while the lower line represents the *V. midae* SY9 16S rRNA sequence. Vertical lines (|) represent homologous bases, while dots (·) represent gaps between the 16S rRNA gene sequences of *E. coli* JM109 and *V. midae* SY9.

6.3.9 Specificity of oligonucleotide probes by Southern hybridization

A Southern hybridization analysis was preformed in order to assess the specificity of the DIG-labelled DNA probes (Table 6.3) to be used in the *in situ* hybridisation experiments.

Table 6.3 5'-DIG-labelled oligonucleotide probes employed in this study.

Probe	Sequence 5' - 3' [a]	Target	Reference
EUB338	X-GCTGCCTCCCGTAGGAGT	All bacteria	Stahl *et al.* (1989)
ECJ109	X-GAGTAAAGTTAATACCTTTGCTCA	*E. coli* JM109	This study
GFP001	X-GTTGAATTAGATGGTGATGTTAATGG	*V. midae* SY9::Tn10.52	This study
GFP002	X-CTACCTGTTCCATGGCCAACACTTG	*V. midae* SY9::Tn10.52	This study
GFP003	X-CAAAATACTCCAATTGGCGATGGCCCTG	*V. midae* SY9::Tn10.52	This study

[a] - X, 5'-DIG label.

6.3.9.1 Specificity of EUB338 and ECJ109 oligonucleotide probes

The 16S rRNA PCR products amplified with the universal eubacterial oligonucleotide primer set fD1/Rp2 (Table 6.2) from chromosomal DNA of *E. coli* JM109, *Pseudoalteromonas* sp. strain C4, *V. midae* SY9, and *V. midae* SY9::Tn10.52 as described in Appendix B.25.3 were used as the DNA template in Southern blots, to assess the specificity of the DIG-labelled oligonucleotide probes EUB338 and ECJ109 (Table 6.3).

The amplified PCR products were analyzed by agarose gel electrophoresis (Appendix B.6), before being purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. A total of 150 ng of each of the amplified PCR products were separated on 1% (w/v) TAE agarose gels (Appendix B.6), and transferred to nylon membranes (Roche) (Appendix B.8.1). Southern hybridizations were performed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) protocol (as described in appendices B.8.5, B.8.6 and B.8.7). The 5'-DIG end labelled oligonucleotides EUB338 and ECJ109 (Table 6.3), respectively, were used as the probes. The hybridization temperature for the DIG-labelled probe EUB338 was 52 °C, while a hybridization temperature of 62 ºC was employed for the ECJ109 probe.

6.3.9.2 Specificity of GFP001, GFP002 and GFP003 oligonucleotide probes

The 717 bp *gfp* PCR product amplified with the *gfp*-specific primer set *gfp*-F/*gfp*-R (Table 6.2) from chromosomal DNA of *V. midae* SY9::Tn10.52, as described in Appendix B.25.1, was used as the DNA template in Southern blots to assess the specificity of the DIG-labelled oligonucleotide probes GFP001, GFP002 and GFP003 (Table 6.3).

The amplified PCR products were analyzed by agarose gel electrophoresis, before being purified using the BioSpin Gel Extraction Kit (BioFlux) according to the manufacturer's instructions. A total of 150 ng of each of the amplified PCR products were separated on 1% (w/v) TAE agarose gels (Appendix B.6), and transferred to nylon membranes (Roche) (Appendix B.8.1). Southern hybridizations were performed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) protocol (as described in appendices B.8.5, B.8.6 and B.8.7). The 5'-DIG end labelled oligonucleotides GFP001, GFP002 and GFP003 (Table 6.3), respectively, were used as the probes. The hybridization temperature for DIG-labelled probes GFP001, GFP002 and GFP003 was 52 °C.

6.3.10 Preparation of experimental ABFEED® S34 diets

The basal diet consisted of ABFEED® S34 weaning chips, as supplied by Marifeed (Pty.) Ltd., Hermanus, South Africa. The experimental diet was prepared by incorporating *V. midae* SY9::Tn10.52 into ABFEED® S34 weaning chips by vacuum impregnation as described in section 5.3.3.2.

6.3.10.1 Cultivation of *V. midae* SY9::Tn10.52 for incorporation into the experimental diet

V. midae SY9::Tn10.52 was grown for 8 hours in a 5 ml starter culture of P-MBM (Appendix A.1.3) supplemented with kanamycin (Appendix A.2.2) to a final concentration of 400 µg ml^{-1} on an orbital shaker (100 rpm) at 22 °C. A flask containing 100 ml P-MBM was inoculated with the entire volume of the 5 ml starter culture and incubated on an orbital shaker (100 rpm) for 12 hours at 22 ºC. The 100 ml culture was inoculated into five litre flasks (in triplicate), containing 1000 ml of P-MBM, to achieve an initial A_{600} of 0.02 and incubated for 24 hours at 22 °C with shaking (100 rpm). Thereafter, the bacterial cells were harvested by centrifugation (8,000x g for 15 minutes at 4 °C), washed with one volume of ASW (Appendix A.2.1) and resuspended in 100 ml of ASW.

6.3.10.2 Incorporation of *V. midae* SY9::Tn10.52 into the ABFEED® S34 experimental diet by vacuum impregnation

The *gfp* chromosomally-tagged probiotic strain, *V. midae* SY9::Tn10.52, was incorporated into the basal diet of ABFEED® S34 weaning chips using vacuum impregnation, to a final concentration of at least 1.0 x 10^8 culturable cells g^{-1} feed. The experimental diet was prepared as described in section 5.3.2.2.

6.3.10.3 Effect of post-vacuum impregnation drying on *V. midae* SY9::Tn10.52 cell viability

The effect of drying the post-impregnated ABFEED® S34 weaning chips at 22ºC for approximately 16 hours on *V. midae* SY9::Tn10.52 viability was assessed as described in section 5.3.2.2.1. Viable cell numbers were determined by spread-plating serial dilutions in ASW (Appendix A.2.1) of the homogenized ABFEED® S34 onto MA (Appendix A.1.2) containing 400 µg ml^{-1} kanamycin (Appendix A.2.2) to culture *V. midae* SY9::Tn10.52. Cell numbers were recorded after incubating *V. midae* SY9::Tn10.52 for 48 hrs at 22 ºC and viability recorded as the number of cfu g^{-1} of ABFEED® S34 weaning chips. For statistical analysis of the cell viability of vacuum impregnated ABFEED® S34 weaning chips, the cell count data was natural log transformed and analysed by Student *t*-test using SigmaStat 3.11.0 (Systat Software, Inc.). Significant differences were established at $P<0.05$.

6.3.10.4 Optimal storage conditions of *V. midae* SY9::Tn10.52 supplemented ABFEED® S34

In order to determine how often *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 weaning chips would need to be prepared, the effect of storing impregnated ABFEED® S34 weaning chips at 4 ºC on bacterial viability was assessed. A batch of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips was prepared as described in sections 5.3.2.2 and stored at 4 ºC in a clean plastic bag for a period of two weeks. Viable bacterial counts were determined at regular intervals, by removing a sample and determining the viable *V. midae* SY9::Tn10.52 cell numbers as described in section 6.3.10.3. Cell viability was recorded as the number of cfu g^{-1} of ABFEED® S34 weaning chips. For statistical analysis of the cell viability of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips stored at 4 ºC, the cell count data was natural log transformed and analysed by one-way ANOVA, followed by the Holm-Sidak multiple comparison procedure to test for significant differences between sample means. Significant differences were established at $P<0.05$.

6.3.10.5 Stability of *V. midae* SY9::Tn10.52 vacuum-impregnated ABFEED® S34

The stability of the probiotic bacteria in vacuum-impregnated ABFEED® S34 weaning chips incubated in flow-through experimental tanks was investigated. This was done to determine how often the impregnated ABFEED® S34 weaning chips would need to be removed from the tanks and replaced with fresh impregnated ABFEED® S34 weaning chips during the course of the experiment.

A batch of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips was prepared, as described in section 5.3.2.2. Approximately 50 g of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips were placed in an otherwise empty basket (30 x 24 x 26 cm) suspended in glass tank (52 x 30 x 30.5 cm). Seawater, pumped directly from the sea, passed through the tank at a rate of approximately 150 litres $hour^{-1}$, and was constantly aerated by means of an air stone suspended inside the tank.

A sample of *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 weaning chips was immediately removed (T = 0), and additional samples were taken after 24, 48 and 72 hours. Viable bacterial cell counts were determined, as described in section 5.3.2.2.3. Viable cell numbers were determined by spread-plating serial dilutions, in ASW (Appendix A.2.1), of the homogenized ABFEED® S34 onto MA (Appendix A.1.2) containing 400 µg ml^{-1} kanamycin (Appendix A.2.2) to culture

V. midae SY9::Tn10.52. Cell numbers were recorded after incubating *V. midae* SY9::Tn10.52 for 48 hours at 22 ºC and viability recorded as the number of cfu g^{-1} of ABFEED® S34. The viable count data was analysed using SigmaStat 3.11.0 (Systat Software, Inc.) by one-way ANOVA. When the results of the one-way ANOVA were significant, the Tukey test was used to analyse for significant differences between the sample means. Statistically significant differences were established at critical levels of $P<0.05$.

V. midae SY9::Tn10.52 vacuum impregnated ABFEED® S34 feed was prepared weekly for the duration of the experiment and stored in clean, plastic bags at 4 ºC until fed to the abalone.

6.3.11 Animals and Experimental Set-up

Juvenile *H. midae* abalone (smaller than 15 mm shell length) were donated by Sea Plant Products (Jacobsbaai, South Africa) and transported to the MCM Research Aquarium, Sea Point, South Africa, where they were maintained for the remainder of this experiment.

The animals were divided into two groups (n = 75) and placed into two plastic mesh baskets in adjacent glass tanks. The tanks were supplied with continuously flowing, aerated, natural sea water (15-18 ºC) that was passed through a mechanical and biological filter before entering the tanks (Fig. 6.3). The room housing the glass tanks was maintained on a 10:14 (light:dark) light-cycle for the duration of the experiment. The animals were acclimatized to the conditions at the aquarium for 3 weeks prior to the start of the experiment. During this acclimatization period abalone were fed to satiation on the basal diet of ABFEED® S34 weaning chips. All uneaten food was removed from the baskets, and the tanks and baskets were thoroughly cleaned every 2 days, before the addition of fresh feed.

Prior to the beginning of the experiment, abalone in both tanks were starved for a period of 24 hours. This was done to ensure that once the experiment was started and the animals were fed, they would immediately begin feeding on the experimental diets provided. At the start of the experiment (Day 0), six randomly selected animals were removed from each tank and immediately sacrificed and processed, as described in section 6.3.12. Thereafter, the remaining abalone in one tank were fed the *V. midae* SY9::Tn10.52-supplemented diet, while those in the other tank were fed the basal ABFEED® S34 diet. The animals were fed to satiation on the respective diets for the duration of the experiment.

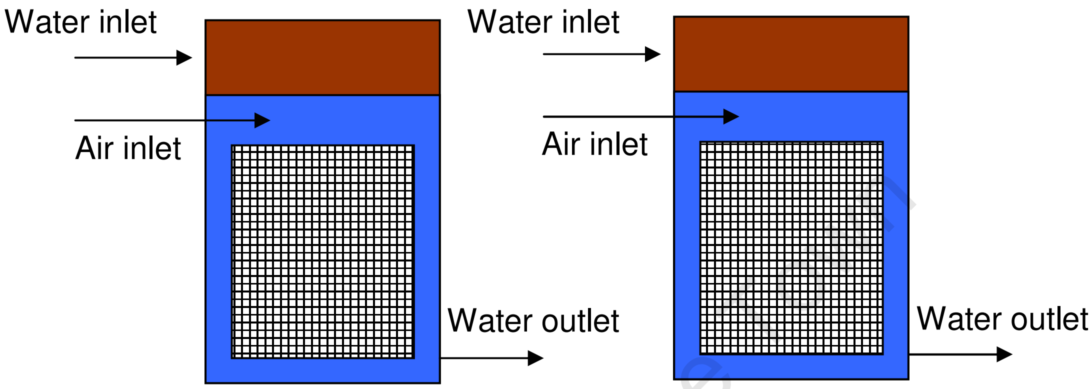

Figure 6.3 Schematic diagram of the experimental tanks used to house juvenile *H. midae*. The brown block (■) represents the mechanical and biological filtration unit, the blue block (■) represents a separate compartment housing the basket (hatched block) containing the animals. The water inlet and outlet pipes are also shown.

6.3.12 Histology

The abalone shells were gently removed by severing the adductor muscle (Fig. 6.4), as close to the shell as possible, with a thin metal spatula. Care was taken to ensure that the digestive tract was not damaged during this process. The animals were placed adductor muscle down in labeled embedding cassettes and fixed in Invertebrate Davidson's solution (Shaw and Battle, 1957) (Appendix A.2.26) for 36 hours at 4 °C, before being transferred to 70% (v/v) ethanol (Appendix A.2.26) at 4 °C. Following fixation, the whole abalone tissue samples were dehydrated through a graded ethanol series to xylene (Saarchem) in a Tissue Trek II tissue processor. The dehydrated tissue samples were embedded in paraffin wax and sectioned at 5 µm on a microtome. Thereafter, the sections were carefully mounted and adhered onto positively charged microscope glass slides (SuperFrost® Plus, Menzel-Gläser) and stored at room temperature in slide boxes until used for Hematoxylin and Eosin (H & E) staining, *in situ* hybridization and immunohistochemistry.

One section from each wax block was deparaffinised by immersion in xylene (section 6.3.12) and stained using standard Harris' H & E stain according to Hayat (1993). The sections were viewed using a Nikon Eclipse 50i Compound Microscope equipped with a Nikon DS Camera Control Unit DS-U2 and DS-5M Camera head with Nikon Software (NIS Elements Documentation and Digital 3D Imaging).

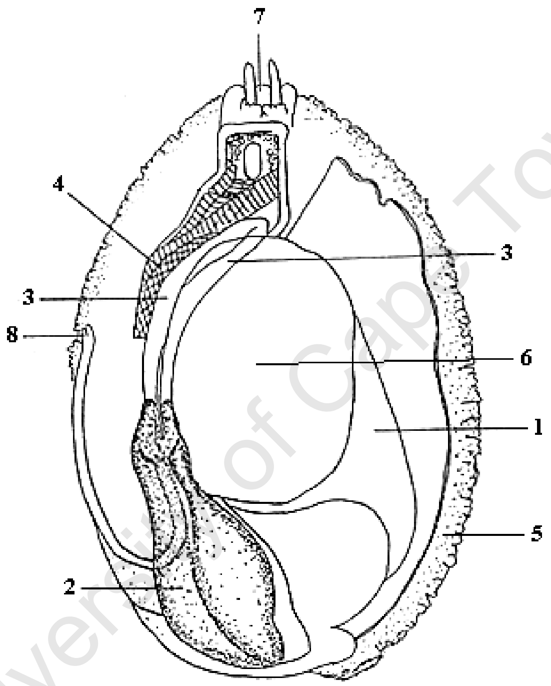

Figure 6.4 Schematic anterior view of the abalone *Haliotis midae* showing major organs. 1 - digestive gland, 2 - crop/stomach, 3 - intestine, 4 - gills, 5 – epipodium, 6 - adductor muscle, 7 – buccal region, 8 – anus (Figure modified from www.fao.org).

6.3.13 *In situ* hybridization

The protocol was adopted from the *in situ* hybridization procedure for detecting *Candidatus* Xenohaliotis californiensis (OIE, 2006) with modifications.

H. midae sections were deparaffinized by immersion in xylene (Saarchem) (3 x 10 min) followed by immersion in two successive absolute ethanol baths for 10 minutes each. Sections were then gently rehydrated through an ethanol series (95, 80, 70, and 50% ethanol; Appendix A.2.27) for 3 minutes each and briefly rinsed in sterile Milli-Q distilled water for 1 minute. The sections were then washed (2 x 5 minutes) in phosphate buffered saline (1x PBS; Appendix A.2.27).

Prehybridization was performed to reduce background due to non-specific hybridization of the probes, in the tissue sections. The tissue sections were covered with pre-warmed *in situ* hybridization buffer (Appendix A.2.27) and pre-hybridized at 42 ºC for 60 minutes without coverslips in a humidity chamber.

After the 60 minute pre-hybridization treatment, the tissue sections were denatured on a heating block (98 ºC for 10 minutes). Thereafter, the slides were rapidly cooled by placing them (section side up) on ice for one minute. The remaining *in situ* hybridization buffer was discarded and the sections briefly rinsed in buffer 1 (Appendix A. 2.27). The sections were probed with the cocktail of *gfp*-specific probes (GFP001, GFP002 and GFP003). Each probe was added at a concentration of approximately 6.66 pmol ml^{-1} of *in situ* hybridization buffer, resulting in a total probe concentration of approximately 20 pmol ml^{-1}. The positive (EUB338 universal eubacterial probe) and the negative (ECJ109 *E. coli* JM109 specific probe) controls were added at a concentration of approximately 20 pmol ml^{-1} of *in situ* hybridization buffer. *In situ* hybridization buffer containing 20 pmol of the DIG-labelled oligonucleotide probes (Table 6.3) was gently layered onto the tissue sections and covered, while ensuring that no air bubbles were trapped, with a coverslip (50 x 24 mm, Menzel-Gläser) and incubated for approximately 16 hours at 40 ºC in a humidity chamber.

Any unbound DIG-labeled probes were removed from the tissue sections by washing twice for 5 minutes in 2x SSC (Appendix A.2.27) at 22 ºC with gentle agitation, twice for 5 minutes in 1x SSC (Appendix A.2.27) at 22 ºC with gentle agitation, and twice for 10 minutes in 0.5x SSC (Appendix A.2.27) at 40 ºC with gentle agitation. The tissue sections were then equilibrated in buffer 1 for 2 minutes at 22 ºC. Thereafter, the sections were placed in blocking buffer (Appendix A.2.27) for 60 minutes at 22 ºC with gentle agitation, to reduce signal associated with non-specific antibody binding. The antibody [anti-digoxigenin-AP] (Roche) vial was centrifuged (13,000x g for 5 minutes at 4 ºC), before the anti-(digoxigenin-AP) was diluted 1:500 in blocking buffer. The antibody [anti-digoxigenin-AP] containing blocking buffer was applied to the sections and incubated for 3 hours, without a coverslip, at 22 ºC in a humidity chamber.

After the three hour incubation, the tissue sections were rinsed with gentle agitation twice in buffer 1 for 5 minutes each, and twice for 5 minutes each in buffer 2 (Appendix B.2.27). The sections were then overlaid with nitroble tetrazolium (NBT) / 5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) colour development solution (Appendix A.2.27) and incubated at 22 ºC in a dark humidity chamber for two hours, or until the signal was clearly visible. The colour development reaction was stopped by washing the tissue sections with TE buffer (Appendix A.2.3) and rinsing them with sterile double distilled water. The sections were counter-stained with 0.05% (w/v) aqueous Methyl Green (Sigma) for 2 minutes to enhance the background tissue morphology relative to the alkaline phosphatase (AP) signal. The slides were then rinsed in distilled water, air dried and mounted with a cover slip (50 x 24 mm, Menzel-Gläser) using phosphate-buffered glycerin jelly (Appendix A.2.27) as the mounting medium and stored in the dark.

Hybridization signals were identified by light microscopy as areas showing a purple precipitate (NBT/BCIP) where the DIG-labelled probe had bound to the target DNA within the tissue on the microscope slide. The stained and mounted sections were viewed and photographed, as previously described in section 6.3.12.

6.3.14 Immunohistochemical localisation

The *H. midae* tissue sections were prepared and deparaffinized as described in section 6.3.13.

The tissue sections were covered with immunohistochemistry hybridization buffer (Appendix A.2.28) and prehybridized for 2 hours at 22 ºC without coverslips in a humidity chamber.

The prehybridization solution was discarded and replaced with fresh immunohistochemistry hybridization buffer (Appendix A.2.28) containing anti-[VmproA] polyclonal antibodies (Chapter 3) that had been pre-absorbed against *E. coli* JM109 cellular extract (Appendix B.17) and abalone tissue powder (Appendix B.18). The sections and antibodies were incubated in a humidity chamber for 24 hours at 4 ºC. Thereafter, any unbound anti-[VmproA] antibodies were removed from the tissue sections by eight washes, with gentle agitation at 50 rpm on an orbital shaker, of 15 minutes each with PBT (Appendix A.2.28) at 22 ºC.

The sections were incubated with fresh immunohistochemistry hybridization buffer containing 1:1000 diluted goat anti-[rabbit AP-conjugated] secondary antibodies (Sigma). The tissue sections and anti-[rabbit AP-conjugated] secondary antibodies were incubated in a humidity chamber for 3 hours at 22 ºC. Thereafter, any unbound anti-[rabbit AP-conjugated] secondary antibodies were removed from the tissue sections by eight washes, with gentle agitation, of 15 minutes each with PBT (Appendix A.2.28) at 22 ºC.

The tissue sections were then rinsed with buffer 1 and buffer 2, as described in section 6.3.13, and then overlaid with NBT/BCIP colour development solution and incubated at 22 ºC in a dark humidity chamber until a signal was clearly visible. The colour development reaction was stopped by washing the tissue sections with TE buffer (Appendix A.2.3) and rinsing them with sterile double distilled water. The sections were counter-stained with 0.05% (w/v) aqueous Methyl Green (Sigma) for 2 minutes to enhance the background tissue morphology relative to the AP signal. The slides were then rinsed in distilled water and mounted, as described in section 6.3.13.

The AP-conjugated anti-rabbit secondary antibodies that recognised and bound to the anti-[VmproA] polyclonal antibodies that had in turn bound to *V. midae* SY9 extracellular detergent resistant protease proteins within the tissue sections were identified by light microscopy as areas showing a purple precipitate (NBT/BCIP).

The stained and mounted sections were viewed and photographed as previously described in section 6.3.12.

6.4 Results

6.4.1 Transposon mutagenesis and chromosomal-tagging of *V. midae* SY9

The mini-Tn10-*gfp-kan* cassette was transferred to recipient *V. midae* SY9Sm^r cells, enabling those harbouring the cassette to grow on VNSS agar supplemented with streptomycin and kanamycin at concentrations of 120 and 400 µg ml^{-1}, respectively. Fifty eight of these potentially *gfp*-chromosomally tagged *V. midae* SY9 strains were screened by PCR for chromosomal integration of the mini-Tn10-*gfp*-kan cassette (data not shown). As a result, three strains were identified as possibly being *gfp* chromosomally-tagged *V. midae* SY9 strains.

A comparative growth curve analysis of the wild-type *V. midae* SY9 and the three mutant strains, *V. midae* SY9::Tn10.11, *V. midae* SY9::Tn10.12 and *V. midae* SY9::Tn10.52, was conducted in P-MBM over a 24 hour period at 22 ºC (Fig. 6.5 A - D). There were no significant differences between the growth profiles of the three chromosomally-tagged strains and *V. midae* SY9.

The level of extracellular protease activity of all three mutant strains (Fig. 6.5) were lower than that of the wild-type *V. midae* SY9 (Fig. 6.5) over the course of the 24 hour growth experiment. However, the extracellular protease activity profile of the mutant strain *V. midae* SY9::Tn10.52, closely resembled that of *V. midae* SY9 and the level of protease activity was higher than that of the other two mutant strains. Therefore, *V. midae* SY9::Tn10.52 was selected as the best *gfp* chromosomally-tagged *V. midae* SY9 strain and was used for further analysis.

Southern hybridization analysis of *V. midae* SY9 and *V. midae* SY9::Tn10.52 chromosomal DNA with an α-^{32}P-labelled 717 bp *gfp* DNA fragment confirmed the integration of the mini-Tn10-*gfp-kan* transposable element into the chromosome of *V. midae* SY9Sm^r (Fig. 6.6). The radiolabelled probe did not hybridise to any DNA fragments generated by restriction digestion of *V. midae* SY9 chromosomal DNA (Fig. 6.6, lanes 1 - 3). The 0.7 kb *gfp* DNA probe hybridized to a single 1.8 kb fragment of *Hin*dIII digested V. *midae* SY9::Tn10.52 chromosomal DNA (Fig. 6.6, lane 4). Single hybridization bands of 13.3 and 20.1 kb were also observed for *V. midae* SY9::Tn10.52 chromosomal DNA digested with *Eco*RI (Fig. 6.6, lane 5) and *Pst*I (Fig. 6.6, lane 6), respectively. The restriction enzymes *Hin*dIII, *Eco*RI and *Pst*I cut once within the transposable element of the mini-Tn10-*gfp*-kan cassette, but not within the 717 bp portion used as the probe, indicating that the transposable element only integrated at a single site in the *V. midae* SY9 chromosome.

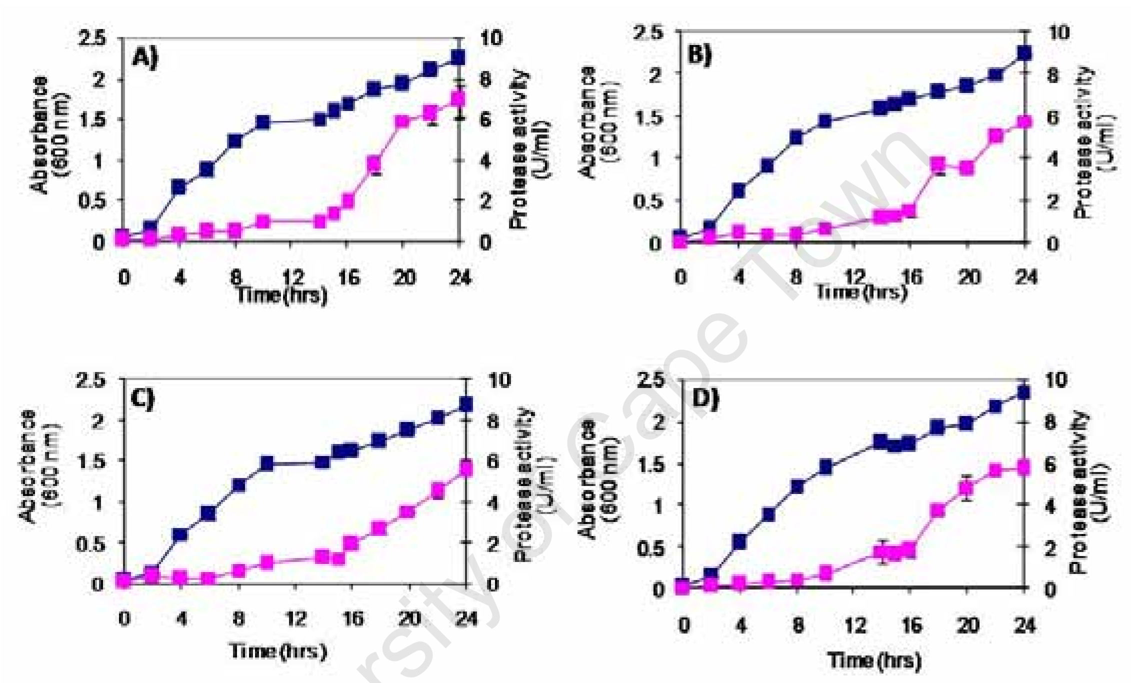

Figure 6.5 Growth and extracellular protease activity profiles of *V. midae* SY9 (A), *V. midae* SY9::Tn10.11 (B), *V. midae* SY9::Tn10.12 (C) and *V. midae* SY9::Tn10.52 (D) in P-MBM at 22 °C on an orbital shaker (100 rpm) over the course of a 24 hour period. Cell growth was monitored by absorbance (600 nm) readings (■) and alkaline protease activity (■) within the culture supernatant was determined using standard assays. Data represents the mean ± standard error (n=3).

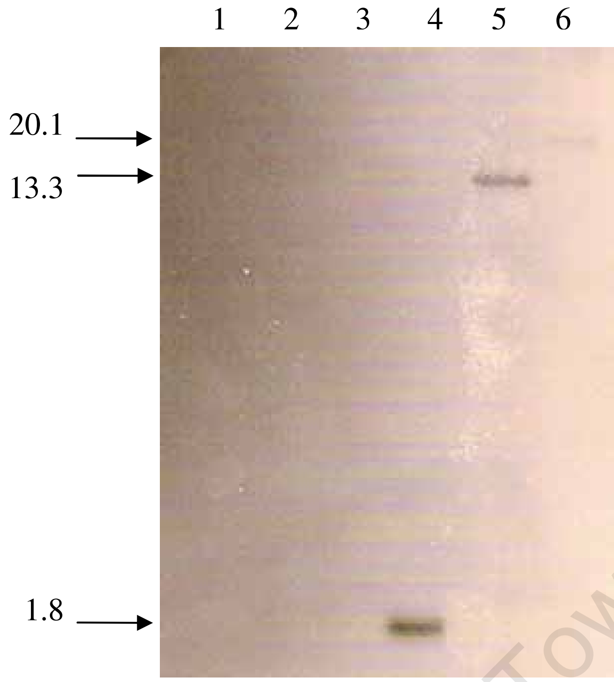

Figure 6.6 Southern hybridisation of the radiolabelled 717 bp DNA fragment PCR amplified from pLofKm*gfp*, using *gfp*-F and *gfp*-R, to 10 µg of *V. midae* SY9 and *V. midae* SY9::Tn10.52 chromosomal DNA. *V. midae* SY9 chromosomal DNA digested with *Hin*dIII (lane 1), *Eco*RI (lane 2) and *Pst*I (lane 3), and *V. midae* SY9::Tn10.52 chromosomal DNA digested with *Hin*dIII (lane 4), *Eco*RI (lane 5) and *Pst*I (lane 6). The arrows indicate the approximate sizes of the bands in kilo base pairs (kb).

6.4.2 Specificity of *in situ* hybridisation probes

A PCR was performed to investigate whether or not the *gfp* tagged strain *V. midae* SY9::Tn10.52 could be specifically differentiated from other bacterial strains on the basis of the presence of the *gfp* gene contained in the mini-Tn10-*gfp*-*kan* cassette. The plasmid pLofKm*gfp* and chromosomal DNA isolated from *V. midae* SY9, *E. coli* JM109, *Pseudoalteromonas* sp. strain C4 and *V. midae* SY9::Tn10.52 were investigated for the presence of the *gfp* gene using the primer set *gfp*-F/*gfp*-R (Fig. 6.7). A single DNA fragment of 717 bp was only amplified from the positive control pLofKm*gfp* and *V. midae* SY9::Tn10.52, indicating that the *gfp* gene used to chromosomally-tag *V. midae* SY9::Tn10.52 is not naturally present in *V. midae* SY9, *E. coli* JM109 or *Pseudoalteromonas* sp. strain C4, and that specific oligonucleotide probes to the *gfp* gene could potentially allow specific *in situ* localisation of *V. midae* SY9::Tn10.52.

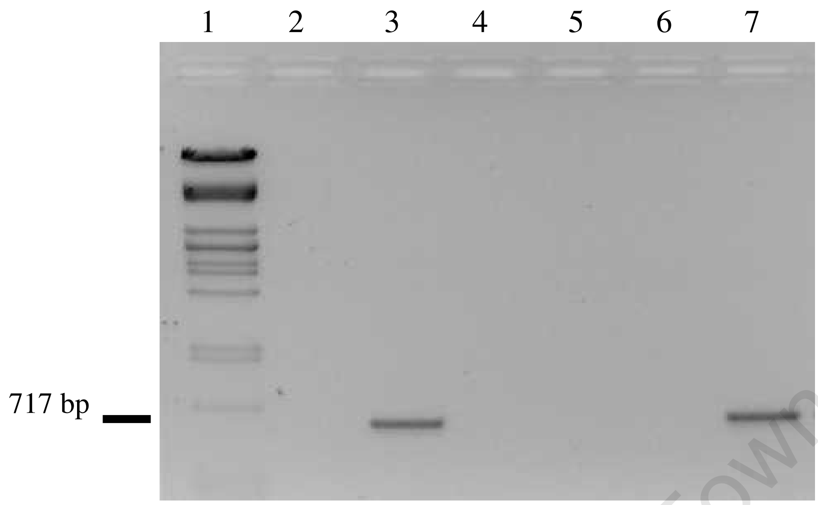

Figure 6.7 PCR confirmation of the integration of the mini-Tn10-*gfp*-*kan* cassette into the chromosome of *V. midae* SY9. The presence of the *gfp* gene is indicated by amplification of a 717 bp DNA fragment of the *gfp* gene from bacterial genomic DNA, using the *gfp* specific primers *gfp*-F and *gfp*-R. Lane 1, λ*Pst*I MW marker; lane 2, No template control; lane 3, pLofKm*gfp* (positive control); lane 4, *E. coli* JM109; lane 5, *Pseudoaltermonas* sp. strain C4; lane 6, *V. midae* SY9 and lane 7, *V. midae* SY9::Tn10.52.

Southern hybridization of the 717 bp DNA fragment of the *gfp* gene amplified (Fig. 6.7) from pLofKm*gfp* and *V. midae* SY9::Tn10.52 with the *gfp* specific oligonucleotide DIG-labelled probes GFP001 (Fig. 6.8 A), GFP002 (Fig. 6.8 B) and GFP003 (Fig. 6.8 C) resulted in a single hybridization band for each of the *gfp*-specific probes against each of the target DNA sequences. Therefore, the *gfp*-specific probes all hybridise to the *gfp* gene used to chromosomally-tag *V. midae* SY9::Tn10.52.

The specificity of the universal 16S rRNA eubacterial probe EUB338 and the *E. coli* JM109 16S rRNA specific oligonucleotide probe ECJ109, were investigated by PCR amplifying a 1.5 kb fragment of the 16S rRNA gene with the primer set fD1/Rp2 from *V. midae* SY9, *E. coli* JM109, *Pseudoalteromonas* sp. strain C4 and *V. midae* SY9::Tn10.52.

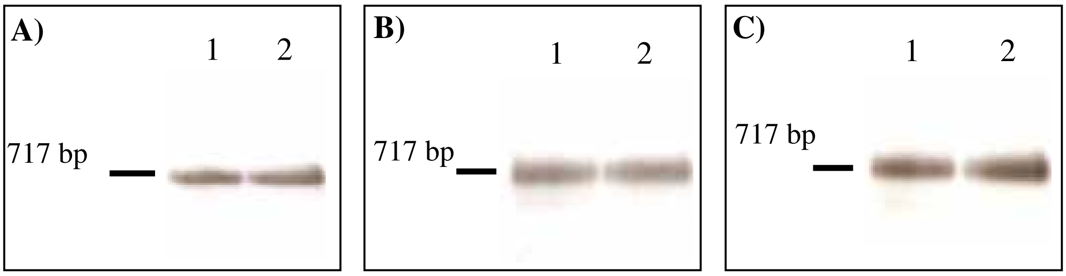

Figure 6.8 Southern hybridization of the DIG-labelled oligonucleotide probes GFP001 (A), GFP002 (B) and GFP003 (C) to 150 ng of the PCR amplified 717 bp DNA fragment of the *gfp* gene from pLOFKm*gfp* (lane 1) and *V. midae* SY9::Tn10.52 (lane 2). The line indicates the approximate size of the bands in base pairs (bp).

Figure 6.9 Southern hybridization of the DIG-labelled *E. coli*-specific 16S rRNA oligonucleotide probe, ECJ109, to 150 ng of the PCR amplified 1.5 kb DNA fragment of the 16S rRNA from *E. coli* JM109 (lane 1), *Pseudoaltermonas* C4 (lane 2), *V. midae* SY9 (lane 3) and *V. midae* SY9::Tn10.52 (lane 4). The line indicates the approximate size of the band in kilo base pairs (kb).

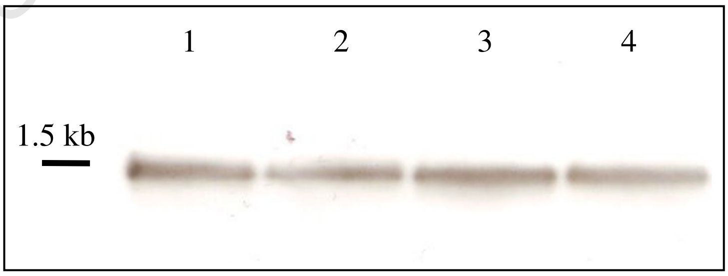

Figure 6.10 Southern hybridization of the DIG-labelled universal 16S rRNA eubacterial oligonucleotide probe, EUB338, to 150 ng of the PCR amplified 1.5 kb DNA fragment of the 16S rRNA from *E. coli* JM109 (lane 1), *Pseudoaltermonas* C4 (lane 2), *V. midae* SY9 (lane 3) and *V. midae* SY9::Tn10.52 (lane 4). The line indicates the approximate size of the bands in kilo base pairs (kb).

Southern hybridization of the 1.5 kb DNA fragment of the 16S rRNA gene amplified from *V. midae* SY9, *E. coli* JM109, *Pseudoalteromonas* sp. strain C4 and *V. midae* SY9::Tn10.52 with the *E. coli* JM109 16S rRNA specific probe ECJ109 (Fig. 6.9), resulted in a single hybridization band for only the amplified *E. coli* JM109 16S rRNA target DNA sequence (Fig. 6.9, lane 1). The DIG-labelled oligonucleotide probe ECJ109 did not hybridise to the 1.5 kb DNA fragment of the 16S rRNA gene from *Pseudoalteromonas* sp. strain C4 (Fig. 6.9, lane 2), *V. midae* SY9 (Fig. 6.9, lane 3) and *V. midae* SY9::Tn10.52 (Fig. 6.9, lane 4). Specific hybridisation of the ECJ109 probe to the amplified 1.5 kb 16S rDNA fragment from *E. coli* JM109 confirms the specificity of this oligonucleotide probe and its application as a negative control for the *in situ* hybridization experiments.

Southern hybridization of the 1.5 kb DNA fragment of the 16S rRNA gene amplified from *V. midae* SY9, *E. coli* JM109, *Pseudoalteromonas* sp. strain C4 and *V. midae* SY9::Tn10.52 with the universal 16S rRNA eubacterial DIG-labelled probe EUB338 (Fig. 6.10), resulted in a single hybridization band for the amplified 16S rRNA target DNA sequences of *E. coli* JM109 (Fig. 6.10, lane 1), *Pseudoalteromonas* sp. strain C4 (Fig. 6.10, lane 2), *V. midae* SY9 (Fig. 6.10, lane 3) and *V. midae* SY9::Tn10.52 (Fig. 6.10, lane 4). The hybridisation of the EUB338 probe to the amplified 1.5 kb 16S rRNA DNA fragments from *E. coli* JM109, *Pseudoalteromonas* sp. strain C4, *V. midae* SY9 and *V. midae* SY9::Tn10.52 confirms the application of this oligonucleotide probe as a eubacterial positive control for the *in situ* hybridization experiments.

6.4.3 Preparation of the experimental diet

Optimal conditions for the incorporation of *V. midae* SY9::Tn10.52 into an ABFEED® S34 diet by vacuum impregnation and the stability of the impregnated feed were investigated to ensure that the abalone received a high concentration of viable probiotic microorganisms for the duration of the experiment.

6.4.3.1 Optimal post- vacuum impregnation drying conditions for *V. midae* SY9 viability

In order to determine the optimal drying conditions for the vacuum impregnated ABFEED® S34 weaning chips, the number of *V. midae* SY9::Tn10.52 culturable cells was determined for impregnated ABFEED® S34 weaning chips dried at 22 ºC for 16 hours. While there was a significant (P<0.05) decrease in the level of viable *V. midae* SY9::Tn10.52 within the vacuum impregnated ABFEED® S34 weaning chips following the drying process, the remaining viable count was greater than 5 x 10^9 cfu g^{-1} of dried ABFEED® S34 weaning chips and was therefore high enough for the purposes of this experiment (Fig. 6.11).

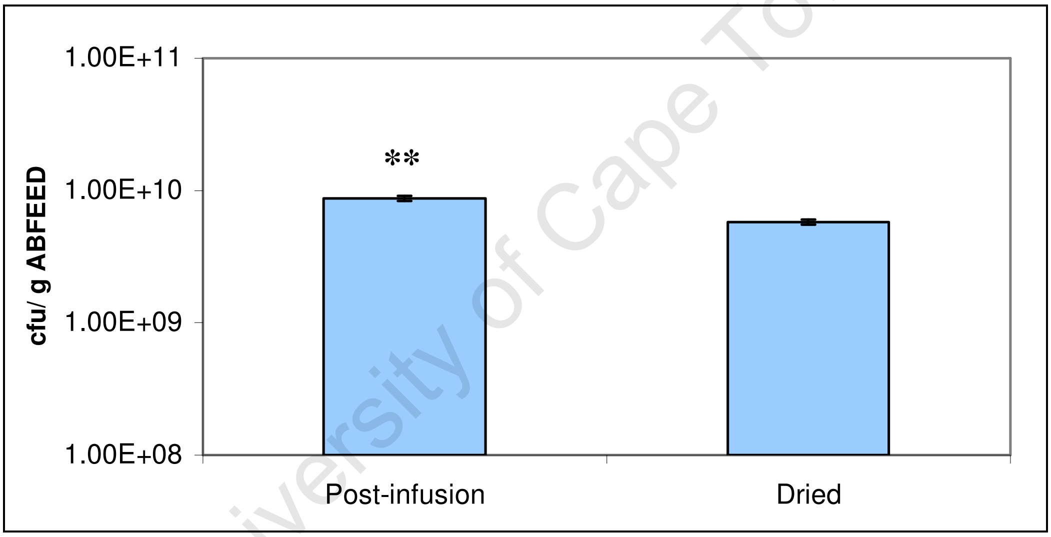

Figure 6.11 The effect of drying *V. midae* SY9::Tn10.52 vacuum-impregnated ABFEED® S34 weaning chips at 22 ºC for 16 hours on cell viability. Data represents the mean ± standard error (n=3). ** (P<0.05; paired Student *t*-test) represent a significant difference between the means of cell viability within freshly *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips and dried ABFEED® S34 weaning chips.

6.4.3.2 Optimal storage temperature for *V. midae* SY9 vacuum impregnated ABFEED® S34

In order to determine the optimal storage temperature for the probiotic supplemented ABFEED® S34 weaning chips, the number of culturable cells was determined for *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips stored at the constant temperature of 4 ºC. The culturable *V. midae* SY9::Tn10.52 cells within the vacuum-impregnated ABFEED® S34 weaning chips increased significantly (P<0.05) within the first 24 and 48 hours of storage at 4 ºC (Fig. 6.12). Thereafter, whilst the number of culturable *V. midae* SY9::Tn10.52 cells remained above 1.0 x 10^{10} culturable cells g^{-1} ABFEED® for the duration of the 7 day period when the impregnated feed was stored at 4 ºC, there was a significant (P<0.05) decrease in the number of culturable *V. midae* SY9::Tn10.52 cells after 3 and 7 days at 4 ºC.

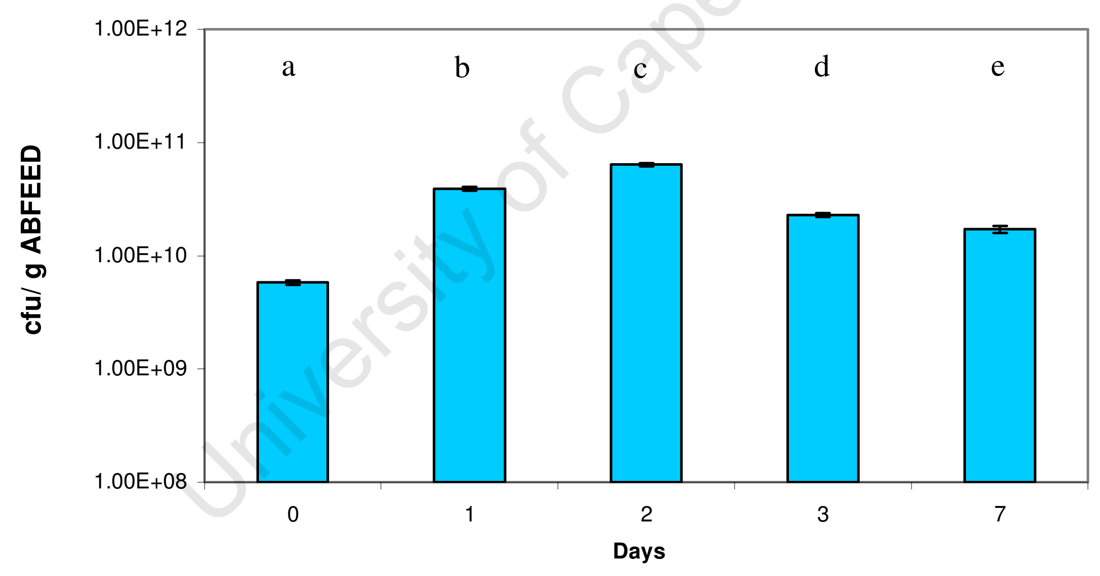

Figure 6.12 Viability of *V. midae* SY9::Tn10.52 within vacuum impregnated ABFEED® S34 weaning chips stored at 4 °C over the course of a 7 day period. Data represents the mean ± standard error (n=3). Different postscripts represent a significant difference (P<0.05) between the average cell viability of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 weaning chips stored at 4 ºC.

6.4.3.3 Leaching of the probiotic *V. midae* SY9::Tn10.52 from vacuum impregnated ABFEED® S34

In order to determine the stability of *V. midae* SY9::Tn10.52 vacuum impregnated ABFEED® S34 in the flowing water of an experimental abalone tank, the number of viable *V. midae* SY9::Tn10.52 cells within supplemented feed was determined over the course of three days for impregnated feed incubated in the experimental tanks. There was no significant difference (*P*>0.05, one-way ANOVA) in the number of culturable *V. midae* SY9::Tn10.52 cells detected after 1 day (Fig. 6.13). There was a significant increase (*P*<0.05) in the level of viable *V. midae* SY9::Tn10.52 cells within the vacuum impregnated ABFEED® S34 weaning chips incubated in the sea water from day 1 to day 2; however, the levels at day 2 were not statistically significant (*P*>0.05) from those observed initially. After 3 days there was a significant decrease in the viable counts of *V. midae* SY9::Tn10.52, where the probiotic associated with the vacuum impregnated feed had declined to 2.5 x 10^{10} (Fig. 6.13).

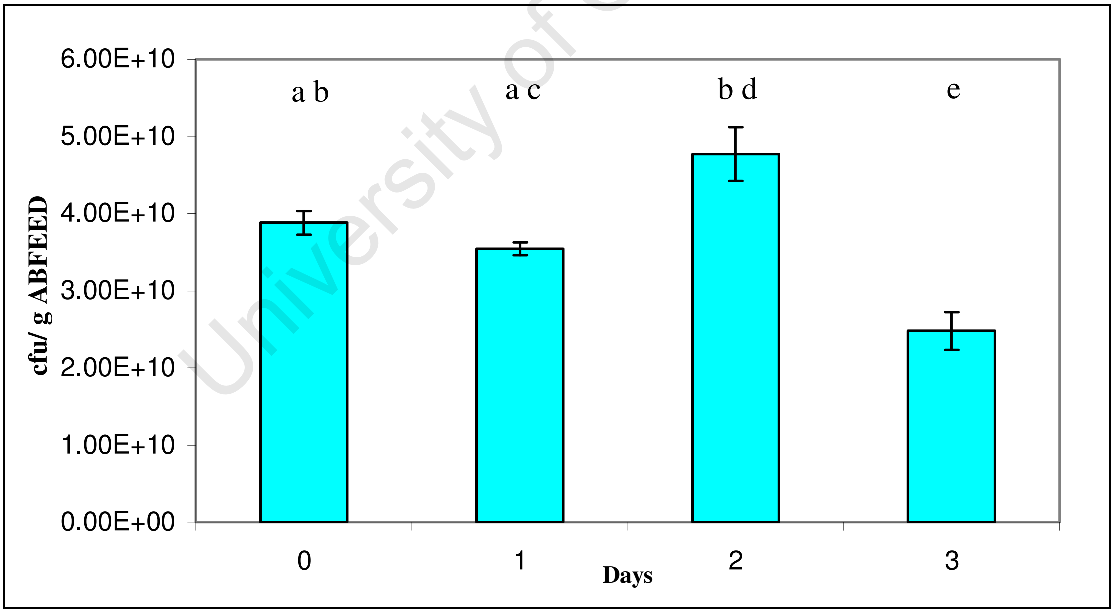

Figure 6.13 Leaching of *V. midae* SY9::Tn10.52 from supplemented ABFEED® S34 weaning chips stored for 3 days within experimental abalone tanks. Data represents the mean ± standard error of three batches of vacuum impregnated ABFEED® S34 weaning chips (n=3). Different postscripts represent a significant difference (*P*<0.05) between the average cell viability of *V. midae* SY9::Tn10.52 vacuum infused ABFEED® S34 weaning chips.

6.4.4 Histological examination of the digestive tract of juvenile *H. midae*

Histological examination of H & E stained *H. midae* tissue sections were conducted for abalone from every sampling point. This was done to assess the quality of the tissue sections. Paraffin-embedded whole abalone sections that appeared to be damaged from shucking, sectioning or handling were discarded and not used for *in situ* hybridization or immunohistochemistry. The H & E stained *H. midae* tissue sections were also inspected for differences between abalone fed the basal diet and those fed the *V. midae* SY9::Tn10.52 supplemented diet.

Histological examination of whole animal sections did not identify any morphological differences between *H. midae* fed the ABFEED® S34 basal diet (Fig. 6.14) and abalone fed the *V. midae* SY9::Tn10.52 supplemented feed (Fig. 6.15) over the course of the 14 day experimental period. The oesophageal region of the alimentary canal was devoid of any food in both groups of animals, while the crop/stomach and intestinal regions were full of mucous and food particles. There was also no clear histological evidence of bacterial colonies indicative of the intracellular *H. midae* Rickettsiales-like prokaryote (Horwitz, 2008) within the digestive gland of the sampled abalone.

6.4.5 *In situ* hybridization and localisation of *V. midae* SY9::Tn10.52 within the digestive tract of *H. midae*

In situ hybridization was performed to localise *V. midae* SY9::Tn10.52 within the digestive tract of probiotically supplemented *H. midae*, by visual identification of specific oligonucleotide probe(s)-to-target hybridization. A cocktail of three *gfp*-specific DIG-labelled oligonucleotide probes (GFP001, GFP002 and GFP003) was used to identify and localise *V. midae* SY9::Tn10.52 within abalone digestive tract sections.

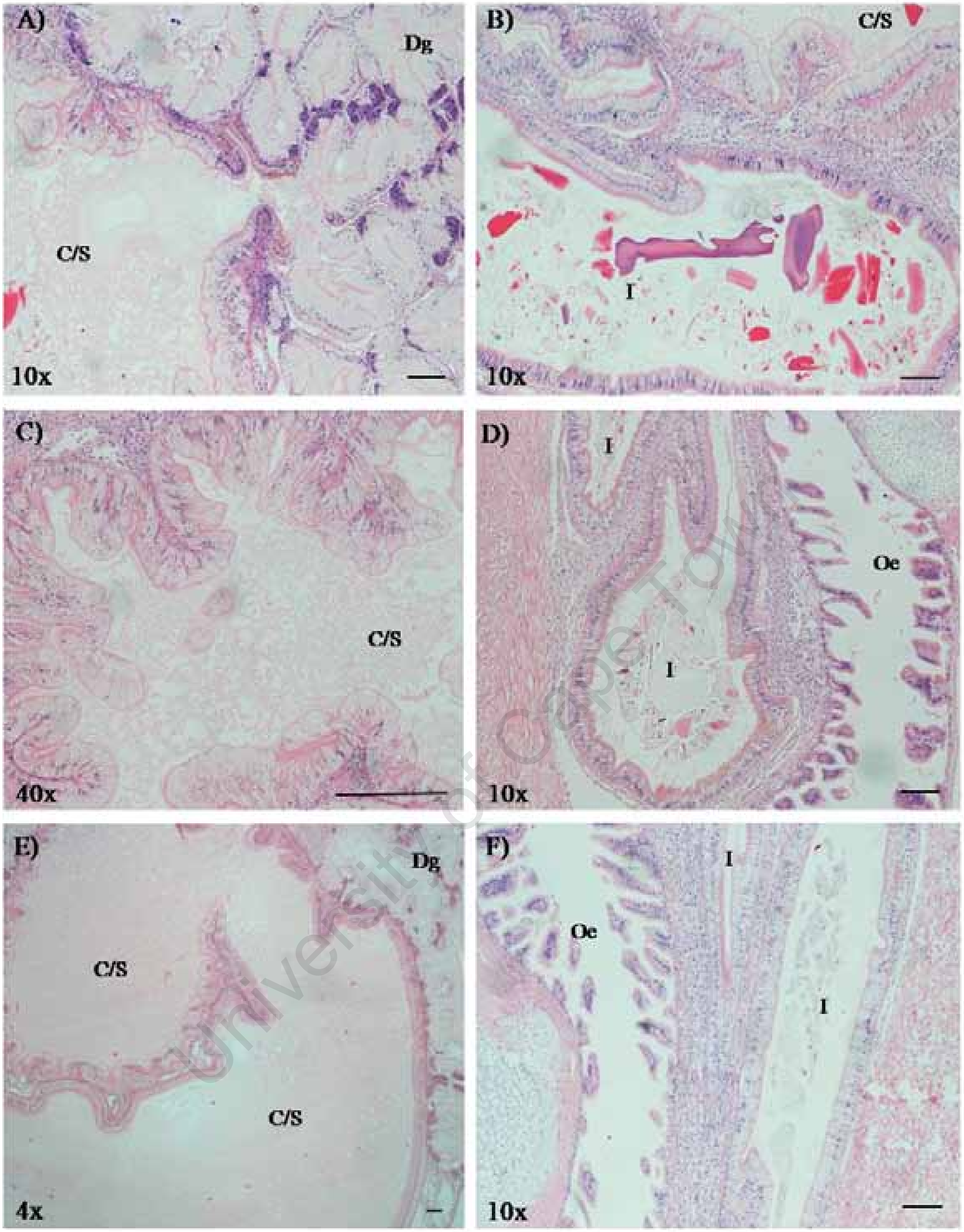

Figure 6.14 Hematoxylin and eosin stained whole abalone sections of juvenile *H. midae* 0 (A and B), 2 (C and D) and 14 days (E and F) after receiving the basal ABFEED® S34 diet (negative control). Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. C/S, Crop/stomach; Dg, Digestive gland; G, Gills; I, Intestine; and Oe, Oesophagus.

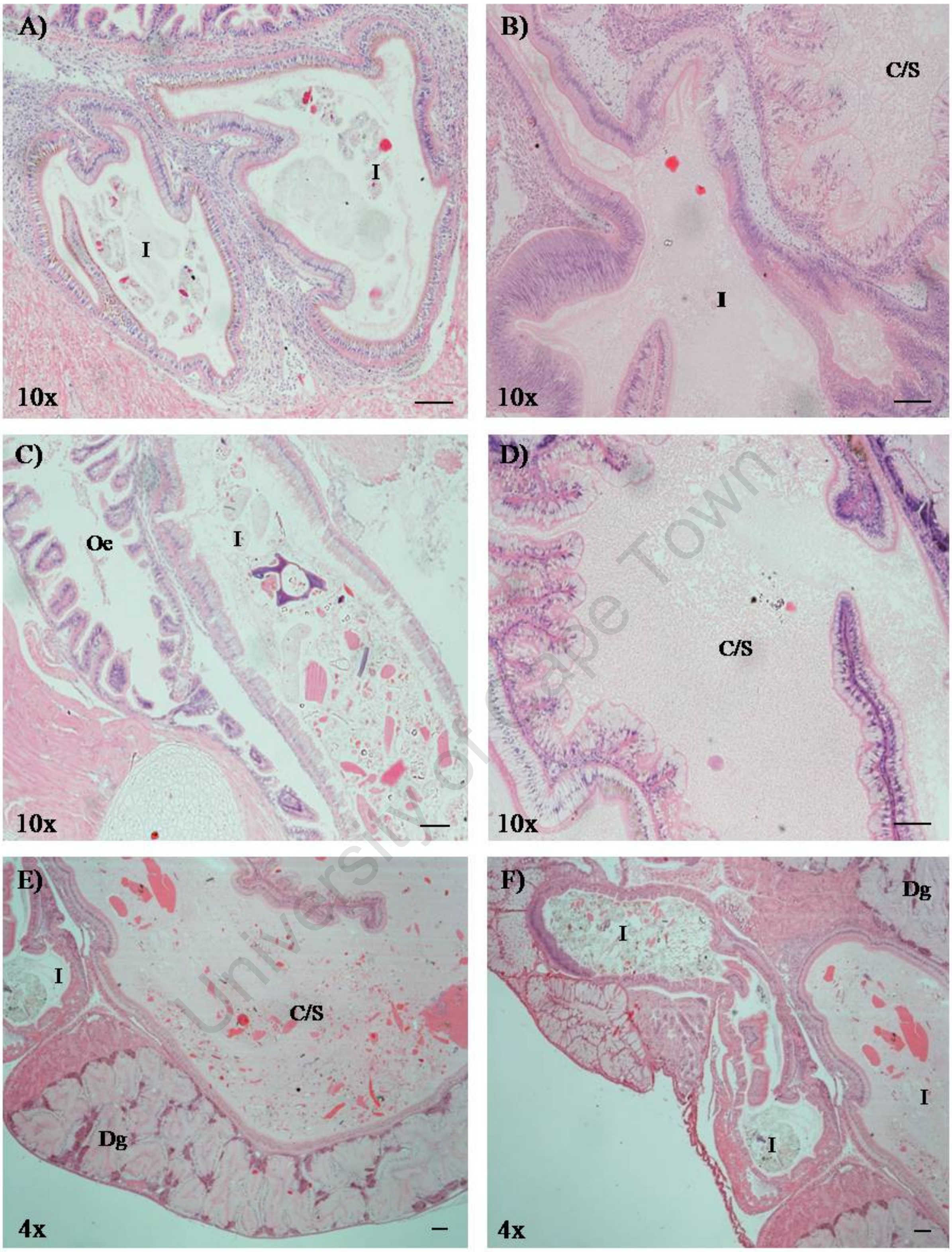

Figure 6.15 Hematoxylin and eosin stained whole abalone sections of juvenile *H. midae* 0 (A and B), 2 (C and D) and 14 days (E and F) after receiving the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 diet. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.

The universal eubacterial probe EUB338 (Stahl *et al.,* 1989) was used as an *in situ* hybridization positive control. The probe ECJ109, designed from a portion of the *E. coli* JM109 16S rRNA gene that was not homologous to the *V. midae* SY9 16S rRNA gene, was used as a negative control. A positive result was indicated by visual identification of the purple-black precipitate identifying the location of the target bacterial cells.

Hybridization signals were not observed within whole-animal tissue sections of *H. midae* hybridized with the *E. coli* JM109-specific DIG-labeled oligonucleotide probe ECJ109, which served as a negative control (Figs. 6.16 and 6.17).

Strong hybridization signals were observed within the crop/stomach and intestinal regions of *H. midae* fed either the basal or the probiotic-supplemented feeds hybridized with the universal eubacterial probe EUB338 (Figs. 6.18 and 6.19). Hybridization signals were not detected within the oesophageal or digestive gland regions of the *H. midae* digestive tract (data not shown). Furthermore, no background hybridization and/or non-specific staining was observed within the surrounding tissues for *in situ* hybridizations performed with the universal oligonucleotide probe EUB338. The *in situ* hybridization signals that were detected with EUB338 appear to be associated with the food and particulate matter within crop/stomach and intestinal regions of the abalone digestive tract (Figs. 6.18 and 6.19).

No hybridization signals were detected following *in situ* hybridization of the cocktail of *gfp*-specific oligonucleotide probes with whole animal sections of abalone fed the basal ABFEED® S34 diet for 0, 2 or 14 days (Fig. 6.20).

Hybridization signals were not detected following *in situ* hybridization with the *gfp*-specific oligonucleotide probes to whole animal sections of abalone fed the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 weaning chips diet at day 0 (Fig. 6.21 A and B).

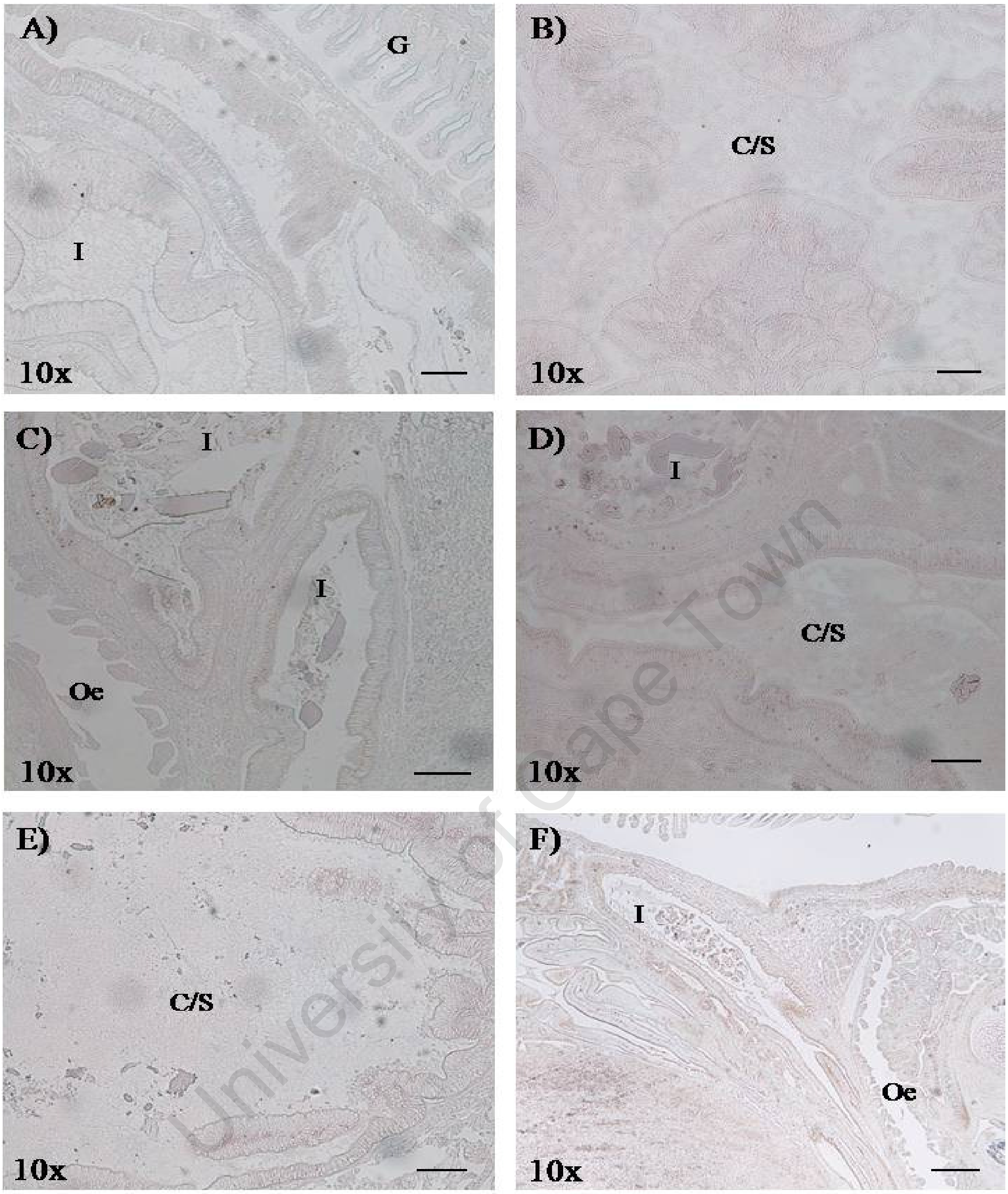

Figure 6.16 ***In situ*** **hybridization of whole abalone tissue sections of juvenile *H. midae* with the *E. coli* JM109 specific oligonucleotide probe ECJ109 0 (A and B), 2 (C and D) and 14 days (E and F) after receiving the basal ABFEED® S34 weaning chips diet. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.**

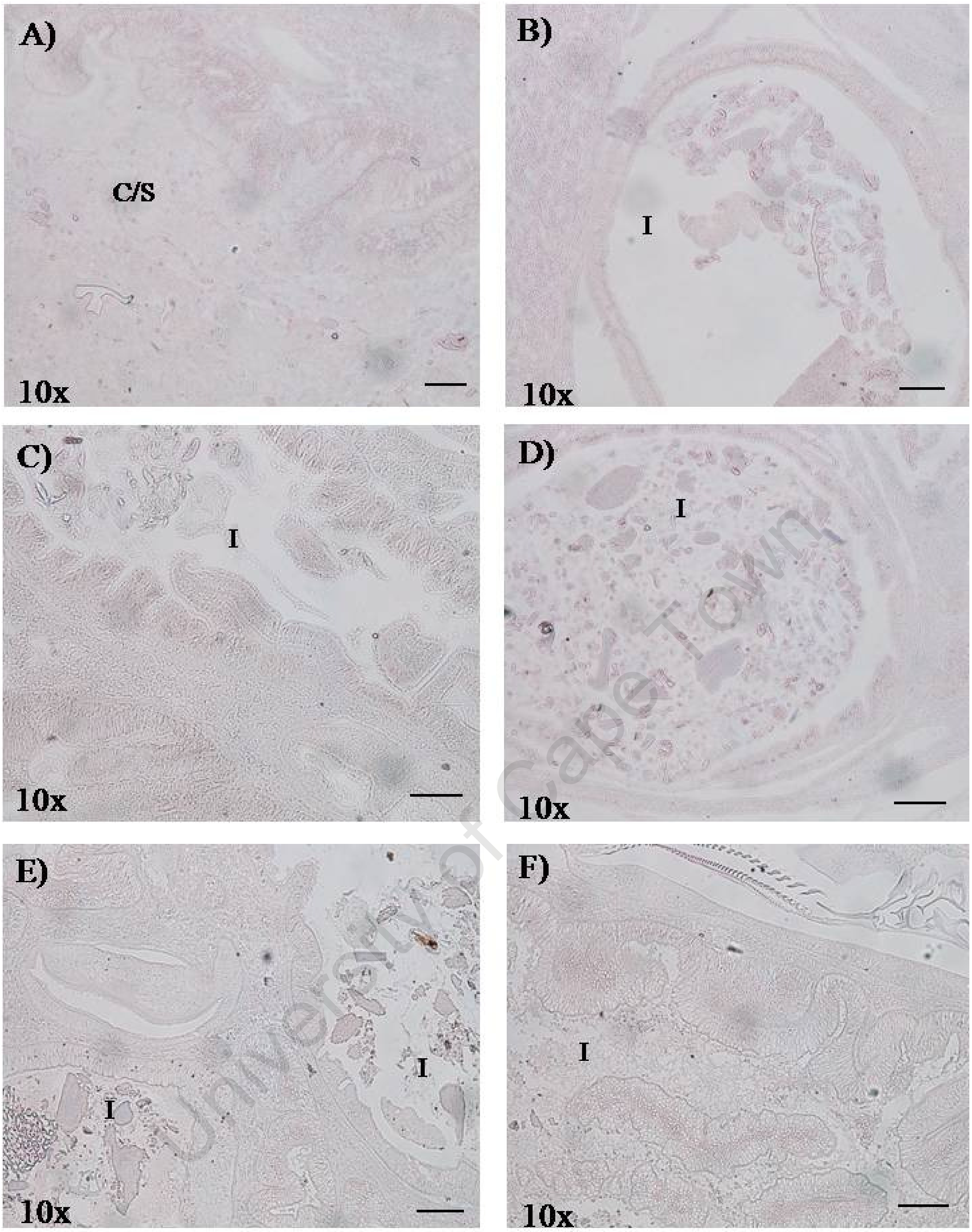

Figure 6.17 *In situ* hybridization of whole abalone tissue sections of juvenile *H. midae* with the *E. coli* JM109 specific oligonucleotide probe ECJ109 0 (A and B), 2 (C and D) and 14 days (E and F) after receiving the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 diet. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.

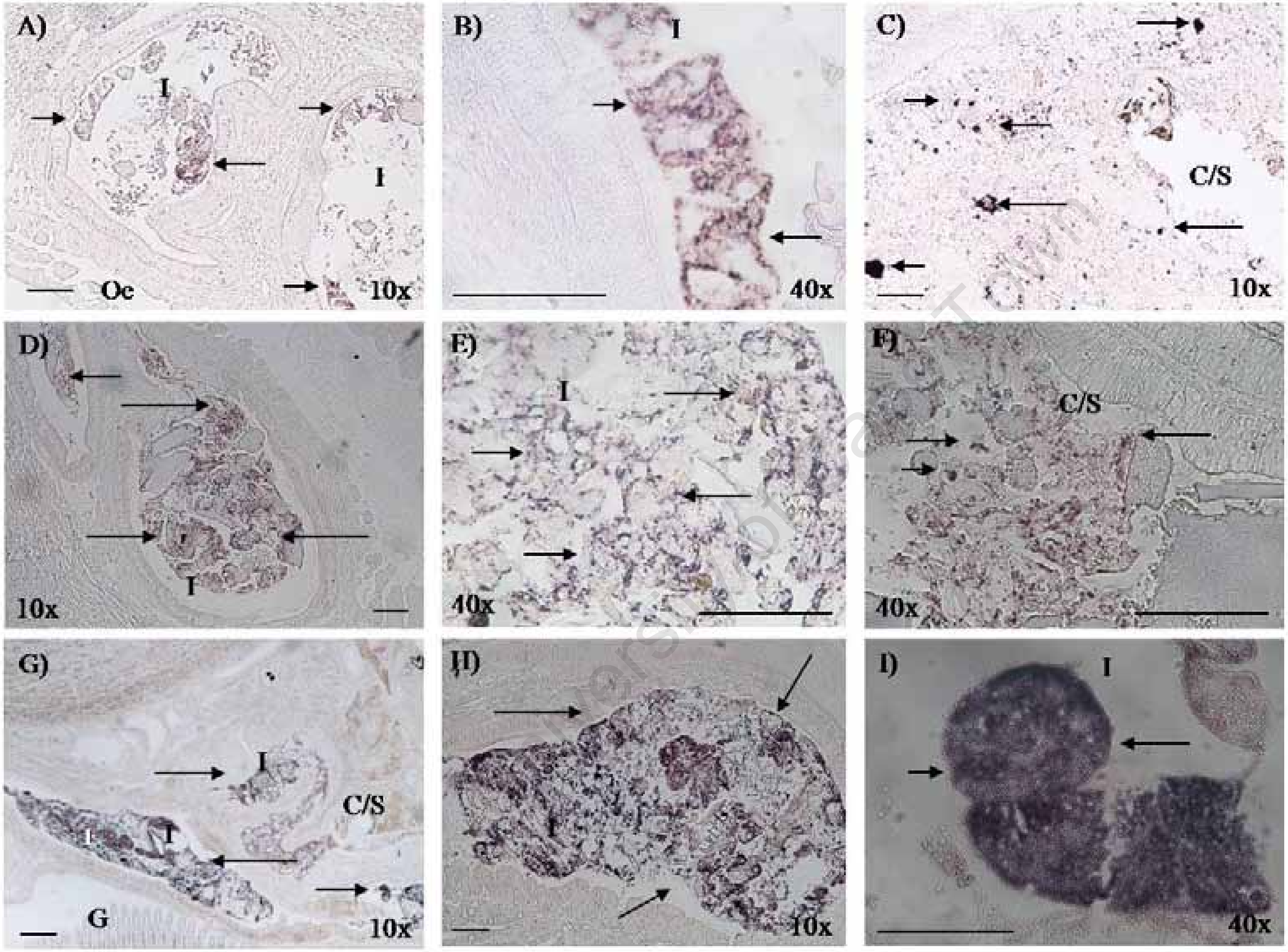

Figure 6.18
In situ hybridization of whole abalone tissue sections of juvenile *H. midae* with the universal eubacterial probe EUB338 0 (A, B and C), 2 (D, E and F) and 14 days (G, H and I) after receiving the ABFEED® S34 basal diet. Arrows indicate some of the regions displaying a positive signal. Scale bar, 100 µm. Magnification depicted at the bottom of each picture. Abbreviations in this figure are the same as in Fig. 6.14.

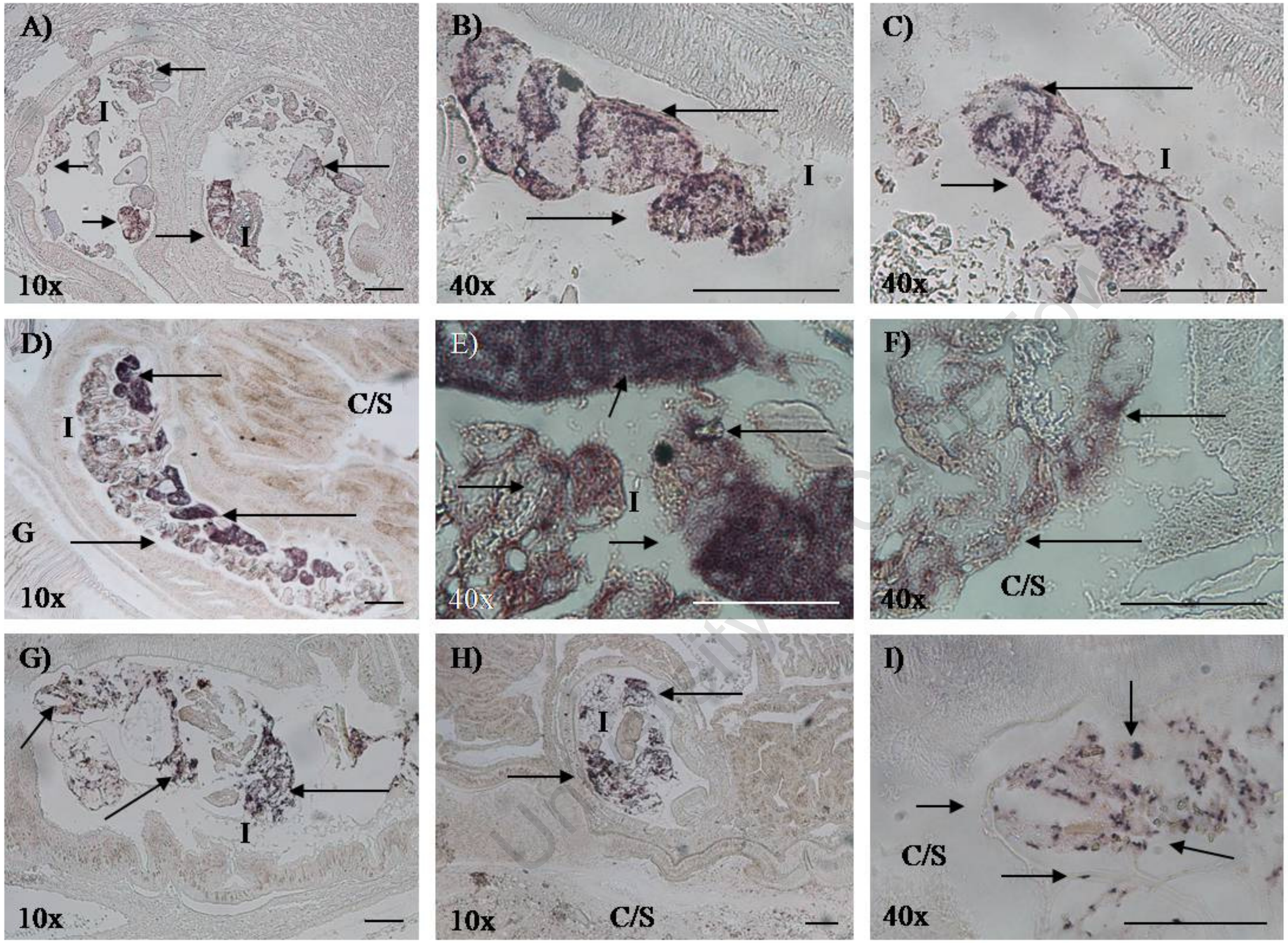

Figure 6.19
In situ **hybridization of whole abalone tissue sections of juvenile *H. midae* with the universal eubacterial probe EUB338, 0 (A, B and C), 2 (D, E and F) and 14 days (G, H and I) after receiving the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 diet. Arrows indicate some of the regions displaying a positive signal. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.**

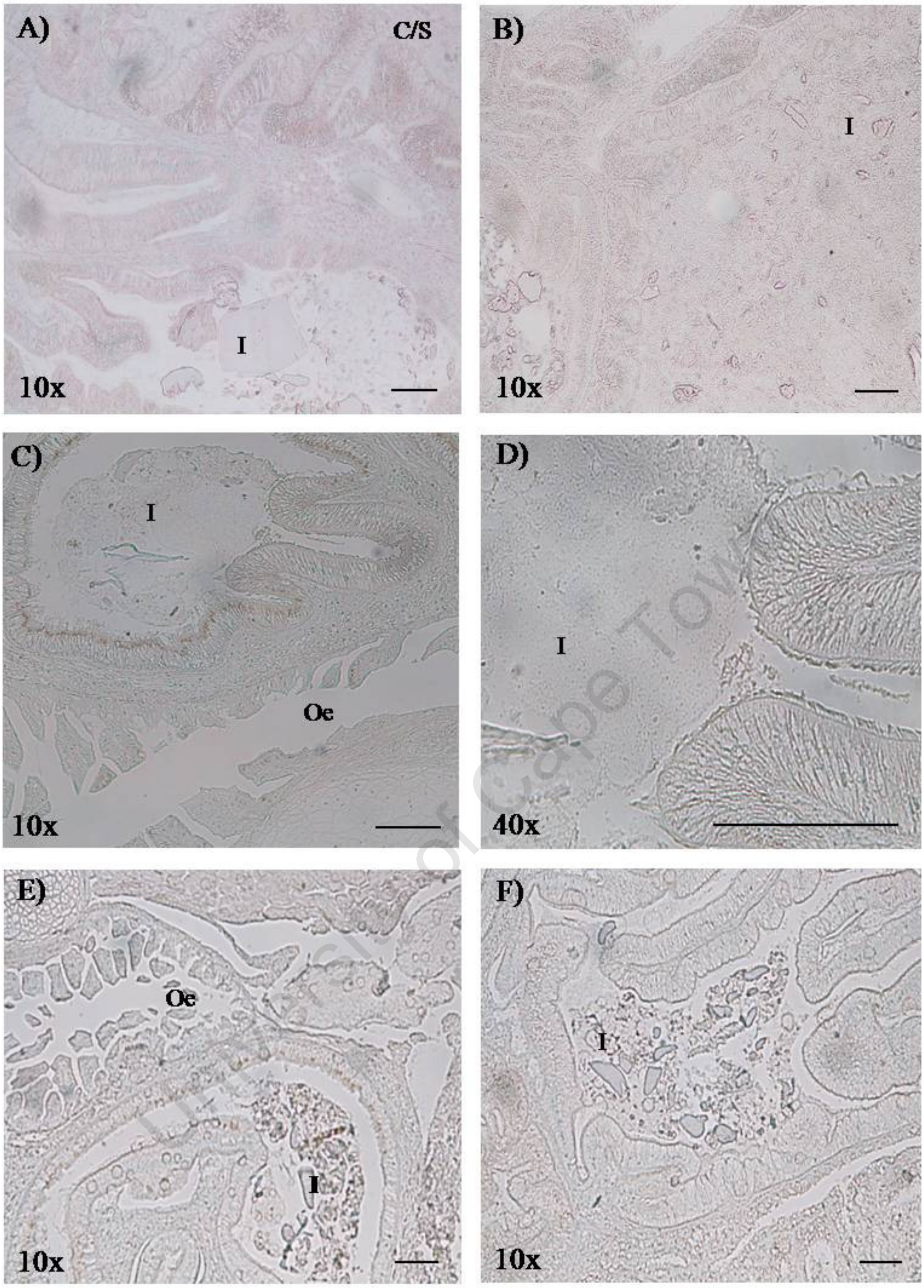

Figure 6.20 ***In situ*** **hybridization of whole abalone tissue sections of juvenile *H. midae* with the cocktail of *gfp*-specific oligonucleotide probes GFP001, GFP002 and GFP003 0 (A and B), 2 (C and D) and 14 days (E and F) after receiving the ABFEED® S34 basal diet. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.**

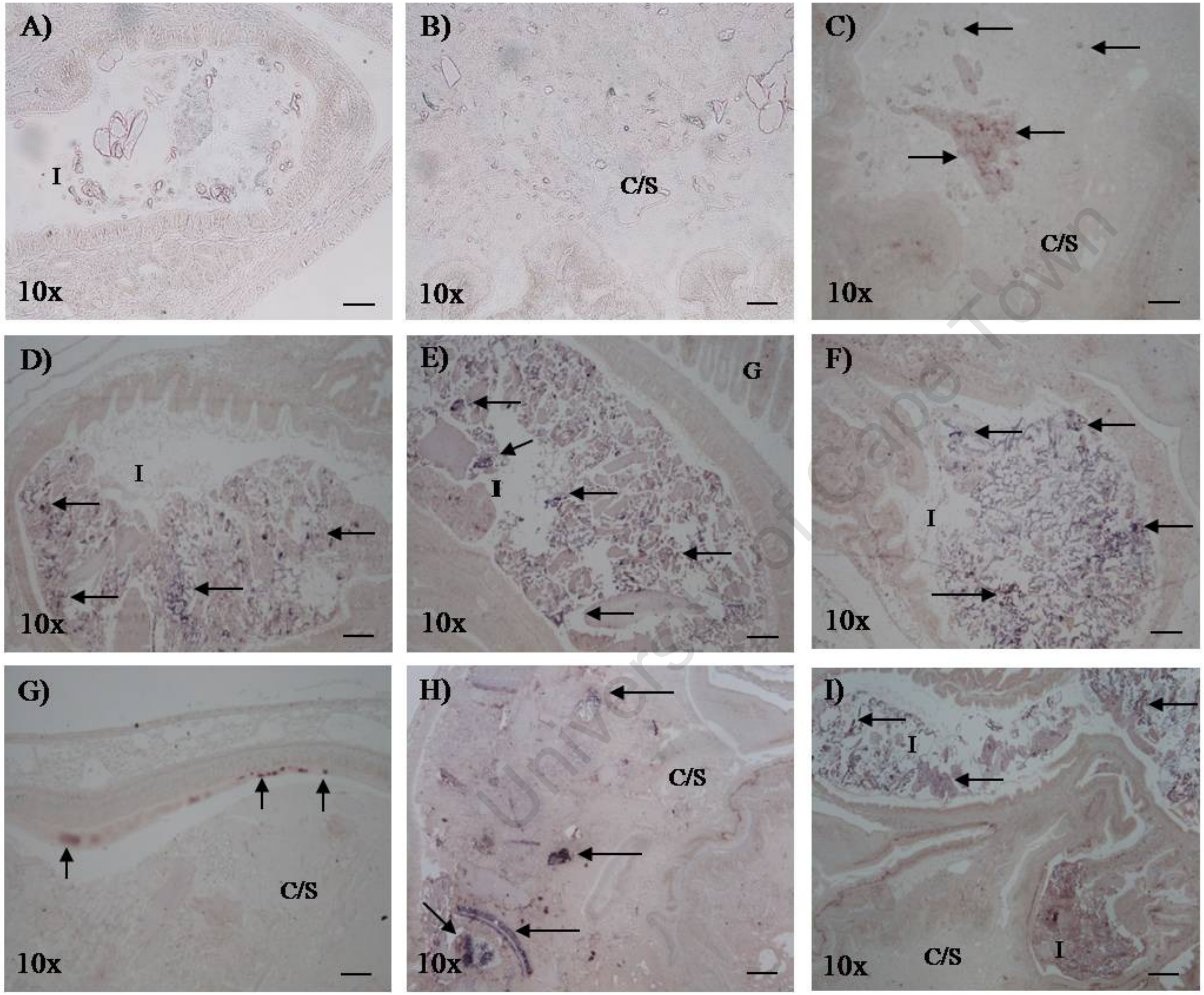

Figure 6.21
In situ **hybridization of whole abalone tissue sections of juvenile *H. midae* with the cocktail of *gfp*-specific oligonucleotide probes GFP001, GFP002 and GFP003 0 (A and B), 2 (C, D and E) and 14 days (F, G, H and I) after receiving the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 diet. Arrows indicate regions displaying a positive signal. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations used in this figure are the same as in Fig. 6.14.**

Hybridization signals were detected following *in situ* hybridization with the *gfp*-specific oligonucleotide probes within the crop/stomach and intestine of whole animal paraffin-embedded sections of *H. midae* fed the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 weaning chips diet after 2 (Fig. 6.21 C, D and E) and 14 days (Fig. 6.21 F - I). As was observed for sections probed with the eubacterial probe (Fig. 6.18 and 6.19), hybridization signals with the *gfp*-specific oligonucleotide probes appear to be associated with feed and/or particulate matter within the *H. midae* crop/stomach and intestinal regions (Fig. 6.21 C, D, E, F, H and I). Hybridization signals were also detected on the lining of the abalone crop/stomach (Fig. 6.21 G).

6.4.6 Immunohistochemical localisation of VmproA within the digestive tract of *H. midae*

We demonstrated in Chapter 5 that alkaline protease activity is significantly improved within the crop/stomach and intestine of abalone fed ABFEED® S34 weaning chips supplemented with *V. midae* SY9. Therefore, in order to examine the specific presence and distribution of VmproA within the digestive tract of *H. midae*, we performed immunohistochemical localisation using paraffin-embedded whole animal sections of juvenile *H. midae*. Anti-VmproA polyclonal antibodies were obtained by immunizing rabbits with purified VmproA protein (Chapter 3).

Immunostaining signals corresponding to the presence of VmproA were not detected in any of the abalone sections prior to the beginning (Day 0) of the feeding experiment (Figs 6.22 and 6.23), although a weak positive signal was observed within the intestinal region of one *H. midae* section (Fig. 6.23 A). Weak immunohistochemical signals were also observed within the intestine of a section from an abalone fed the basal diet for two days (Fig. 6.22 E). Immunostaining signals were not detected within the surrounding tissues of paraffin-embedded whole animal sections of *H. midae* fed either diet throughout the course of the 14 day period (Figs. 6.22 and 6.23). Controls for non-specific binding of secondary antibodies to abalone tissues indicated that no immunohistochemical reactions were detected for tissue sections of abalone sampled after being fed the basal or probiotic supplemented diets for 14 days (data not shown).

Immunohistochemical signals corresponding to the presence of anti-VmproA polyclonal antibodies indicates that VmproA was associated with food and/or other particulate matter within the crop/stomach and intestine of *H. midae* fed *V. midae* SY9::Tn10.52 supplemented ABFEED® for 2 and 14 days (Fig. 6.23 D - I).

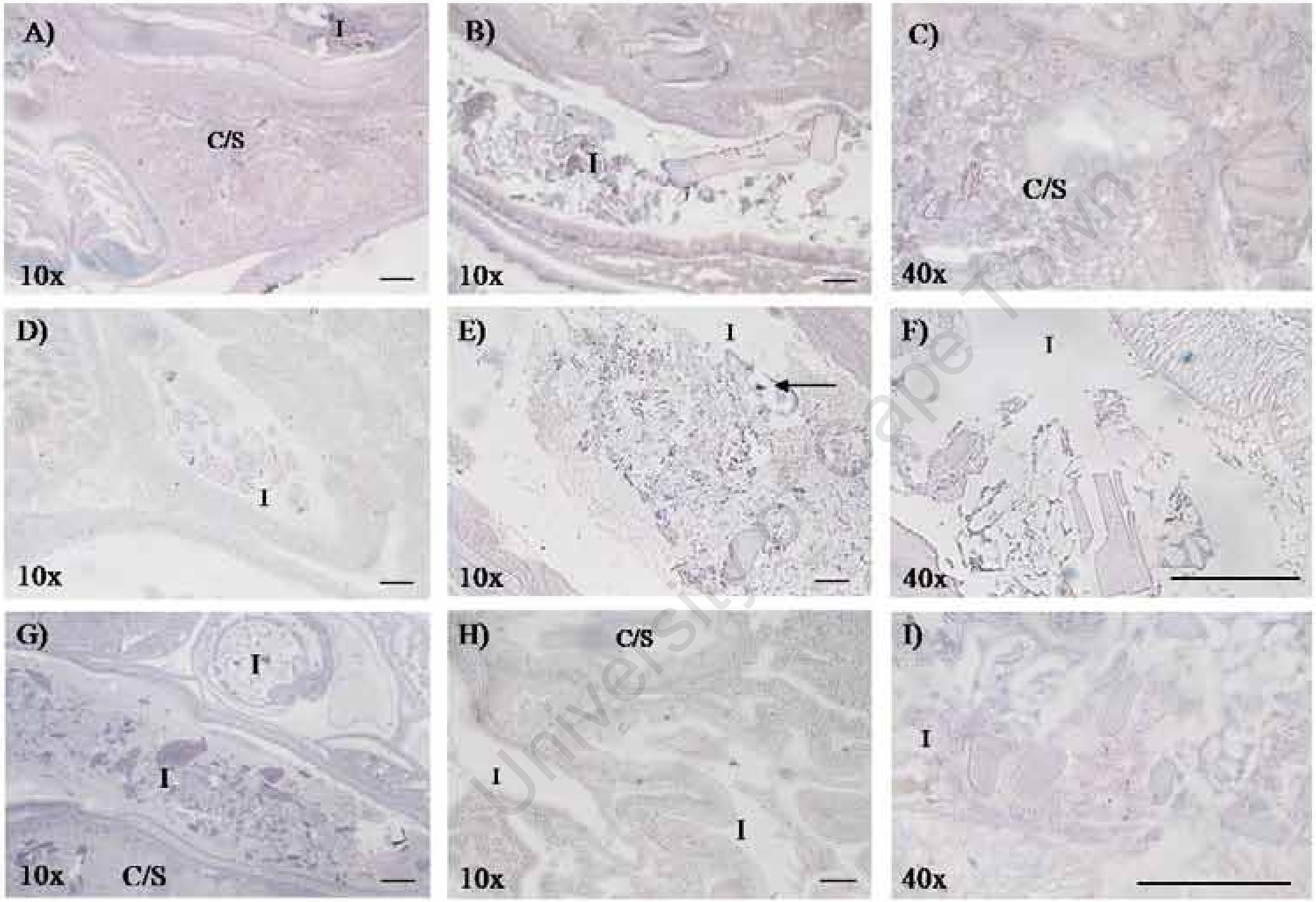

Figure 6.22 Immunohistochemical detection and localization of *V. midae* SY9 secreted VmproA within whole animal sections of juvenile *H. midae* 0, (A, B and C) 2 (D, E and F) and 14 days (G, H and I) after receiving the basal ABFEED® S34 diet. Arrow indicates a region showing a positive signal. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations in this figure are the same as in Fig. 6.14.

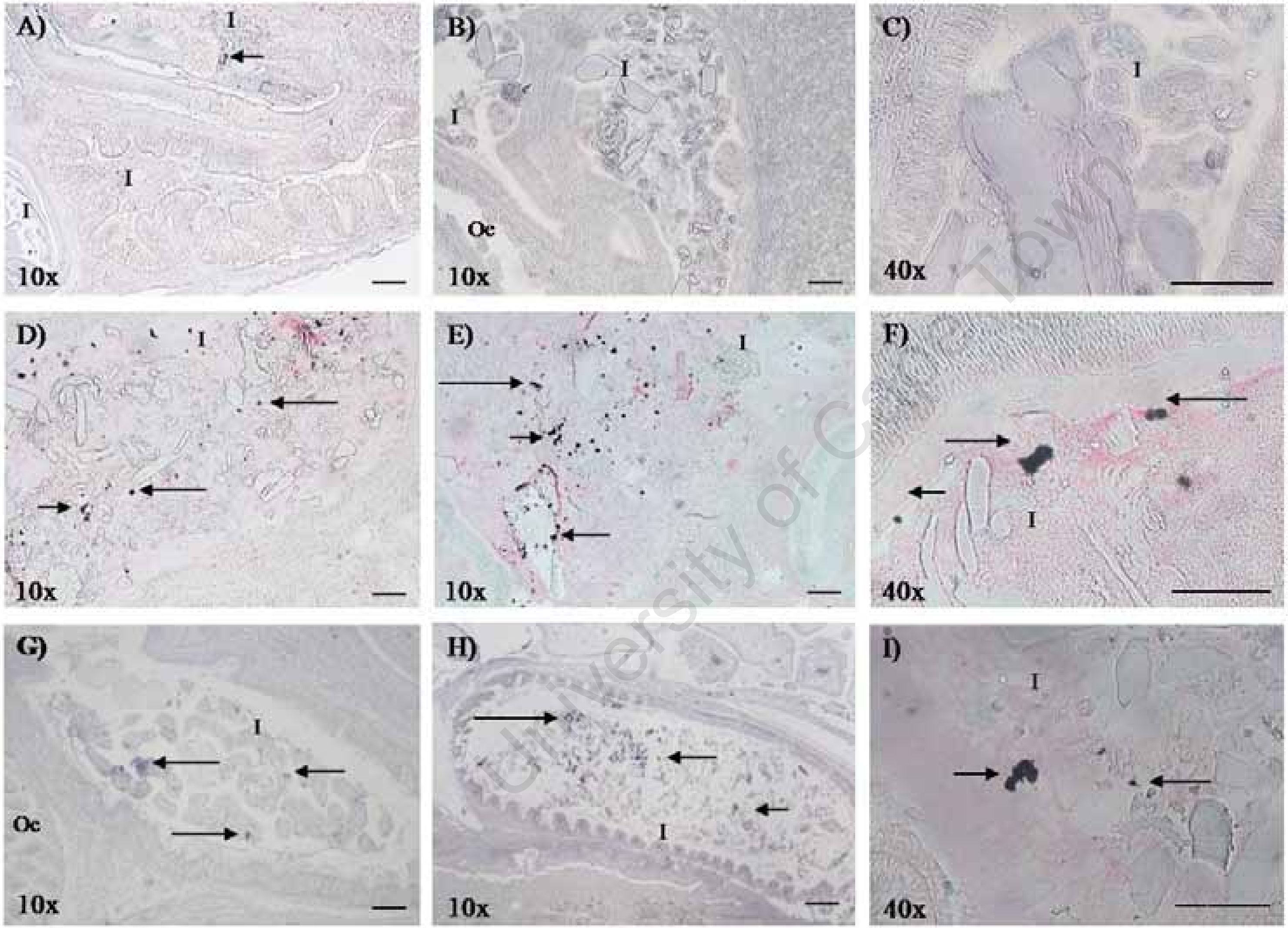

Figure 6.23
Immunohistochemical detection and localization of *V. midae* SY9 secreted VmproA within whole animal sections of *H. midae* 0, (A, B and C) 2 (D, E and F) and 14 days (G, H and I) after receiving the *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 diet. Arrows indicate regions showing a positive signal. Scale bar, 100 µm. Magnification depicted in bottom left corner of each picture. Abbreviations in this figure are the same as in Fig. 6.14.

6.5 Discussion

Macey and Coyne (2005) investigated the effect of supplementing a high-protein artificial diet with a mixture of three probiotic strains, including *V. midae* SY9, on *in situ* protease activity and protein digestion within abalone digestive tract regions. Abalone fed a diet supplemented with the probiotic strains had enhanced levels of intestinal *in situ* alkaline protease activity in comparison with animals fed a basal ABFEED® S34 diet (Macey and Coyne, 2005). They also observed a significant improvement in the digestion and absorption of proteins within the intestinal region of abalone fed the probiotic supplemented diet (Macey and Coyne, 2005). Similarly, in this study (Chapter 5), we showed that *H. midae* fed an ABFEED® S34 diet supplemented with a single probiotic strain, *V. midae* SY9, had significantly enhanced *in situ* alkaline protease activity levels within both the crop/stomach and intestinal digestive tract regions in comparison to abalone fed the basal diet. A mutant strain *V. midae* SY9Mut2, deficient in the predominant extracellular alkaline protease VmproA, was constructed using an insertional inactivation strategy (Chapter 4). *H. midae* fed ABFEED® S34 weaning chips supplemented with *V. midae* SY9Mut2 had equivalent *in situ* alkaline protease activity levels within both the crop/stomach and intestinal regions when compared to abalone fed the basal control diet (Chapter 5). Therefore, the difference in *in situ* alkaline protease activity levels of abalone fed the *V. midae* SY9 supplemented diet and those fed the basal diet is most likely due to the production and secretion of VmproA by *V. midae* SY9 within the abalone digestive tract.

As *V. midae* SY9 was initially isolated from the digestive tract of cultured *H. midae* (Macey, 2005), it is likely that this bacterial strain may be a common enteric bacterium of cultured *H. midae*. Therefore, it was necessary to construct a strain of *V. midae* SY9 that would enable the specific identification of probiotic cells ingested by *H. midae* from supplemented feed and not *V. midae* SY9 cells present within the natural digestive microbiota of *H. midae*. Macey and Coyne (2006) constructed chromosomally tagged strains of *V. midae* SY9 by using the mini-Tn*10-gfp-kan* transposon mutagenesis system. This was done in order to track the persistence of ingested *V. midae* SY9 within the digestive tract of *H. midae*. A mutant strain

V. midae SY9.8 was employed in *H. midae* colonisation experiments. The mini-Tn*10*-*gfp*-*kan* cassette was shown to have randomly integrated into a single position in the chromosome of *V. midae* SY9. Rengpipat *et al.* (2009) transformed *Bacillus* S11 with the constitutive GFP-expressing plasmid pAD44-12 as a marker for fluorescent *in situ* localisation of *Bacillus* S11 within the intestine of *P. monodon*. However, Macey (2005) observed that relative GFP expression and fluorescence in the *V. midae* SY9 mini-Tn*10*-*gfp*-*kan* transconjugants was inadequate for fluorescent *in situ* detection of *V. midae* SY9 within the abalone digestive tract. Never the less, Macey and Coyne (2006) demonstrated that a 1.3 kb *kan*-*gfp* fragment of the mini-Tn*10*-*gfp*-*kan* transposon cassette could be used as a specific DNA probe to detect *V. midae* SY9.8 in the digestive tract of *H. midae*. Therefore, *in situ* hybridization using specific DNA probes was investigated as an alternative approach to the localisation of *V. midae* SY9 within *H. midae*. Horwitz (2008) successfully localised an intracellular bacterium infecting *H. midae* using specific DIG-labeled oligonucleotide DNA probes. Therefore, in this study *gfp* chromosomally- tagged strains of *V. midae* SY9 were constructed using the mini-Tn*10*-*gfp*-*kan* transposon system, as the chromosomally integrated mini-Tn*10*-*gfp*-*kan* cassette was identified as a suitable target for specific DNA probes to enable localisation of ingested probiotic cells within *H. midae*.

Macey (2005) reported that chromosomal integration of the mini-Tn*10*-*gfp*-*kan* transposon cassette had no significant influence on the growth of the mutant strain relative to *V. midae* SY9. Similarly, Rengpipat *et al.* (2009) demonstrated that the properties of a GFP-expressing mutant strain of a shrimp probiotic *Bacillus* S11-GFP were not significantly different to that of the wild-type strain. In this study, chromosomal integration of the mini-Tn*10*-*gfp*-*kan* transposon had no significant impact on the growth and protease activity of the *gfp* chromosomally-tagged *V. midae* SY9 strains. As a result, any of the three strains constructed could have been used to investigate and localise *V. midae* SY9 within the digestive tract of *H. midae* by *in situ* hybridisation.

As previously discussed (Chapter 5), an important consideration in studies of this nature is that the probiotic microorganisms need to be viably, stably and consistently incorporated into the experimental feed. Therefore, ensuring that the manufacturing, drying and storage conditions are optimized, in turn ensures that live probiotic strains supplemented into a formulation feed remain viable and retain their functionality through the feed preparation and storage processes. Vacuum impregnation has been shown to be a viable, stable and consistent method for supplementation of ABFEED® S34 weaning chips with *V. midae* SY9 strains (Chapter 5). Similar to results obtained for post-impregnation drying of probiotic supplemented ABFEED® (Chapter 5), there was a significant (P<0.05) decline in *V. midae* SY9::Tn10.52 viability within supplemented ABFEED® S34 following post-vacuum impregnation drying at 22 ºC for 16 hours. However, the level of viable probiotic cells within the supplemented feed following post-vacuum impregnation drying remained above the previously discussed (Chapter 5) minimum level of 10^6 cfu g^{-1} ABFEED® S34.

A storage temperature of 4 ºC was investigated to ensure that ABFEED® S34 weaning chips supplemented with *V. midae* SY9::Tn10.52 could be stored for a maximum of 7 days without a significant loss in the probiotic cell viability below the pre-determined threshold of approximately 10^6 cfu g^{-1}ABFEED®. Investigation of a longer storage period was not considered as the abalone in this experiment were only fed probiotically supplemented feed for 14 days. Therefore, being able to store the feed for a minimum period of 7 days meant that probiotically supplemented ABFEED® S34 weaning chips only had to be prepared twice during the 14 day experimental period. The viable cell count within the stored vacuum impregnated feed increased over the course of the 7 day storage period with the highest titre recorded after 2 days. Previous investigations of this nature with *V. midae* SY9 strains in Chapter 5 and by Macey and Coyne (2005), did not determine cell viability within supplemented feed on such a regular basis within a relatively short (7 day) period. Instead, the focus of those investigations into probiotic storage was on long-term storage of probiotic-supplemented ABFEED® S34 for use in long-term *H. midae* growth trials.

As was previously discussed (Chapter 5), the stability of vacuum impregnated *V. midae* SY9::Tn10.52 within the flow-through abalone tanks was of concern, as we wanted to ensure that the abalone had access to ABFEED® containing probiotics at a level of at least 10^6 cfu g^{-1}. Therefore, the number of viable *V. midae* SY9::Tn10.52 probiotic cells in supplemented ABFEED® S34 immersed in the abalone tanks was monitored over a three day period. Viable *V. midae* SY9::Tn10.52 cells from vacuum impregnated ABFEED® S34 remained at levels greater than 10^{10} cfu g^{-1} for the duration of the three-day period. Therefore, the abalone tanks were cleaned, any uneaten food removed and *V. midae* SY9::Tn10.52 supplemented feed replaced every 2-3 days for the duration of the experimental period.

The colonisation experiment conducted by Macey and Coyne (2006) was based on the isolation of total genomic DNA from the digestive tract of *H. midae* fed an ABFEED® diet supplemented with the chromosomally-tagged strain, *V. midae* SY9.8. The levels of *V. midae* SY9.8 within the *H. midae* digestive tract was monitored by *in situ* Southern blot hybridization analysis. Macey and Coyne (2006) observed a significant decline in the levels of *V. midae* SY9.8 within the digestive tract of *H. midae* two days after cessation of feeding the probiotic-supplemented experimental diet. Furthermore, this drop in detectable *V. midae* SY9.8 was correlated with a significant decline in the intestinal *in situ* alkaline protease activity levels of abalone that had previously been orally administered probiotic supplemented feed.

Previous investigations have identified a variety of enteric bacterial isolates from the gut of abalone (Erasmus, 1996; Erasmus *et al.,* 1997; Macey, 2005; Sawabe et al., 2004b). Therefore, it was not surprising to detect strong hybridization signals for whole animal sections probed with a eubacterial probe within both the crop/stomach and intestinal regions of *H. midae* irrespective of their diet. Bacteria are thought to occur throughout the digestive tract of aquatic invertebrates (Harris *et al.,* 1998a) and hybridization of the eubacterial probe to targets within the crop/stomach and intestine provides further direct evidence for the presence of bacteria throughout the digestive tract of *H. midae*. Erasmus *et al.* (1997) demonstrated that comparatively, the intestine contained the largest number and greatest diversity of culturable enteric bacteria within the digestive system of *H. midae*. Similarly, in this study the hybridization signals associated with the intestinal regions of the digestive tract appeared to be more intense than those signals observed for the crop/stomach, indicating a greater load of enteric bacteria within the latter regions of the abalone digestive tract.

In situ hybridization of whole animal tissue sections enabled specific localisation and visualization of dietary-supplemented *V. midae* SY9::Tn10.52 within the crop/stomach and intestinal regions of the *H. midae* digestive tract. In contrast, hybridization signals were not detected in abalone fed the basal ABFEED® S34 weaning chips. Similar results have been reported in related studies investigating the colonisation potential of probiotics for shrimp (Rengpipat *et al.,* 2009) and abalone (Macey and Coyne, 2006), by means of *gfp*-tagged bacterial strains. Macey and Coyne (2006) detected the chromosomally-tagged strain *V. midae* SY9.8 using both culture- and *in situ* hybridization-based approaches, within both the crop/stomach and intestine of *H. midae* fed probiotic supplemented feed. However, the probiotic strains could not be identified from control abalone fed a basal (unsupplemented) diet (Macey and Coyne, 2006).

Rengpipat *et al.* (2009) detected GFP-tagged *Bacillus* S11 (S11-GFP) cells attached to the intestinal mucous lining of the black tiger shrimp, *P. monodon*. Similarly, we observed distinct regions of positive signal corresponding to the presence of *V. midae* SY9::Tn10.52 along the inner surface of the crop/stomach of *H. midae* fed the probiotically-supplemented ABFEED® diet for 14 days. Additionally, strong *V. midae* SY9::Tn10.52 hybridization signals were observed to be associated with the surfaces of feed or other particulate matter within the crop/stomach and intestine of *H. midae* fed the probiotically supplemented diet.

Immunological analysis showed that VmproA could be detected within the crop/stomach and intestinal regions of *H. midae* fed ABFEED® S34 supplemented with *V. midae* SY9::Tn10.52. This corroborates the increased *in situ* alkaline protease activity detected within the crop/stomach and intestine of *H. midae* fed *V. midae* SY9 supplemented ABFEED® S34 in comparison to abalone fed the basal diet (Chapter 5). On the whole, there was no immunohistochemical evidence of VmproA within the digestive tract regions of *H. midae* prior to their being fed the probiotically supplemented diet, or within abalone fed the basal diet over the course of the 14 day experimental period. However, a single positive hybridization signal was detected within a tissue section from a basal fed animal sampled at 2 days following the start of the experiment. Macey (2005) isolated *V. midae* SY9 from the digestive tract of *H. midae* fed ABFEED® S34. Therefore, *V. midae* SY9 may be a common bacterium within the gut of cultured *H. midae* where it may express VmproA in response to high-protein formulation feeds, such as ABFEED® S34, explaining our detection of VmproA in some of the sections prepared from abalone fed the basal diet.

Fewer regions of positive immuno-staining for VmproA were observed within the crop/stomach of abalone fed *V. midae* SY9::Tn10.52 supplemented feed, compared to intestinal regions. Similarly, the enhanced *in situ* alkaline protease activity detected within the crop/stomach in abalone fed with ABFEED® supplemented with *V. midae* SY9 was not as pronounced as the increased protease activity observed within the intestinal regions (Chapter 5), implying that perhaps there are lower levels of VmproA within the crop/stomach. As already mentioned, *in situ* hybridization with the *gfp*-specific probes showed lower levels of *V. midae* SY9::Tn10.52 within the crop/stomach in comparison to the intestine of *H. midae*. Therefore, the lower number of *V. midae* SY9 cells present within the crop/stomach may influence the amount of VmproA present in that particular region of the digestive tract. The pH of the crop/stomach may also not be conducive to the activity of VmproA, since it has been shown that the crop of *H. midae* has a pH of approximately 5.2 (Knauer *et al.,* 1996), and the crop of *Haliotis laevigata* Donovan has been shown to have a pH of 5.28 (Harris *et al.*, 1998a). We have shown that a pH of approximately 9 is optimal for VmproA activity (Chapter 3).

The areas displaying positive immunoreactions with VmproA appeared to be generally associated with the feed and/or particulate matter within the intestine of *H. midae* fed *V. midae* SY9::Tn10.52 supplemented ABFEED® S34 weaning chips. Therefore, immunohistochemical localisation of VmproA within paraffin-embedded *H. midae* tissue sections are similar to the *in situ* localisation of *V. midae* SY9::Tn10.52 within abalone tissue sections hybridized with the *gfp* oligonucleotide DNA probes. Thus, it could be hypothesised that viable *V. midae* SY9 cells, ingested with the supplemented artificial feed, remain attached to the surface of the digestive tract and associated with food particles and/or other particulate matter within the abalone digestive tract. Presumably, proximity to the feed induces *V. Midae* SY9 to secrete hydrolytic enzymes, such as VmproA, within the digestive tract of *H. midae*.

CHAPTER 7

GENERAL DISCUSSION

Abalone are a sought after seafood delicacy, particularly in Asian countries where they are used in traditional ceremonies and cuisine. The South African abalone aquaculture industry developed rapidly throughout the 1990s and is founded on land-based cultivation of *Haliotis midae*, known locally as ‘perlemoen’ (Britz, 1995; Sales and Britz, 2001). One of the major challenges facing the abalone aquaculture industry is the relatively slow growth rate of abalone. However, dietary supplementation with probiotic microorganisms has been shown to improve the growth rate of a variety of aquacultured animals. Probiotics are thought to influence the health and growth of aquatic host organisms through several possible modes of action (as discussed in Chapter 1), including improved host digestion efficiency through the secretion of extracellular enzymes which contribute to the host’s pool of digestive enzymes. Recent investigations have shown that dietary supplementation with probiotic strains capable of producing and secreting hydrolytic enzymes can significantly improve the growth rate of *H. midae* (Macey and Coyne, 2005; ten Doeschate and Coyne, 2008).

Macey and Coyne (2005) isolated *Vibrio midae* SY9 from the digestive tract of *H. midae* as it displayed broad-substrate extracellular proteolytic activity. Dietary supplementation of a high-protein artificial diet with *V. midae* SY9 and other probiotic strains resulted in an increase in the levels of alkaline protease activity and protein digestion within the *H. midae* digestive tract. Furthermore, the survival and growth rate of *H. midae* fed a diet supplemented with a mixture of probiotic strains, including *V. midae* SY9, was significantly improved in comparison to abalone fed an unsupplemented basal diet. Therefore, the primary aim of this study was to investigate the specific role of the predominant *V. midae* SY9 extracellular protease in the previously observed increased growth rate and digestive protease activity of cultured *H. midae* fed a formulated diet supplemented with *V. midae* SY9 (Macey and Coyne, 2005). In order to achieve these aims and answer the research questions highlighted in chapter one, the initial steps of the project involved cloning, sequencing and molecular characterisation of the predominant *V. midae* SY9 extracellular protease, VmproA.

During the course of this study the fragment of *V. midae* SY9 chromosomal DNA containing the gene encoding VmproA was cloned and sequenced. Nucleotide sequencing indicated that *vmproA* is encoded by a 1605 bp ORF producing a predicted 534 amino acid protein that had high sequence identity to extracellular alkaline serine proteases produced by an assortment of *Vibrio* species. Three conserved domains, including a catalytic domain containing three active site residues (D^{180}, H^{213} and S^{363}) comprising the catalytic triad, were identified within the deduced 534 amino acid sequence. The active site residues are arranged in the same order as for other related subtilase serine proteases, while the catalytic domain had homology to members of the subtilisin-like serine proteases. Based on the conserved domain architecture, VmproA may be expressed and translated in precursor form with both pre- and pro-domains. Furthermore, bioinformatic and phylogenetic analyses indicated that VmproA may be a member of the proteinase K-like subfamily of the subtilase superfamily of serine proteases. In addition, we concluded that VmproA is the proteolytic enzyme purified from the culture supernatant of *V. midae* SY9, and that VmproA is an alkaline detergent-stable extracellular serine protease.

VmproA was shown to have similar properties to related proteolytic enzymes, as *V. midae* SY9 culture supernatant containing VmproA produced a broad band of streaking when electrophoresed at 4 ºC in a polyacrylamide gel containing SDS and gelatine as a co-polymerised substrate. The zone of clearing is indicative of gelatine hydrolysis during electrophoresis and indicates that VmproA is stable and active in the presence of the detergent SDS.

In order to further characterise VmproA produced and secreted by *V. midae* SY9, experiments were conducted in which *V. midae* SY9 was cultured in a variety of growth media and under a range of incubation temperatures. The results indicated that *V. midae* SY9 extracellular alkaline protease activity was influenced by incubation temperature, growth medium and growth phase of the culture. Furthermore, *V. midae* SY9 extracellular alkaline protease(s) may also be regulated by catabolite and end-product repression.

In an attempt to elucidate the role of VmproA in the previously observed growth advantage of probiotically supplemented *H. midae* (Macey and Coyne, 2005), *V. midae* SY9 mutant strains were constructed. Insertional inactivation of the *vmproA* gene resulted in a *V. midae* SY9 mutant strain, *V. midae* SY9Mut2, that was unable to produce the distinctive detergent-stable extracellular protease, VmproA, and displayed significantly reduced levels of alkaline extracellular protease activity. A *vmproA* gene duplication mutant, *V. midae* SY9Pro2, was constructed in an attempt to generate a mutant strain capable of producing increased levels of VmproA in comparison to *V. midae* SY9. However, chromosomal *vmproA* duplication did not enhance extracellular protease activity in the abalone digestive tract as expected, despite the strain having shown increased alkaline extracellular protease activity during batch cultivation in a minimal medium supplemented with additional protein. *In vitro* batch cultivation was the only method at our disposal to assess the growth and extracellular protease production characteristics of the mutant strains constructed in this study. Therefore, *in vitro* cultivation may provide an indication of the potential growth characteristics of strains such as *V. midae* SY9Pro2, however, it cannot be used as an accurate measure of the potential *in vivo* growth and VmproA activity levels within the abalone digestive tract.

The effect of dietary supplementation with *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2 on abalone growth parameters was investigated in a growth trial. The overall results of the *H. midae* growth trial were not what we had expected. The initial hypothesis for this study was that viable *V. midae* SY9 ingested from probiotic supplemented feed would produce and secrete VmproA within the digestive tract of *H. midae*, thereby enhancing digestion of the protein portion of ABFEED® S34, and ultimately resulting in increased abalone growth as observed by Macey and Coyne (2005). Based on this hypothesis the expected outcome for the growth trial was that the highest growth rate would be recorded for *H. midae* fed either *V. midae* SY9 or *V. midae* SY9Pro2 supplemented diets, while those animals receiving the *V. midae* SY9Mut2 supplemented diet would have had the next fastest growth rate, compared to the abalone fed the unsupplemented basal diet which would be expected to have the slowest growth rate. However, we observed significantly enhanced growth of abalone fed either *V. midae* SY9Mut2 or *V. midae* SY9Pro2 supplemented ABFEED® S34, relative to the growth of animals fed the basal diet and the basal diet supplemented with *V. midae* SY9. The growth of *H. midae* receiving *V. midae* SY9 supplemented feed was enhanced in comparison to that of abalone fed the basal diet, but the improvement in growth was not statistically significant. Macey and Coyne (2005) observed a significant improvement in growth rate for abalone fed a diet containing a mixture of probiotic microorganisms which included *V. midae* SY9. Therefore, the lack of a significant enhancement in growth of animals fed *V. midae* SY9 supplemented ABFEED® S34 was surprising. The decline in the overall growth rate between 111 and 180 days of the growth trial for abalone fed *V. midae* SY9 supplemented feed was particularly unexpected and may be partially responsible for the lack of a significant enhancement in growth over the entire growth trial period. We are unsure of the exact cause of this shift in the abalone growth parameters.

Interestingly, the levels of protease activity within the crop/stomach and intestinal regions of abalone fed the experimental diets in this study generally corresponded with our initial hypothesis of the potential contribution of VmproA to *in situ* protease activity. However, increased levels of *in situ* protease activity did not correspond with improved growth rates as we initially hypothesized it would. Instead, abalone fed ABFEED® S34 supplemented with either of the two mutant strains had a lower *in situ* enzyme activity than that of abalone fed wild type supplemented ABFEED®, and yet had significantly improved growth rates. It is likely that the decreased alkaline protease activity may have directly contributed to the enhanced growth rate of abalone fed these mutant probiotic diets. Therefore we hypothesize that it is possible that VmproA may influence the colonisation potential of *V. midae* SY9 within the abalone digestive tract and/or that the probiotic effect on abalone growth may be due to another presently unknown *V. midae* SY9 extracellular protease such as those additional extracellular proteases observed in Chapters 3 and 4.

Unfortunately, the effect of dietary supplementation with the complemented mutant *V. midae* SY9Mut2.Comp on abalone growth was not examined during the course of this study. The inclusion of *H. midae* fed *V. midae* SY9Mut2.Comp supplemented ABFEED® S34 weaning chips in the growth trial may have helped to address some of the questions that arose from the initial results of the growth trial, in which *H. midae* fed an artificial diet supplemented with either *V. midae* SY9Pro2 or *V. midae* SY9Mut2 displayed enhanced growth. The primary reason for not including *V. midae* SY9Mut2.Comp and/or duplicate tanks containing a larger size class of abalone was due to space constraints at the research aquarium where the growth trial experiment was conducted. Unlike Macey and Coyne (2005) and ten Doeschate and Coyne (2008), we were unable to conduct the growth trial on a large commercial abalone farm as the mutant strains, *V. midae* SY9Pro2 and *V. midae* SY9Mut2, contain antibiotic resistance genes. We wanted to limit the exposure of natural marine microbial populations to these mutant strains and the antibiotic markers that they harbour, particularly within a commercial aquaculture facility.

The growth trial period of 180 days is relatively short in comparison to the 4 - 5 year grow-out phase of *H. midae* within a commercial abalone aquaculture facility. Macey and Coyne (2005) conducted their farm-based growth trial on smaller post-weaning *H. midae* for a period of 252 days. In a similar study ten Doeschate and Coyne (2008) conducted a farm-based growth trial to investigate *Pseudoalteromonas* sp. strain C4 supplementation of a kelp-based *H. midae* feed for 241 days. Perhaps the growth trial period within the present study should have been longer. This may have enabled us to obtain additional information which would assist in clarifying the role of *vmproA* in the previously observed growth advantage on cultured *H. midae*. Furthermore, the effect of altering the concentration of viable probiotic cells on abalone growth warrants further investigation, as it is conceivable that improved growth parameters may result from increasing the levels of viable probiotic cells within abalone feeds.

Polyclonal antibodies against purified VmproA were employed to further explore the contribution of VmproA to the observed *in situ* protease activity (Chapter 5) and improved protein digestion of abalone fed probiotically supplemented feed (Macey and Coyne, 2005). Immunohistochemistry using anti-VmproA antibodies enabled *in situ* localisation of VmproA to the crop/stomach and intestine of *H. midae* where the enzyme appeared to be associated with feed and/or other particulate matter. Therefore, VmproA is present within the digestive tract of *H. midae* following dietary supplementation with *V. midae* SY9, confirming its contribution to the enhanced *in situ* alkaline protease activity detected within the crop/stomach and intestine of probiotic supplemented abalone.

The *in situ* hybridization experiments successfully confirmed the ingestion and colonisation of dietary supplemented *V. midae* SY9::Tn10.52 cells within the digestive tract of *H. midae*. The use of a chromosomally-tagged *V. midae* SY9 strain effectively prevented the detection of any naturally occurring enteric *V. midae* strains and enabled specific localisation of dietary supplemented probiotic microorganisms. Furthermore, we showed that *V. midae* SY9::Tn10.52 cells are generally associated with feed and/or other particulate matter within the *H. midae* digestive tract. However, hybridization signals were also detected along the surface of the crop/stomach of abalone fed the probiotically-supplemented diet. This suggests that *V. midae* SY9 may colonise and/or adhere to the mucous lining of the abalone gut, as well as, being associated with the surfaces of ingested food particles passing through the digestive tract. Therefore, future studies should attempt to characterise the colonisation potential of *V. midae* SY9 to surfaces within the abalone gut.

The success of the *in situ* hybridization experiment in this study has led to a planned follow-up investigation. The objective will be to visually localise *V. midae* SY9Pro2 and *V. midae* SY9Mut2, as well as evaluate the colonisation potential of these strains, within the digestive tract of *H. midae* fed artificial feed supplemented with these mutant strains. Assessing the colonisation potential of all the *V. midae* SY9 strains employed in the growth trial may help to further address the enhanced growth rate of *H. midae* receiving *V. midae* SY9Pro2 or *V. midae* SY9Mut2 supplemented diets, as these strains may exhibit improved colonisation of the *H. midae* digestive tract in comparison to that of the wild-type strain.

Although Macey and Coyne (2005) suggested that the growth advantage observed for *H. midae* fed a formulation feed supplemented with *V. midae* SY9 may have been the result of proteolytic enzyme contribution to digestion, the results of this study suggest that production and secretion of VmproA by *V. midae* SY9 may not be the primary factor responsible for enhanced growth. The results of this investigation as a whole suggest that the relationship between probiotic microorganisms and their host is complex and, although a single hydrolytic enzyme may indeed play a role in providing a growth advantage, the overall effect on the health and/or growth of the host is likely to be due to a multitude of interacting factors and thus an individual enzyme should not be completely studied in isolation. Therefore, the increased growth rate of abalone in this study receiving the artificial diet supplemented with either *V. midae* SY9Mut2 or *V. midae* SY9Pro2 may have been due to any number of factors, including altered survival or colonisation within the abalone digestive tract. Nevertheless, it seems most likely that by mutating *vmproA* we have improved the ability of these *V. midae* SY9 strains to function as growth promoting probiotics for cultured *H. midae*.

Finally, the ongoing development of growth and health enhancing probiotics is critical to the future advancement of not only abalone aquaculture, but aquaculture in general. Probiotics offer some of the most feasible solutions to many of the challenges faced by the aquaculture industry as a whole. Successful identification of the factor(s) responsible for the significant enhancement in abalone growth following dietary supplementation with *V. midae* SY9Mut2 and *V. midae* SY9Pro2 may reveal which traits are necessary for probiotic induced abalone growth enhancement. Future studies of this nature may aid in a better understanding of the complex relationship between probiotic microorganisms and their abalone hosts, and therefore, assist in the selection and development of future probiotic strains.

APPENDIX A

MEDIA AND SOLUTIONS

CONTENTS

All media were autoclaved at 121 ºC for 20 minutes prior to use, unless otherwise specified. Distilled water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system. Ultrapure water used was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system.

A.1 Media

A.1.1 Marine Broth (MB)

D-glucose (Saarchem)	2.0 g
Casamino Acids (Difco)	5.0 g
Yeast extract (Biolab)	1.0 g
NaCl (Saarchem)	30.0 g
$MgCl_2.6H_2O$ (Saarchem)	2.3 g
KCl (Saarchem)	0.3 g
water to	1000 ml

A.1.2 Marine Agar (MA)

D-glucose	2.0 g
Casamino acids	5.0 g
Yeast extract	1.0 g
NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Agar (Biolab)	20.0 g
water to	1000 ml

A.1.3 Peptone-Marine Basal Medium (P-MBM)

Peptone (Biolab)	10.0 g
Yeast extract	1.0 g
NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
water to	1000 ml

A.1.4 P-MBM supplemented with 0.2% glucose

Peptone (Biolab)	10.0 g
Yeast extract	1.0 g
NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
water to	990 ml

P-MBM was prepared and autoclaved, before 10 ml of 20% (w/v) glucose (Appendix A.2.3) was asceptically added.

A.1.5 Luria-Bertani broth (LB)

Tryptone (Biolab)	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
water to	1000 ml

A.1.6 Luria-Bertani Agar (LA)

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Agar	15.0 g
water to	1000 ml

A.1.7 Ψ-Broth

Tryptone	20.0 g
Yeast extract	5.0 g
$MgSO_4.7H_2O$ (Saarchem)	4.0 g
KCl	0.75 g
water to	1000 ml

A.1.8 Media for Protease activity test

A.1.8.1 Double strength (2X) MA

D-glucose	2.0 g
Casamino acids	5.0 g
Yeast extract	1.0 g
NaCl	30.0 g
$MgCl_2$	2.3 g
KCl	0.3 g
Agar	20.0 g
dH_2O to	500 ml

Media made up and autoclaved in a 1000 ml Schott bottle.

A.1.8.2 Double strength (2X) LA

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Agar	15.0 g
water to	500 ml

Media made up and autoclaved in a 1000 ml Schott bottle.

A.1.8.3 4% (w/v) Skim milk

Skim milk powder (Elite)	20.0 g
water to	500 ml

4% Skim milk autoclaved for 5 minutes at 121 ºC and then mixed with autoclaved 2X MA (Appendix A.1.8.1) or 2x LA (Appendix A.1.8.2).

A.1.9 Media for filter mating

- LB-10

NaCl	5.0 g
Tryptone	5.0 g
Yeast extract	2.5 g
water to	500 ml

Adjust the pH to 7.5 (Beckman Φ70 pH meter) and make the volume up to 500 ml.

- LA-20

NaCl	10.0 g
Tryptone	5.0 g
Yeast extract	2.5 g
Agar	7.5 g
water to	500 ml

Adjust the pH to 7.5 (Beckman Φ70 pH meter), add the agar and make the volume up to 500 ml.

- Nine Salts Solution (NSS)

NaCl	17.6 g
Na_2SO_4 (Saarchem)	1.47 g
$NaHCO_3$ (Saarchem)	0.08 g
KCl	0.25 g
KBr (Saarchem)	0.04 g
$MgCl_2.6H_2O$	1.87 g
$CaCl_2.2H_2O$ (Saarchem)	0.41 g
$SrCl_2.6H_2O$ (Saarchem)	0.008 g
H_3BO_3 (Saarchem)	0.008 g
water to	1000 ml

Adjust the pH to 7.0 (Beckman Φ70 pH meter) and make up to 1000 ml.

- VNSS Broth

Peptone	1.0 g
Yeast extract	0.5 g
D-glucose	0.5 g
$FeSO_4.7H_2O$ (Saarchem)	0.01 g
Na_2HPO_4 (Saarchem)	0.01 g
NSS to	1000 ml

- VNSS Agar

Peptone	1.0 g
Yeast extract	0.5 g
D-glucose	0.5 g
$FeSO_4.7H_2O$	0.01 g
Na_2HPO_4	0.01 g
Agar	15.0 g
NSS to	1000 ml

A.1.10 Minimal Marine Medium (MMM)

- Trace metal solution

Na_2EDTA	3 g
$MnCl_2.4H_2O$	430 mg
$FeCl_3.6H_2O$	380 mg
H_3BO_3	340 mg
$ZnSO_4.7H_2O$	66 mg
$CoCl_2.6H_2O$	2 mg
$CuSO_4.5H_2O$	0.37 mg
water to	1000 ml

Filter sterilize, through a 0.22 µm filter, and store aliquots at 4 ºC.

- Vitamin solution

Thiamine	0.5 g
Biotin	1 mg
Vitamin B_{12}	0.2 mg
water to	1000 ml

Filter sterilize, through a 0.22 µm filter, and store aliquots at 4 ºC.

- MMM

NaCl	18 g
$MgSO_4$	5 g
NH_4Cl	1 g
KCl	0.6 g
Glucose	1 g
K_2HPO_4	5 mg
$NaNO_3$	0.05 g
$NaSiO_3.9H_2O$	0.15 g
Tris buffer (1.45 M Tris/HCl, pH 7.6)	8 ml
Trace metal solution	10 µl
Vitamin solution	1 ml
Water to	1000 ml

A.1.11 MMM, 1% (w/v) Peptone (MMM-P)

NaCl	18 g
$MgSO_4$	5 g
NH_4Cl	1 g
KCl	0.6 g
Glucose	1 g
K_2HPO_4	5 mg
$NaNO_3$	0.05 g
$NaSiO_3.9H_2O$	0.15 g
Tris buffer (1.45 M Tris/HCl, pH 7.6)	8 ml
Trace metal solution	10 µl
Vitamin solution	1 ml
Peptone	10 g
Water to	1000 ml

A.2 Solutions

A.2.1 Artificial seawater (ASW)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
water to	1000 ml

A.2.2 Antibiotic solutions in growth media

- Ampicillin (Sigma) (100 mg ml^{-1})
  Dissolve 2 g in 20 ml water. Filter sterilize, through a 0.22 µm syringe filter, and store aliquots at -20 ºC. Dilute 1:1000 into media to a final concentration of 100 µg ml^{-1}.

- Chloramphenicol (30 mg ml^{-1})
  Dissolve 0.6 g in 20 ml methanol. Filter sterilize, through a 0.22 µm syringe filter, and store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 30 µg ml^{-1}.

- Kanamycin (Sigma) (30 mg ml^{-1})
  Dissolve 0.6 g in 20 ml water. Filter sterilize, through a 0.22 µm syringe filter, and store aliquots at -20 °C. Dilute 1:1000 into media to a final concentration of 30 µg ml^{-1}, unless otherwise indicated.

- Streptomycin (100 mg ml^{-1})
  Dissolve 2 g in 20 ml water. Filter sterilize, through a 0.22 µm syringe filter, and store aliquots at -20 ºC. Dilute 1:1000 into media to a final concentration of 100 µg ml^{-1}.

A.2.3 General stock solutions

- 20% (w/v) Glucose

Glucose (Saarchem)	20 g
water to	100 ml

Sterilize by autoclaving. Dilute into media, to achieve the desired final concentration of glucose within the growth medium.

- 100 mM IPTG

IPTG (Fermentas)	119.15 mg
water to	5 ml

Filter sterilize, through a 0.22 µm filter, and store aliquots at 4 °C. Dilute 1:200 into media, to a final concentration of 0.5 mM.

- 1 N NaOH

NaOH (Saarchem)	4 g
water to	100 ml

Store in a plastic bottle.

- 0.5 M EDTA

EDTA (Saarchem)	93.05 g
NaOH (Saarchem)	10 g
water to	500 ml

Dissolve the EDTA and the NaOH in 400 ml water, adjust the pH to 8 (Beckman Φ70 pH meter) and make up to a final volume of 500 ml.

- 1 M HCl

37% HCl (Saarchem)	8.4 ml
water to	100 ml

Store in a foil covered glass bottle.

- 100 mM EGTA

EGTA (Calbiochem)	1.902 g
1 N NaOH to	50 ml

Dissolve the EGTA in 40 ml 1 N NaOH and then make up to a final volume of 50 ml with 1 N NaOH.

- 1 M Tris base

Tris	12.1 g
water to	100 ml

- 1 M Tris-HCl

Tris	12.1 g
water to	100 ml

Dissolve the Tris in 80 ml water and adjust pH (Beckman Φ70 pH meter) to required level with concentrated HCl (Saarchem). Finally make up to a final volume of 100 ml.

- TE buffer (Tris-EDTA)

1 M Tris-HCl (pH 7.6)	1 ml
0.5 M EDTA	200 µl
water to	100 ml

- Ethidium Bromide (10 mg ml^{-1})

Ethidium Bromide (Sigma)	0.1 g
water to	10 ml

Shake well to dissolve. Do not autoclave. Store in the dark, in a foil covered bottle. Powerful mutagen; wear gloves and clean spills with isopropanol.

- 1 M NaCl

NaCl	5.84 g
water to	100 ml

- 20% (w/v) Sodium dodecyl sulphate (SDS)

SDS (Saarchem)	20 g
water to	100 ml

Stir on a warm plate to dissolve, and do not overheat. Do not autoclave.

- 70% (v/v) Ethanol (EtOH)

Absolute EtOH (Merck)	70 ml
water to	100 ml

Do not autoclave.

- 10x Phosphate buffered saline (PBS)

NaCl	87 g
Na_2PO_4 (Merck)	22.5 g
KH_2PO_4 (Saarchem)	2 g
water to	1 l

Dissolve NaCl, Na_2PO_4 and KH_2PO_4 in 900 ml water. Adjust pH to 7.4 (Beckman Φ70 pH meter) and make up to 1 l with water.

- 1x PBS

(dilute 10x PBS 1:10 with water)

- 3 M Sodium acetate (pH 5.2)

Na-acetate	204.05 g
water to	500 ml

Dissolve sodium acetate in 400 ml water, adjust pH with glacial acetic acid to 5.2 (Beckman Φ70 pH meter) and make volume up to 500 ml.

- 1M $MgCl_2$

$MgCl_2.6H_2O$	20.3 g
water to	100 ml

- 50% (v/v) Glycerol

Glycerol	50 ml
water to	100 ml

Autoclave.

- 20% (v/v) Triton X-100

Triton X-100 (Saarchem)	2 ml
sterile water to	10 ml

- 20% (v/v) Tween-20

Tween-20 (Saarchem)	2 ml
sterile water to	10 ml

A.2.4 Solutions for chromosomal DNA extractions

- 10% (w/v) SDS

SDS	10 g
water to	100 ml

Stir on a warm plate to dissolve, and do not overheat. Do not autoclave.

- Proteinase K (20 mg ml^{-1})

Proteinase K (Sigma)	20 mg
sterile water to	1 ml

Do not autoclave. Store at -20 ºC.

- 5 M NaCl

NaCl	29.22 g
water to	100 ml

- CTAB/NaCl

NaCl	4.1 g
CTAB (USB)	10 g
water to	100 ml

Dissolve the NaCl in 80 ml of water. Slowly add the CTAB (cetryltrimethylammonium bromide). Heat while stirring slowly. If necessary, heat to 65 ºC to dissolve. Adjust to a final volume of 100 ml with water.

- Lysozyme buffer

100 mM Tris-HCl (pH 7.0)	0.25 ml
100 mM Glucose	0.5 ml
0.5 M EDTA	0.02 ml
Proteinase K (20 mg ml^{-1})	0.01 ml
Lysozyme (Sigma)	0.02 g
Sterile water to	1 ml

Do not autoclave. Make up fresh before each use, and store on ice.

- Chloroform (Merck) / isoamyl alcohol (Merck)

Mix at a ratio of 24:1. Store in the dark, in a foil covered bottle.

- Phenol / chloroform (Merck)

Mix the phenol (pH 8.0) and the chloroform at a ratio of 1:1. Store in a foil covered bottle.

- RNase A (10 mg ml^{-1})

RNase A (Sigma)	0.1 g
1 M Tris-HCl (pH 7.5)	100 µl
5 M NaCl	3 ml
water to	10 ml

Heat for 15 minutes at 100 ºC and allow to cool slowly to room temperature. Do not autoclave. Aliquot into sterile microfuge tubes and store at -20 ºC.

- TE buffer containing RNase

RNase A	10 µl
TE buffer	990 µl

A.2.5 Solutions for *E. coli* competent cell preparation

- 0.1 M $MgCl_2$

$MgCl_2$	2.03 g
water to	100 ml

Autoclave. Chill to 4 ºC, before use.

- 1 M RbCl

RbCl	6.05 g
water to	50 ml

- 0.1 M $CaCl_2$

$CaCl_2.2H_2O$	1.472 g
water to	100 ml

Autoclave. Chill to 4 ºC, before use.

- 750 mM $CaCl_2.2H_2O$

$CaCl_2.2H_2O$	5.52 g
water to	50 ml

- 100 mM MOPS (pH 7)

MOPS	1.05 g
water to	50 ml

Adjust the pH of the MOPS buffer to 7 with NaOH (Beckman Φ70 pH meter).

- TFB 1

1 M RbCl	5 ml
$MnCl_2.4H_2O$	0.495 g
KOAc	0.147 g
750 mM $CaCl_2.2H_2O$	0.67 ml
50% Glycerol	15 ml
water to	50 ml

Adjust to pH 5.8 with Glacial Acetic acid (Beckman Φ70 pH meter), make to volume and filter sterilize. Make up fresh before each use, and store on ice.

- TFB 2

100 mM MOPS (pH 7)	5 ml
1 M RbCl	0.5 ml
750 mM $CaCl_2.2H_2O$	5 ml
50% Glycerol	15 ml
water to	50 ml

Make up fresh before each use, and store on ice.

A.2.6 Solution for agarose gel electrophoresis

- 50x TAE (Tris-acetate buffer)

Tris	242 g
Glacial acetic acid (Merck)	57.1 ml
0.5 M EDTA	100 ml
water to	1000 ml

- Tracking Dye

Bromophenol blue	62.5 g
Sucrose	10 g
0.5 M EDTA	1 ml
water to	25 ml

Autoclave.

- 1x TAE

Dilute 50x TAE 1:50 with water.

- λ*Pst*I DNA Molecular Weight Marker

Lambda (λ) phage genomic DNA (Promega)	40 µl
*Pst*I (Roche)	20 U
10x Buffer R (Roche)	20 µl
sterile water to	200 µl

Perform a standard restriction digest at 37 ºC overnight. Add 40 µl tracking dye to stop the reaction, and load 15 - 20 µl per gel lane as a DNA molecular weight marker.

A.2.7 Solutions for preparation of plasmid DNA

- Solution 1

1 M Tris-Cl (pH 8)	25 ml
D-glucose	20 g
0.5 M EDTA	20 ml
water to	100 ml

Combine the Tris-Cl and EDTA with 15 ml water, autoclave and add filter sterilized glucose to the mixture. Adjust the volume to 100 ml with sterilized water.

- Solution 2

NaOH	0.8 g
SDS	1 g
sterile water to	100 ml

Make fresh weekly.

- Solution 3

KOA	147 g
water to	500 ml

Dissolve potassium acetate in 250 ml water, pH to 4.8 using Glacial acetic acid (Merck) (Beckman Φ70 pH meter) and adjust the volume to 500 ml.

- Salt saturated isopropanol

5 M NaCl	29.22 g
TE buffer	300 ml
Isopropanol to	600 ml

Shake up and stand overnight. Do not autoclave.

A.2.8 Solutions for Southern hybridization analysis

- 0.25 M HCl

HCl	21.35 ml
water to	1 l

Do not autoclave.

- 20x SSC (Sodium chloride tri-sodium citrate)

NaCl	17.5 g
Tri-Na Citrate	8.82 g
water to	100 ml

Dissolve NaCl and tri-Na citrate in 80 ml water, adjust pH to 7.4 with NaOH (Beckman Φ70 pH meter) and make up to 100 ml with water.

- 2x SSC

Dilute 20 x SSC 1:10 with water.

- 0.5x SSC

Dilute 20 x SSC 1:40 with water.

- STE (Sodium chloride-Tris EDTA)

0.1 M NaCl	2.92 g
TE buffer	500 ml

- PB stock solution (1 M Na_2HPO_4, pH 7.2)

$Na_2HPO_4 . 7H_2O$	134 g
85% H_3PO_4	4 ml
water to	1 l

- Sephadex G-50

Sepahdex G-50 (medium)	30 g
TE buffer	250 ml

Autoclave in order to swell the beads, allow to cool to room temperature, and store at 4 °C.

- Tracking dye

Dextran Blue 2000	0.3 g
Orange G	0.1 g
50 mM NaCl	10 ml

Dissolve the Dextran Blue in the 50 mM NaCl, before dissolving the Orange G in the same solution.

- 0.4 M NaOH

NaOH	16 g
water to	1 l

- 0.4 M NaOH / 1 M NaCl

NaOH	16 g
NaCl	58.44 g
water to	1 l

- 25% (w/v) SDS (Sodium dodecyl sulphate)

SDS	250 g
water to	1 l

Stir on a warm plate and do not overheat. Do not autoclave.

- Church pre-hybridization buffer (pre CHB)

Non-fat dry milk	0.5 g
PB stock solution	50 ml
0.5 M EDTA	0.2 ml
25% SDS	28 ml
water to	100 ml

- Church hybridization buffer (CHB)

PB stock solution	50 ml
0.5 M EDTA	0.2 ml
25% SDS	28 ml
water to	100 ml

- Wash buffer A (WBA)

PB stock solution	20 ml
0.5 M EDTA	1 ml
25% SDS	100 ml
water to	500 ml

- Wash buffer B (WBB)

PB stock solution	40 ml
0.5 M EDTA	2 ml
25% SDS	40 ml
water to	1 l

- 2x SSC / 0.1% (w/v) SDS

20 x SSC	10 ml
10% SDS	1 ml
water to	100 ml

- 0.5x SSC / 0.1% (w/v) SDS

20 x SSC	2.5 ml
10% SDS	1 ml
water to	100 ml

- 5x Maleic acid

Maleic acid (Sigma)	58.0 g
NaOH	35.0 g
NaCl	43.8 g
water to	1 l

Dissolve the Maleic acid, NaOH, and NaCl in 800 ml water, adjust the pH to 7.5 with NaOH and make the solution up to a final volume of 1000 ml with water. Autoclave.

- 1x Maleic acid

Dilute 5x maleic acid 1:5 with water.

- Washing buffer

5 x Maleic acid	20 ml
Tween-20 (Saarchem)	0.3 ml
water to	100 ml

- Blocking solution

1 x Maleic acid	18 ml
10 x Blocking buffer (Roche)	2 ml

- Antibody solution

Blocking solution	20 ml
Anti-digoxigenin-AP (Roche) (0.75 U μl^{-1})	2 µl

- Detection buffer

Tris	1.21 g
NaCl	0.58 g
water to	100 ml

Dissolve the Tris and NaCl in 80 ml of water, adjust the pH to 9.5 with concentrated HCl (Saarchem) and make the solution up to a final volume of 100 ml with water. Autoclave.

A.2.9 Solutions for RNA extractions

All solutions were either DEPC treated and autoclaved twice, or made up in baked glass bottles using sterile DEPC water.

- Sterile DEPC treated water

DEPC	1 ml
water to	1 l

Allow to stand overnight, with periodic mixing, prior to autoclaving twice.

- 0.5 M EDTA

EDTA	93.05 g
NaOH	10 g
DEPC	0.5 ml
water to	500 ml

Dissolve the EDTA and the NaOH in 400 ml water, adjust the pH to 8 (Beckman Φ70 pH meter) and make up to a final volume of 500 ml after adding DEPC.

- 1 M Tris-HCl

Tris	12.1 g
DEPC	0.1 ml
water to	100 ml

Dissolve the Tris in 80 ml water and adjust pH to required level with concentrated HCl (Beckman Φ70 pH meter). Add DEPC and make up to a final volume of 100 ml.

- TE buffer (Tris-EDTA)

1 M Tris-HCl (pH 7.6)	1 ml
0.5 M EDTA	200 µl
DEPC	0.1 ml
water to	100 ml

- 1 M Sucrose

Sucrose	34.23 g
DEPC treated water to	100 ml

- Ethidium bromide (10 mg ml^{-1})

Ethidium bromide	0.1 g
DEPC treated water to	10 ml

Shake well to dissolve. Do not autoclave. Powerful mutagen; wear gloves and clean spills with isopropanol.

- 70% (v/v) EtOH

Absolute EtOH	70 ml
DEPC treated water to	100 ml

Do not autoclave. Store at -20 ºC.

- Protoplast buffer

1 M Tris-Cl (pH 8.0)	1.5 ml
1 M Sucrose	45 ml
0.5 M EDTA	1.58 ml
DEPC treated water to	100 ml

Do not autoclave. Store at 4 ºC.

- Lysozyme (80 mg ml^{-1})

Lysozyme	0.8 g
DEPC treated water to	10 ml

Do not autoclave. Aliquot the lysozyme out into sterile microfuge tubes and store at -20 ºC.

- Lysis buffer

1 M Tris-Cl (pH 8.0)	1 ml
NaCl (Saarchem)	58 mg
Na citrate (1 mM)	29 mg
SDS	1.5 g
DEPC	0.1 ml
water to	100 ml

Store at 22 °C.

- Saturated NaCl

NaCl (Saarchem)	40 g
DEPC	0.1 mg
water to	100 ml

Stir until solution reaches saturation.

- Phenol / chloroform / iso-amyl alcohol (25:24:1)

Mix (phenol (pH 4.0): Chloroform: iso-amyl alcohol) at a ratio of 25:24:1.

Do not autoclave. Store at 4 ºC in a foil covered bottle.

- 3 M Sodium acetate

Na-acetate	204.05 g
DEPC	0.5 ml
water to	500 ml

Dissolve sodium acetate in 400 ml water and adjust pH with glacial acetic acid to 5.2 (Beckman Φ70 pH meter). Add DEPC and make volume up to 500 ml.

A.2.10 Solutions for RNA formaldehyde agarose gel electrophoresis

- Ethidium Bromide for RNA gel electrophoresis (10 mg ml^{-1})

Ethidium Bromide (Sigma)	0.1 g
sterile DEPC treated MIlli-Q water to	10 ml

Shake well to dissolve. Dissolve and store in RNA-treated glassware. Do not autoclave. Powerful mutagen; wear gloves and clean spills with isopropanol.

- 10x MOPS buffer, pH 6.6 - 7.8

MOPS	10.0 g
NaOAc	0.5 g
0.5 M EDTA	5 ml
sterile DEPC treated Milli-Q water to	250 ml

Adjust the pH to 7.0 with NaOH, filter sterilise and store in the dark at 4 ºC.

- RNA gel running buffer

1x MOPS buffer

Dilute 10x MOPS buffer 1:10 with sterile DEPC treated water.

- RNA sample application buffer

10x MOPS	0.3 ml
37% Formaldehyde	0.08 ml
Formamide	0.9 ml
Ethidium bromide (10 mg ml^{-1})	2 µl
sterile DEPC treated Milli-Q water to	2.2 ml

Store in a foil-covered sterile microfuge tube at 4 ºC.

A.2.11 Solutions for northern blot hybridization analysis

- 20x SSC

NaCl	17.5 g
Tri-Na Citrate	8.82 g
DEPC-treated water to	100 ml

Dissolve NaCl and tri-Na citrate in 80 ml water, adjust pH to 7.4 with NaOH (Beckman Φ70 pH meter) and make up to 100 ml with DEPC-treated water. Double autoclave.

- 1x SSC

Dilute 20 x SSC 1:20 with sterile DEPC-treated water.

- 2x SSC/0.1% (w/v) SDS

20x SSC (RNA solution)	10 ml
10% SDS	1 ml
DEPC-treated water to	100 ml

- 7.5 mM NaOH

NaOH	0.03 g
water to	100 ml

Double autoclave to sterilise.

A.2.12 Solutions for filter mating

- 10 mM $MgSO_4$

$MgSO_4$	0.1205 g
water to	10 ml

- Wash solution

10 mM $MgSO_4$	5 ml
NSS	5 ml

- 300 mM IPTG

IPTG	357.45 mg
water to	5 ml

Filter sterilize, through a 0.22 µm syringe filter, and store aliquots at 4 ºC. Dilute into media to a final concentration of 3.0 mM.

A.2.13 Solution for the Bradford assay for determining protein concentration

- Bovine serum albumin (BSA) (1 mg ml^{-1})

BSA (Roche)	0.01 g
sterile water to	10 ml

Do not autoclave. Aliquot and store at -20 ºC.

- Bradford's Reagent

Dye Reagent Concentrate (Bio-Rad)	1 ml
sterile water to	5 ml

Make up fresh, before every use.

A.2.14 Solutions for protein purification

- Dialysis buffer (50 mM Tris-HCl, pH 9.0)

50 mM Tris	6.07 g
water to	1000 ml

Dissolve Tris in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

- 50 mM NaCl Elution buffer

50 mM Tris	6.07 g
NaCl (Saarchem)	2.92 g
water to	1000 ml

Dissolve Tris and NaCl in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

- 100 mM NaCl Elution buffer

50 mM Tris	6.07 g
NaCl (Saarchem)	5.84 g
water to	1000 ml

Dissolve Tris and NaCl in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

- 250 mM NaCl Elution buffer

50 mM Tris	6.07 g
NaCl (Saarchem)	11.68 g
water to	1000 ml

Dissolve Tris and NaCl in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

- 500 mM NaCl Elution buffer

50 mM Tris	6.07 g
NaCl (Saarchem)	23.37 g
water to	1000 ml

Dissolve Tris and NaCl in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

- Gel-filtration buffer (50 mM Tris-HCl (pH 9.0) 300 mM NaCl)

50 mM Tris	6.07 g
NaCl (Saarchem)	17.52 g
water to	1000 ml

Dissolve Tris and NaCl in 800 ml water and adjust pH (Beckman Φ70 pH meter) to 9.0 with 32% HCl (Saarchem). Finally make volume up to 1000 ml. Autoclave and store at 4 ºC.

A.2.15 Solutions for SDS-PAGE

- Stacking gel buffer

0.5 M Tris-HCl, pH 6.8

- Seperating gel buffer

1.5 M Tris-HCl, pH 8.8

- 10x SDS-PAGE running buffer

Glycine	150 g
Tris	30 g
SDS	20 g
water to	1000 ml

- 1x SDS-PAGE running buffer

Dilute 10x SDS-PAGE running buffer 1:10 with water before use.

- 10% (w/v) Ammonium persulfate (AMPS)

AMPS	1 g
water to	10 ml

Do not autoclave. Filter sterilize before aliquoting into sterile microfuge tubes and store at -20 ºC.

- 2x SDS-PAGE Sample Application Buffer (SAB)

Glycerol	2 ml
10% SDS	2 ml
Bromophenol blue	0.25 mg
0.5 M Tris-HCl, pH 6.8	2.5 ml
β-mercaptoethanol	0.5 ml
water to	10 ml

A.2.16 Solutions for coomassie staining of polyacrylamide gels

- Coomassie SDS-PAGE gel stain

Coomasie brilliant blue R250 (Sigma)	1 g
Methanol	225 ml
Glacial acetic acid	50 ml
water to	500 ml

- Coomassie SDS-PAGE gel destain

Methanol	250 ml
Glacial acetic acid	70 ml
water to	500 ml

- SDS-PAGE Gel storage solution

Glacial acetic acid	7 ml
water to	100 ml

A.2.17 Solutions for silver staining of polyacrylamide gels

- Fix solution

Methanol	100 ml
Glacial acetic acid	24 ml
37% Formaldehyde solution	100 µl
ultrapure water to	200 ml

Do not autoclave.

- Wash solution I

Absolute ethanol	100 ml
ultrapure water	200 ml

- Pre-treatment solution

$Na_2S_2O_3.5H_2O$	40 mg
ultrapure water to	200 ml

- Wash solution II

Methanol	50 ml
ultrapure water to	100 ml

- Impregnating solution

$AgNO_3$ (Merck)	400 mg
ultrapure water to	199.85 ml

Formaldehyde [150 µl of a 37% solution] was added just before use. Do not autoclave.

- Developing solution

Na_2CO_3	12 g
Pre-treatment solution	4 ml
ultrapure water to	199 ml

Formaldehyde [100 µl of a 37% solution] was added just before use. Do not autoclave.

- Stop solution

Methanol (Merck)	50 ml
Glacial acetic acid	12 ml
ultrapure water to	100 ml

- Gel storage solution

Glacial acetic acid	1 ml
ultrapure water to	100 ml

Place gels in the gel storage solution, in a sealed clean plastic bag and store at 4 ºC, until photographed or scanned.

A.2.18 Solutions for gelatine zymography

- 5% (w/v) Gelatine solution

Gelatine (Merck)	1 g
water to	20 ml

Heated in a water bath (approximately 60 °C), with regular mixing to dissolve. Make up fresh before use.

- 2.5% SDS

SDS	2.5 g
water to	100 ml

Stir on a warm plate to dissolve, and do not overheat. Do not autoclave.

- 2% Glycerol

Glycerol	2 ml
water to	100 ml

Stir on a warm plate to dissolve. Sterilize by autoclaving.

- 2x Zymogram sample application buffer

Bromophenol blue	0.25 mg
10% (w/v) SDS	4 ml
Glycerol	2 ml
1M Tris, pH 6.8	1.25 ml
water to	10 ml

- 1 M $CaCl_2$

$CaCl_2$	11.1 g
water to	100 ml

- Incubation buffer (0.1 M Glycine buffer, pH 9.0 and 10 mM $CaCl_2$)

Glycine (Saarchem)	7.507 g
1 M $CaCl_2$	10 ml
water to	1000 ml

Adjust the pH to 9.0 (Beckman Φ70 pH meter), and make up to a final volume of 1000 ml.

- Wash solution [2.5% (v/v) Triton X-100 and 10 mM $CaCl_2$]

Triton X-100	2.5 ml
1 M $CaCl_2$	1 ml
water to	100 ml

Gently heat with regular mixing to dissolve the Triton X-100.

- Amido black staining solution [1% (w/v) Amido black in 7% (v/v) acetic acid]

Amido Black 10B	10 g
Glacial acetic acid	70 ml
water to	1000 ml

- Amido black destaining solution [7% (v/v) acetic acid]

Glacial acetic acid	70 ml
water to	1000 ml

A.2.19 Solutions for casein zymography

- Wash solution (2.5% Triton X-100)

Triton X-100	2.5 ml
ultrapure water to	100 ml

Stir on a warm plate to dissolve, and do not overheat. Do not autoclave. Chill at 4°C before use.

- Casein penetration solution

Casein	1.05 g
100 mM Tris-HCl (pH 9.0) to	35 ml

Stir to dissolve. Do not autoclave. Use fresh, and chill at 4 ºC before use.

A.2.20 Solutions for Azo-casien protease assays

- 1 M Tris-HCl

Tris	6.05 g
water to	50 ml

Dissolve the Tris in 40 ml water and adjust to required pH (Beckman Φ70 pH meter) with concentrated HCl (Saarchem). Finally make up to a final volume of 50 ml.

- 1 M MES

MES (Sigma)	9.76 g
water to	50 ml

Dissolve the MES in 40 ml water and adjust to required pH (Beckman Φ70 pH meter) with 50% (v/v) H_2SO_4 (Saarchem). Finally make up to a final volume of 50 ml.

- 1 M CAPS

CAPS (Sigma)	11.07 g
water to	50 ml

Dissolve the Tris in 80 ml water and adjust to required pH (Beckman Φ70 pH meter) with 1 N NaOH (Saarchem). Finally make up to a final volume of 50 ml.

- Azo-casein solution

Azo-casein (Sigma)	2 g
1 M NaCl	40 ml
1 M $CaCl_2$	0.2 ml
1 M buffer (Tris, MES or CAPS)	10 ml
sterile water to	100 ml

Do not autoclave and store at 4 °C in the dark (light sensitive).

- 10% (w/v) Trichloroacetic acid (TCA)

TCA (Merck)	10 g
water to	100 ml

Do not autoclave and store at 4 °C.

- 0.5 M NaOH

NaOH (Saarchem)	4 g
water to	200 ml

Store in a plastic bottle.

- 100 mM Phenylmethylsulphonyl fluoride (PMSF)

PMSF (Roche)	174 mg
Absolute ethanol	10 ml

Aliquot and store -20 ºC.

- 100 mM 1,10-Phenanthroline

1,10-Phenanthroline (Sigma)	180 mg
Absolute ethanol	10 ml

A.2.21 Solution for antigen preparation

- Freund's incomplete adjuvant

Mannide monoleate (Sigma)	0.5 ml
Liquid paraffin (BDH)	4.5 ml

Mannide monooleate mixed at a ratio of 1:9 (v:v) with light, colourless, liquid paraffine. Mixed in a 1:1 (v:v) ratio with the antigen and emulsified (Rybicki, 1979) prior to injection.

A.2.22 Solutions for ELISA assay

- TBS

1 M NaCl	10 ml
100 mM Tris-HCl (pH 7.5)	10 ml
water to	100 ml

- TBS / Tween 20 (TBST)

1 M NaCl	10 ml
100 mM Tris-HCl (pH 7.5)	10 ml
Tween-20 (Saarchem)	100 µl
water to	100 ml

- ELISA Blocking buffer

1 M NaCl (Saarchem)	10 ml
100 mM Tris-HCl (pH 7.5)	10 ml
BSA	3 g
Tween-20	100 µl
water to	100 ml

Dissolve the BSA in 50 ml of water, before adding the remaining solutions and make up to a final volume of 100 ml. Do not autoclave. Make up fresh before each use.

- 10% Diethanlamine/ 0.5 mM $MgCl_2$

- Substrate (pH 9.6)

10% Diethanlamine/ 0.5 mM $MgCl_2$	10 ml
pNPP (BDH Laboratory Supplies)	10 mg

Store at 4°C in the dark.

A.2.23 Solutions for the PEG precipitation of polyclonal antibodies

- Borate-buffered Saline

Boric acid	2.16 g
NaCl (Saarchem)	2.19 g
NaOH (Saarchem)	0.7 g
37% HCl	620 µl
sterile distilled water to	1000 ml

Dissolve the Boric acid, NaCl, NaOH and HCl in 800 ml sterile distilled water. Adjust the pH to 8.6 (Beckman Φ70 pH meter) with NaOH and make up to a final volume of 1000 ml with sterile distilled water.

- 1x PBS

10 x PBS	10 ml
distilled water to	100 ml

Autoclave to sterilise.

- PBS containing 60% (v/v) glycerol

10 x PBS	1 ml
Glycerol (Saarchem)	6 ml
sterile distilled water to	10 ml

A.2.24 Solutions for western blot analysis

- 10% (v/v) Tween-20

Tween-20 (Saarchem)	2 ml
sterile distilled water to	20 ml

Store in the dark.

- Blocking solution

Non-fat dried skim milk powder	5 g
10x PBS	10 ml
10% Tween-20	1 ml
sterile water to	100 ml

Dissolve the skim milk powder before adding Tween-20. Do not autoclave. Make up fresh before each use.

- Wash solution (PBT)

10x PBS	10 ml
10% Tween-20	1 ml
sterile water to	100 ml

Do not autoclave. Make up fresh before each use.

A.2.25 Solution for electroblotting proteins onto nitrocellulose membrane

- Towbin blotting buffer

Tris	3.0 g
Glycine (Saarchem)	14.4 g
Methanol	200 ml
sterile distilled water to	1000 ml

Dissolve the Tris and Glycine in 750 ml water, before the addition of the methanol. Make up to 1000 ml with water and store at 4°C before use. Do not autoclave. Discard if the solution becomes yellow.

A.2.26 Solutions for tissue fixation

- 95% EtOH

Absolute EtOH (Merck)	95 ml
water to	100 ml

Do not autoclave.

- Davidson's fixative

95% EtOH	600 ml
Glacial acetic acid (Saarchem)	200 ml
Formalin (Saarchem)	400 ml
water to	1800 ml

- 70% (v/v) EtOH

Do not autoclave. Make-up fresh before every use.

A.2.27 Solutions for *in situ* hybridization analysis

- 95% (v/v) EtOH

Absolute ethanol	95 ml
Sterile milli-Q water to	100 ml

Do not autoclave. Store at -20 ºC.

- 80% (v/v) EtOH

Absolute ethanol	80 ml
Sterile milli-Q water to	100 ml

Do not autoclave. Store at -20 ºC.

- 70% (v/v) EtOH

Do not autoclave. Store at -20 ºC.

- 50% (v/v) EtOH

Absolute ethanol	50 ml
Sterile milli-Q water to	100 ml

Do not autoclave. Store at -20 ºC.

- 10 x Phosphate-buffered Saline (PBS)

NaCl	75.97 g
$Na_2HPO_4.2H_2O$ (Saarchem)	12.46 g
$NaH_2PO_4.2H_2O$ (Saarchem)	4.8 g
Milli-Q water to	1000 ml

Dissolve in 800 ml Milli-Q water, adjust the pH to 7.0 and make up to a final volume of 1000 ml. Autoclave.

- 1 x PBS

Dilute 10 x PBS 1:10 with sterile Milli-Q water.

- 2x SSC

Dilute 20x SSC 1:10 with water.

- 1x SSC

Dilute 20x SSC 1:20 with water.

- 0.5x SSC

Dilute 20x SSC 1:40 with water.

- 5x Denhardt's solution

Ficoll® (Sigma)	100 mg
Polyvinylpyrrolidone (PVP-10) (Sigma)	100 mg
BSA	100 mg
sterile Milli-Q water to	100 ml

Do not autoclave. Store at -20 ºC.

- *In situ* hybridization buffer

20x SSC	0.2 ml
Formamide (Saarchem)	0.5 ml
50x Denhardt's solution	0.02 ml
Yeast tRNA (Sigma)	0.05 ml
Heat denatured Herring Sperm DNA (10 mg ml^{-1}, Sigma)	0.05 ml
sterile water to	1 ml

Make up fresh before each use. Pre-warm to required hybridization temperature before use.

- 1 M $MgCl_2$

$MgCl_2.6H_2O$	20.3 g
Milli-Q water to	100 ml

- Buffer 1

1 M Tris-HCl (pH 7.5)	10 ml
5 M NaCl	3 ml
Sterile milli-Q water to	100 ml

- Blocking buffer

1 M Tris-HCl (pH 7.5)	1 ml
5 M NaCl	0.3 ml
Triton X-100 (Sigma)	0.03 ml
Feotal calf serum (Invitrogen)	0.2 ml
Sterile milli-Q water to	10 ml

Make up fresh before each use.

- Buffer 2

1 M Tris-HCl (pH 9.5)	10 ml
5 M NaCl	2 ml
1 M $MgCl_2$	5 ml
Sterile milli-Q water to	100 ml

- NBT/BCIP colour development solution

NBT (Roche)	3.375 µl
BCIP (Roche)	3.5 µl
Buffer 2 to	1 ml

- Phosphate-buffered glycerin jelly

1 M PB (pH 7.0)	5 ml
Gelatine (Merck)	0.75 g
Glycerol (Saarchem)	5 ml

Slowly dissolve the gelatine in the phosphate buffer with gentle heating and regular mixing. When dissolved, add the glycerol and mix well. Filter through glass wool while still hot.

A.2.28 Solutions for immunohistochemistry

- 20% (v/v) Tween 20

Tween 20 (Saarchem)	4 ml
Sterile milli-Q water to	20 ml

Do not autoclave. Store in the dark.

- Immunohistochemistry hybridization buffer

10x PBS	1 ml
20% Tween-20	0.05 ml
BSA	20 mg
Feotal calf serum	0.5 ml
DMSO (Merck)	0.1 ml
Sterile milli-Q water to	10 ml

Make up fresh, before each use.

- PBT

10x PBS	100 ml
20% Tween-20	10 ml
Sterile milli-Q water to	1000 ml

A.2.29 Solutions for Dinitrosalicyclic Acid (DNS) assay for reducing sugars

- DNS Reagent

3,5-Dinitrosalicyclic acid (Sigma)	2.65 g
NaOH	4.95 g
NaK-tartrate (Merck)	76.5 g
Phenol (Merck)	1.9 g
Na-Metabisulphate (Sigma)	2.075 g
Sterile milli-Q water to	354 ml

Dissolve the 3,5-Dinitrosalicyclic acid, NaOH and NaK-tartrate in the water, before adding and dissolving the other constituents in turn. The phenol was melted at 50 ºC. A three ml aliquot of the solution must be titrated to an endpoint with 5-6 ml of 0.1M HCl using phenolphthalein as an endpoint indicator. If the pH is correct, the pinkish colour will be lost after adding the 5-6 ml of 0.1M HCl.

- 0.5% (w/v) Glucose

Glucose (Saarchem)	0.5 g
Sterile milli-Q water to	100 ml

Prepare fresh on the day. Do not autoclave.

APPENDIX B

STANDARD METHODS

CONTENTS

B.1 Preparation of *E. coli* competent cells

B.1.1 Preparation of *E. coli* competent cells by $CaCl_2$ shock treatment
(Dagert and Ehrlich, 1979)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate, into 5 ml Ψ-broth (Appendix A.1.7) and incubate for 16 hours at 37 °C with shaking. Inoculate this starter culture into 100 ml pre-warmed Ψ-broth and incubate as before, until the A_{600} reaches between 0.3 - 0.6. Transfer the 100 ml culture to a pre-chilled (-20 ºC) GSA centrifuge tube (Beckman) and harvest the cells by centrifugation (3,000x g for 5 minutes at 4 °C). Discard the supernatant fraction, gently resuspend the cells in 100 ml of ice-cold 0.1 M $MgCl_2$ (Appendix A.2.5) and incubate on ice for one minute. Collect the cells as before, gently resuspend them in 50 ml of ice-cold 0.1 M $CaCl_2$ (Appendix A.2.5) and incubate on ice for approximately 2 hours. Harvest the cells as before, and gently resuspend them in 10 ml ice cold 0.1 M $CaCl_2$. Aliquot (0.1 ml) into sterile microfuge tubes, on ice, and transform with plasmid DNA (Appendix B.2).

B.1.2 Preparation of *E. coli* competent cells by the RbCl method
(Draper *et al.,* 1988)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate, into 5 ml Ψ-broth (Appendix A.1.7) and incubate for 16 hours at 37 °C with shaking. Inoculate this starter culture into 100 ml pre-warmed Ψ-broth and incubate, as before, until the A_{600} reaches 0.35 (the culture contains approximately 3.5 – 4.0 x 10^7 cells ml^{-1}). Transfer the 100 ml culture to a pre-chilled (-20 ºC) GSA centrifuge tube and chill on ice for 15 minutes. Collect the cells by centrifugation (3,000x g for 5 minutes at 4 °C) and discard the supernatant fraction. Gently resuspend the cells in 21 ml ice-cold TFB 1 (Appendix A.2.5) and incubate on ice for 90 minutes. Collect the cells as before and resuspend in 3.5 ml ice-cold TFB 2 solution (Appendix A.2.5). Aliquot (100 µl) resuspended competent cells into sterile microfuge tubes, rapidly freeze in liquid nitrogen and store at -70 °C.

B.2 Transformation of competent *E. coli* cells

Thaw the 0.1 ml aliquots of frozen *E. coli* competent cells (Appendix B.1.2) on ice for 10 minutes until just molten, or incubate freshly prepared 0.1 ml aliquots of *E. coli* competent cells (Appendix B.1.1) on ice for 10 minutes. Add 10 ng of plasmid DNA to 100 µl of thawed competent cells and incubate on ice for 20 minutes. Heat shock cells at 37 ºC for 1 minute and immediately incubate on ice for 2 minutes, before adding 0.8 ml Ψ-broth (Appendix A.1.7) and incubating at 37 ºC, with shaking, for 30 - 60 minutes to allow expression. Thereafter, plate 100 µl aliquots of the *E. coli* cells onto Luria-Bertani solid media (LA) (Appendix A.1.6) containing an appropriate antibiotic selection (Appendix A.2.2) and incubate for 16 hours at 37 ºC.

B.3 Small-scale preparation of plasmid DNA

(Ish-Horowicz and Burke, 1981)

Inoculate a single bacterial colony off a freshly streaked *E. coli* plate into 5 ml Luria-Bertani broth (LB) (Appendix A.1.5) with antibiotic selection (Appendix A.2.2), and incubate for 16 hours at 37 °C with shaking. Harvest the cells by centrifugation (13,000x g for 1 minute at 22 ºC), resuspend the bacterial pellet in 0.2 ml Solution 1 (Appendix A.2.7) and incubate for 10 minutes at 22 ºC. Lyse the cells by adding 0.4 ml Solution 2 (Appendix A.2.7), gently mix by inverting the microfuge tube several times and incubate at 22 ºC for 5 minutes. To this mixture, add 0.3 ml of ice-cold Solution 3 (Appendix A.2.7) and incubate on ice for 10 minutes. Pellet the cell debris by centrifugation (13,000x g for 5 minutes at 22 ºC), recover the supernatant fraction containing the plasmid DNA and transfer it to a sterile microfuge tube. Precipitate the plasmid DNA by the addition of 0.7 volumes of isopropanol. Mix the solution and centrifuge (13,000x g for 15 minutes at 4 °C) to pellet the plasmid DNA. Gently remove the supernatant, wash the pellet in 70% (v/v) ethanol (Appendix A.2.3) and centrifuge as before. Remove the supernatant and air-dry the pellet before resuspending it in 30 µl of TE buffer containing RNAse (Appendix A.2.4).

B.4 Preparation of bacterial chromosomal DNA

B.4.1 Large-scale preparation of bacterial chromosomal DNA

Grow a 100 ml culture of the bacterial strain for 16 hours. Harvest the cells by centrifugation (6,000x g for 10 minutes at 4 °C) and discard the supernatant fraction. Resuspend the bacterial pellet in 9.5 ml TE buffer (Appendix A.2.3), to this add 0.5 ml 10% (w/v) SDS (Appendix A.2.4) and 0.1 ml of 20 mg ml^{-1} proteinase K (Appendix A.2.4). Mix gently and incubate at 37 °C for 60 minutes. Add 1.8 ml 5 M NaCl (Appendix A.2.4) and mix thoroughly. Add 1.5 ml pre-warmed CTAB/NaCl solution (Appendix A.2.4), mix thoroughly and incubate at 65 °C for 20 minutes. Extract with an equal volume of chloroform/iso-amyl alcohol (Appendix A.2.4), and separate the phases by centrifugation (8,000x g for 10 minutes at 22 °C). Remove the aqueous phase to a clean centrifuge tube and precipitate the chromosomal DNA by the addition of 0.6 volumes of isopropanol. Mix the solution and centrifuge (13,000x g for 15 minutes at 4 °C) to pellet the chromosomal DNA. Gently remove the supernatant, wash the pellet in 70% (v/v) ethanol (Appendix A.2.3) and centrifuge as before. Remove the supernatant and air-dry the pellet before resuspending it in 1 ml of TE buffer containing RNAse (Appendix A.2.4). Quantitate the chromosomal DNA using the Nanodrop® ND-

100 spectrophotometer (Nano Drop Technologies, Inc.) and assess the integrity of the chromosomal DNA by electrophoresis of 1 µg of DNA on a 0.7% (w/v) TAE (Appendix A.2.6) agarose gel (Appendix B.6).

B.4.2 Small-scale bacterial chromosomal DNA extraction
(adapted from Wang *et al.,* 1996)

Grow a 5 ml culture of the bacterial strain for 16 hours. Harvest the cells by centrifugation (8,000x g for 5 minutes at 22 ºC) and resuspend the bacterial pellet in 0.5 ml lysozyme buffer (Appendix A.2.4). Incubate the resuspended bacterial cells for 30 minutes at 37 ºC. Add 0.05 ml 10% SDS (Appendix A.2.4), mix gently and incubate for 30 minutes at 65 °C. Extract with an equal volume of phenol (pH 8.0) (Sigma) and separate the phases by centrifugation (8,000x g for 5 minutes at 22 °C). Remove the aqueous phase to a clean centrifuge tube and repeat the phenol extraction as before. Thereafter, remove the aqueous phase to a clean centrifuge tube and extract with an equal volume of chloroform/iso-amyl alcohol (Appendix A.2.4), and separate the phases by centrifugation (8,000x g for 5 minutes at 22 °C). Remove the aqueous phase to a clean centrifuge tube and precipitate the chromosomal DNA by the addition of one volume of isopropanol. Gently mix the solution and centrifuge (13,000x g for 5 minutes at 4 °C) to pellet the chromosomal DNA. Thereafter, gently remove the supernatant and air-dry the pellet before resuspending it in 0.1 ml of TE buffer containing RNase A (Appendix A.2.4). Incubate at 37 ºC for 60 minutes and extract with an equal volume of phenol/chloroform (Appendix A.2.4). Precipitate the chromosomal DNA by the addition of one volume of isopropanol. Gently mix the solution and centrifuge (13,000x g for 5 minutes at 4 °C) to pellet the chromosomal DNA. Gently remove the supernatant and air-dry the pellet before resuspending it in 50 µl TE buffer (Appendix A.2.3) at 4 ºC. Quantitate the chromosomal DNA using the Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.) and assess the integrity of the chromosomal DNA by electrophoresis of 1 µg of DNA on a 0.7% (w/v) TAE (Appendix A.2.6) agarose gel (Appendix B.6).

B.5 Restriction endonuclease digestions
(Ausubel *et al*., 1989 unit 3.1; www.fermentas.com)

All restriction enzymes and their respective buffers were obtained from Fermentas and Roche. Carefully pipette 0.5 to 10 µg of either plasmid or chromosomal DNA into a sterile microfuge tube. The appropriate restriction enzyme buffer was added to a final concentration of 1x, or where required 2x (as recommended by the manufacturer) and adjust the volume to 18 µl with sterile water. Add restriction endonuclease (1 to 5 U μg^{-1} DNA) and make up to a final volume of 20 µl with sterile water. Pulse tube briefly in a bench-top centrifuge and incubate the reaction mixture for 16 hours in a water bath at the appropriate temperature (ºC). Stop the restriction endonuclease reaction by heat inactivation (as recommended by the manufacturer) and/or adding 4 µl tracking dye (Appendix A.2.6).

B.6 Agarose gel electrophoresis

(Ausubel *et al*., 1989 unit 2.5)

Melt the agarose (Hispanagar D1 LE) in 1x TAE (Appendix A.2.6) by heating in a microwave. Agarose concentrations can vary from 2% (w/v) for separating small DNA fragments to 0.7% (w/v) for separating larger DNA fragments such as restriction enzyme digested chromosomal DNA. Add ethidium bromide (Appendix A.2.3) solution to the melted agarose to a final concentration of 0.5 µg ml^{-1}. Allow agarose to cool before pouring into a gel-casting platform that has been sealed with masking tape and has a gel comb approximately 1 cm from the top of the gel-casting platform. After the gel has hardened, remove the masking tape from the gel-casting platform and carefully withdraw the comb ensuring that the wells are not damaged in the process. Place the gel-casting platform containing the set gel into an electrophoresis tank and add sufficient 1x TAE buffer to cover the gel. Load DNA samples into the wells of the gel. Attach leads so that DNA migrates into the gel towards the anode. Electrophorese the gel at 1 to 10 V cm^{-1} until the dye reaches the end of the gel. Visualize the DNA on a Gel Doc XR (Bio-Rad) system using Quantity One Version 4.5.2 Software.

B.7 Total RNA isolation from Gram-negative bacteria

(Ausubel *et al*., 1989 unit 4.4)

Incubate a bacterial culture for approximately 16 hours before collecting 10 ml of cells by centrifugation (10,000x g for 10 minutes at 4 ºC). Discard the supernatant fraction, resuspend the harvested cells in 10 ml protoplast buffer (Appendix A.2.10) and incubate on ice for 15 minutes. Collect the resulting protoplasts by centrifugation (8,000x g for 5 minutes at 4 ºC) and resuspend in 0.5 ml lysis buffer (Appendix A.2.9) and 15 µl DEPC (Sigma). Thereafter, incubate the tubes at 37 ºC for 5 minutes before adding salt-saturated NaCl (Appendix A.2.9) to precipitate most of the SDS contained within the lysis buffer. Remove the white precipitate by centrifugation (13,000x g for 10 minutes). Precipitate the nucleic acids within the supernatant by adding ice-cold absolute ethanol, followed by centrifugation at 13,000x g for 10 minutes at 4 ºC to collect the precipitated nucleic acids. Remove any remaining salts by washing the nucleic acid pellet in ice-cold 70% (v/v) ethanol (Appendix A.2.9) and resuspend in 20 µl of sterile DEPC treated water (Appendix A.2.9).

Treat the total RNA samples with DNAse I (Promega) to remove any contaminating chromosomal and/or plasmid DNA, by adding 10 U of DNAse I to the RNA sample in a total volume of 50 µl and incubating at 37 ºC for 3 hours. Recover the RNA by adding an equal volume of phenol (pH 4.0)/chloroform/isoamylalcohol (25:24:1) (Appendix. A.2.9), separate the phases by centrifugation (13,000x g for 10 minutes at 22 ºC) and transfer the aqueous phase to a clean sterile microfuge tube. Add 5 µl of 3 M sodium acetate (Appendix A.2.9) and 150 µl ice-cold absolute ethanol (Merck) to the RNA sample and centrifuge the mixture (13,000x g for 10 minutes) to precipitate the RNA. Resuspend the RNA sample in 20 µl sterile DEPC water (Appendix A.2.9) and quantitate using the Nanodrop® ND-100 spectrophotometer (Nano Drop Technologies, Inc.). Assess the RNA integrity by electrophoresis of 1 µg of total RNA on a 1.5% (w/v) agarose formaldehyde gel (Appendices B.13.1 and B.13.2) and visualize on a Gel Doc XR (Bio-Rad) system using Quantity One Version 4.5.2 Software.

B.8 Southern hybridization

B.8.1 Southern transfer of DNA from an agarose gel onto nitrocellulose membrane
(Coyne *et al*., 2002)

Soak the agarose gel in 0.25 M HCl (Appendix A.2.8) for 5 minutes at 22 ºC, before rinsing the gel in sterile ultrapure water. Saturate 10 sheets Whatman 3MM paper with 0.4 M NaOH (Appendix A.2.8) and place these sheets on top of an inverted gel-casting tray which has been placed in a tray covered with Cling wrap (Glad). Add 0.4 M NaOH/1 M NaCl (Appendix A.2.8) to the tray so that the ends of the Whatman paper are flooded. Invert the gel and place on top of the saturated Whatman paper, ensuring that no air bubbles remain trapped under the gel. Cut Hybond N+ nylon membrane (Amersham Pharmacia Biotech) to the size of the gel (15 x 20 cm). Wet the membrane in water and place on gel, ensuring that all the air bubbles are removed. Cover the edges of the membrane with Cling wrap. Place 3 x sheets (15 x 20 cm) Whatman 3MM paper over the membrane, followed by a 10 cm stack of dry absorbent paper towel. Place a glass plate on top of the towels, followed by a 0.2 - 0.4 kg weight and blot for 3 - 16 hours. Mark the position of the wells of the gel on the membrane with a blunt pencil and rinse the membrane in 2x SSC (Appendix A.2.8) for 5 minutes at 22 ºC. Air-dry the membrane on dry Whatman 3MM paper and store between 2 sheets of Whatman 3MM paper at 22 ºC.

B.8.2 Radiolabelling DNA by random prime labelling
(Protocol: Roche Random Primed DNA Labelling Kit)

All reagents used in this protocol were supplied in the Random primed DNA labelling kit (Roche). Transfer 25 ng of DNA to a sterile microfuge tube and denature the DNA fragments by heating for 10 minutes at 95 ºC, before rapidly cooling on ice. Add 3 µl of a dATP, dGTP, dTTP mixture (consisting of 1 µl of each dNTP) and 2 µl of reaction mixture. Add 50 µCi [α-^{32}P] dCTP and make up the reaction volume to 19 µl with sterile water. Add 2U Klenow enzyme and incubate the reaction mixture at 37 ºC for 30 minutes. Separate the labelled DNA from the unincorporated nucleotides using a spin column (Appendix B.8.3).

B.8.3 Separation of radioisotope labelled DNA from unincorporated nucleotides using spin columns

(Ausubel *et al*., 1989 unit 3.4)

Plug the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool and place the syringe in a disposable bench top centrifuge tube. Prepare a Sephadex G-50 (Appendix A.2.9) column with a bed volume of 0.9 ml in the syringe. Wash the column with 0.1 ml STE buffer (Appendix A.2.8). Add 10 µl tracking dye (Appendix A.2.8) and 40 µl STE buffer (Appendix A.2.8) to the labelled DNA sample. Place a microfuge tube (with its cap removed) at the bottom of the centrifuge tube to collect the labelled probe from the spin column. Load the DNA onto the spin column and centrifuge at 1,600x g for 4 minutes. The unincorporated nucleotides migrate with the Orange G on the column, while the Dextran Blue is eluted off the column together with the labelled DNA probe. Determine the specific activity of the radio-labelled DNA by counting 1 µl of probe in 2 ml of scintillation fluid (Appendix A.2.8) on a scintillation counter (Beckman LS1701). Specific activity is expressed as counts per minute (cpm) per µg DNA.

B.8.4 Prehybridisation, hybridisation and washing of radioactive southern blots

(Church and Gilbert, 1984)

Place the Hybond N^+ membrane containing the transferred DNA in a hybridization bottle (Ammersham). Add approximately 20 ml of Church pre-hybridization buffer (pre-CHB, Appendix A.2.8) so that the membrane is covered. Close the hybridization bottle and incubate for 30 minutes at 65 ºC with gentle agitation. Denature the radio-labelled probe by heating for 10 minutes at 95 ºC followed by rapid cooling on ice. Remove the pre-CHB from the hybridization bottle and add approximately 20 ml of fresh Church hybridization buffer (CHB, Appendix A.2.8) to the membrane so that the membrane is covered. Add 1.0×10^6 cpm of labelled probe per ml of CHB and incubate overnight at 65 ºC with gentle agitation.

Discard the CHB containing the radio-labelled probe and wash the membrane in Wash buffer A (WBA; Appendix A.2.8), followed by Wash buffer B (WBB; Appendix A.2.8) at 65 ºC for 10 minutes. Monitor the radioactivity on the membrane after each wash using a Geiger counter. If the radiation on the membrane reaches 200 to 500 cpm, stop washing. Seal the membrane in a plastic bag and place it in an X-ray cassette containing enhancer screens. Expose the membrane to X-ray film

(Hyperfilm, Amersham) at -70 ºC and develop manually. Rinse the developed X-ray with tap water and air-dry for approximately 30 minutes.

B.8.5 DIG-labelling DNA by random primer labelling

(Protocol: Roche DIG High Prime DNA Labelling and Detection Starter Kit II)

All reagents used in this protocol were supplied in the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). Transfer 10 ng to 3 µg of template DNA and sterile water in a maximum volume of 16 µl to a sterile microfuge tube and denature the DNA fragments by heating for 10 minutes at 95 ºC, before cooling rapidly on ice. Add 4 µl of the DIG-High prime (vial 1), mix and incubate at 37 ºC for 1 hour to 20 hours. Stop the labelling reaction by adding 2 µl of 0.2 M EDTA (pH 8.0) (Appendix A.2.3) and heating to 65 ºC for 10 minutes. Store the labelled probe DNA in a sterile microfuge tube at 4 ºC.

B.8.6 Determination of DIG-labelling efficiency

(Protocol: Roche DIG High Prime DNA Labelling and Detection Starter Kit II)

Prepare a dilution series (10^{-1} to 10^{-10}) of the DIG-labelled probe DNA in sterile distilled water. Spot 1 µl aliquots of each dilution onto a small piece of nylon membrane and dry at 22 ºC for 15 minutes. Block the membrane by incubating in a small volume of blocking solution (Appendix A.2.8) for 30 minutes with gentle agitation at 22 ºC. Centrifuge the anti-digoxigenin-AP (Roche) antibody vial (13,000x g for 5 minutes at 4 ºC) and add the appropriate volume to the blocking solution. Incubate the membrane for 30 minutes in the antibody [anti-digoxigenin-AP (Roche)] containing blocking solution with gentle agitation at 22 ºC. Thereafter, wash the membrane twice for 15 minutes in ample washing buffer and equilibrate for 5 minutes at 22 ºC in 20 ml detection buffer (Appendix A.2.8).

Visualize the hybridized DIG-labelled probes by placing each membrane (DNA side up) in an opened hybridization bag and applying 1 ml of the defrosted chemiluminescence substrate CSPD (Roche). Spread the substrate evenly over the membrane by folding over the opened bag. Remove excess substrate, after incubation for 5 minutes at 22 ºC, by squeezing it out of the hybridization bag. Seal the edges of the bag to prevent the membrane drying out. Incubate the damp membrane for 10 minutes at 37 °C to enhance the luminescent reaction. Expose the membrane to X-ray film (Hyperfilm, Amersham) at 22 °C in an X-ray cassette. Develop the X-ray film manually as described in Appendix B.8.4. Visualize the results and compare the intensities of the dilution series of the DIG-lebelled probe to determine the appropriate dilution to be used in the hybridization with the target DNA.

B.8.7 Prehybridization, hybridization and washing of DIG-labelled southern blots

(Protocol: Roche DIG High Prime DNA Labelling and Detection Starter Kit II)

Pre-hybridize the nylon membrane in DIG Easy Hyb (Roche) at 42 ºC for 45 minutes with gentle agitation. Add the DIG-labelled DNA probe to pre-heated DIG Easy Hyb (3.5 ml/100 cm^2 membrane) at a concentration of 2.5 ng ml^{-1} of hybridization buffer and mix well without foaming as bubbles may lead to background. Discard the pre-hybridization solution and add the probe-containing DIG Easy Hyb mixture to the membrane. Hybridize overnight with gentle agitation at the desired hybridization temperature.

Wash the membrane twice for 5 minutes in 2× SSC, 0.1 % SDS (Appendix A.2.8) at 22 ºC with constant agitation. Thereafter, wash the membrane twice for 15 minutes in 0.5× SSC, 0.1% SDS (pre-warmed to 65 °C) (Appendix A.2.8) at 65 °C with constant gentle agitation. Rinse the membrane for 5 minutes in washing buffer (Appendix A.2.8), before blocking the membrane by incubating in approximately 25 ml of blocking solution (Appendix A.2.8) for 2 hours with gentle agitation. Centrifuge the anti-digoxigenin-AP (Roche) antibody vial (13,000x g for 5 minutes at 22 ºC) and add the appropriate volume of it to blocking solution. Incubate the membrane for 30 minutes in the antibody [anti-digoxigenin-AP (Roche)] solution (Appendix A.2.8) with gentle agitation. Wash the membrane twice for 15 minutes in ample washing buffer and equilibrate for 5 minutes in 20 ml of detection buffer (Appendix A.2.8).

Visualize the hybridized DIG-labelled probes by placing each membrane (DNA side up) in an opened hybridization bag and applying 1 ml of the defrosted chemiluminescence substrate CSPD (Roche). Spread the substrate evenly spread over the membrane by folding over the opened bag. Remove excess substrate after incubation for 5 minutes at 22 ºC by squeezing it out of the hybridization bag. Seal the edges of the bag to prevent the membrane drying out. Incubate the damp membrane for 10 minutes at 37 °C to enhance the luminescent reaction. Expose the membrane to X-ray film (Hyperfilm, Amersham) at 22 °C in an X-ray cassette containing enhancer screens. Develop the X-ray film manually as described in Appendix B.8.4.

B.9 Repairing 3' and 5' overhanging ends to generate blunt ends

(Ausubel *et al.*, 1989 unit 3.5.8)

Prepare the reaction mixture by adding the restriction enzyme digested DNA (1 - 4 µg), 2 µl of 10x Klenow Fragment reaction buffer, 5 U Klenow Fragment and 1 µl of 0.5 mM dNTP mix into a sterile microfuge tube and make up to a total volume of 20µl with nuclease-free water. Incubate the reaction mixture at 37 °C for 30 minutes and stop the reaction by incubating the reaction mix at 75 °C for 10 minutes.

B.10 Dephosphorylation of vector DNA

(Coyne *et al.,* 2002)

Calf intestinal phosphatase (CIP) is commonly used to enzymatically remove the 5'-phosphate groups from linearised DNA. This prevents linearised vector DNA recircularising in an intermolecular ligation.

Heat-inactivate the restriction enzyme digest according to the restriction enzyme manufacture's instructions. Prepare the reaction mixture by adding the heat inactivated restriction enzyme digested DNA (1 -20 pmol ends), 3 µl of 10x CIP buffer, 0.1 U CIP into a sterile microfuge tube and make up to a total volume of 30µl with sterile water. Incubate the reaction mixture at 37 °C for 30 minutes and then stop the reaction by incubating the reaction mix at 65 °C for 10 minutes. Prepare the DNA for ligation by ethanol precipitation (Appendix B.11) or electrophoresis on an agarose gel followed by gel extraction using a commercial kit.

B.11 Ethanol precipitation of DNA

(Coyne *et al.,* 2002)

Adjust the salt concentration of the DNA sample by adding 1/10 volume of 3 M sodium acetate, pH 5.2 (Appendix A.2.3) and mix well. Add 2 - 2.5 volumes of ice-cold absolute ethanol, mix well and add 5 - 10 µg of yeast tRNA (Sigma). Incubate this mixture at -20 ºC for 30 minutes and pellet the DNA by centrifugation (13,000x g for 15 minutes at 4 ºC). Wash the pellet with 1 ml of ice-cold 70% (v/v) ethanol (Appendix A.2.3) and resuspend the DNA in sterile distilled water or TE buffer (Appendix A.2.3).

B.12 Ligations

(Coyne *et al.,* 2002)

B.12.1 Intramolecular ligations

In order to recircularize plasmid DNA for the construction of deletion subclones, use approximately 1 pmol of plasmid DNA. Add 2 µl of 10x T4 ligase buffer (Fermentas) to the plasmid DNA in a sterile microfuge tube. Adjust the volume to 18 µl with sterile nuclease-free water, add 2 U of T4 ligase (Fermentas) and incubate the ligation reaction for approximately 16 hours at 15 ºC.

B.12.2 Intermolecular ligations

In order to ligate two different DNA fragments (vector and insert) the total DNA concentration should not exceed 10 pmol and the ratio of vector to insert (V:I) should be in the range of 1:1 to 1:4 pmol. Add 2 µl of 10x T4 ligase buffer (Fermentas) to the DNA fragments in a sterile microfuge tube. Adjust the volume to 18 µl with sterile nuclease-free water, and add 2 U of T4 ligase (Fermentas). For the ligation of DNA fragments with cohesive ends, incubate the reaction overnight at 15 ºC. For the ligation of DNA fragments with blunt ends, use 10x more T4 ligase enzyme and incubate the reaction for 4 to 16 hours at 22 ºC.

B.13 Northern hybridization

All the solutions, apparatus and glassware were treated as recommended by Sambrook *et al.* (1989) for RNA applications.

When working with RNA, clean the gel electrophoresis apparatus by rinsing the equipment with 1% SDS, DEPC treated water, and a brief rinse with absolute ethanol. Thereafter, the electrophoresis tank and gel tray should be soaked in 3% H_2O_2 for 10 minutes, before being rinsed with DEPC treated water (Appendix A.2.9).

B.13.1 Preparation of RNA for electrophoresis
(Sambrook *et al.* 1989, unit 7.43)

Mix the RNA sample and the RNA sample application buffer (Appendix A.2.10) in a 1:1 ratio. RNA secondary structures are denatured by heating the sample at 65 ºC for 15 minutes and rapidly cooling on ice before loading the gel.

B.13.2 Preparation and electrophoresis of RNA formaldehyde gels
(Ausubel et al., 1989, unit 2.5)

For a 1.2% (w/v) agarose gel melt 0.72 g of agarose, and for a 1% (w/v) agarose gel melt 0.6 g of agarose, in 43.92 ml DEPC treated water (Appendix A.2.9). Allow to cool to approximately 60 °C before adding 10 ml of 37% formaldehyde and 6 ml 10x MOPS buffer (pH 7.0) (Appendix A.2.10). Allow the melted agarose to cool to approximately 55 ºC before pouring the gel and allowing it to set in a fume-cupboard. Once the gel has set, place it in an electrophoresis tank containing sufficient 1x MOPS buffer (Appendix A.2.10) to cover the gel.

Load the RNA samples and a RNA molecular weight marker (Promega) into the wells of the gel. Electrophorese the RNA formaldehyde gel at 3-4 V cm^{-1} for approximately 1.5 hours, before visualizing the RNA on a Gel Doc XR (Bio-Rad) system using Quantity One Version 4.5.2 Software.

B.13.3 Northern transfer of RNA onto nitrocellulose membrane

(Reed and Mann, 1985; and Sambrook et al. 1989, unit 7.46)

Soak the RNA formaldehyde gel in 2x volumes sterile DEPC treated water (Appendix A.2.9) at 22 ºC for 30-60 minutes, with gentle shaking to remove the formaldehyde. Thereafter, rinse the gel several times in 2x volumes sterile DEPC treated water and then soak the gel in 2x volumes 7.5 mM NaOH (Appendix A.2.11) for 20 minutes. Soak the gel in 2x volumes 20x SSC (Appendix A.2.11) for 45 minutes and saturate 10 sheets (10 x 15 cm) Whatman 3MM paper with 1x SSC (Appendix A.2.11). Place these sheets on top of an inverted gel-casting tray which has been placed in a tray covered with cling wrap. Add 1x SSC to the tray so that the ends of the Whatman paper are submerged. Invert the gel and place on top of the saturated Whatman paper, ensuring that no air bubbles are trapped under the gel. Cut Hybond N^+ nylon membrane (9 x 12 cm) (Amersham). Wet the membrane in distilled water and place on top of the gel, ensuring that no air bubbles are trapped between the gel and the membrane. Cover the edges of the membrane with cling wrap, to prevent a short circuit. Cover the membrane with 3 sheets of Whatman 3MM paper (9 x 12 cm) and place a 10 cm stack of dry absorbant paper towel on top. Place a glass plate on top of the towels, followed by a 0.2 to 0.4 kg weight and blot for 2 to 16 hours.

Mark the wells of the gel on the membrane with a blunt pencil and rinse the membrane in 2x SSC / 0.1% SDS (Appendix A.2.11) for 5 minutes at 22 ºC. Air-dry the membrane on dry Whatman 3MM paper and then UV crosslink the RNA onto the membrane (Ultraviolet Crosslinker, Amersham Life Science). The membrane is stored between two sheets of Whatman 3MM paper.

B.13.4 Prehybridization, hybridization and washing of Northern blots

As described in Appendix B.8.7.

B.14 Preparation of soluble bacterial cell extracts by sonication

Grow the bacterial culture of interest in an appropriate growth medium under the desired culture conditions. Harvest the bacterial cells by centrifugation (8,000x g for 15 minutes at 4 ºC) and wash once with 1x PBS (Appendix A.2.3) to remove any contaminating media components. Resuspend the bacterial cells in 1/10th culture volume of 1x PBS and transfer to a standard container. Place this standard container, holding the resuspended bacterial cells, in a beaker containing a mixture of ice and 70% ethanol (Appendix A.2.3) to prevent heat denaturation of proteins during sonication. The sonication protocol involves four bursts of 20 seconds each, at a power setting of approximately 25% (Virsonic Sonicator), with a 20 second pause between each burst to ensure the cellular extracts do not heat up and the proteins get denaturated. Thereafter, pellet the insoluble cell debris by centrifugation (13,000x g for 5 minutes at 4 ºC) retain the supernatant containing the soluble intracellular proteins and store at -20 ºC.

B.15 ELISA method

Coat a 96-well polysorb ELISA plate (Nunc™) with 100 µl per well of the antigen. Cover the polysorb plate with Cling Wrap and incubate for approximately 16 hours at 4 °C to allow the antigen to bind and coat the wells of the ELISA plate. Discard excess unbound antigen and gently rinse the wells in the polysorb plate with three sequential washes of 200 µl TBS Tween (TBST) (Appendix A.2.22). Block the wells of the ELISA plate to prevent non-specific binding with 200 µl of ELISA blocking solution (Appendix A.2.22) for 1 hour at 22 ºC. Thereafter, discard the blocking solution and rinse the wells of the polysorb ELISA plate with three brief TBST washes. Prepare a serial dilution of primary antibody (1×10^{-1} to 1×10^{-8}) in TBST. Add 100 µl of serially diluted primary antibody to each well and incubate at 22 ºC for 1 hour. The microtitre plate reader (TitreTek Multiscan Machine) reads an entire row at a time and therefore the first row of the microtitre plate serves as a blank. As a result, no primary antibodies should be added to the first row of wells. After the primary antibody incubation step, discard the excess primary antibody dilutions and rinse the wells as described above. Add 100 µl of secondary antibody [1:2,500 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in TBST] to each well and incubate at 22 ºC for 30 minutes. After the secondary antibody incubation step, discard the excess secondary antibody and rinse the wells as before. Equilibrate the wells, briefly, with 100 µl of 10% Diethanolamine, 0.5 mM $MgCl_2$ (Appendix A.2.22), before incubating the wells with 100 µl of ELISA substrate (Appendix A.2.22) at 22 ºC for 30 to 60 minutes in the dark. As soon as a positive yellow signal is clearly visible, scan (405 nm) the microtitre plate on the TitreTek Multiscan machine.

B.16 PEG precipitation for purification of IgG polyclonal antibodies

(Adapted from Polson *et al.,* 1964)

Add two volumes of borate-buffered saline (Appendix A.2.23) to one volume of serum. Crush some PEG 6000 (Sigma) with a sterile pestle and mortar and add the ground PEG 6000 to the diluted serum to a final concentration of 14% (w/v). Gently mix and dissolve the PEG 6000 by inversion. Centrifuge the mixture (13,000x g for 10 minutes at 4 °C) and dissolve the pellet in the original serum volume of 1x PBS (Appendix A.2.23). Once again, add ground PEG 6000 to a final concentration of 14% (w/v), mix gently and dissolve by inversion. Centrifuge the mixture (13,000x g for 10 minutes at 4 °C), re-dissolve the pellet in 1x PBS containing 60% (v/v) glycerol (Appendix A.2.23) and store in aliquots at -20 °C.

B.17 Pre-absorbing polyclonal antibodies against bacterial cell extracts

Grow an overnight bacterial culture of the strain of interest and prepare the soluble bacterial cell extracts by sonication as described in Appendix B.14. Prepare an 8% SDS-PAGE gel (Appendix B.19). Separate the soluble cell extract proteins by electrophoresis at a constant current of 30 mA for approximately 2 hours at 4 ºC as described in Appendix B.19. After electrophoresis, remove the gels from the assembled gel plates and transfer the proteins to nitrocellulose membrane, as described in Appendix B.21.1. Following electroblotting, dismantle the transfer apparatus and place the membrane in blocking solution (Appendix A.2.24) for 1 hour at 22 ºC with gentle shaking. Discard the blocking solution and incubate the membrane in primary antibody [1:1000 dilution of (PEG-precipitated and purified IgG antibodies, as described in Appendix B.16) in blocking buffer] at 4 ºC with gentle agitation for approximately 16 hours. Use the blocking buffer, containing the pre-absorbed antibodies, as the primary antibody mixture for western blots and/or immunohistochemical experiments.

B.18 Pre-absorbing polyclonal antibodies against abalone

(Adapted from Streit and Stern, 2001)

B.18.1 Preparation of abalone powder

Sacrifice twenty *H. midae* spat (<10 mm shell length) that have been maintained on ABFEED® S34 weaning chips for approximately 3 weeks, by incubating directly on ice for 30 minutes. Remove their shells with the back of a metal spatula. Place these shucked abalone in an SS34 centrifuge tube (Beckman) containing 10 ml ice-cold 1x PBS (Appendix A.2.3) and thoroughly homogenize them using a tissue disruptor. Add 40 ml of ice-cold acetone (Saarchem), gently mix and incubate on ice for

30 minutes. Collect the abalone homogenate by centrifugation (13,000x g for 10 minutes at 4 ºC), before washing the protein pellet with 40 ml of ice-cold acetone. Remove the pelleted abalone homogenate from the centrifuge tube, grind it into a fine powder and spread it out on a piece of Whatman 3MM paper to air-dry. Once dry the abalone powder was stored in a sterile plastic bottle at 4 ºC.

B.18.2 Pre-absorbing polyclonal antibodies against abalone powder

To pre-absorb the anti-VmproA polyclonal antibodies against abalone tissues, weigh out 3 mg of abalone powder (Appendix B.18.1) for every microliter of the PEG precipitated anti-VmproA polyclonal antibody stock (Appendix B.16) to be pre-absorbed. Add 1 ml of TBST (Appendix A.2.22) to the weighed out abalone powder, mix well by vortexing and incubate at 70 ºC for 30 minutes. Thereafter, mix again by vortexing and centrifuge (3,000x g for 1 minute at 22 ºC) to pellet the insoluble debris. Discard the supernatant and wash the pellet, as before, until the supernatant is clear. After the final wash resuspend the pellet in 1 ml of immunohistochemical buffer (Appendix A.2.28) and the desired dilution of PEG precipitated anti-VmproA polyclonal antibodies. Incubate this mixture for 2-3 hours at 22 ºC with gentle agitation. Harvest the ant-VmproA polyclonal antibody-containing supernatant by centrifugation (13,000x g for 5 minutes at 4 ºC), adjust to the final required dilution of antibody using immunohistochemical buffer and store at 4 ºC.

B.19 Preparing and running denaturing SDS-PAGE gels
(Ausubel *et al.,* 1989, unit 10.2)

Combine the following reagents for an 8% separating gel in a clean glass beaker:

1.5 M Tris-HCl, pH 8.8 (Appendix A.2.15)	5 ml
40% Acrylogel (Sigma)	4 ml
20% SDS (Appendix A.2.3)	0.1 ml
10% ammonium persulphate (Appendix A.2.15)	0.1 ml
TEMED	0.01 ml
Sterile water to	20 ml

Once the separating gel layer has been poured, gently layer isopropanol (Merck) on top to ensure an even interface between the separating and stacking gels. Once the separating gel has polymerized, remove the isopropanol and any unpolymerized acrylamide with several gentle rinses of stacking gel buffer (Appendix A.2.15), before pouring the stacking gel.

Combine the following reagents for a 4% stacking gel in a clean glass beaker:

0.5 M Tris-HCl, pH 6.8 (Appendix A.2.15)	1.25 ml
40% Acrylogel (Sigma)	0.5 ml
20% SDS (Appendix A.2.3)	0.025 ml
10% ammonium persulphate (Appendix A.2.15)	0.025 ml
TEMED	0.005 ml
Sterile water to	5 ml

Pour the stacking gel, insert the comb, and allow the stacking gel to polymerize. Once the stacking gel has polymerized, remove any unpolymerized acrylamide with several gentle rinses of stacking gel buffer (Appendix A.2.15). The polymerized polyacrylamide gels are clamped into the electrophoresis apparatus (Bio-Rad) and the reservoir tank filled with SDS-PAGE running buffer (Appendix A.2.15) that has been pre-chilled to 4 ºC.

Prepare the protein samples by adding 6x SDS-PAGE sample application buffer (Appendix A.2.15), before loading into the wells of the polyacrylamide gel. Electrophorese the gel at a constant current of 30 mA. After electrophoresis, remove the gels from the assembled gel plates and visualize the protein bands in the gel by staining with Coomassie SDS-PAGE stain (Appendix A.2.16) for 30 minutes at 37 ºC. Destain the gel in Coomassie SDS-PAGE gel destain solution (Appendix A.2.16) and store in gel storage solution (Appendix A.2.16).

B.19.1 Silver staining of polyacrylamide gels

(Blum *et al.,* 1987)

Remove the gels from the assembled gel plates and fix for 1 hour to 16 hours in Fix solution (Appendix A.2.17). Discard the fix solution and wash the gels with the first wash solution (Appendix A.2.17) three times for 20 minutes each. Thereafter, incubate the gels in pretreatment solution (Appendix A.2.17) for one minute, before washing the gels three times for 20 seconds each in the second wash solution (Appendix A.2.17). Rinse the gels twice for 20 seconds with distilled water. The protein bands on the gel are visualized by incubating the gels in the Develop solution (Appendix A.2.17) until the bands are clearly visible. The reaction is stopped by rinsing the gels in water twice for 2 minutes and then in stop buffer (Appendix A.2.17) for 10 minutes. Store the gel in 1% (v/v) Glacial acetic acid until photographed or scanned.

B.20 Preparing and running substrate SDS-PAGE gels (Zymograms)

B.20.1 Preparing and running SDS-Gelatine-PAGE gels

Combine the following reagents for an 8% separating gel in a clean glass beaker:

1.5 M Tris-HCl, pH 8.8 (Appendix A.2.15)	5 ml
5% Gelatine (Appendix A.2.18)	4 ml
40% Acrylogel (Sigma)	4 ml
20% SDS (Appendix A.2.3)	0.1 ml
10% ammonium persulphate (Appendix A.2.15)	0.1 ml
TEMED	0.01 ml
Sterile water to	20 ml

Pour the separating gel layer, gently layer isopropanol on top to ensure an even interface between the separating and stacking gels. Once the separating gel has polymerized, remove the isopropanol and unpolymerized acrylamide with several gentle rinses of stacking gel buffer (Appendix A.2.15), before the stacking gel is poured.

Prepare and pour the 4% stacking gel as described in Appendix B.19.

Clamp the polymerized gels into the Mighty Small II SE250 electrophoresis apparatus (Hoefer Scientific Instruments) and fill the reservoir tanks with SDS-PAGE running buffer (Appendix A.2.15) that has been pre-chilled to 4 ºC. Prepare the protein samples by filtering culture supernatant through a 0.22 µm syringe filter, before adding 2.5% (w/v) SDS (Appendix A.2.18) and 2% (v/v) glycerol (Appendix A.2.18) and incubating the mixture at 37 ºC for 30 minutes. Add zymogram sample application buffer (Appendix A.2.18) to a final 1x concentration, before loading the gel and separating the proteins at a constant current of 30 mA at 4 ºC. After electrophoresis, remove the gels from the assembled gel plates and incubate in Triton X-100 wash solution (Appendix A.2.18) for 1 hour at 22 ºC to remove the SDS. Thereafter, incubate the gels in Glycine incubation buffer (Appendix A.2.19) for 3 hours at 37 ºC. Visualize zones of proteolytic activity in the gel by staining the gel with Amido black staining solution (Appendix A.2.18) for 10 minutes at 22 ºC and destaining in Amido black destaining solution (Appendix A.2.18). Photograph or scan the gel and store in gel storage solution (Appendix A.2.16).

B.20.2 Preparing and running SDS-PAGE Casein zymograms

(Based on Saborowski *et al.,* 2004)

Prepare the SDS-PAGE gel according to the method outlined in Appendix B.19.

Prepare and electrophorese the protein samples as described in Appendix B.20.1, with the exception that the samples are loaded in duplicate on opposite sides of the polyacrylamide gel. After electrophoresis, remove the gels from the assembled gel plates, and cut in half with a sharp scalpel blade. Stain one half of the polyacrylamide gel with coomassie stain, as described in Appendix B.19, in order to assess the integrity and size of the protein samples. Incubate the other half in Triton X-100 wash solution (Appendix A.2.19) for 1 hour at 22 ºC to remove the SDS. Thereafter, rinse briefly in 100 mM Tris-HCl (pH 9.0) before incubating in casein penetrating solution (Appendix A.2.19) at 4 ºC for 30 minutes. Incubate the gel in the casein penetrating solution for a further 60 minutes at 22 ºC. Wash the gel thoroughly with sterile distilled water to remove any adhering casein, before visualizing any zones of proteolytic activity in the gel by staining the gel with coomassie stain, as outlined in Appendix B.19. The gel is photographed or scanned and stored in gel storage solution (Appendix A.2.16).

B.21 Western blot Analysis

B.21.1 Electroblotting of proteins onto nitrocellulose membrane

(Towbin *et al.,* 1979)

Remove the SDS-PAGE gel from the glass plates of the electrophoresis apparatus and soak the gel in Towbin blotting buffer (Appendix A.2.25) for 1 hour. Cut four sheets of Whatman 3MM filter paper and nitrocellulose membrane (Protran) to the size of the gel. Pre-wet the nitrocellulose membrane and Whatman 3MM filter paper pieces in Towbin blotting buffer (Appendix A.2.25) and place the gel on the membrane, ensuring that no air bubbles are trapped between the gel and membrane. Sandwich the membrane and gel between the filter paper and clamp the sandwich between the perforated perspex leaves of the transfer apparatus (Bio-Rad). Load the entire assembly into the transfer apparatus, containing the ice-pack and ensure that the chamber is filled with Towbin blotting buffer. Transfer at 100 V for 1 hour at room temperature.

B.21.2 Chemiluminescent detection of western Blots

Remove the membrane from the transfer apparatus and place it in blocking solution (Appendix A.2.24) for 1 hour at 22 ºC with gentle shaking. Discard the blocking solution and incubate the membrane for 3 to 16 hours in primary antibody (1:5000 dilution in blocking buffer) at 4 ºC. Thereafter, wash the membrane 4x 15 minutes in PBT wash solution (Appendix A.2.24) at 22 ºC with gentle agitation. Incubate the membrane for 3 hours in secondary antibody [1:10,000 dilution of goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (KPL, USA) in blocking buffer]. Thereafter, wash the membrane 4 x 15 minutes in PBT wash solution at 22 ºC with gentle agitation. Detection is carried out using the chemiluminescent substrate (LumiGLO®, KPL, USA) according to the manufacturer's instructions. The fluorescent signal is detected on X-ray film (HyperFilm, Amersham) at 22 °C in an X-ray cassette containing enhancer screens. Visualise the result following manual X-ray film development and air drying the film for approximately 30 minutes.

B.22 Azo-casein protease assay

(Long *et al.,* 1981)

Transfer 1 ml of culture to a microfuge tube and pellet the cells by centrifugation (13,000x g for 5 minutes at 22 ºC). Transfer 100 µl aliquots of culture supernatant to three separate microfuge tubes. Add 100 µl of the Azo-casein solution (Appendix A.2.20) to each of the microfuge tubes. Immediately add 200 µl of ice-cold 10% (w/v) TCA (Appendix A.2.20) to one of the three tubes containing culture supernatant and Azo-casein solution, place on ice (this is the control tube, t = 0) and incubate the remaining two tubes at 37 °C, unless otherwise indicated, for 30 minutes. After this incubation step, add 200 µl ice-cold 10% (w/v) TCA to each of the remaining two microfuge tubes and incubate on ice for 30 minutes.

Pellet the precipitated Azo-casein by centrifugation (13,000x g for 5 minutes at 22 ºC). Carefully transfer 0.3 ml of each supernatant to a 1.5 ml plastic disposable cuvette. Add 0.3 ml of 0.5 M NaOH (Appendix A.2.20) to each sample, mix and determine the absorbance of the solutions at 440 nm, using the control tube as a blank.

One unit of protease activity per ml of culture supernatant (U ml^{-1}) is an increase in absorbance of 0.1 OD units at 440 nm after 30 minutes at 37 °C.

B.23 DNS reducing sugar assay

(Miller, 1959)

The standard protocol for the DNS reducing sugar assay is used to determine the glucose concentration in growth media and cell-free supernatant. Briefly, add 50 µl of cell-free culture supernatant to 150 µl of DNS reagent (Appendix A.2.29) and boil for 5 minutes, before rapidly cooling on ice. Following the addition of 800 µl of distilled water, the amount of reducing sugar present in the supernatant is determined by measuring the absorbance at 510 nm (Beckman DU-64 spectrophotometer). For the assay blank, 50 µl of distilled water is added instead of culture supernatant, and the assay is preformed as described above. A reducing sugar standard curve is constructed by plotting light absorbance at 510 nm against known concentrations of glucose standards (Appendix A.2.29).

B.24 Bradford protein assay for protein quantitation

Aliquot (in triplicate) the following amounts of the 10 mg ml^{-1} BSA stock (Appendix A.2.13) and sterile distilled water into sterile microfuge tubes:

Tube no.	BSA (µl)	Distilled water (µl)	Final BSA concentration (µg ml^{-1})
Blank	0	100	0
1	2.5	97.5	2.5
2	5	95	5
3	10	90	10
4	15	85	15
5	20	80	20

Add 20 µl of the protein sample (in triplicate) to a sterile microfuge tube. Add 1 ml of the diluted Bradford's Reagent (Appendix A.2.13) to the standard and the sample tubes, and mix by vortexing briefly. Allow the microfuge tubes to stand for between 5 and 60 minutes at 22ºC. Following the incubation, the amount of protein within the samples is determined by measuring the absorbance at 595 nm (Beckman DU-64 spectrophotometer). For the assay blank, 100 µl of distilled water is added and the assay preformed as described above. A standard curve of protein concentration is constructed by plotting light absorbance at 595 nm against known concentrations of BSA protein standards (Appendix A.2.13). The standard curve is used to calculate the protein concentration of the unknown sample.

B.25 PCR Protocols

B.25.1 PCR amplification of *gfp* fragment

B.25.1.1 Primers

gfp-F 5' - GATTTCTAGATTTAAGAAGC - 3'
gfp-R 5' - TCATATTTGTATAGTTCATCC - 3'

B.25.1.2 PCR Protocol

This is an optimized PCR protocol for 50 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
Chromosomal DNA (50 ng μl^{-1}), or pLOFKm*gfp* plasmid DNA (5 ng μl^{-1})	2
$MgCl_2$ (25 mM)	6
PCR buffer (10x)	5
Primer *gfp*-F (10 µM)	5
Primer *gfp*-R (10 µM)	5
Taq Polymerase (1 U μl^{-1})	1
dNTPs (2.5 mM)	4
Sterile dH_2O	22
TOTAL	50

The PCR cycle profile:

Temperature (ºC)	**Time (s)**	**Cycles**
94	300	1
94	60	30
45	60	
72	60	
72	420	1

B.25.2 PCR amplification of the kanamycin resistance gene from pGreenII or to screen for the presence of the kanamycin resistance gene in *V. midae* SY9 insertional inactivation mutant strains

B.25.2.1 Primers

KanFwd 5' - CTCACGTTAAGCGGCCGCATTTTGG - 3'

KanRev 5' - CAAAGCCACGGAATTCGTGTCTC - 3'

B.25.2.2 PCR Protocol

This an optimized PCR protocol for 50 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
Chromosomal DNA (50 ng μl^{-1}), or pGreenII plasmid DNA (5 ng μl^{-1})	2
$MgSO_4$ (25 mM)	4
PCR buffer (10x)	5
Primer KanFwd (10 µM)	5
Primer KanRev (10 µM)	5
Pfu Polymerase (2.5 U μl^{-1})	1
dNTPs (2.5 mM)	4
Sterile dH_2O	24
TOTAL	50

The PCR cycle profile:

Temperature (ºC)	**Time (s)**	**Cycles**
94	180	1
94	60	
54	60	30
72	120	
72	420	1

B.25.3 Universal eubacterial 16S rRNA PCR

(Weisburg *et al.,* 1991)

B.25.3.1 Primers

fD1 5' - AGAGTTTGATCCTGGCTCAG - 3'

Rp2 5' - ACGGCTACCTTGTTACGACTT - 3'

B.25.3.2 PCR Protocol

This an optimized PCR protocol for 25 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
Chromosomal DNA (50 ng μl^{-1})	1
$MgSO_4$ (25 mM)	3
PCR buffer (10x)	2.5
Primer fD1 (10 µM)	2.5
Primer Rp2 (10 µM)	2.5
Pfu Polymerase (2.5 U μl^{-1})	0.5
dNTPs (2.5 mM)	2
Sterile dH_2O	11
TOTAL	25

The PCR cycle profile:

Temperature (°C)	**Time (s)**	**Cycles**
95	180	1
95	45	35
56	30	
72	195	
72	300	1

B.25.4 PCR of StopFwd/StopRev internal fragment of the detergent-stable protease gene from pMR11

B.25.4.1 Primers

StopFwd 5' - CGTC**TAG**CTTGTCGGCGTG - 3'

StopRev 5' - CTCC**CTA**AGCAACGCGTGC - 3'

Note: The bold red letters indicate the bases that were altered to form in-frame stop codons.

B.25.4.2 PCR Protocol

This an optimized PCR protocol for 50 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
pMR11 plasmid DNA (5 ng μl^{-1})	2
$MgSO_4$ (25 mM)	6
PCR buffer (10x)	5
Primer StopFwd (10 µM)	5
Primer StopRev (10 µM)	5
Pfu Polymerase (2.5 U μl^{-1})	1
dNTPs (2.5 mM)	4
Sterile dH_2O	22
TOTAL	50

The PCR cycle profile:

Temperature (°C)	**Time (s)**	**Cycles**
95	300	1
95	60	25
62	60	
72	120	
72	600	1

B.25.5 *E. coli* SM10λpir colony PCR to screen for the StopFwd/StopRev internal detergent-stable protease gene fragment in recombinant pGP704

B.25.5.1 Primers

StopFwd 5' - CGTCTAGCTTGTCGGCGTG - 3'
StopRev 5' - CTCCCTAAGCAACGCGTGC - 3'

B.25.5.2 PCR Protocol

A single colony was picked from the LA solid media, supplemented with 100 µg ml^{-1} ampicillin, with a sterile toothpick and resuspended in 10 µl of sterile water. This bacterial suspension was thoroughly mixed, and 1 µl was included in the PCR reaction as the template DNA.

This is an optimized PCR protocol for 10 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
Template DNA (Bacterial suspension)	1
$MgCl_2$ (25 mM)	1.2
PCR buffer (10x)	1
Primer StopFwd (10 µM)	0.5
Primer StopRev (10 µM)	0.5
Taq Polymerase (1 U µl^{-1})	0.2
dNTPs (2.5 mM)	0.8
Sterile dH_2O	4.8
TOTAL	10

The PCR cycle profile:

Temperature (ºC)	**Time (s)**	**Cycles**
95	300	1
95	30	
62	30	30
72	30	
72	420	1

B.25.6 *E. coli* SY327λpir colony PCR to screen for the presence of the kanamycin resistance gene in recombinant pGPro.1

B.25.6.1 Primers

KanFwd 5' - CTCACGTTAAGCGGCCGCATTTTGG - 3'

KanRev 5' - CAAAGCCACGGAATTCGTGTCTC - 3'

B.25.6.2 PCR Protocol

A single colony was picked from the LA solid media, supplemented with 120 µg ml^{-1} kanamycin, with a sterile toothpick and resuspended in 10 µl of sterile water. This bacterial suspension was thoroughly mixed, and 1 µl was included in the PCR reaction as the template DNA.

This PCR protocol was optimized for 10 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	Volume (µl)
Template DNA (Bacterial suspension)	1
$MgCl_2$ (25 mM)	1.2
PCR buffer (10x)	1
Primer KanFwd (10 µM)	0.5
Primer KanRev (10 µM)	0.5
Taq Polymerase (1 U µl^{-1})	0.2
dNTPs (2.5 mM)	0.8
Sterile dH_2O	4.8
TOTAL	10

The PCR cycle profile:

Temperature (ºC)	Time (s)	Cycles
94	300	1
94	30	30
54	30	
72	45	
72	420	1

B.25.7 PCR amplification of full-length detergent-stable protease gene from *V. midae* SY9 strains

B.25.7.1 Primers

ProMutFwd 5' - GCTTGGTTATTGATCATGTTTCTG - 3'

ProMutRev 5' - GAATAGCGTCTCCTCGTTAACAC - 3'

B.25.7.2 PCR Protocol

This is an optimized PCR protocol for 50 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
Chromosomal DNA (50 ng μl^{-1}), or pMR11 plasmid DNA (5 ng μl^{-1})	2
$MgCl_2$ (25 mM)	8
PCR buffer (10x)	5
Primer ProMutFwd (10 µM)	5
Primer ProMutRev (10 µM)	5
Taq Polymerase (1 U μl^{-1})	1
dNTPs (2.5 mM)	4
Sterile dH_2O	20
TOTAL	50

The PCR cycle profile:

Temperature (ºC)	**Time (s)**	**Cycles**
94	300	1
94	45	35
54	90	
72	60	
72	420	1

B.25.8 PCR of internal *vmproA* DNA fragment (Northern hybridization probe)

B.25.8.1 Primers

ProFwd1 5' - CTGTCGGCTCGACCAC- 3'
ProRev1 5' - CGCGGCTAACGATCAA- 3'

B.25.8.2 PCR Protocol

This is an optimized PCR protocol for 50 µl reaction volumes.

The PCR mix for each reaction was prepared as follows:

Reagents	**Volume (µl)**
pMR11 plasmid DNA (5 ng μl^{-1})	2
$MgCl_2$ (25 mM)	68
PCR buffer (10x)	5
Primer ProFwd1 (10 µM)	5
Primer ProRev1 (10 µM)	5
Taq Polymerase (1 U μl^{-1})	1
dNTPs (2.5 mM)	4
Sterile dH_2O	22
TOTAL	50

The PCR cycle profile:

<table>
<tr><th>Temperature
(ºC)</th><th>Time
(s)</th><th>Cycles</th></tr>
<tr><td>94</td><td>300</td><td>1</td></tr>
<tr><td>94</td><td>60</td><td rowspan="3">30</td></tr>
<tr><td>50</td><td>60</td></tr>
<tr><td>72</td><td>60</td></tr>
<tr><td>72</td><td>420</td><td>1</td></tr>
</table>

B.26 Sequencing

B.26.1 Automated sequencing

The clones (pMR11, pDel11, pSub11 and pProH3) were sequenced in both the forward and reverse direction using the M13Fwd and M13Rev vector primers (Yanisch-Perron *et al.,* 1985), and the sequence specific sequencing primers PSeqFwd and PSeqRev.

B.26.1.1 PCR primers for cycle sequencing

M13Fwd 5' - CGCCAGGGTTTTCCCAGTCACGAC - 3'
M13Rev 5' - GAGCGGATAACAATTTCACACAGG - 3'
PSeqFwd 5' - CCAGCACGGACGTACGTTCGAG - 3'
PSeqRev 5' - CCAGCACGGACGTACGTTCGAG - 3'

B.26.1.2 Automated sequencing protocol

The ABI Prism cycle sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) was used for sequencing, using BIOLINE Half Dye Mix. All reactions were performed according to the manufacturer's instructions.

The sequencing reactions were prepared as follows:

Reagents	**Volume (µl)**
Sequencing primer (5 µM)	2
Template (200 ng)	4
Big Dye Buffer (10x)	2
Big Dye Terminator Ready Reaction mix	2
TOTAL	10

The sequencing PCR cycle profile:

Temperature (ºC)	Time (s)	Cycles
96	60	1
94	30	
50	10	25
60	240	
72	180	1

Sequencing was performed in both the forward and reverse directions using the corresponding primers. All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700, Perkin Elmer, Applied Biosystems. Sequencing reactions of the clones were run on a MegaBACE 500 Automated Capillary DNA Sequencing System (AmershamBiosciences, Amersham and Pharmacia Biotech Molecular Dynamics) using LPA long-read gel matrix and analysed using the MegaBACE 500 Sequence Analyser v2.4 software, by the MCB Sequencing Unit, University of Cape Town.

The sequences were edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft) and Chromas version 2.01 (Technelysium).

B.26.2 Primer extension

B.26.2.1 Oligonucleotide primer for primer extension

The oligonucleotide primers PExt and PExtCy5 were synthesized by the MCB Oligonucleotide Synthesis Unit, University of Cape Town.

PExt: 5' - GCACTGTCCGCAATCTCATTGG - 3'
PExtCy5: 5' - *GCACTGTCCGCAATCTCATTGG - 3'
* - PExtCy5 was 5' - end labelled with Cy5-Far Red flouroscein.

B.26.2.2 Sequencing protocol

The sequencing reactions were prepared as follows:

Reagents	Volume (µl)
Sequencing primer (2 µM)	1
Template (500 ng)	4
Buffer (3.5x)	7.2
Sequitherm polymerase (5 U µl^{-1})	1

The reaction mixture was gently mixed by vortexing, before 3 µl was aliquoted into each of four thin-walled PCR tubes containing either ddATP, ddCTP, ddGTP or ddTTP. These sequencing reactions were mixed and cycle sequenced as described below.

The sequencing PCR cycle profile:

Temperature (ºC)	Time (s)	Cycles
94	60	1
94	30	
50	15	30
70	60	
70	180	1

Sequencing was performed using the 5'-Cy5 labelled primer, PExtCy5. All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700, Perkin Elmer, Applied Biosystems. Primer extension sequencing reactions were stopped by the addition of 3 µl of stop buffer and denatured at 70 ºC for 3 minutes, before being electrophoresed on a 5% (v/v) LongRanger® Gel Solution (Lonza, USA) polyacrylamide gel on an ALFExpress™ DNA Sequencer (Pharmacia Biotech), by the MCB Sequencing Unit, University of Cape Town. The whole volume of the primer extension sample was loaded on the gel in the G-lane, with 2 µl A (0.45 mM ddATP and 45 µM each of dATP, dCTP, dTTP and 7deaza dGTP), C (0.3 mM ddCTP and 45 µM each of dATP, dCTP, dTTP and 7deaza dGTP), and T (0.45 mM ddTTP and 45 µM each of dATP, dCTP, dTTP and 7deaza dGTP) from a pMR11 sequencing reaction in the corresponding flanking lanes. At the same time 2 µl of A (ddATP), C (ddCTP), G (0.3 mM ddGTP and 45 µM each of dATP, dCTP, dTTP and 7deaza dGTP) and T (ddTTP) of an additional pMR11 sequencing reaction were run in the corresponding lanes on either side of the primer extension reaction, and the sequencing gel run for 13 hours at 750 V and 24 Watts according to the manufacturer's instructions.

Data was analysed using the AlfWin AM version 3.0 software. The sequence was edited and assembled using DNAMAN version 4.13 (Lynnon BioSoft) and Chromas version 2.01 (Technelysium).

APPENDIX C

STATISTICAL ANALYSES SUPPLEMENT TO CHAPTER 5

CONTENTS

C 1 Analysis of cell viability

Table 1 Analysis of cell viability (CFU g^{-1} ABFEED®) of ABFEED® S34 vacuum impregnated with *Vibrio midae* SY9, *Vibrio midae* SY9Mut2 and *Vibrio midae* SY9Pro2 and stored at 4 ºC over a 29 day period. The cell viability was determined for each strain after 7, 14, 22 and 29 days of storage and the loss of viability analyzed by paired student *t*-tests.

Days of storage at 4 ºC	**Student *t*-test *P*-values**		
	***V. midae* SY9**	***V. midae* SY9Mut2**	***V. midae* SY9Pro2**
7	<0.001*	<0.001*	<0.001*
14	0.002*	<0.001*	0.001*
22	0.086	0.015*	0.043*
29	0.017*	0.006*	0.002*

The student *t*-test for pairwise comparisons (significance level = 0.05) was performed on normally distributed data. * ($P<0.05$) represents a significant difference between the mean viability values compared to the previous time point.

C 2 Analyses of abalone growth parameters

Table 2 Pairwise multiple comparison analysis (Tukey Test) of *Haliotis midae* shell length (mm) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	Dietary treatment (n)	Tukey Test *P*-values			
		Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
0	Basal (57)		0.986	1.00	0.992
	V. midae SY9 (57)			0.992	0.919
	V. midae SY9Pro2 (62)				0.984
	V. midae SY9Mut2 (62)				
56	Basal (57)		0.795	0.826	0.697
	V. midae SY9 (57)			1.00	0.999
	V. midae SY9Pro2 (62)				0.996
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.639	0.018*	0.009*
	V. midae SY9 (57)			0.317	0.494
	V. midae SY9Pro2 (62)				0.996
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.342	0.008*	0.033*
	V. midae SY9 (57)			0.444	0.739
	V. midae SY9Pro2 (62)				0.963
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time (P<0.05). * (P<0.05) represents a significant difference between the mean values. Values in parentheses are numbers of animals tested from each treatment group.

Table 3 Pairwise multiple comparison analysis (Tukey Test) of *Haliotis midae* shell width (mm) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	Dietary treatment (n)	Tukey Test *P*-values			
		Basal	*V. midae* SY9	*V. midae* SY9Pro2	*V. midae* SY9Mut2
0	Basal (57)		0.987	0.997	0.950
	V. midae SY9 (57)			0.999	0.814
	V. midae SY9Pro2 (62)				0.875
	V. midae SY9Mut2 (62)				
56	Basal (57)		0.918	0.882	0.839
	V. midae SY9 (57)			1.000	0.998
	V. midae SY9Pro2 (62)				1.000
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.286	0.021*	0.014*
	V. midae SY9 (57)			0.707	0.633
	V. midae SY9Pro2 (62)				0.999
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.102	<0.001*	0.003*
	V. midae SY9 (57)			0.267	0.669
	V. midae SY9Pro2 (62)				0.899
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values. Values in parentheses (n) are numbers of animals tested from each treatment group.

Table 4 Pairwise multiple comparison analysis (Tukey Test) of mean *Haliotis midae* weight over a 180 day period, after feeding the abalone a basal ABFEED® S34 weaning chips diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	**Dietary treatment (n)**	**Tukey Test *P*-values**			
		Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
0	Basal (57)		0.988	0.999	1.000
	V. midae SY9 (57)			0.997	0.973
	V. midae SY9Pro2 (62)				0.996
	V. midae SY9Mut2 (62)				
56	Basal (57)		0.883	0.915	0.809
	V. midae SY9 (57)			1.000	0.999
	V. midae SY9Pro2 (62)				0.995
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.626	0.017*	0.019*
	V. midae SY9 (57)			0.314	0.330
	V. midae SY9Pro2 (62)				1.000
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.888	0.003*	0.040*
	V. midae SY9 (57)			0.032*	0.223
	V. midae SY9Pro2 (62)				0.835
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values. Values in parentheses (n) are numbers of animals tested from each treatment group.

Table 5 Pairwise multiple comparison analysis (Tukey Test) of the growth rate in terms of shell length of *Haliotis midae* (mm month^{-1}) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	**Dietary treatment (n)**	**Tukey Test *P*-values**			
		Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
56	Basal (57)		0.092	0.058	<0.001*
	V. midae SY9 (57)			0.999	0.191
	V. midae SY9Pro2 (62)				0.230
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.126	<0.001*	<0.001*
	V. midae SY9 (57)			0.088	0.006*
	V. midae SY9Pro2 (62)				0.775
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.560	0.011*	0.016*
	V. midae SY9 (57)			0.297	0.357
	V. midae SY9Pro2 (62)				0.999
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values. Values in parentheses are numbers of animals tested from each treatment group.

Table 6 Pairwise multiple comparison analysis (Tukey Test) of the overall growth rate in shell length of *H. midae* (mm month^{-1}) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *V. midae* SY9, *V. midae* SY9Pro2 or *V. midae* SY9Mut2.

Dietary treatment	**Tukey Test *P*-values**			
	Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
Basal		0.106	<0.001*	<0.001*
V. midae SY9			0.351	0.036*
V. midae SY9Pro2				0.711
V. midae SY9Mut2				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with feed (P<0.05). * (P<0.05) represents a significant difference between the mean values.

Table 7 Pairwise multiple comparison analysis (Tukey Test) of the growth rate in shell width of *Haliotis midae* (mm month^{-1}) after a 56, 111 and 180 day period, while feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	Dietary treatment (n)	Tukey Test *P*-values			
		Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
56	Basal (57)		0.490	0.290	<0.001*
	V. midae SY9 (57)			0.989	0.012*
	V. midae SY9Pro2 (62)				0.026*
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.080	<0.001*	<0.001*
	V. midae SY9 (57)			0.180	0.003*
	V. midae SY9Pro2 (62)				0.465
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.191	<0.001*	<0.001*
	V. midae SY9 (57)			0.186	0.165
	V. midae SY9Pro2 (62)				1.000
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values. Values in parentheses (n) are numbers of animals tested from each treatment group.

Table 8 Pairwise multiple comparison analysis (Tukey Test) of the overall growth rate in shell width of *Haliotis midae* (mm $month^{-1}$) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Dietary treatment	**Tukey Test *P*-values**			
	Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
Basal		0.115	<0.001*	<0.001*
V. midae SY9			0.338	0.005*
V. midae SY9Pro2				0.343
V. midae SY9Mut2				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with feed ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values.

Table 9 Pairwise multiple comparison analysis (Tukey Test) of the growth rate in weight of *Haliotis midae* (g month^{-1}) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Time (Days)	**Dietary treatment (n)**	**Tukey *P*-values**			
		Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
56	Basal (57)		0.495	0.473	0.031*
	V. midae SY9 (57)			1.000	0.559
	V. midae SY9Pro2 (62)				0.546
	V. midae SY9Mut2 (62)				
111	Basal (57)		0.614	<0.001*	<0.001*
	V. midae SY9 (57)			0.036*	0.016*
	V. midae SY9Pro2 (62)				0.992
	V. midae SY9Mut2 (62)				
180	Basal (57)		0.987	0.020*	0.083
	V. midae SY9 (57)			0.054	0.179
	V. midae SY9Pro2 (62)				0.953
	V. midae SY9Mut2 (62)				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time ($P<0.05$). * ($P<0.05$) represents a significant difference between the mean values. Values in parentheses (n) are numbers of animals tested from each treatment group.

Table 10 Pairwise multiple comparison analysis (Tukey Test) of the growth rate in terms of *Haliotis midae* weight (g $month^{-1}$) over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Dietary treatment	**Tukey Test *P*-values**			
	Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
Basal		0.702	0.224	0.004*
V. midae SY9			0.224	0.099*
V. midae SY9Pro2				0.979
V. midae SY9Mut2				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time (P<0.05). * (P<0.05) represents a significant difference between the mean values.

Table 11 Pairwise multiple comparison analysis (Tukey Test) of the *Haliotis midae* condition factor over a 180 day period, after feeding the abalone a basal diet, or the basal diet supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Pro2 or *Vibrio midae* SY9Mut2.

Dietary treatment	**Tukey Test *P*-values**			
	Basal	***V. midae* SY9**	***V. midae* SY9Pro2**	***V. midae* SY9Mut2**
Basal		0.368	0.952	0.707
V. midae SY9			0.675	0.938
V. midae SY9Pro2				0.945
V. midae SY9Mut2				

The Tukey method for multiple comparisons (significance level = 0.05) was performed only on data sets for which the Repeated Measures two-way ANOVA showed a significant interaction with time (*P*<0.05). * (*P*<0.05) represents a significant difference between the mean values.

C 3 Analyses of in situ protease activity of H. midae digestive tract samples

Table 12 Pairwise multiple comparison analysis of the *in situ* specific protease activity of crop/stomach samples from *Haliotis midae* fed a basal ABFEED® S34 diet, or ABFEED® S34 supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Mut2 or *Vibrio midae* SY9Pro2.

Feed	**Holm-Sidak *P*-values**			
	Basal	***V. midae* SY9**	***V. midae* SY9Mut2**	***V. midae* SY9Pro2**
Basal		<0.001*	0.313	0.095
***V. midae* SY9**			<0.001*	<0.001*
***V. midae* SY9Mut2**				0.008*
***V. midae* SY9Pro2**				

The Holm-Sidak method for multiple comparisons (significance level = 0.05) was performed on normally distributed and equally variant data, for which one-way ANOVA showed a significant effect due to feed (P<0.05). * (P<0.05) represents a significant difference between the mean values of the *in situ* specific protease activity.

Table 13 Pairwise multiple comparison analysis of the *in situ* protease activity of intestinal samples from *Haliotis midae* fed a basal ABFEED® S34 diet, or ABFEED® S34 supplemented with either *Vibrio midae* SY9, *Vibrio midae* SY9Mut2 or *Vibrio midae* SY9Pro2.

Feed	Student *t*-test *P*-values			
	Basal	***V. midae* SY9**	***V. midae* SY9Mut2**	***V. midae* SY9Pro2**
Basal		<0.001*	0.429	<0.001*
***V. midae* SY9**			<0.001*	<0.001*
***V. midae* SY9Mut2**				<0.001*
***V. midae* SY9Pro2**				

The paired student *t*-test for pairwise comparisons (significance level = 0.05) was performed on normally distributed, but not equally variant data, for which the Kruskal-Wallis one-way ANOVA showed a significant effect due to feed (P<0.05). * (P<0.05) represents a significant difference between the mean values of the *in situ* specific protease activity.

LITERATURE CITED

Abd El-Rhman, A.M., Khattab, Y.A.E., and Shalaby, A.M.E. (2009) *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. *Fish and Shellfish Immunology* **27:** 175–180.

Abraham, L.D., and Breuil, C. (1995) Factors affecting autolysis of a subtilisin-like serine proteinase secreted by *Ophiostoma piceae* and identification of the cleavage site. *Biochimica et Biophysica Acta* **1245:** 76-84.

Akinbowale, O.L., Peng,H., and Barton, M.D. (2006) Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *Journal of Applied Microbiology* **100:** 1103-1113.

Albertson, N., Nystrom, T., and Kjelleberg, S. (1990) Exoprotease activity of two marine bacteria during starvation. *Applied and Environmental Microbiology* **56:** 218-223.

Albertson, N.H., Stretton, S., Pongpattanakitshote, S., Östling, J., Marshal, K.C., Goodman, A.E., and Kjelleberg, S. (1996) Construction and use of a new vector/transposon, pLBT::mini-Tn10:*lac*:*kan*, to identify environmentally responsive genes in marine bacteria. *FEMS Microbiology Letters* **140:** 287-294.

Albrecht, U., Keller, H., Gebauer, W., and Markl, J. (2001) Rhogocytes (pore cells) as the site of hemocyanin biosynthesis in the marine gastropod *Haliotis tuberculata. Cell Tissue Research* **304:** 455-462.

Al-Dohail, M.A., Hashim, R., and Aliyu-Paiko, M. (2009) Effects of the probiotic, *Lactobacillus acidophilus*, on the growth performance, haematology parameters and immunoglobulin concentration in African Catfish (*Clarias gariepinus*, Burchell 1822) fingerling. *Aquaculture Research* **40:** 1642-1652.

Allan, G.L., and Maguire, G.B. (1992) Effects of stocking density on production of *Penaeus monodon* Fabricus in model farming ponds. *Aquaculture* **107:** 49-66.

Altschul, S., Gish, W., Miller, W., Meyers, E., and Lipman, D. (1989) Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389-3402.

Alzamora, S. M., Salvatori, D., Tapia, M.S., López-Malo, A., Welti-Chanes, J., and Fito, P. (2005) Novel functional foods from vegetable matrices impregnated with biologically active compounds. *Journal of Food Engineering* **67:** 205-214.

Amann, R.I., Ludwig, and W., Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbial Reviews* **59:** 143-169.

Antonio, D.B., Andree, K.B., Moore, J.D., Friedman, C.S., and Hedrick, R.P. (2000) Detection of *Ricketsiales*-like prokaryotes by *in situ* hybridisation in black abalone, *Haliotis cracherodii*, with withering syndrome. *Journal of Invertebrate Pathology* **75:** 180-182.

Araki, T., Hayakawa, M., Lu, Z., Karita, S., and Morishita, T. (1998) Purification and characterisation of agarases from a marine bacterium, *Vibrio* sp. PO-303. *Journal of Marine Biotechnology* **6:** 260-265.

Arellano, C.F., and Olmos, S.J. (2002) Thermostable α-1,4- and α-1,6-glucosidase enzymes from *Bacillus* sp. isolated from a marine environment. *World Journal of Microbiology and Biotechnology* **18:** 791-795.

Arnórsdóttir, J., Smáradottir, R.B., Magnússon, O.T., Thorbjarnardóttir, S.H., Eggertsson, G., and Kristjánsson, M.M. (2002) Characterization of a cloned subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species. *European Journal of Biochemistry* **269:** 5536-5546.

Asfie, M., Yoshijima, T., and Sugita, H. (2003) Characterization of the goldfish fecal microflora by the fluorescent *in situ* hybridization method. *Fisheries Science* **69:** 21-26.

Austin B., Stuckley L.F., Robertson P.A.W., Effendi I., and Griffith D.R.W. (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases* **18:** 93-96.

Ausubel, J.F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989) *Current Protocols in Molecular Biology*. Green Publishing Associates and Wiley-Interscience, Harvard Medial School, USA.

Babé, L.M., and Schmidt, B. (1998) Purification and biochemical analysis of WprA, a 52-kDa serine protease secreted by *B. subtilis* as an active complex with its 23-kDa propeptide. *Biochimica et Biophysica Acta* **1386:** 211-219.

Bairagi, A., Ghosh, K.S., Sen, K.S., and Ray, A.K. (2002) Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International* **10:** 109-121.

Balcázar J.L., de Blas I., Ruiz-Zarzuela I., Cunningham D., Vendrell D., and Múzquiz J.L. (2006) The role of probiotics in aquaculture. *Veterinary microbiology* **114:** 173-186.

Balcázar J.L., Rojas-Luna, T., and Cunningham, D.P. (2007) Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *Journal of Invertebrate Pathology* **96:** 147-150.

Baranyi, J., and Pin, C. (1999) Estimating bacterial growth parameters by means of detection times. *Applied and Environmental Microbiology* **65:** 732-736.

Barkai, R., and Griffiths, C.L. (1986) Diet of the South African abalone *Haliotis midae*. *South African Journal of Marine Science* **4:** 37-44.

Bautista-Teruel, M.N., and Millamena, O.M. (1999) Diet development and evaluation for juvenile abalone, *Haliotis asinina*: protein/energy levels. *Aquaculture* **178:** 117-126.

Bautista-Teruel, M.N., Fermin, A.C., and Koshio, S.S. (2003) Diet development and evaluation for juvenile abalone, *Haliotis asinina*: animal and plant protein sources. *Aquaculture* **219:** 645-653.

Beg, Q.K., and Gupta, R. (2003) Purification and characterisation of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technology* **32:** 294-304.

Berger, D., and Altmann, T. (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes and Development* **14:** 1119-1131.

Betoret, N., Puente, L., Díaz, M.J., Pagán, M.J., García, M.J., Gras, M.J., Martínez-Monzó, J., and Fito, P. (2003) Development of probiotic-enriched dried fruits by vacuum impregnation. *Journal of Food Engineering* **56:** 273-277.

Bevelander, G. (1988) *Abalone: Gross and fine structure*. The Boxwood Press, Pacific Grove, California.

Blum, H., Beier, H., and Gross, H.J. (1987) Improved staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:** 93–99.

Branch, G.M., and Branch, M.L. (1982) *The living shores of Southern Africa.* Struik Publishers, South Africa.

Branch, G.M., Griffiths, C.L., Branch, M.L., and Beckley, L.E. (2007) *Two Oceans. A guide to the marine life of Southern Africa.* Struik Publishers, South Africa.

Britz, P.J. (1990) Global status of abalone aquaculture. *In:* Cook, P. **(Ed.)**, *Perlemoen farming in South Africa. Mariculture association of South Africa*. pp 20–26.

Britz, P.J. (1994) The development of an artificial feed for abalone farming. *South African Journal of Science* **90:** 6-7.

Britz, P.J. (1995) The nutritional requirements of *Haliotis midae* and development of a practical diet for abalone aquaculture. *PhD Thesis, Rhodes University, South Africa*.

Britz, P.J. (1996) Effect of dietary protein level on growth performance of South African abalone, *Haliotis midae*, fed fishmeal-based semi-purified diets. *Aquaculture* **140:** 55-61.

Britz, P.J., and Hecht, T. (1997) Effect of dietary protein and energy level on growth and body composition of South African abalone, *Haliotis midae*. *Aquaculture* **156:** 195-210.

Britz P.J., Hecht T., and Mangold S. (1997) The effect of temperature on growth, feed consumption and nutritional indices of *Haliotis midae* fed a formulated diet. *Aquaculture* **157:** 191-203.

Brummett, R.E., Lazard, J., and Moehl, J. (2008) African aquaculture: Realizing the potential. *Food Policy* **33:** 371-385.

Bryan, P., Wang, L., Hoskines, J., Ruvinev, S., Stausberg, S., Alexander, P., Almog, O., Gilliland, G., and Gallagher, T. (1995) Catalysis of a protein folding reaction: Mechanistic implications of the 2.0 Å structure of the subtilisin-prodomain complex. *Biochemistry* **34:** 10310-10318.

Burr, G., and Gatlin, D. (2005) Microbial Ecology of the Gastrointestinal Tract of Fish and the Potential Application of Prebiotics and Probiotics to Finfish Aquaculture. *Journal of the World Aquaculture Society* **36:** 425-436.

Cai, S.H., Wu, Z.H., Jian, J.C., and Lu, Y.S. (2007) Cloning and expression of the gene encoding an extracellular alkaline serine protease from *Vibrio alginolyticus* strain HY9901, the causative agent of vibriosis in *Lutjanus erythopterus* (Bloch). *Journal of Fish Diseases* **30:** 493-500.

Campa-Córdova, A.I., Gonzalez-Ocampo, H., Luna-González, A., Mazón-Suástegui, J.M., and Ascencio, F. (2009) Growth, survival, and superoxide dismutase activity in juvenile *Crassostrea corteziensis* (Hertlein, 1951) treated with probiotics. *Hidrobiológica* **19:** 151-157.

Campbell, J.L. (1965) The structure and function of the alimentary canal of the Black Abalone, *Haliotis cracherodii* Leach. *Transactions of the American Microscopical Society* **83:** 376-395.

Capinpin, E.C., Toledo, J.D., and Doi, E.M. (1999) Density dependent growth of the tropical abalone *Haliotis asinia* in cage culture. *Aquaculture* **171:** 227-235.

Castex, M., Chim, L., Pham, D., Lemaire, P., Wabete, N., Nicolas, J-L., Schmidely, P., and Mariojouls, C. (2008) Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. *Aquaculture* **275:** 182-193.

Castex, M., Lemaire, P., Wabete, N., and Chim, L. (2009) Effect of dietary probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress status of shrimp *Litopenaeus stylirostris*. *Aquaculture* **294:** 306-313.

Chougule, N.P., Doyle, E., Fitches, E., and Gatehouse, J.A. (2008) Biochemical characterization of midgut digestive proteases from *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. *Journal of Insect Physiology* **54:** 563-572.

Chuang, Y.C., Chang, T.M., and Chang, M.C. (1997) Cloning and characterization of the gene (*empV*) encoding extracellular metalloprotease from *Vibrio vulnificus*. *Gene* **189:** 163-168.

Chung, S.S., Kim, H.J., Shin, Y.U., Jin, G.H., and Lee, H.H. (2001) Molecular characterisation of gene encoding an extracellular alkaline protease in *Vibrio metschnikovii*. *Biotechnology Letters* **23:** 1175-1182.

Church, G., and Gilbert, W. (1984) Genomic sequencing. *Proceeding of the National Academy of Sciences*, USA. **81**: 1991-1995.

Coleman, J.E., and Vallee, B.L. (1960) Metallocarboxypeptidases. *Journal of Biological Chemistry* **235:** 390-395.

Cook, P.A. (1990) Potential of abalone culture in South Africa. *In:* Cook, P. A. **(Ed.)**, *Perlemoen farming in South Africa. Mariculture association of South Africa* pp 27-32.

Coons, A.H., Creech, H.J., and Jones, R.N. (1941) Immunological properties of an antibody containing fluorescent group. *Proceedings of the Society Experimental Bioliogy and Medicine* **47:** 200-202.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996) FACS – optimized mutants of the green fluorescent protein (GFP). *Gene* **173:** 33–38.

Coyne, V.E., James, M.D., Reid, S.J., and Rybicki, E.P. (2002) *Molecular Biology Techniques Manual, 6th edition*. Department of Molecular and Cell Biology, University of Cape Town, South Africa.

Dagert, M., and Ehrlich, S.D. (1979) Prolonged incubation in calcium chloride improved the competence of *Escherichia coli* cells. *Gene* **6:** 23-28.

Dautin, N., and Bernstein, H.D. (2007) Protein secretion in gram-negative bacteria via the autotransporter pathway. *Annual Review of Microbiology* **61:** 89-112.

Deane, S.M. (1989) Molecular biology studies on the extracellular serine proteases of *Vibrio alginolyticus*. *PhD thesis, University of Cape Town, South Africa.*

Deane, S.M., Robb, F.T., and Woods, D.R. (1986) Isolation and characterization of a *Vibrio alginolyticus* mutant that overproduces extracellular proteases. *Journal of General Microbiolgy* **132:** 391-398.

Deane, S.M., Maharaj, R., Robb, F.T., and Woods, D.R. (1987a) Cloning, expression and release of a *Vibrio alginolyticus* SDS-resistant Ca^{2+}-dependent exoprotease in *Escherichia coli. Journal of General Microbiology* **133:** 2295-2302.

Deane, S.M., Robb, F.T., and Woods, D.R. (1987b) Production and activation of an SDS-resistant alkaline serine exoprotease of *Vibrio alginolyticus*. *Journal of General Microbiology* **133:** 391-398.

Deane, S.M., Robb, F.T., Robb, S.M., and Woods, D.R. (1989) Nucleotide sequence of the *Vibrio alginolyticus* calcium-dependent, detergent-resistant alkaline serine exoprotease A. *Gene* **76:** 281-288.

Decamp, O., and Moriaty, D.J.W. (2006) Probiotics as alternative to antimicrobials: limitations and potential. *World Aquaculture*. **37:** 60-62.

De Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram negative Eubacteria. *Journal of Bacteriology* **172:** 6568-6572.

De Schrijver, R., and Ollevier, F. (2000) Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. *Aquaculture* **183:** 107-116.

Dlaza, T.S. (2006) Growth of juvenile abalone under aquaculture conditions. *MSc Thesis, University of the Western Cape, South Africa.*

Douillet, P.A. (2000a) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions I. Use of single and multiple bacterial probiotics. *Aquaculture* **182:** 241-248.

Douillet, P.A. (2000b) Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions II. Evaluation of commercial products and pure isolates. *Aquaculture* **182:** 249-260.

Douillet, P.A., and Langdon, C.J. (1994) Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). *Aquaculture* **119:** 25 - 40.

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988) Vectors for the transformation of plant cell using *Agrobacterium*, p. 47. *In* Plant Genetic Transformation and Gene Expression. A Laboratory Manual. Blackwell Scientific Publications.

Durham, D.R., Stewart, D.B., and Stellwag, E.J. (1987) Novel alkaline- and heat-stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. *Journal of Bacteriology* **169:** 2762-2768.

Eberl, L., Schulze, R., Ammendola, A., Geisenberger, O., Erhart, R., Sternberg, C., Molin, S., and Amann, R. (1997) Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. *FEMS Microbiology Letters* **149:** 77-83.

Eder, J., Rheinecker, M., and Fresht, A.R. (1993) Folding of subtilisin BPN': role of the pro-sequence. *Journal of Molecular Biology* **233:** 293-304.

Edwards, S., and Condon, C. (2001) Digestive protease characterization and adaption in blacklip abalone (*Haliotis rubra* Leach). *Aquaculture Research* **32:** 95-102.

Egan, S., James, S., and Kjelleberg, S. (2002) Identification and characterization of a putative transcriptional regulator controlling the expression of fouling inhibitors in *Pseudoalteromonas tunicate*. *Applied and Environmental Microbiology* **68:** 372-378.

Eikmanns, B., Thumb-Shmitz L., Eggeling K., Lüdtke., and Sahm, H. (1994) Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. *Microbiology* **140:** 1817–1828.

Elston, R., and Lockwood, G.S. (1983) Pathogenesis of vibriosis in cultured juvenile Red abalone, *Haliotis rufescens* Swainson. *Journal of Fish Diseases* **6:** 111-128.

Englard, S., and Seifter, S. (1990) Precipitation techniques. *Methods in Enzymology* **182:** 285-300

Erasmus, J.H. (1996) The role of enteric bacteria in the abalone, *Haliotis midae*. *MSc thesis, University of Cape Town, South Africa.*

Erasmus, J., Cook, P., and Coyne, V. (1997) The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture* **155:** 377-386.

Farrell, D.H., and Crosa, J.H. (1991) Purification and characterisation of a secreted protease from the pathogenic marine bacterium, *Vibrio anguillarum*. *Biochemistry* **30:** 3432-3436.

Farto, R., Pérez, M.J., Fernández-Briera, A., and Nieto, T.P. (2002) Purification and partial characterization of a fish letha; extracellular protease from *Vibrio pelagius*. *Veterinary Microbiology* **89:** 181-194.

Farzanfar, A. (2006) The use of probiotics in shrimp aquaculture. *FEMS Immunology and Medical Microbiology* **48:** 149-158.

FAO (2000) The State of World Fishies and Aquaculture 2000. Electronic edition http://www.fao.org/docrep/003/x8002e/x8002e00.htm.

FAO (2004) The State of the World Fisheries and Aquaculture 2004. FAO, Rome.

FAO (2007) FAO Fishery Information, Data and Statistics Unit (FIDI) c2002. Fishery Statistical Collections. FIGIS Data Collection. FAO, Rome. Updated March 2007. Available via FIGIS from: http://www.fao.org/figis/servlet/static?dom=collection&xml=global-aquaculture-production.xml.

Felsenstein, J. (1985) An approach to using the bootstrap. *Evolution* **39:** 783–791.

Finkelstein, R.A., and Hanne, L.F. (1982) Purification and characterization of the soluble hemagglutinin (cholera lectin) produced by *Vibrio cholerae*. *Infection and Immunity* **36:** 1199-1208.

Fito, P. (1994) Modelling of vacuum osmotic dehydration of foods. *Journal of Food Engineering* **23:** 313-328.

Fleming, A.E. (1995) Growth, intake, feed conversion efficiency and chemosensory preference of the Australian abalone, *Haliotis rubra*. *Aquaculture* **132:** 297-311.

Fleming A.E., Van Barneveld R.J., and Hone R.W. (1996) The development of artificial diets for abalone: A review and future directions. *Aquaculture* **140:** 5-53.

Focareta, T., and Manning, P.A. (1987) Extracellular proteins of *Vibrio cholerae*: molecular cloning, nucleotide sequence and characterization of the deoxyribonuclease (DNase) together with its periplasmic localization in *Escherichia coli* K-12. *Gene* **53:** 31-40.

Fuller, R. (1989) Probiotics in man and animals. *Journal of Applied Bacteriology* **66:** 365-378.

Fuller, R. (1992) *In: Probiotics – The Scientific Basis* Chapman and Hall, London. pp 1-8.

Fuqua, W.C. (1992) An improved chloramphenicol resistance gene cassette for site-directed marker replacement mutagenesis. *Biotechniques* **12:** 223–225.

Garcia-Carreño, L.F., Navarrte del Toro, M.A., and Serviere-Zaragoza, E. (2003) Digestive enzymes in juvenile green abalone, *Haliotis fulgens*, fed natural foods. *Comparative Biochemistry and Physiology* **134:** 143-150.

Garcia-Esquivel, Z., and Felbeck, H. (2006) Activity of digestive enzymes along the gut of juvenile red abalone, Haliotis rufescens, fed natural and balanced diets. *Aquaculture* **261:** 615-625.

Garriques, D., and Arevalo, G. (1995) An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae n Ecuador. *In:* Browdy, C.L., and Hopkins, J.S. **(Eds.)**, *Swimming through troubled water, proceedings of the special session of shrimp farming*. World Aquaculture Society. pp 53–59.

Gatesoupe, F.J. (1991) The effect of three strains of lactic bacteria on the production rate of rotifers, *Brachionus plicatilis*, and their dietary value for turbot larvae, *Scophthalmus maximus*. *Aquaculture* **96:** 335-342.

Gatesoupe, F.J. (1999) The use of probiotics in aquaculture. *Aquaculture* **180:** 147-165.

Gavine, F., Ingram, B., and Doroudi, M. (2009) Development of management strategies for herpes-like virus infection in abalone. *Fisheries Research and Development Corporation and State Government of Victoria (FRDC Project no.: 2006/243).*

Genade, A.B., Hirst, A.L., and Smit, C.J. (1985) Observations on the spawing and rearing of the South African abalone *Haliotis midae* Linn. Fisheries Development Corporation, Knysna, South Africa.

Genade, A.B., Hirst, A.L., and Smit, C.J. (1988) Observations on the spawning, development and rearing of the South African abalone *Haliotis midae* Linn. *South African Journal of Marine Science* **6:** 3-12.

Ghosh, S., Sinha, A., and Sahu, C. (2007a) Dietary supplementation in growth and health of live-bearing ornamental fishes. *Aquaculture Nutrition* **13:** 1 - 11.

Ghosh, S., Sinha, A., and Sahu, C. (2007b) Effect of probiotic on reproductive performance in female livebearing ornamental fish. *Aquaculture Research* **38:** 518-526.

Gibson, L.F., Woodworth, J., and George, A.M. (1998) Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii. Aquaculture* **169:** 111-120.

Gildberg, A., Mikkelsen, H., Sandaker, E., and Ringõ, E. (1997) Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry and Atlantic cod (*Gadus morhua*). *Hydrobiologia* **352:** 279-285.

Gildberg, A., and Mikkelsen, H. (1998) Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulatory peptides during a challenge trial with *Vibrio anguillarum*. *Aquaculture* **167:** 103-113.

Giovannoni, S.J., Delong, E.F., Olsen, G.J., and Pace, N.R. (1988) Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. *Journal of Bacteriology* **170:** 720-726.

Glenn A.R. (1976) Production of extracellular proteins by bacteria. *Annual Reviews of Microbiology* **30:** 41-62.

Gomez-Gil, B., Roque, A., and Turnbull, J.F. (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* **191:** 259-270.

Gordon, H.R., and Cook, P.A. (2004) World abalone fisheries and aquaculture update: supply and market dynamics. *Journal of Shellfish Research* **23:** 935-939.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., and Nielsen, T.F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Applied and Environmental Microbiology* **65:** 969-973.

Guarner, F., and Schaafsma, G.J. (1998) Probiotics. *International Journal of Food Microbiology* **39:** 237-238.

Gullian, M., Thompson, F., and Rodriguez, J. (2004) Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture* **233:** 1-14.

Guo, J.-J., Liu, K.-F., Cheng, S.-H., Chang, C.-I., Lay, J.-J., Hsu, Y.-O., Yang, J.-Y., and Chen, T.-I. (2009) Selection of probiotic bacteria for use in shrimp larviculture. *Aquaculture Research* **40:** 609-618.

Hadley, N.H., and Manzi, J.J. (1984) Growth of seed clams, *Mercenaria mercenaria*, at various densities in a commercial scale nursery system. *Aquaculture* **36:** 369-378.

Hahn, K.O. (1989a) Nutrition and growth of abalone. *In:* Hahn, K.O. **(Ed.)**, *Handbook of culture of abalone and other marine gastropods*. CRC Press, Florida, pp.135-156.

Hahn, K.O. (1989b) Abalone aquaculture in Japan. *In:* Hahn, K.O. **(Ed.)**, *Handbook of culture of abalone and other marine gastropods*. CRC Press, Florida, pp. 185-194.

Hai, N.V., and Fotedar, R. (2009) Comparison of the effects of the probiotics (Bio-Mos® and β-1,3-D-glucan) and the customized probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of the juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture* **289:** 310–316.

Hamid, A., Sakata, T., and Kakimoto, D. (1978) Microflora in the alimentary tract of grey mullet: 2. A comprison of the mullet intestinal microflora in fresh and sea water. *Bulletin of the Japanese Society of Scientific Fisheries* **44:** 53-57.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology.* **166:** 557-580.

Hanlon, G.W., and Hodges, N.A. (1981) Requirement for glucose during production of extracellular serine protease by cultures of *Bacillus licheniformis*. *FEMS Microbiology* **11:** 51-54.

Hare, P., Long, S., Robb, F.T. and Woods, D.R. (1981) Regulation of exoprotease production by temperature and oxygen in *Vibrio alginolyticus* . *Archives of Microbiology* **130:** 276-280.

Hare, P., Scott-Burden, T., and Woods, D.R. (1983) Characterization of extracellular alkaline proteases and collagenase induction in *Vibrio alginolyticus*. *Journal of General Microbiology* **129:** 1141-1147.

Harris, J. (1993) The presence, nature, and role of gut microflora in aquatic invertebrates: a synthesis. *Microbial Ecology* **25:** 195–231.

Harris, J.O., Burke, C.M., and Maquire, G.B. (1998a) Characterisation of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. I. Morphology and histology. *Journal of Shellfish Research.* **17:** 979-988.

Harris, J.O., Burke, C.M., and Maquire, G.B. (1998b) Characterisation of the digestive tract of greenlip abalone, *Haliotis laevigata* Donovan. II. Microenvironment and bacterial flora. *Journal of Shellfish Research*. **17:** 989-994.

Harris, J.O., Maguire, G.B., Edwards, S.J., and Johns, D.R. (1999) Low dissolved oxygen reduces growth rate and oxygen consumption rate of juvenile greenlip abalone, *Haliotis laevigata* Donovan. *Aquaculture* **174:** 265-278.

Häse, C.C., and Finkelstein, R.A. (1991) Cloning and nucleotide sequence of the *Vibrio cholerae* Hemagglutinin/Protease (HA/Protease) gene and construction of an HA/Protease-negative strain. *Journal of Bacteriology* **173:** 3311-3317.

Hauck, M., and Sweijd, N.A. (1999) A case study of abalone poaching in South Africa and its impact on fisheries management. *ICES Journal of Marine Science* **56:** 1024-1032.

Hayat, M.A. (1993) *Stains and Cytochemical methods*. Plenum Press. New York and London. pp 63-64.

Hecht, T. (1994) Behavioural thermoregulation of the abalone, *Haliotis midae*, and implications for intensive culture. *Aquaculture* **126:** 171–181.

Heim, R., Cubitt, A.B., and Tsien, R.Y. (1995) Improved green fluorescence. *Nature* **373:** 663–664.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S., and Millineaux, P.M. (2000) pGreen: A versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42:** 819–832.

Hensel, M., and Holden, D.W. (1996) Molecular genetic markers for the study of virulence in both pathogenic bacteria and fungi. *Microbiology* **142:** 1049-1058.

Hernández-Santoyo, A., Hernández-Arana, A., Arreguín-Esoinoza, R., and Rodríguez-Romero, A. (1998) Purification and characterisation of several digestive proteases from the blue abalone, *Haliotis fulgens*. *Aquaculture* **159:** 203–216.

Herrero M., De Lorenzo V., and Timmis K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *Journal of Bacteriology* **172:** 6557–6567.

Herry, A., Diouris, M., and Le Pennec, M. (1989) Chemoautotrophic symbionts and translocation of fixed carbon from bacteria to host tissues in the littoral bivalve *Loripes lucinalis* (Lucinidae). *Marine Biology* **101:** 305-312.

Heussen, C., and Dowdle, E.B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. *Analytical Biochemistry* **102:** 196-202.

Hjelm, M., Bergh, Ø., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., Duncan, H., Ahrens, P., Birkbeck, H., and Gram, L. (2004) Selection and identification of autochthonous potential probiotic bacteria from Turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology* **27:** 360-371.

Høj, L., Bourne, D.G., and Hall, M.R. (2009) Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment. *Aquaculture* **293:** 278-285.

Holben, W.E., Williams, P., Gilbert, M.A., Saarinen, M., Sarkilahti, L.K., and Apajalahti, J.H. (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* **44:** 175-185.

Holliday, J.E., Allan, G.L. and Nell, J.A. (1993) Effects of stocking density on juvenile Sydney rock oysters, *Saccostrea commercialis* (Iredale and Roughley), in cylinders. *Aquaculture* **109:** 13-26.

Holzapfel, W.H., Haberer, P., Snel, J., Schillinger, U., and Huis in't Veld, J. (1998) Overview of gut flora and probiotics. *International Journal of Food Microbiology* **41:** 85-101.

Horwitz, R. (2008) Characterization of an intracellular bacterium infecting the digestive gland of the South African abalone, *Haliotis midae*. *MSc Thesis, University of Cape Town, South Africa*.

Howard, S.P., and Buckley, J.T. (1986) Molecular cloning and expression in *Escherichia coli* of the structural gene for the haemolytic toxin aerolysin from *Aeromonas hydrophila*. *Molecular and General Genetics.* **204:** 289-295.

Ichige, A., Oishi, K., and Mizushima, S. (1988) Isolation and characterization of mutant in a marine *Vibrio* strain that are defective in production of extracellular proteins. *Journal of Bacteriology* **170:** 3537-3542.

Iehata, S., Inagaki, T., Okunishi, S., Nakano, M., Tanaka, R., and Maeda, H. (2009) Colonization and probiotic effects of lactic acid bacteria in the gut of the abalone *Haliotis gigantea*. *Fisheries Science* **75:** 1285-1293.

Inamura, H., Nakai, T., and Muroga, K. (1985) An extracellular protease produced by *Vibrio anguillarum*. *Bulletin of the Japanese Society of Scientific Fisheries* **51:** 1915-1920.

Imai, T. (1977) *Aquaculture in shallow seas: Progress in shallow sea culture*. Part IV. Amerind Pub., New Dehli.

Irianto, A., and Austin, B. (2002a) Probiotics in aquaculture. *Journal of Fish Diseases* **25:** 633-642.

Irianto, A., and Austin, B. (2002b) Use of probiotics to control furnuculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **25:** 1-10.

Ish-Horowicz, D., and Burke, J.F. (1981) Rapid and efficient cosmid cloning. *Nucleic Acids Research* **9:** 2989-2998.

Jacobs, I., Eliasson, M., Uhlen, M., and Flock, J.I. (1985) Cloning, sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*. *Nucleic Acids Research* **13:** 8913-8926.

Jeong, H.S., Jeong, K.C., Choi, H.K., Park, K.-J., Lee, K.-H., Rhee, J.H., and Choi, S.H. (2001) Differential expression of *Vibrio vulnificus* elastase gene in a growth phase-dependent manner by two different types of promoters. *The Journal of Biological Chemistry* **276:** 13875-13880.

Jeong, K.C., Jeong, H.S. Rhee, J.H., Lee, S.E., Chung, S.S., Starks, A.M., Escudero, G.M., Gulig, P.A., and Choi, S.H. (2000) Construction and phenotypic evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. *Infection and Immunity* **68:** 5096-5106.

Jobling, M.G., and Holmes, R.K. (1997) Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/Protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Molecular Microbiology* **26:** 1023-1036.

Jolly, C., Berland, S., Milet, C., Borzeix, S., Lopez, E., and Doumenc, D. (2004) Zonal localization of shell matrix proteins in mantle of *Haliotis tuberculata* (Mollusca, Gastropoda). *Marine Biotechnology* **6:** 541-551.

Jory, D. (1998) Use of probiotics in penaeid shrimp growout. *Aquaculture Magazine* **24:** 62-67.

Junfeng, L., Zhenming, C., Hongfung, L., and Xianghong, W. (2008) Characterization of a mutant of *Alteromonas aurantia* A18 and its application in mariculture. *Journal of Ocean University of China* **7:** 55-59.

Kalisz, H. (1988) Microbial proteinases. *Biotechnology* **36:** 1-65.

Kannan, Y., Koga, Y., Inoue, Y., Haruki, M., Takagi, M., Imanaka, T., Morikawa, M., and Kanaya, S. (2001) Active subtilisin-like protease from a hyperthermophilic archaeon in a form with a putative prosequence. *Applied and Environmental Microbiology* **67:** 2445-2452.

Kar, N., and Ghosh, K. (2008) Enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* (Hamilton) and *Channa punctatus* (Bloch). *Turkish Journal of Fisheries and Aquatic Sciences* **8:** 115-120.

Karunasagar, I., Pai, R., Malathi, G.R., and Karunasagar, I. (1994) Mass mortality of *Peneaus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* **128:** 203-209.

Kato, T., Yamagato, Y., Arai, T., and Ichishima, E. (1992) Purification of a new extracellular 90-kDa serine proteinase with isoelectric point of 3.9 from *Bacillus subtilis* (*natto*) and elucidation of its distinct mode of action. *Bioscience, Biotechnology, and Biochemistry* **56:** 1166-1168.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., and Gibson, L. (2008) Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* **274:** 1-14.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., and Gibson, L. (2009) Screening for probiotics of Greenshell™ mussel larvae, *Perna canaliculus*, using a larval challenge bioassay. *Aquaculture* **296:** 159-164.

Kim, C.M., Kang, S.M., Jeon, H.J., and Shin, S.H. (2007) Production of *Vibrio vulnificus* metalloprotease VvpE begins during the early growth phase: Usefulness of gelatine-zymography. *Journal of Microbiological Methods* **70:** 96-102.

Knauer, J., Britz, P.J., and Hecht, T (1996) Comparative growth performance and digestive enzyme activity of juvenile South African abalone, *Haliotis midae*, fed on diatoms and a practical diet. *Aquaculture* **140:** 75-85.

Kothary, M.H., and Kreger, A.S. (1985) Production and partial characterization of an elastolytic protease of *Vibrio vulnificus*. *Infection and Immunity* **50:** 534-540.

Kothary, M.H., and Kreger, A.S. (1987) Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. *Journal of General Microbiology* **133:** 1783-1791.

Kraut, J. (1977) Serine proteases: Structure and mechanism of catalysis. *Annual Review of Biochemistry* **46:** 331-358.

Kumar, C.G., Tiwari, M.P., and Jany, K.D. (1999) Novel alkaline serine proteases from alkilophilic *Bacillus* spp.: purification and some properties. *Process Biochemistry* **34:** 441-449.

Kumar, S., Tamura, K., and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5:** 150-163.

Kwon, Y.T., Moon, S.Y., Kim, J.O., Kho, Y.H., and Rho, H.M. (1992) Characterisation of extracellular proteases from *Vibrio* sp. strain RH530. *Korean Journal of Microbiology and Biotechnology* **30:** 501-506.

Kwon, Y. T., Moon, S. Y., Lee, H. H., and Rho, H. M. (1994) Extracellular alkaline proteases from alkalophilic *Vibrio metschnikovii* strain RH530. *Biotechnology Letters* **16:** 413-418.

Kwon, Y.T., Kim, J.O., Moon, S.Y., Yoo, Y.D., and Rho, H.M. (1995) Cloning and characterization of the gene encoding an extracellular alkaline serine protease from *Vibrio metschnikovii* strain RH530. *Gene* **152:** 59-63.

Lara-Flores, M., Olvera_Novoa, M.A., Guzmán-Méndez, B.E., and López-Madrid, W. (2003) Use of bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture* **216:** 193-201.

Lalloo, R., Ramchuran, S., Ramduth, D., Görgens, J., and Gardiner, N. (2007) Isolation and selection of *Bacillus* spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. *Journal of Applied Microbiology* pg.: 1-8.

Landau, M. (1991) *Introduction to Aquaculture*. John Wiley and Sons, Inc.

Lee, J-H., Ahn, S-H., Lee, E-M., Kim, Y-O., Lee, S-J., and Kong, I-S. (2003) Characterization of the enzyme activity of an extracellular metalloprotease (VMC) from *Vibrio mimicus* and its C-terminal deletions. *FEMS Microbiology Letters* **223:** 293-300.

Lee, C.Y., Cheng, M.F., Yu, M.S., and Pan, M.J. (2002) Purification and characterization of a putative virulence factor, serine protease, from *Vibrio parahaemolyticus*. *FEMS Microbiology Letters* **209:** 31-37.

Lee, K.-K., Yu, S.-R., and Liu, P.-C. (1997) Alkaline serine protease is an exotoxin of *Vibrio alginolyticus* in Kuruma Prawn, *Penaeus japonicus*. *Current Microbiology* **34:** 110-117.

Lee, S., Malone, C., and Kemp, P.F. (1993) Use of multiple 16S rRNA targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. *Marine Ecology Progress Series* **101**:193–201.

Lee, Y.C., Miyata, Y., Terada, I., Ohta, T., and Matsuzawa, H. (1991) Involvement of NH_2-terminal pro-sequence in the production of active aqualysin I (a thermophilic serine protease) in *Escherichia coli*. *Agricultural and Biological Chemistry* **55:** 3027-3032.

Lee, Y.C., Ohta, T., and Matsuzawa, H. (1992) A non-covalent NH2-terminal pro-region aids the production of active aqualysin I (a thermophilic protease) without the COOH-terminal pro-sequence in *Escherichia coli*. *FEMS Microbiology Letters* **92:** 73-78.

Lee, Y-K., and Salminen, S (1995) The coming of age of probiotics. *Trends in Food Science and Technology* **6:** 241-245.

Leighton, D., and Boolootian, R.A. (1963) Diet and growth in the Black Abalone, *Haliotis cracerodi*. *Ecology* **44:** 228-238.

Leighton, D.L., and Lewis, C.A. (1982) Experimental hybridization in abalones. *International Journal of Invertebrate Reproduction* **5:** 273-282.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. (2007) Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Applied and Environmental Microbiology* **73:** 777-784.

Lésel, R. (1990) Thermal effect on bacterial flora in the gut of rainbow trout and African catfish. *In: Microbiology in Poecilotherms* **(Ed.)** Lésel, R.. Elsevier, Amsterdam, pp. 33-38.

Li, J., Tan, B., and Mai, K. (2009) Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune parameters and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). *Aquaculture* **291:** 35–40.

Li, U., Tan, B., Mai, K., Ai, Q., Zhang, W., Xu, W., Liufu, Z., and Ma, H. (2006) Comparative study between probiotic bacterium *Arthrobacter* XE-7 and chloramphenicol on protection of *Penaeus chinensis* post-larvae from pathogenic vibrios. *Aquaculture* **253:** 140-147.

Lin, A.Y.-M., Chen, P.-Y., and Meyers, M.A. (2008) The growth of nacre in the abalone shell. *Acta Biomaterialia* **4:** 131-138.

Lindberg, D.R. (1992) Evolution, distribution and systematics of Haliotidae. *In:* Shepherd, S.A., Tegner, M.J., and Guzman Del Proo, S.A. **(Eds.)**, *Abalone of the World.* pp. 3-18.

Litwin, C.M., Boyko, S.A., and Calderwood, S.B. (1992) Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. *Journal of Bacteriology* **174:** 1897 - 1903.

Liu, P.C., Chen, Y.C., and Lee, K.K. (2001) Pathogenicity of *Vibrio alginolyticus* isolated from diseased small abalone *Haliotis diversicolor* supertaxa. *Microbios* **104:** 71-77.

Long, S., Mothibeli, M.A., Robb, F.T., and Woods, D.R. (1981) Regulation of extracellular alkaline protease activity by histidine in a collagenolytic *Vibrio alginolyticus* strain. *Journal of General Microbiology* **127:** 193–199.

Louie, K., and Conrad, P. (1999) Characterization of a cDNA encoding a subtilisin-like serine protease (NC-p65) of *Neospora caninum*. *Molecular and Biochemical Parasitology* **103:** 211-223.

Macey, B.M. (2005) Probiotic effect of *Vibrio midae* SY9, *Cryptococcus* sp. SS1 amd *Debaryomyces hansenii* AY1 on the growth and disease resistance of farmed *Haliotis midae*. *PhD thesis, University of Cape Town, South Africa.*

Macey, B.M., and Coyne, V.E. (2005) Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* **245:** 249-261.

Macey, B.M., and Coyne, V.E. (2006) Colonization of the gastrointestinal tract of the farmed South African abalone *Haliotis midae* by the probionts *Vibrio midae* SY9, *Cryptococcus* sp. SS1, and *Debaryomyces hansenii* AY1. *Marine Biotechnology* **8:** 246-259.

Maguiña, G., Tapia, M.S., Briceño, A.G., Rodríguez, C., Sánchez, D., Roa, V., and López-Malo, A. (2002) Incoporation of *Bifidobacterium* spp. una matriz porosa de fruta por el mecanismo hidrodinámico (PF-08). *In: Actas del 2º Congreso Español de Ingeniería de Alimentos (pp. 1-4). Universitat de Lleida, Lleida, España.*

Mai, K. (1998) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino. VII. Effects of dietary vitamin C on survival, growth and tissue concentration of ascorbic acid. *Aquaculture* **161:** 383–392.

Makridis, P., Martins, S., Vercauteren, T., Van Driessche, K., Decamp, O., and Dinis, M.T. (2005) Evaluation of candidate probiotic strains for gilthead sea bream larvae (*Sparus aurata*) using an *in vivo* approach. *Letters in Applied Microbiology* **40:** 274-277.

Maloy, S., Stewart, V., and Taylor, R. (1996) *Genetic Analysis of Bacterial Pathogens*. Cold Spring Harbour Laboratory Press, New York.

Marcello, A., Loregian, A., De Filippis, V., Fontana, A., Hirst, T.R., and Palú, G. (1996) Identification and characterisation of an extracellular protease activity produced by the marine *Vibrio* sp. 60. *FEMS Microbiology Letters* **136:** 39-44.

Marchler-Bauer, A., Anderson, J.B., Cherukuri, P.F., DeWeese-Scott, C., Geer, L.Y., Gwadz, M., He, S., Hurwitz, D.I. Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Marchler, G.H., Mullokandov, M., Shoemaker, B.A., Simonyan, V., Song, J.S., Thiessen, P.A., Yamashita, R.A., Yin, J.J., Zhang, D., and Bryant, S.H. (2005) CDD: a Conserved Domain Database for protein classification. *Nucleic Acids Research* **33:** (Database issue) D192-D196.

Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., De-Weese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., He., S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Tasneem, A., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N., and Bryant, S.H. (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acid Research* **7:** (Database issue) D205-D210.

Marchler-Bauer, A., and Bryant, S.H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Research* **32:** (Web server issue) W327-W331.

Matthysse, A.G., Stretton, S., Dandie, C., McClure, N.C., and Goodman, A.E. (1996) Construction of GFP vectors for use in Gram-negative bacteria other than *Escherichia coli*. *FEMS Microbiology Letters* **145:** 87-94.

McConn, J.D., Tsuru, D., and Yasunobu, K.T. (1964) *Bacillis subtilis* neutral proteinase. *Journal of Biological Chemistry* **239:** 3706-3715.

McLean, N. (1970) Digestion in *Haliotis rufescens* Swainson (Gastropoda: Prosobranchia). *Journal of Experimental Zoology* **173:** 303-318.

Mei, C., and Jiang, X. (2005) A novel surfactant- and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL33-51. *Process Biochemistry* **40:** 2167-2172.

Meichtry, J., Amrhein, N., and Schaller, A. (1999) Characterization of the subtilase gene family in tomato (*Lycopersicon esculentum* Mill.). *Plant Molecular Biology* **39:** 749-760.

Meng, X. C., Stanton, C., Fitzgerald, G.F., Daly, C., and Ross, R.P. (2008) Anhydrobiotics: The challenges of drying probiotic cultures. *Food Chemistry* **106:** 1406-1416.

Mercurio, A., and Manning, P.A. (1985) Cellular localization and export of the soluble haemolysin of *Vibrio cholerae* El Tor. *Molecular and General Genetics* **200:** 472-475.

Merrifield, D.L., Bradley, G., Baker, R.T.M., and Davies, S.J. (2009) Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum) II. Effects of growth performance, feed utilization, intestinal microbiota and related health criteria postantibiotic treatment. *Aquaculture Nutrition* (doi: 10.1111/j.1365-2095.2009.00688.x).

Metchnikoff, E. (1907) The prolongation of life. Optimistic Studies. William Heinemann, London.

Mgaya, Y.D., and Mercer, J.P. (1995) The effects of size grading and stocking density on growth performance of juvenile abalone, *Haliotis tuberculata* Linnaeus. *Aquaculture* **136:** 297-312.

Miller, G.L. (1959) The use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31**:426-428.

Miller, V.L., and Mekalanos, J.J. (1988) A novel suicide vector and its use in the construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *V. cholera* requires ToxR. *Journal of Bacteriology* **170**: 2575-2583.

Milton, D.L., Norqvist, A., and Wolf-Watz, H. (1992) Cloning of a metalloprotease gene in the virulence mechanism of *Vibrio anguillarum*. *Journal of Bacteriology* **174:** 7235-7244.

Mintz, K.P., Brissette, C., and Fives-Taylor, P.M. (2002) A recombinase A-deficient strain of *Actinobacillus actinomycetemcomitans* constructed by insertional mutagenesis using a mobilizable plasmid. *FEMS Microbiology Letters* **206:** 87-92.

Miyamoto, T. (1962) *Ama; The Women Sea Divers in Japan*. Chunichi, Tokyo.

Miyoshi, N., Shimizu, C., Miyoshi, S., and Shinoda, S. (1987) Purification and characterization of *Vibrio vulnificus* protease. *Microbiology and Immunology* **31:** 13-25.

Moriarty, D.J.W. (1990) Interactions of microorganisms and aquatic animals, particularily the nutritional role of the gut flora. *In:* Lésel, R **(Ed.)**, *Microbiology of Poecilotherms*. Elsevier, Amsterdam, pp. 217-222.

Moriarty, D.J.W. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* **164:** 351-358.

Morita, J., and Kusuda, R. (1994) Protease production profiles of the fish pathogen *Listonella anguillara* based on substrate specificities. *Fisheries Science* **60:** 741-745.

Moter, A., and Gobel, U.B. (2000) Fluorescence *in situ* hybridization for direct visualization of microorganisms. *Journal of Microbial Methods* **41:** 85-112.

Muller, S. (1986) Taxonomy of the genus *Haliotis* in South Africa. *Transactions of the Royal Society of South Africa* **46:** 69–77.

Naik, A.T.R., Murthy, H.S., and Ramesha, T.J. (1999) Effects of graded levels of G-probiotic on growth, survival and feed conversion of tilapia, *Oreochromis mossambicus*. *Fish Technology* **36:** 63-66.

Nakada, H.I., and Sweeny, P.C. (1967) Alginic acid degradation by eliminases from abalone hepatopancreas. *Journal of Biological Chemistry* **242:** 845-851.

Nicolas, J.L., Basuyaux, O., Mazurie, J., and Thebault, A. (2002) *Vibrio carchariae*, a pathogen of the abalone *Haliotis tuberculata*. *Diseases of Aquatic Organisms* **50:** 35-43.

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S., and Lilius, E-M. (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish and Shellfish Immunology* **15:** 443-452.

Nishibuchi, M., and Kaper, J.B. (1985) Nucleotide sequence of the thermostable direct haemolysin gene of *Vibrio parahaemolyticus*. *Journal of Bacteriology* **162:** 558-564.

Nishimori, E., Hasegawa, O., Numata, T., and Wakabayashi, H. (1998) *Vibrio carchariae* causes mass mortalities in Japanese abalone, *Sulculus diversicolor supratexta*. *Fish Pathology* **33:** 495-502.

Norqvist, A., Norman, B., and Wolf-Watz, H. (1990) Identification and characterization of a zinc metalloprotease associated with invasion by the fish pathogen *Vibrio anguillarum*. *Infection and Immunity* **58:** 3731-3736.

Ochoa, S.J.L., and Olmos, S.J. (2006) The functional property of *Bacillus* for shrimp feeds. *Food Microbiology* **23:** 519-525.

OIE (World Organisation for Animal Health) (2006) *Diagnostic Manual for Aquatic Animal Diseases.* Office Interational Des Epizooties, Paris, France. (http://www.oie.int/eng/normes/fmanual/Asummary.html)

Oishi, K., Yokoshima, S., Tomiyama, T., and Aida, K. (1979) Exohemagglutinins: new products of vibrios. *Applied Environmental Microbiology* **38:** 169-172.

Okuda, J., and Nishibuchi, M. (1998) Manifestation of the Kanagawa phenomenon, the virulence-associated phenotype, of *Vibrio parahaemolyticus* depends on a particular single base change in the promoter of the thermostable direct haemolysin gene. *Molecular Microbiology* **30:** 499-511.

Olafsen, J.A. (2001) Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* **200:** 223-247.

Palmieri, G., Casbarra, A., Marino, G., Catara, G., Ruggiero, G., Capasso, A., and Rossi, M. (2005) High cleavage specificity of a subtilisin-like protease from a hyperthermophilic archaeon under extreme conditions. *Enzyme and Microbial Technology* **37:** 745-749.

Parsons, G.C., and Dadswell, M.J. (1992) Effect of stocking density on growth, production, and survival of the giant scallop, *Placopecten magellanicus*, held in intermediate suspension culture in Passamaquoddy Bay, New Brunswick. *Aquaculture* **103:** 291-309.

Paul, L.V. (2006) The role of melanin production in the survival of *Vibrio cholerae* in the marine environment. *PhD thesis, University of Cape Town, South Africa.*

Pearson, G.D.N., and Mekalanos, J.J. (1982) Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. *Proceedings of the National Academy of Sciences, USA* **79:** 2976 - 2980.

Picos-García, C., García-Carreño, F.L., and Serviere-Zaragoza, E. (2000) Digestive proteases in juvenile Mexican green abalone, *Haliotis fulgens*. *Aquaculture* **181:** 157-170.

Prieur, D., Mével, G., Nicolas, J.L., Plusquellec, A., and Vigneulle, M. (1990) Interactions between bivalve molluscs and bacteria in the marine environment. *Oceanography and Marine Biology: An Annual Review* **28:** 277-352.

Polak, J.M., and McGee, J.O.D. (1998) *In situ hybridization, principles and practice.* Oxford University Press.

Pollock, M.R. (1962) Exoenzymes. *In:* Gunsalus, J.C., and Stanier, R.Y. **(Eds.)**, *The Bacteria.* Academic Press Inc., London and New York. pp. 121-178.

Polson, A., Potgieter, G.M., Largier, J.F., Mears, G.E.F., and Joubert, F.J. (1964) The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochimica et Biophysica Acta* **82:** 463–475.

Popovič, T., Puizdar, V., and Brzin, J. (2002) A novel subtilase from common bean leaves. *FEBS Letters* **530:** 163-168.

Prasher, D.C. (1995) Using GFP to see the light. *Trends in Genetics* **11:** 320-323.

Preece, M.A., and Mladenov, P.V. (1999) Growth and mortality of the New Zealand abalone *Haliotis iris* Martyn 1784 cultured in offshore structures and fed artificial diets. *Aquaculture Research* **30:** 865-877.

Ramos-Vara, J.A. (2005) Technical aspects of immunohistochemistry. *Veterinary Pathology* **42:** 405-426.

Raser, K.J., Posner, A., and Wang, K.K. (1995) Casein zymography: a method to study μ-calpain, Mcalpain, and their inhibitory agents. *Archives of Biochemistry and Biophysics* **319:** 211-216.

Rattray, M., and Michael, G.J. (1999) Oligonucleotide probes for *in situ* hybridization. *In:* Wilkinson, D. G. **(Ed.)**, *In situ hybridization - A practical approach*. Oxford University Press, Oxford. pp 23-66.

Rawlings, N.D., and Barrett, A.J. (1994) Families of serine peptidases. *Methods in Enzymology* **244:** 19-61.

Rawlings, N.D., O'Brien, E., and Barret, A.J. (2002) MEROPS: the protease database. *Nucleic Acids Research* **30:** 343-346.

Raychaudhuri, S., Jain, V., and Dongre, M. (2006) Identification of a constitutively active variant of LuxO that affects production of HA/Protease and biofilm development in a non-O1, non-O139 *Vibrio cholerae* O110. *Gene* **369:** 126-133.

Reed, K.C., and Mann, D.A. (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Research* **13**: 7207-7221.

Reid, G.C., Woods, D.R., and Robb, F.T. (1980) Peptone induction and rifampicin insensitive collagenase production by *Vibrio alginolyticus. Journal of Bacteriology* **142:** 447-454.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., and Menasveta, P. (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* **167:** 301-313.

Rengpipat, S., Pusiririt, S., and Rukpratanporn, S. (2008) Differentiating between isolates of *Vibrio vulnificus* with monoclonal antibodies. *Journal of Microbiological Methods* **75:** 398-404

Rengpipat, S., Tunyamum, A., Fast, A.W., Piyatiratitivorakul, S., and Menasveta, P. (2003) Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. *Diseases of Aquatic Organisms* **55:** 169-173.

Rengpipat, S., Wongtangprasert, N., and Palaga, T. (2009) The use of green fluorescent protein as a marker for monitoring a probiotic *Bacillus* S11 in the black tiger shrimp *Penaeus monodon*. *Aquaculture Nutrition* **15:** 297-305.

Reyrat, J-M., Pelicic, V., Gicquel, B., and Rappuoli, R. (1998) Counterselectable Markers: Untapped Tools for Bacterial Genetics and Pathogenesis. *Infection and Immunity* **66:** 4011-4017.

Ringø, E., Birkbeck, T.H., Munro, P.D., Vadstein, O., and Hjelmeland, K. (1996) The effects of early exposure to *Vibrio pelagius* on the aerobic bacterial flora of turbot, *Scophthalmus maximus* (L) larvae. *Journal of Applied Bacteriology* **81:** 207-211.

Ringø, E., and Strøm, E. (1994) Microflora of Arctic charr, *Salvelinus alpinus* (L.): gastrointestinal microflora of free-living fish and effect of diet and salinity on intestinal microflora. *Aquaculture and Fisheries Management* **25:** 623-629.

Riquelme, C., Araya, R., Vergara, N., Rojas, A., Guarita, M., and Candia, M. (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Aquaculture* **154:** 17-26.

Robertson, P.A.W., O'Dowl, C., Burrels, C., Williams, P., and Austin, B. (2000) Use of *Carnobacterium* sp. as a probiotic for atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture* **185:** 235-243.

Rodríguez, M.I. (1998) Estudio de la penetración de microogansimos en frutas mediante el modelo Hidrodinámico (HDM). *Thesis, Instituto de Ciencia y Tecnología de Alimentas, Universidad Cemtral de Venezuela.*

Rybicki, E. (1979) The serology of the Bromoviruses. *M.Sc. Thesis, University of Cape Town, South Africa.*

Saborowski, R., Sahling, G., Navarette del Toro, M.A., Walter, I., and García-Carreño, F.I. (2004) Stability and effects of organic solvents on endopeptidases from the gastric fluid of the marine crab *Cancer pagurus*. *Journal of Molecular Catalysis B: Enzymatic* **30:** 109-118.

Sakata, T. (1990) Microflora in the digestive tract of fish and shellfish. *In* Lesel, R (**Ed.**) *Microbiology in poecilotherms*. Elsevier, Amsterdam, pp 171-176.

Sakata, T., Okabayashi, J., and Kakimoto, D. (1980) Variations in the intestinal microflora of Tilapia reared in fresh and sea water. *Bulletin of the Japanese Society of Scientific Fisheries* **46:** 313-317.

Sakurai, J., Matsuzaki, A., and Miwatani, T. (1973) Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infect. Immun*. **8:** 775-780.

Sales, J., and Britz, P. J. (2001) Research on abalone (*Haliotis midae* L.) cultivation in South Africa. *Aquaculture Research* **32:** 863-874.

Sales, J., Truter, P.J., and Britz, P.J. (2003) Optimum dietary crude protein level for growth in South African abalone (*Haliotis midae* L.). *Aquaculture Nutrition* **9:** 85-89.

Salinas, I., Cuesta, A., Esteban, M.A., and Mesegeur, J. (2005) Dietary administration of *Lactobacillus delbrüeckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish and Shellfish Immunology* **19:** 67-77.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning – A laboratory manual, 2nd Edition*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74:** 5463-5467

Sáenz de Rodrigáñez, M.A., Díaz-Rosales, P., Chabrillón, M., Smidt, H., Arijo, S., León-Rubio, J.M., Alarcón, F.J., Balebona, M.C., Moriñigo, M.A., Cara, J.B., and Moyano, F.J. (2009) Effect of dietary administration of probiotics on growth and intestine functionality of juvenile Senegalese sole (*Solea senegalensis,* Kaup 1858). *Aquaculture Nutrition* **15:** 177-185.

Saitou, N., and Nei, M. (1987) The neighbour-joining method a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

Sawabe, T., Hayashi, K., Moriwaki, J., Thompson, F., Swings, J., Potin, P., Christen, R., and Ezura, Y. (2004a) *Vibrio gallicus* sp. nov., isolated from the gut of the French abalone *Haliotis tuberculata. International Journal of Systematic and Evolutionary Microbiology* **54:** 843-846.

Sawabe, T., Hayashi, K., Moriwaki, J., Fukui, Y., Thompson, F., Swings, J., and Christen, R. (2004b) *Vibrio neonatus* sp. nov. and *Vibrio ezurae* sp. nov. Isolated from the gut of Japanese abalones. *Systematic and Applied Microbiology* **27:** 527-534.

Sawabe, T., Sugimura, I., Ohtsuka, M., Nakano, K., Tajima, K., Ezura, Y., and Christen, R. (1998) *Vibrio halioticoli* sp. nov., a non-motile alginolytic marine bacterium isolated from the gut of the abalone *Haliotis discus hannai*. *International Journal of Systematic Bacteriology* **48:** 573-580.

Sawabe, T., Oda, Y., Shiomi, Y., Ezura, Y. (1995) Alginate degradation by bacteria isolated from the gut of sea urchins and abalones. *Microbial Ecology* **30:** 193–202.

Schaffer, H.E., and Sederoff, R.R. (1981) Improved estimation of DNA fragment lengths from agarose gels. *Analytical Biochemistry* **115:** 113-122.

Scholz, U., Garcia Diaz, G., Ricque, D., Guz Suarez, L. E., Varqas Albores, F., and Latchford, J. (1999) Enhancement of vibriosis resistance in juvenile *Penaeus vannamei* by supplementation of diets with different yeast products. *Aquaculture* **176:** 271-283.

Schroeder, D.C., Jaffer, M.A., and Coyne, V.E. (2003) Investigation of the role of a β(1-4) agarose produced by *Pseudomonas gracilis* B9 in eliciting disease symptoms in the red alga *Gracilaria gracilis*. *Microbiology* **149:** 2919-2929.

Schwab, C., Walter, J., Tannock, G.W., Vogel, R.F., and Gänzle, M.G. (2007) Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri*. *Systematic and Applied Microbiology* **30:** 433-443.

Schwarz, S., Kehrenberg, C., and Walsh, T.R. (2001) Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents* **17:** 431-437.

Scott, K.P., Mercer, D.K., Richardson, A.J., Melville, C.M., Glover, L.A., and Flint, H.J. (2000) Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. *FEMS Microbiology Letters* **182:** 23-27.

Scott, M.E., Dossani, Z.Y., and Sandkvist, M. (2001) Directed polar secretion of protease from single cells of *Vibrio cholerae* via the type II secretion pathway. *PNAS* **98:** 13978 13983.

Segers, R., Butt, T.M., Carder, J.H., Keen, J.W., Kerry, B.R., and Peberdy, J.F. (1999) The subtilisins of fungal pathogens of insects, nematodes and plants: distribution and variation. *Mycological Research* **103:** 395-402.

Serviere-Zaragoza, E., Navarrete del Toro, M.A., and García-Carreño, F.L. (1997) Protein-hydrolyzing enzymes in the digestive systems of the adult Mexican blue abalone, *Haliotis fulgens* (Gastropoda). *Aquaculture* **157:** 325-336.

Sharifuzzaman, S.M., and Austin, B. (2009) Influence of probiotic feeding duration on disease resistance and immune parameters on disease resistance and immune parameters in rainbow trout. *Fish and Shellfish Immunology* **27:** 440-445.

Shaw, B.L., and Battle, H.I. (1957) The gross and fine microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Canadian Journal of Zoology* **35:** 325-347.

Shi, J., Coyne, V.E., and Weiner, R.M. (1997) Identification of an alkaline metalloproteases produced by the hydrothermal vent bacterium *Hypomonas jannachiana* VP3. *Microbios* **91:** 15-26.

Shieh, W.Y., Chen, Y.-W., Chaw, S.-M., and Chiu, H.-H. (2003) *Vibrio ruber* sp. nov., a red, facultatively anaerobic, marine bacterium isolated from sea water. *International Journal of Systematic and Evolutionary Microbiology* **53:** 479-484.

Shinde, U., and Inouye, M. (1993) Intramolecular chaperone and protein folding. *Trends in Biochemical Sciences* **18:** 442-446.

Shinde, U., and Inouye, M. (2000) Intramolecular chaperones: polypeptide extensions that modulate protein folding. *Seminars in Cell and Developmental Biology* **11:** 35-44.

Shinde, U.P., Liu, J.J., and Inouye, M. (1997) Protein memory through altered folding mediated by intramolecular chaperones. *Nature* **389:** 520-522.

Shipton, T.A. (1999) The protein requirements of the South African abalone, *Haliotis midae*. *PhD Thesis, Rhodes University, South Africa.*

Shipton, T. A., and Britz, P. J. (2001) The effect of animal size on the ability of *Haliotis midae* L. to utilize selected dietary protein sources. *Aquaculture Research* **32:** 393-403.

Short, J., Fernandez, J., Sorge, J., and Huse, W. (1988) λZAP: a bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acid Research* **16:** 7583-7600.

Siezen, R.J., de Vos, W.M., Leunissen, J.A.M., and Dijkstra, B.W. (1991) Homology modelling and engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Engineering* **4:** 719-737.

Siezen, R.J., Creemers, J.W.M., and Van De Ven, W.J.M. (1994) Homology modelling of the catalytic domain of human furin - A model for the eukaryotic subtilisin-like proprotein convertases. *European Journal of Biochemistry* **222:** 255-266.

Siezen, R.J., and Leunissen, J.A.M. (1997) Subtilases: The superfamily of subtilisin-like serine proteases. *Protein Science* **6:** 501-523.

Simon, C.A., and McQuaid, C. (1999) Extracellular digestion in two co-occurring intertidal mussels (*Perna perna* (L.) and *Choromytilus meridionalis* (Kr)) and the role of enteric bacteria in their digestive ecology. *Journal of Experimental Marine Biology and Ecology* **234:** 59-81.

Simon, R., Preifer, U., and Puler, A. (1983) A broad range mobilization system for *in vivo* genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1:** 784-791.

Simpson, B.J.A. (1994) An investigation of diet management strategies for culture of South African abalone, *Haliotis midae*. *M.Sc. thesis, University of Cape Town, South Africa.*

Singh, J., Vohra, R.M., and Sahoo, D.K. (2004) Enhanced production of alkaline proteases by *Bacillus spaericus* using fed-batch culture. *Process Biochemistry* **39:** 1093-1101.

Smith, G.C., and Merkel, J.R. (1982) Collagenolytic activity of *Vibrio vulnificus*: potential contribution to its invasiveness. *Infection and Immunity* **35:** 1155-1156.

Sohaskey, C.D., Im, H., and Schauer, A.T. (1992) Construction and application of plasmid- and transposon-based promoter-probe vectors for *Streptomyces* spp. that employ a *Vibrio harveyii* luciferase reporter cassette. *Journal of Bacteriology* **174:** 367-376.

Spanggaard, B., Huber, I., Nielsen, J., Sick, E.B., Pipper, C.B., Martinussen, T., Slierendrecht, W.J., and Gram, L. (2001) The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environmental Microbiology* **3:** 755-765.

Spory, A., Bosserhoff, A., von Rhein, C., Goebel, W., and Ludwig, A. (2002) Differential regulation of multiple proteins of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium by the transcriptional regulator SlyA. *Journal of Bacteriology* **184:** 3549-3559.

Stahl, D.A., Devereux, R., Amann, R.I., Flesher, B., Lin, C., and Stromley, J. (1989) Ribosomal RNA based studies of natural microbial diversity and ecology. *In:* Hattori, T., Ishida, Y., Maruyama,Y., Morita, R., and Uchida, A. **(Eds.)**, *Recent advances in microbial ecology* Japan Scientific Societies Press, Tokyo, Japan. pp 669-673.

Stanford, J. (2004) Aquaculture ambition: South African aquaculture industry is heading for an export boom. *Engineering News*, **August 6-12**: 16-17.

Stearns, R.E.C. (1869) The Haliotis, or Pearly Ear-Shell. *The American Naturalist*, **3:** 250-256.

Stewart, B.J., and McCarter, L.L. (2003) Lateral flagellar gene system of *Vibrio parahaemolyticus*. *Journal of Bacteriology* **185:** 4508-4518.

Stewart-Tull, D.E.S., Bleakley, C.R., and Galloway, T.S. (2004) Characteristics of *Vibrio cholerae* proteinases: potential, candidate vaccine antigens. *Vaccine* **22:** 3026-3043.

Stormo, G.D., Schneider, T.D., and Gold, L.M. (1982) Characterization of translational initiation sites in *E. coli*. *Nucleic Acids Research* **10:** 2971-2996.

Streit, A., and Stern, C.D. (2001) Combined whole-mount *in situ* hybridization and immunohistochemistry in avian embryos. *Methods* **23:** 339-344.

Stretton, S., Techkarnjanaruk, S., McLennan, A.M., and Goodman, A.E. (1998) Use of green fluorescent protein to tag and investigate gene expression in marine bacteria. *Applied and Environmental Microbiology* **64:** 2554-2559.

Sudheesh, P.S., and Xu, H.S. (2001) Pathogenicity of *Vibrio parahaemolyticus* in tiger prawn *Penaeus monodon* Fabricius: possible role of extracellular proteases. *Aquaculture* **196:** 37-46.

Sugano, Y., Matsumoto, T., Kodama, H., and Noma, M. (1993) Cloning and sequencing of *agaA*, a unique agarose 0107 gene from a marine bacterium, *Vibrio* sp. Strain JT0107. *Applied and Environmental Microbiology* **59:** 3750-3756.

Suzer, C., Çoban, D., Kamaci, H.O., Saka, Ş., Firat, K., Otgucuoğlu, Ö., and Küçüksari, H. (2008) *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effect son growth performance and digestive enzyme activities. *Aquaculture* **280:** 140-145.

Suzuki, M., Taguchi, S., Yamada, S., Kajima, S., Miura, K.I., and Momose, H. (1997) A novel member of the subtilisin-like protease family from *Streptomyces albogriseolus*. *Journal of Bacteriology* **179:** 430-438.

Swartzman, E., Silverman, M., and Meighen, E.A. (1992) The *luxR* gene product of *Vibrio harveyi* is a transcriptional activator of the *lux* promoter. *Journal of Bacteriology* **174:** 7490-7493.

Takagi, H., and Takahashi, M. (2003) A new approach for alteration of protease functions: Pro-sequence engineering. *Applied Microbiology and Biotechnology* **63:** 1-9.

Tanaka, R., Ootsubo, M., Sawabe, T., Ezura, Y., and Tajima, K. (2004) Biodiversity and in situ abundance of gut micrflora of abalone (*Haliotis discus hannai*) determined by culture-independent techniques. *Aquaculture* **241:** 453-463.

Tanaka, R., Sugimura, I., Sawabe, T., Yoshimizu, M., and Ezura, Y. (2003) Gut microflora of abalone *Haliotis discus hannai* in culture changes coincident with a change in diet. *Fisheries Science* **69:** 951-958.

Taniguchi, H., Ohta, H., Ogawa, M., and Mizuguchi, Y. (1985) Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile hemolysin genes. *Journal of Bacteriology* **162:** 510-515.

Tannock, G.W., Ghazally, S., Walter, J., Loach, D., Brooks, H., Cook, G., Surette, M., Simmers, C., Bremer, P., Dal Bello, F., and Hertel, C. (2005) Ecological behaviour of *Lactobacillus reuteri* 100-23 is affected by mutation of the LuxS gene. *Applied and Environmental Microbiology* **71:** 8419-8425.

Taylor, M. (2002) Construction of a genomic library and isolation of putative proteolytic clones of the marine bacterium *Shewanella* sp. strain SY9. *Honours thesis, University of Cape Town, South Africa.*

Teo, J.W.P., Zhang, L.-H., and Poh, C.L. (2003) Cloning and characterization of a metalloprotease from *Vibrio harveyi* strain AP6. *Gene* **303:** 147-156.

ten Doeschate, K. (2005) *Pseudomonas* sp. strain C4 as a probiotic for farmed South African abalone, *Haliotis midae*. *PhD thesis, University of Cape Town, South Africa.*

ten Doeschate, K., and Coyne, V.E. (2008) Improved growth rate in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* **284:** 174–179.

Thangam, E.B., and Rajkumar, G.S. (2002) Purification and characterization of alkaline protease from *Alcaligenes faecalis*. *Biotechnology and Applied Biochemistry* **35:** 149-154.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* **25:** 4876–4882.

Troell, M., Robertson-Andersson, D., Anderson, R.J., Bolton, J.J., Maneveldt, G., Halling, C., and Probyn, T. (2006) Abalone farming in South Africa: An overview with perspectives on kelp resources, abalone feed, potential for on-farm seaweed production and socio-economic importance. *Aquaculture* **257:** 266-281.

Tseng, D.-Y., Ho, P.-L., Huang, S.-Y., Cheng, S.-C., Shiu, Y.-L., Chiu, C.S., and Liu, C.-H. (2009) Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. *Fish and Shellfish Immunology* **26:** 339-344.

Tovar, D., Zambonino, J., Cahn, C., Gatesoupe, F.J., Vázquez-Juárez, R., and Lésel, R. (2002) Effect of live yeast incorportation in compound diet on digestive enzyme activity in sea badd (*Dicentrachus labrax*) larvae. *Aquaculture* **204:** 113-123.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76:** 4350-4354.

Tsujino, I., and Saito, T. (1962) Studies on alginase. Part II. A new unsaturated uronide isolated from alginase hydrolysate. *Agricultural and Biological Chemistry* **26:** 115-118.

Valbuzzi, A., Ferrari, E., and Albertini, A.M. (1999) A novel member of the subtilisin-like protease family from *Bacillus subtilis*. *Microbiology* **145:** 3121-3127.

Van den Bogaard, A.E., and Stobberingh, E.E. (2000) Epidemiology of resistance to antibiotics: links between animals and humans. *International Journal of Antimicrobial Agents.* **14:** 327-335.

Vaseeharan, B., and Ramasamy, P. (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon. Letters in Applied Microbiology* **36:** 83-87.

Venugopal, M., and Saramma, A.V. (2006) Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolate from mangrove sediment sample and its application as a laundry detergent additive. *Process Biochemistry* **41:** 1239-1243.

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64:** 655-671.

Vosloo, A., and Vosloo, D. (2006) Routes of water loss in South African abalone (*haliotis midae*) during aerial exposure. *Aquaculture* **261:** 670-677.

Walter, J., Chagnaud, P., Tannock, G.W., Loach, D.M., Dal Bello, F., Jenkinson, H.F., Hammes, W.P., and Hertel., C. (2005) A high-molecular surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological performance of *Lactobacillus reuteri* in the murine gut. *Applied and Environmental Microbiology* **71**: 979-986.

Wang, S-L., Chio, Y-H., Yen, Y-H., and Wang, C-L. (2007) Two novel surfactant-stable alkaline proteases from *Vibrio fluvialis* TKU005 and their applications. *Enzyme and Microbial Technology* **40:** 1213-1220.

Wang, Y.-B. (2007) Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture* **269:** 259-264.

Wang, Y., Zhang, Z., and Ruan, J. (1996) A proposal to transfer *Microbicrobispora bispora* (Lechevalier 1965) to a new genus, *Thermobispora* gen. nov., and *Thermobispora bispora* comb. nov. *International Journal of Systematic Bacteriology* **46:** 933-938.

Watanabe, H., Miyoshi, S., Kawase, T., Tomochika, K., and Shinoda, S. (2004) High growing ability of *Vibrio vulnificus* biotype 1 is essential for production of a toxic metalloprotease causing systematic diseases in humans. *Microbial Pathogenesis* **36:** 117-123.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*. **173:** 697-703.

Wells, J.A., Ferrari, E., Henner, D.J., Estell, D.A., and Chen, E.Y. (1983) Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. *Nucleic Acids Research* **11:** 7911-7925.

Welton, R.L., and Woods, D.R. (1973) Halotolerant collagenolytic activity of *Archromobacter iophagus*. *Journal of General Microbiology* **75:** 191-196.

Wilkinson, D.G. (1999) The theory and practise of *in situ* hybridization. *In:* Wilkinson, D.G. **(Ed.)** *In situ hybridization - A practical approach*. Oxford University Press, Oxford, pp. 1-21.

Wong, S.-L., and Doi, R.H. (1986) Determination of the signal peptidase cleavage site in the preprosubtilisin of *Bacillus subtilis*. *The Journal of Biological Chemistry* **261:** 10176-10181.

Yanbo, W., and Zirong, X. (2006) Effects of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Animal Feed Science and Technology* **127:** 283-292.

Yanisch-Perron, C., Viera, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33:** 103-119.

Yeats, C., Bentley, S., and Bateman, A. (2003) New knowledge from old: *In silico* discovery of novel protein domains in *Streptomyces coelicolor*. *BMC Microbiology* **3:** 1-20.

Young, D.B., and Broadbent, D.A. (1982) Biochemical characterisation of extracellular proteases from *Vibrio cholerae*. *Infection and Immunity* **37:** 875-883.

Zhou, Q., Li, K., Jun, X., and Bo, L. (2009) Role and functions of beneficial microorganisms in sustainable aquaculture. *Bioresource Technology* **100:** 3780-3786.

Ziaei-Nejad, S., Rezaei, M.H., Takami, G.A., Lovett, D.L., Mirvaghefi, A-R., and Shakouri, M. (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture* **252:** 516-524.

Zischler, H., Nanda, I., Schafer, R., Schmid, M., and Epplen, J.T. (1989) Digoxygenated oligonucleotide probes specific for simple repeats in DNA fingerprinting and hybridization *in situ*. *Human Genetics* **82:** 227-233.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and van 'T Riet, K. (1990) Modelling of the bacterial growth curve. *Applied and Environmental Microbiology* **56:** 1875-1881.