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Pseudoalteromonas sp. strain C4 as a Probiotic for farmed South African Abalone, _Haliotis midae_

by

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University of Cape Town

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa.

Cape Town

January 2005

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For Eddie – my soul mate, my
best friend, my strength, my
comfort, my joy, my love –thank-
you for your love and support,
this wouldn't be possible without
you. Miss you much and forever
in my heart, until we meet again.

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ABSTRACT

The South African abalone, *Haliotis midae*, is an economically important commercial species and is farmed intensively in shore-based tanks along the South African coast. Abalone, like other aquacultured species, are subject to numerous stresses that can negatively impact growth and reduce the immunocompetence of the animal, increasing their susceptibility to pathogenic infections. Recently, the use of probiotics as a means of increasing growth and preventing disease in aquacultured species such as shrimps, oysters and fish has been demonstrated. The objective of this study was to identify a potential probiotic bacterium that increased the growth rate and decreased the susceptibility of farmed abalone to pathogenic bacterial infection. The mechanism by which the probiotic is able to increase growth rates and reduced susceptibility to pathogen infection was also investigated.

A number of bacterial strains were isolated from the digestive tract of *Haliotis midae* that are capable of degrading a wide variety of different polysaccharides (Erasmus, 1996). Strain C4 was selected for further investigation as a result of its ability to degrade alginate since *H. midae* is predominately fed a kelp diet of which the major component is alginate. Strain C4 was identified as a member of the *Pseudoalteromonas* genus based on phylogenetic analysis and biochemical characteristics and was designated *Pseudoalteromonas* sp. strain C4.

The growth characteristics of strain C4 and its production of alginate lyase were tested in various media. Both extracellular and intracellular alginate lyase activity was induced in the presence of alginate. The addition of glucose to the culture media did not affect extracellular alginate lyase activity although intracellular alginate lyase activity was significantly reduced. Extracellular activity was optimised between 22°C and 37°C and between a pH of 6 and 6.5.

Strain C4 was successfully added to a kelp-based feed at concentrations of approximately 2.4×10^{10} $cfu.g^{-1}$ feed. Abalone fed a kelp-based diet supplemented with strain C4 showed increased alginate lyase activity in the digestive tract compared to abalone fed a standard kelp diet. When feeding the supplemented diet was halted, increased levels of alginate lyase activity were observed in the crop and stomach for up to three days and in the intestine for up to one day compared to abalone continually fed a standard kelp diet. Strain C4 was labeled with ^{14}C-sodium acetate and orally inoculated into abalone. Strain C4 proteins were shown to be incorporated into abalone tissue. Incorporation was predominantly in the hepatopancreas, but also in the gills, foot muscle, adductor muscle and intestine. Abalone fed a kelp-based diet supplemented with strain C4 had significantly higher growth rates compared to abalone fed a standard kelp diet both under laboratory conditions and on the abalone farm. The increased growth rate can potentially reduce the grow-out phase by up to 17 months over a 4 year period. The increase of growth rate may be the result of strain C4 contributing to the pool of enzymes available for digestion of seaweed polysaccharides in the digestive tract of *H. midae* as well as strain C4 itself acting as a protein supplement to the diet of *H. midae*.

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Strain C4 was shown to inhibit the growth of the pathogens *Vibrio alginolyticus* and *Vibrio anguillarum* when co-cultured *in vitro*. Strain C4 also reduced the *in vitro* ability of *V. anguillarum* to adhere to mucous isolated from the oesophagus of *H. midae*. Haemolymph isolated from *H. midae* fed a kelp-based diet supplemented with strain C4 displayed increased phagocytic abilities and increased intracellular superoxide anion production compared to abalone fed a standard kelp diet. Abalone fed a strain C4 supplemented diet and infected with *V. anguillarum* initially showed higher levels of intracellular superoxide anion production and phagocytic activity and were able to eliminate *V. anguillarum* from the haemolymph more rapidly compared to abalone fed a standard diet. Mortalities were reduced from 21% in abalone fed a standard kelp diet to 6% in abalone fed a strain C4 supplemented diet when abalone were infected with *V. anguillarum*. Therefore, addition of strain C4 to the diet of *H. midae* reduces the susceptibility of the abalone to infection by the bacterial

pathogen *V. anguillarum*. Strain C4 may compete with the pathogen for both nutrients and adhesion sites. The severity of the infection may also be reduced due to the elevated immune response (phagocytic activity and superoxide anion production) observed in abalone fed a strain C4 supplemented diet and the greater efficiency with which these abalone are able to eliminate *V. anguillarum* from the haemolymph compared to abalone fed a standard diet.

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ABBREVIATIONS

α	alpha
β	beta
ρ	rho
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees Celsius
%	percentage
$\sum$	sum of
$	United States dollars
A	adenine
A_{600}	absorbance at 600 nm
Ab	antibiotics
AB	alginate broth
BMA	basal marine agar
BMB	basal marine broth
bp	base pairs
C	cytosine
cfu	colony forming units
Ci	Curie
cm	centimetre(s)
CPM	counts per minute
d	day(s)
DNA	deoxyribonucleic acid
DPM	disintegrations per minute

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EM	electron microscope
et al.	and others (*et alii*)
EtOH	ethanol
G	guanine
g	gram(s)
g	centrifugal force
h	hour(s)
kb	kilobase(s)
kDa	kilodalton(s)
kJ	kilojoule(s)
L	litre
M	molar
mA	milli-Ampere(s)
MA	marine agar
MB	marine broth
mg	milligram
min	minute(s)
ml	millilitre
mM	millimolar
mm	millimetre
mmol	nanomoles
mol	mole(s)
NADP	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometre

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OD	optical density
p	probability
PCR	polymerase chain reaction
rDNA	ribosomal deoxynucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RSA	Republic of South Africa
s	second(s)
SE	standard error
sp.	species
SSS	sterile sea salts
t	tonne(s)
T	thymine
t	time
TEMED	N,N,N',N'-tetramethylethylenediamine
U	unit(s)
UV	ultra violet
V	volt(s)
W	Watt(s)
w/v	weight per volume
wt.	weight
ZAR	South African Rand(s)

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CHAPTER 1
GENERAL INTRODUCTION
CONTENTS

1.1 ABALONE AQUACULTURE

1.2 THE DEVELOPMENT OF PROBIOTIC CONCEPTS

1.3 MARINE INVERTEBRATE IMMUNE RESPONSES

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1.1 ABALONE AQUACULTURE

Abalone are members of the phylum Mollusca, classed as Gastropoda and assigned to the family Haliotidae and genus *Haliotis*. This makes abalone distantly related to clams, scallops, octopus and squid and more closely related to sea slugs, snails and whelks. There are approximately ninety described species of abalone worldwide. Six species have been described for the southern African coast, namely *Haliotis midae, Haliotis spadicea, Haliotis parva, Haliotis queketti, Haliotis speciosa* and *Haliotis pustulata*. *Haliotis midae* is the largest and most abundant of the southern African species and occurs from St. Helena Bay (Western Cape, RSA) to Port St. Johns (Eastern Cape, RSA). As a result, *H. midae* is the only species in South Africa upon which a commercial fishery and aquaculture has been based.

The world market for cultured abalone is primarily for live specimens in the 50–100 mm size range (Oakes and Ponte, 1996). Accordingly most cultured abalone are harvested at the end of their third year when they are approximately 70 mm in length. These smaller abalone are referred to as cocktail abalone to distinguish them from the larger naturally occurring abalone. The great demand for cocktail abalone has resulted in the establishment of abalone aquaculture across the world including California, USA, Japan, China, Taiwan, Australia, Chile and South Africa. Between 1989 and 1999 world abalone fisheries declined by about 30% while the production of cultured abalone has increased by over 600% (Gordon and Cook, 2001). Asia is by far the largest producer of cultured abalone, producing 5, 500 t or 75% of the world total. Within Asia, China and Japan produce the vast majority of cultured abalone. Abalone are a sought after delicacy, especially in eastern Asia, and fetch prices of approximately \$34 - \$36/kg for cocktail sized abalone (Stanford, 2004). The current world supply of abalone is 22 000 t per year. Land–based abalone aquaculture has expanded in South Africa over the last decade, and there are now at least nine farms in various stages of production. The South African abalone aquaculture industry produced approximately 300 t of abalone in 2001, an increase from 28 t in 1999, at a value of ZAR 5.94 million. South Africa produces between 800 and 900 t of farmed abalone and about 300 t of abalone are harvested from the wild (Stanford, 2004). Production is expected to increase exponentially within the next few years as more farms are started and others become fully operational. Live abalone are packed in cool boxes and flown overnight to markets in Hong Kong, Singapore and Tokyo. Abalone are then sold from live tanks to supermarkets and restaurants.

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In the 1960s and 1970s researchers from academic, government and private sectors developed the technology to spawn and raise juvenile abalone in captivity. The basic culture techniques were developed in Japan with a view to restocking overexploited natural habitats. Currently, in South Africa, abalone are grown intensively in land-based systems with seawater obtained from shore based pumping. The advantage of this type of culture is that complete control can be exerted over all phases of the production cycle. Most farms have vertically integrated a hatchery, nursery and grow out phase, thereby removing the necessity of purchasing spat to stock the grow out phase. Male and female adult abalones are induced to spawn by hydrogen peroxide treatment or UV irradiation of the water (Figure 1). Egg and sperm are mixed in the correct proportions for fertilisation. Fertilised eggs are transferred to hatching tanks where hatch–out occurs 10–24 h post fertilisation. Trocophore larvae are held for 7–9 days in a rearing chamber. The pre–settlement or veliger larvae feed on yolk of the egg. Plastic plates covered in suitable microalgae are used as settlement surfaces. Abalone metamorphose into the first feeding stage and feed on bacteria and fungal spores. When abalone measure approximately 8 mm they are weaned onto macroalgae, for example kelp, and then transferred to the grow out facility. The grow out phase makes use of large modular cellular raceway systems with directional flow coupled with vigorous aeration serving to remove debris and faeces. This system allows for minimum labour intensity that is limited to food addition, growth rate monitoring and grouping animals by size. When abalone reach cocktail size (50-80 mm) they are harvested and exported, usually frozen or canned, although an increasing proportion are exported live.

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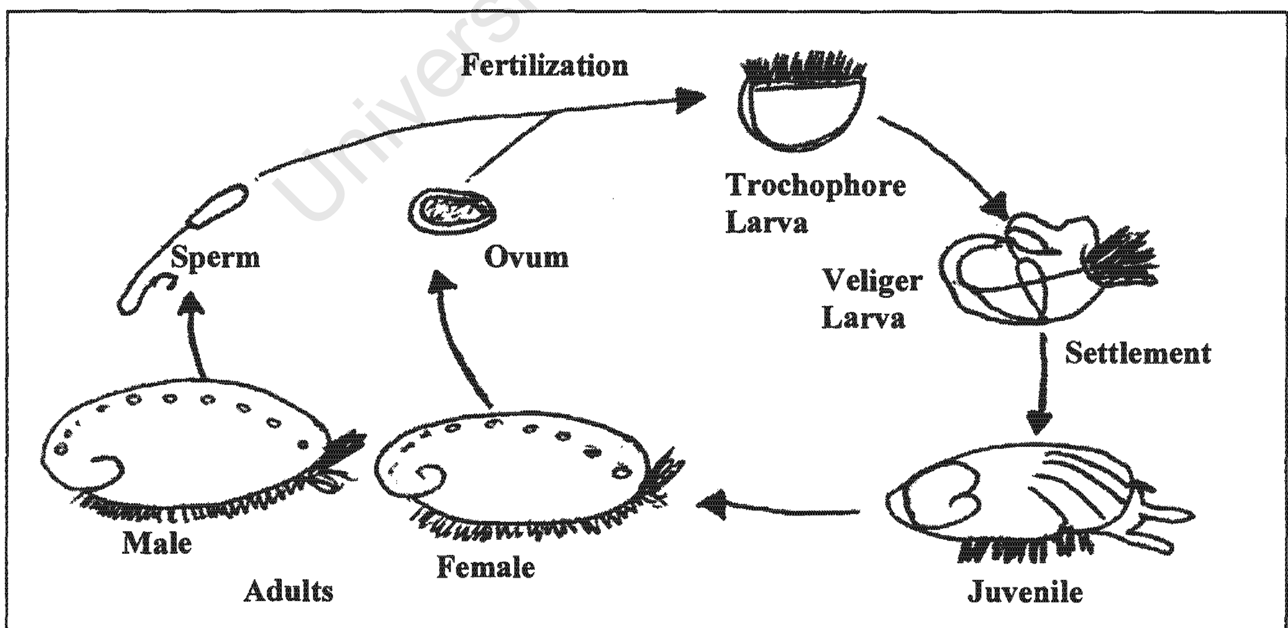

Figure 1. Life-cycle of abalone in the wild. Male and female abalone are induced to spawn by hydrogen peroxide treatment or UV irradiation of the water under farming conditions (www.fishtech.com).

Abalone aquaculture is subject to a number of constraints that negatively effect growth rates, promote disease outbreak and thereby decrease the yield of produce. These constraints include high stocking densities, poor quality feeds and increased stress. A major problem facing abalone farms is obtaining high growth rates. High water temperatures or increased food conversion efficiency has been shown to increase growth rates (Britz *et al.*, 1997). Selective breeding programs whereby two fast growing abalone are hybridised may also increase growth rate. Californian species of abalone have successfully been hybridised and have shown higher growth rates or higher larval survival for some of the crosses compared to either of the parent species (Leighton and Lewis, 1982). Another problem facing abalone aquaculture, as in agriculture, is disease (Olafsen, 2001). Introduction of a pathogen into an abalone farm has the potential to spread and destroy the entire stock. Chemotherapeutic agents and vaccines have been used to protect fish against different bacterial diseases (Nikoskelainen *et al.*, 2001). However chemotherapeutic agents can lead to the development of resistant bacteria, vaccines are stressful for fish, and both methods are expensive. The use of vaccines in abalone aquaculture has not been investigated since an adaptive immune system does not occur in abalone. The use of antibiotics in aquaculture is subject to controversy. Residues of the drug may remain active after use and could be excreted by the animal into the water or remain within the animal (Gibson *et al.*, 1998). The excreted residues or unused free antibiotic (such as oxolinic acid) can enter the environment and be taken up by wild aquatic fauna. The use of antibiotics has also resulted in adverse effects on bivalve larval survival and growth (Douillet and Langdon, 1994). Antibiotics may also dramatically change the intestinal microflora of the animal, and thus, impair its first–line of defence against infection (Olafsen, 2001). Antibiotics have been used in French scallop cultures to prevent disease (Robert *et al.,* 1996), however the use of antibiotics can lead to the acquirement of multi-drug resistance in bacteria (Riquelme *et al.,* 1996). There is also evidence that antibiotics increased the colonisation of the chicken gut by *Salmonellae*. Anti–additive lobbyists have presented antibiotics as foreign substances that should not be present in the food chain. Probiotics, on the other hand, are microorganisms that have been included in food for years without any adverse effects or are microorganisms that are present in the digestive tract of healthy animals and are possible alternatives to antibiotics and chemotherapeutic agents for controlling disease and may potentially increase the growth rates in cultured abalone.

1.2 THE DEVELOPMENT OF PROBIOTIC CONCEPTS

The generally accepted definition of a probiotic is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). However, in aquatic environments, host-microbe interactions are not limited to the intestinal tract.

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In an aquatic environment, microorganisms and their hosts share the same ecosystem, with the result that microorganisms can live in association with the host, either intestinally, or on its skin or gills, or it can share its environment. Thus, the traditional definition of a probiotic in a terrestrial environment needs to be revised for an aquatic environment. Numerous revised definitions have been suggested for probiotics in aquaculture. Gatesoupe (1999) suggested that probiotics are "microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health". Gram *et al.* (1999) removed the restriction to the intestine, to give "a live microbial supplement which beneficially affects the host animal by improving its microbial balance". Verschuere *et al.* (2000) proposed the following, broader definition of a probiotic: "a probiotic is defined 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". Jory (1998) defined probiotics as "cultures (single or mixed) of selected strains of bacteria that are used in culture and production systems (tanks, ponds and other) to modify or manipulate the microbial communities in water and sediment, reduce or eliminate selected pathogenic species of microorganisms, and generally improve growth and survival of the targeted species". This definition seems to be the most applicable to aquaculture.

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Farming practices such as sorting, grading and transporting of animals can be stressful and impact negatively on growth and immunocompetence of the cultured animal. These negative effects may be partially due to the disruption of the intestinal microflora caused by farming practices and the difficulty of establishing a stable intestinal microbial community under farm conditions. As mentioned in the previous section, a number of methods have been tested to increase growth rates and control pathogenic infection under farm conditions. These methods are however often expensive, time-consuming and impractical simply because of the large number of cultured animals that need to be treated. Probiotics offer an alternative that is cost-effective, easily administered, environmentally safe and do not pose a threat to the consumer who will ultimately consume the product. Probiotics have been used on farms since the late 1960s when King (1968) obtained significant growth stimulation of pigs by supplementing their feed with *Lactobacillus acidophilus*. The use of probiotics was stimulated in 1969 after the Swann Committee recommended that antibiotics in animal feed be restricted to those not used therapeutically (Fuller, 1999) and this created a void that probiotics began to fill. Even though probiotics have been in existence for decades the 'science' associated with probiotics has been remarkably weak. Basic research on the use of marine bacteria as probiotics is not well developed (Riquelme *et al.,* 2000) and studies on the

use of probiotics in aquaculture are rare and often inconclusive. Scientists must now search for experimental evidence to support long–held beliefs associated with probiotics already available. Probiotic research is burdened with a reliance on *in vitro* experimentation and much effort has been directed at screening bacterial isolates for properties deemed appropriate for a probiotic strain (Tannock, 1999). These are mostly characteristics that enable the probiotic to survive passage through the digestive tract. Selection of the best microorganisms for inclusion in a probiotic product is dependent on how the probiotic effect is produced. Future development of probiotics should direct research efforts at elucidating the probiotic mechanism so that probiotic activity can be recognised and assessed without resorting to lengthy and costly animal feeding trials (Fuller, 1999).

Theoretically, an ideal probiotic would establish itself permanently in the gut after a single dose. However, this is improbable as evidence suggests that the intestinal microflora is in a continuous state of flux and that a succession of different strains of the same species colonise the gut of the same animal (Fuller, 1999). This is especially true in marine invertebrates where animals are dependent on the external environment due to the water flow passing through the digestive tract and where microbes from water and food continually intrude on the gut (Gatesoupe, 1999). The most active probiotics in ruminants are unable to grow in the rumen, however a few hours of viability is all that is required for the manifestation of the probiotic effect (Fuller, 1999) and therefore the concept that a probiotic should establish itself in the host's gut should be re-evaluated. Residence time in the digestive tract should however be maximised to allow probiotic organisms time to manifest their effect (Kirjavainen *et al.,* 1998). Adherence to the gut wall may also be of importance as this could lead to competitive exclusion of potential pathogens by preventing pathogens from finding an ecological niche on the gut epithelium. Colonisation of a host's digestive tract is complex, and the stability and effect of a probiotic treatment will depend on several factors (Skjermo and Vadstein, 1999). Different bacteria have different mechanisms for establishing themselves in the gastrointestinal tract, and probionts should either be able to colonise the gut or exist transiently in the host's gut in order to improve the nutritional status of the host by improving the function of the gut. Probionts should be isolated from healthy animals so as to reduce the possibility of using a potentially harmful microbe. In aquaculture systems probiotics may have many beneficial effects on the host animal such as increased resistance to infectious disease by competitive exclusion of pathogens or producing pathogen inhibiting substances, stimulation of phagocytosis, increased growth rate, improved feed conversion, improved digestion through the supply of essential enzymes, better absorption of nutrients and provision of essential

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nutrients (Jory, 1998, Fuller, 1999, Tannock, 1999). These beneficial effects would therefore be expected to improve both the health and the growth rate of the host animal (Fuller, 1999).

1.3 MARINE INVERTEBRATE IMMUNE RESPONSES

The use of probiotics to control disease outbreaks in cultured animals is becoming an increasingly attractive alternative to chemotherapeutic agents and antibiotics as a result of the cost-effectiveness and low risk of probiotics. Until recently it was believed that invertebrates had a strictly innate immune system which would make vaccines ineffective, however the existence of an adaptive immune system in invertebrates has recently emerged although it is believed to be both quantitatively and qualitatively different from vertebrates (Olafsen, 2001). Limited knowledge exists regarding the immune defence systems of invertebrates but since the existence of invertebrates pre-dates vertebrates, for example some species of shrimp have existed 30 million years, it is obvious that they must possess an efficient system of defence. What is known is that the marine invertebrate immune defence mechanisms involve a cellular response and a humoral response. Cellular responses include phagocytosis and haemocyte reactions such as the production of both intracellular and extracellular superoxide anions (Rengpipat *et al.*, 2000). The humoral response involves the production of soluble factors, including antibacterial activity, agglutination and clotting factors, by the haemocytes, (Lacoste *et al.*, 2002). The haemocytes play a key role in both cellular and humoral responses.

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Elevation in total blood cell numbers seems to be a common response to environmental stressors and is reversible. For example, *Mytilus edulis* exposed to fluoranthene (Coles *et al.*, 1994), *Crassostrea virginica* exposed to cadmium (Cheng, 1988), *Ruditapes phillipinarium* and *Ruditapes decussates* exposed to pathogenic bacteria (Oubella *et al.*, 1993) exhibited an increase in total blood cell numbers upon exposure that decreased again on withdrawal of the repressor. These relatively rapid reversible responses suggest that increased total numbers of circulating haemocytes occurs by stimulation of migration of the cells from tissues, rather than by blood cell proliferation. The diverse nature of the stimulants suggests that this is a general response to an environmental stressor rather than a reaction to a specific challenge (Coles *et al.*, 1995). In bivalve mollucs, haemocytes constitute the first line of defence against invaders. Agranular haemocytes (hyalinocytes) and granular haemocytes (granulocytes) are classically distinguished and considered by some authors as two distinct cell types (Bachère *et al.*, 1995). However, haemocytes constitute very morphologically heterogenous cell populations which are not accurately characterised in terms of cell type and lineages, nor in terms of their respective defence functions. Circulating haemocytes

can mount phagocytic, cytotoxic or inflammatory responses that broaden the number of immune reactions invertebrates can employ in response to microbial invasion. Some intracellular enzymes and the secretion of lytic and cytotoxic molecules can be induced, largely in a non–specific manner. Large-granule haemocytes in *M. edulis* were shown to be eosinophilic and the small-granule cells basophilic by electron microscopy (Pipe *et al.*, 1997). The large-granular cells are a form of lysosome as they contain hydrolytic enzymes, including proteinases, glycosidases and sulphatases. These cells were most active during phagocytosis and release of reactive oxygen metabolites and demonstrated higher levels of activity for phenol oxidase, peroxidase and arylsulphatase.

1.3.1 The Cellular Response

The cellular response involves elimination of foreign particles through cellular action and includes phagocytosis and superoxide anion production. Phagocytosis is an important phenomenon shared by all animals and is considered an important way to control and eliminate foreign particles (Bachère *et al.*, 1995, Mialhe *et al.*, 1995a). Not only involved in nutrition, the phagocytic capacity of the circulating cells was more recently suspected as part of a complex defence system. The purpose of such a system is to neutralise and eliminate all foreign materials, including inorganic particles, living organisms (pathogenic or non–pathogenic) and modified self cells.

Recognition of foreign particles is achieved by means of membrane and secreted molecules that are termed opsonins (Figure 2). Recognition of foreign particles stimulates the migration of haemocytes, followed by phagocytosis in which foreign particles are internalised and enclosed in a primary phagosome. Intracellular degradation of the pathogen occurs by means of lytic enzymes or the production of highly reactive oxygen metabolites (Roch, 1999). Phagocytic processes in both vertebrates and invertebrates are linked to microbiocidal activities that rely on both oxygen–independent systems and oxidative metabolism (Bland *et al.,* 2001). These oxidants are potent tumoricidal and antimicrobial agents that react with internalised particles in processes associated with photon-generating oxidation–reduction reactions. The 'oxidative or respiratory burst' comprises a metabolic pathway, generally dormant in resting cells, in which oxygen is partially reduced to a number of highly reactive, microbiocidal metabolites (Pipe, 1992, Bachère *et al.*, 1995) such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals. Contact with the pathogen activates the host's NAD(P)H–oxidase which, in turn, increases oxygen consumption and produces superoxides, hydroxyl radicals, singlet oxygen, and hydrogen peroxide. All these short lived compounds can be directly toxic to pathogens, act in concert with hypohalides and halidamines generated by peroxidases, or exert synergistic effects with several lysozymal enzymes.

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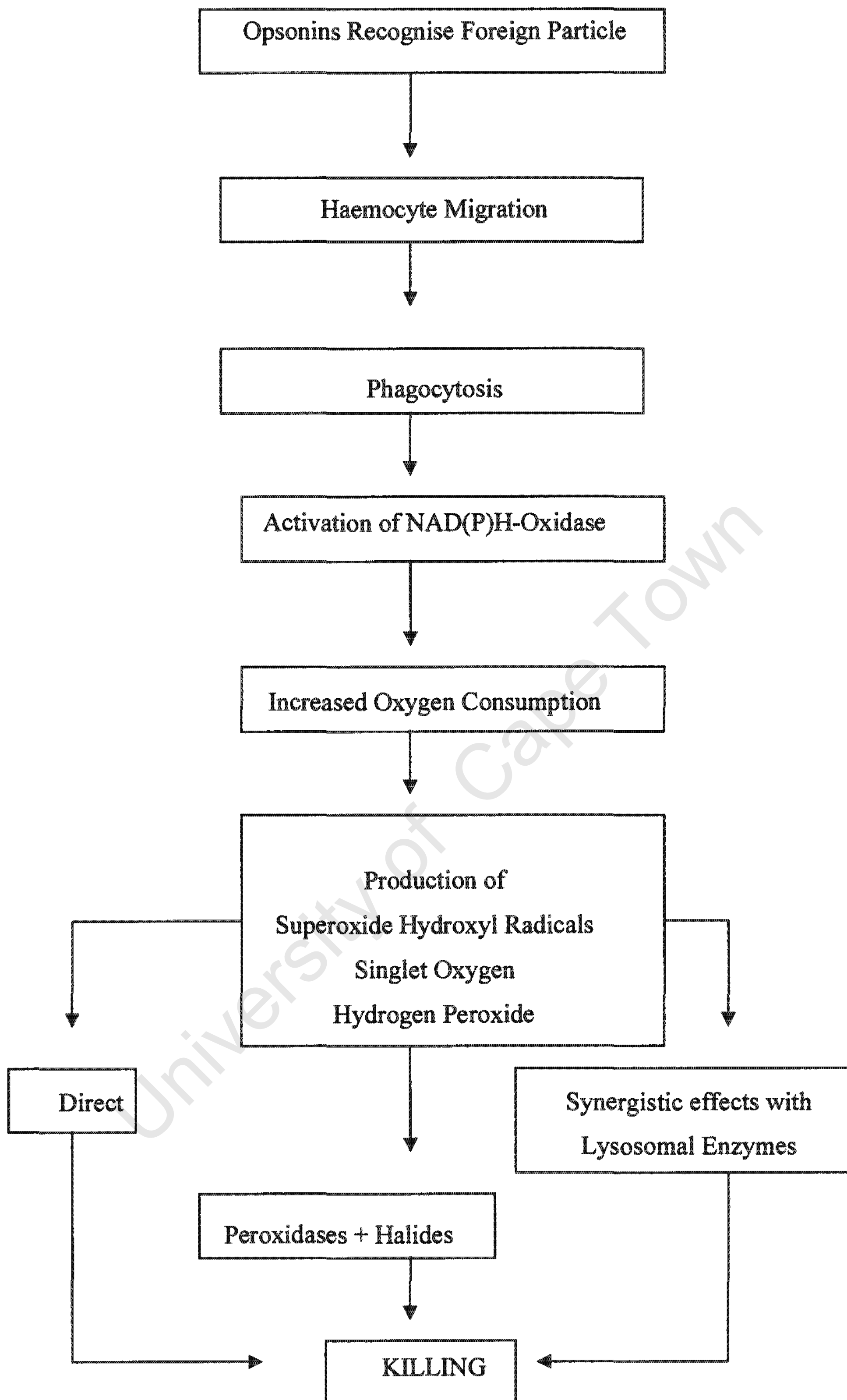

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Figure 2. Schematic representation of the cellular immune response in invertebrates (Adapted from Roch, 1999).

Reactive oxygen intermediates can be dismutated spontaneously or via catalysis by superoxide dismutase to H_2O_2. Excess H_2O_2 may be broken down to H_2O and O_2 by catalase or converted to hypochlorous acid (HOCl) via the myeloperoxidase system, which also plays an important role in the antimicrobial activity of the phagocytic cells. The production of reactive oxygen intermediates (ROIs) associated with phagocytosis has been shown to occur in scallops (Nakamura *et al.*, 1985, Le Gall *et al.*, 1991), mussels (Pipe, 1992, Noël *et al.*, 1993, Winston *et al.*, 1996), oysters (Larson *et al.*, 1989, Bachère *et al.*, 1991, Torreilles *et al.*, 1997) and some clams (Anderson, 1994). However, some clams, such as the Manila clam *Ruditapes decussates*, do not possess detectable ROIs coupled with phagocytosis (Lopez *et al.*, 1994) and some pathogens inhibit the production of ROIs by the haemocytes (Le Gall and Mialhe, 1992). Oxidative metabolism represents one of two mechanisms employed during phagocytosis, the other being enzymatic processes. Degradation may also occur externally by means of soluble factors, some of which are produced by haemocytes, as part of the humoral response (Bachère *et al.*, 1995).

1.3.2 The Humoral Response

The humoral response involves the elimination of pathogens by soluble factors present in the haemolymph and is characterised by a temporary increase in antimicrobial activity in the cell free haemolymph (Bangrak *et al.*, 2004). In many invertebrate species, several kinds of immune–related humoral activities have been reported (Roch, 1999). Bivalves possess various types of non–specific humoral defence molecules including agglutinins, opsonizing lectins, bactericidins, lysozymes and serine proteases. Several proteins and peptides, such as antilipopolysaccharide, clotting factors and antimicrobial substances, are synthesised and stored in shrimp haemocytes and released into the haemolymph upon infection (Supungul *et al.*, 2002). Humoral factors in invertebrates were initially believed to be innate and non–inducible although activities such as lysozyme, lysosomal enzymes, lectins or antibacterial components respond to challenge (Roch, 1999).

Recently a number of genes thought to be involved in marine invertebrate defence mechanisms have been cloned and characterised using expressed sequence tags (ESTs) generated from haemocytes (Supungul *et al.*, 2002; Roux *et al.*, 2002; Bangrak *et al.*, 2002; Gueguen *et al.*, 2003; Bangrak *et al.*, 2004). Fifty-five gene homologues involved in defense mechanisms were identified from *Penaeus monodon* ESTs (Supungul *et al.*, 2002). These included putative components of the clotting system, the prophenoloxidase system, antioxidative enzymes, antibacterial peptides and

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serine proteinase inhibitors. Three heat shock proteins were also identified. Heat shock proteins play an important role during stress where denaturation of proteins in the cell triggers the synthesis of heat shock proteins intracellularly. Heat shock proteins prevent stress-induced protein aggregation and improve regeneration of denatured proteins. Bangrak *et al.* (2002, 2004) identified a translationally controlled tumour protein (TCTP) in *P. monodon* and discovered that it may play a role in the anti-apoptosis process in shrimp infected with white spot syndrome virus (WSSV). Further studies showed that severe systemic illness correlated with the loss of TCTP transcripts and the authors proposed that TCTP may be the key factor in allowing WSSV-infected haemocytes to survive. Expressed sequence tags generated from the haemocytes of bacteria-challenged *Crassostrea gigas* resulted in the identification of 20 genes that may be implicated in immunity (Gueguen *et al.*, 2003). Some of these genes encoded for proteases and protease inhibitors which are important in invertebrate anti-infectious response and may be expressed as part of the humoral response to inhibit proteases produced by invading pathogens. A second group of proteins was termed adhesive proteins and contained genes encoding proteins involved in non self recognition, opsonisation or encapsulation. The stress proteins had homologies to proteins involved in detoxification processes triggered by stress. A fourth group exhibited homology to Vav proteins which transmit signals to the Rel/NF-κB cascade which is considered to be the central mediator of the immune response in mammals.

The only antibacterial or antiviral peptide molecules reported in molluscs until very recently, are proteins of 56–250 kDa found in gastropods, the giant African snail *Achatina fulica* (Obara *et al.*, 1992) and the sea hare *Dolabella auricularia* (Kisugi *et al.*, 1992). Previously the presence of cytolytic molecules in haemolymph and their secretion by haemocytes has been demonstrated in the mussel *M. edulis* (Wittke and Renwrantz, 1984, Leippe and Renwrantz, 1988), but their biochemical nature was unknown. Also, the haemolymph of the Mediterranean mussel, *M. galloprovincilis*, was shown to contain cytotoxic activity against both vertebrate and protozoan cells (Hubert *et al.*, 1996). Haemocytes of the horseshoe crab, *Limulas polyphemus*, contain a family of arthropod peptide antibiotics, named tachyplesins or polyphemusins (Miyata *et al.*, 1989), and an antibacterial protein named anti–LPS factor (Muta *et al.*, 1987). Anti-lipopolysaccharide binds and neutralises bacterial endotoxin and has a strong antibacterial effect, especially on the growth of Gram-negative R-type bacteria. Bactericidal activity has also been demonstrated in abalone (Cushing *et al.*, 1971). Recently, studies have led to the discovery of numerous antimicrobial peptides. Low levels of anti–microbial activity, probably mediated by a large protein of 23 kDa, was found in the haemolymph of the Pacific oyster *C. gigas* (Hubert *et al.*, 1996). Three molecules, panaeidin 1,2, and 3 were isolated from haemocytes and plasma collected from the shrimp, *Penaeus*

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vannamei, and found to contain anti–fungal and antibacterial activities (Destoumieux *et al.*, 1997). Humoral lectins are found in the body fluids of most invertebrates, however their biological role is not yet firmly established. They may act as opsonins that take part in recognition of nonself ligands and facilitate the uptake of microorganisms by phagocytic cells. Antimicrobial peptides with sequence similarity to arthropod defensins and a novel antibacterial peptide designated mytilins and an antifungal peptide designated mytimycin were detected in the haemolymph of bacteria-challenged and unchallenged *Mytilus edulis* (Charlet *et al.*, 1996). Bacterial challenge did not induce any new antimicrobial substances in the haemolymph of mussels suggesting a constitutive presence. Lysozymes are responsible for the killing mechanism, acting either intracellularly or extracellularly to destroy and digest foreign particles (Bachère *et al.*, 1995) and are believed to be involved in internal defence mechanisms and digestion. Haemocytes of *M. edulis*, reported as being toxic to bacteria, contained lysozyme–like activity (Pipe, 1990). Several lysosomal enzymes have been identified in bivalve haemocytes including acid phosphatase, lysozyme and β-glucuronidase, arylsulphatase, elastase and cathepsin B and G.

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1.3.3 The Effect of Probiotics on the Immune Response of Cultured Marine Species

The main technique for disease prevention in vertebrates is vaccination. Invertebrates cannot be efficiently protected by vaccination as they lack antibody–mediated immune responses (Mialhe *et al.*, 1995a, Roch, 1999). Infectious disease outbreaks are suspected of being caused by environmantal, nutritional, physiological and pathological factors and are considered a major limitation to production in terms of quality, quantity, and regularity but also of continuity. The aquaculture industry is plagued by many disease problems and the use of probiotic preparations may be able to prevent disease possibly by either out competing (competition for binding sites and nutrients) pathogens, the production of substances antagonistic towards pathogens, or providing health benefits from factors secreted by the probiotics (Fuller, 1999, Skjermo and Vadstein, 1999, Olafsen, 2001). Increasing evidence suggests that microflora manipulation or addition of probiotic microorgaanisms may improve the health and survival of cultured marine species. However, the complex relationship between marine invertebrates and their microflora is not fully understood and virtually nothing is known of their immune defence. Knowledge of invertebrate immune defence systems and development of multiple strategies for promoting disease resistance needs to be developed in order to fight severe epizootic infections and stabilise production (Roch, 1999).

A number of recent studies have demonstrated the use of bacteria and other immunostimulants to prevent disease in the aquaculture of animals. Douillet and Langdon (1994) showed that survival rates of larval *Crassostrea gigas* were greatly increased after the addition of strain CA2 compared to larvae that had not received the bacterium. Gibson *et al.* (1998) demonstrated the production of a bacteriocin–like inhibitory substance by *Aeromonas media* that is antagonistic towards pathogens and can prevent the death of *C. gigas* oyster larvae when challenged with the pathogen *Vibrio tubiashii*. *Aeromonas media* does not remain in the host for more than 4 days and this necessitates the introduction of fresh cultures at intermittent intervals during the life of the oyster. Riquelme *et al.* (1997) isolated bacterial strains from scallop larvae and the surrounding sea water that were able to protect scallop larvae against infection by *Vibrio anguillarum*–related pathogens. An inhibitor producing bacterium, strain 77, became the dominant bacterium in the Chilean scallop, *Argopecten purpuratus*, and was able to prevent pathogen colonisation of the digestive tract (Riquelme *et al.*, 2000). Probiotics are commonly used in Thailand as an alternative to antibiotics to promote disease resistance in shrimp (Meunpol *et al.*, 2003). *Vibrio alginolyticus* has been tested for use as a probiotic in the production of shrimp, *Penaeus vannamei*, and has been shown to increase growth and survival of postlarval *P. vannamei* (Garriques and Arevalo, 1995). Gomez-Gil *et al.* (2002) demonstrated protection against disease in shrimp treated with *Vibrio alginolyticus* and suggested that *V. alginolyticus* may offer protection by overgrowing potential pathogens. Chythanya *et al.* (2002) demonstrated the ability of *Pseudomonas aeruginosa* strain I-2 to produce inhibitory compounds against shrimp pathogens including *V. harveyi, V. fluvialis, V. parahaemolyticus, V. damsela* and *V. vulnificus* and suggested the potential of this strain in shrimp aquaculture for controlling pathogenic bacteria. *Bacillus* S11 was used as a probiotic in the treatment of *Penaeus monodon*. Challenge with *Vibrio harveyi* D331 resulted in significantly higher mortalities in untreated control groups compared to those treated with the probiotic (Rengpipat *et al.*, 1998). No external pathology was observed in treatment groups, whereas guts from the control group had a pale colour and deformed texture. The percentage phagocytic haemocytes, total haemocytes and phenoloxidase activity were significantly higher in shrimp treated with *Bacillus* S11 compared to a control group of untreated shrimp (Rengipipat *et al.*, 2000). Infection also resulted in a significant decrease in total haemocytes and an increase in antibacterial activity in the treatment groups indicating the initiation of cellular defence reactions (Rengpipat *et al.*, 2000). *Bacillus* S11 was able to displace both *Vibrio* spp. and other bacteria in the culture water and in the shrimp gut and Rengpipat *et al.* (2000) suggest that the lower mortalities resulted from competitive exclusion of the *Vibrio* spp. by the probiont. Guillian *et al.* (2004) isolated a number of bacterial strains from the hepatopancreas of adult shrimp, *P. vannamei* that showed potential probiotic action. *Bacillus* P64 and *Vibrio* P62 reduced the number of *Vibrio harveyii* present in the hepatopancreas, activated the

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phenoloxidase system and altered the diferential haemocyte count indicating immune alert. Although the granulated cell numbers remained constant they demonstrated an increase in phenoloxidase activity indicating that they were highly stimulated. Guillian *et al.* (2004) state that disease prevention is a result of competitive exclusion of the pathogen by the probionts or stimulation of a defence reaction in the host. Probiotics intended for human use were shown to compete with fish pathogens for nutrients and reduced the growth of *Alteromonas salmonicida* and *V. anguillarum*, but did not produce significant antimicrobial activity (Nikoskelainen *et al.*, 2001). One of these probiotics, *Lactobacillus rhamnosus*, reduced mortality in rainbow trout, *Oncorhynchus mykiss*, infected with *A. salmonicida* (Nikoskelainen *et al.*, 2001) and this may be due to stimulation of the respiratory burst response by the probiotic (Nikoskelainen *et al.*, 2003). Bactericidal activity also increased significantly in serum isolated from fish fed the probiotic strain. The addition of *Carnobacterium divergens* to the feed of Atlantic cod, *Gadus morhua*, significantly decreased the amount of mortalities compared to cod fry that did not receive the bacterium (Gildberg *et al.*, 1997). *Carnobacterium divergens* isolated from the digestive tract of juvenile Atlantic salmon is able to inhibit growth of fish pathogens (*Vibrio anguillarum, Vibrio salmonicida* and *Aeromonas salmonicida*) both when the pathogens are cultured in the presence of spent culture media and in the presence of viable bacteria (Ringø and Birkbeck 1999, Ringø *et al.*, 2000).

Use of non–specific immunstimulants has been shown to enhance viability of halibut yolk sac larvae during 4 weeks incubation. The immunstimulant used was alginate rich in mannuronic acid polymers, which are stimulatory towards human and fish monocytes (Skjermo and Vadstein, 1999). β-Glucan prepared from spent brewer's yeast was shown to increase phenoloxidase activity in *P. monodon* haemocytes both *in vitro* and *in vivo* (Suphantharika *et al.*, 2003). A substance containing alginate was shown to increase the ability of the stripped snakehead *Channa striata* serum and macrophages to inhibit growth of the pathogen *Aphanomyces invadans* (Miles *et al.*, 2001). Supplementation of the stripped snakeheads diets with vitamins C, E and some B vitamins increased the ability of serum to inhibit growth and reduced mortality upon infection with *A. invadans*. Although these immunostimulants are not included in the definition of probiotics their effects on immune stimulation and disease prevention in cultured marine species allows for a better understanding of how particular characterisitics of potential probiotics could stimulate the immune system or prevent infectious disease outbreaks.

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1.4 DIGESTION AND NUTRITION IN HALIOTIS MIDAE

The digestive tract of the abalone (Figure 3) begins at the mouth and runs towards the rear through the oesophagus (Ino, 1953). Inside the mouth is a long, file-like tongue called the radula, which scrapes algal matter to a size that can be ingested. The tissue of the oesophagus consists of a layer of intermixed high columnar and epithelial cells with many cilia on the epithelial cells. The oesophagus may also act as a crop, storing certain amounts of food. The oesophagus leads into the stomach which does not have any mucous cells. The crop, also known as the first stomach, is responsible for storing food. The stomach is the site of extracellular digestion, enzymes required for digestion are produced by the salivary glands, oesophagul pouches and hepatopancreas. The hepatopancreas is the major digestive gland and has the same function as the pancreas in vertebrates. The stomach then enters the intestine where mucous cells are present. The wall of the intestine consists of a layer of ciliated epithelium intermixed with many mucous cells. The intestine ends in the anus from which waste products are expelled.

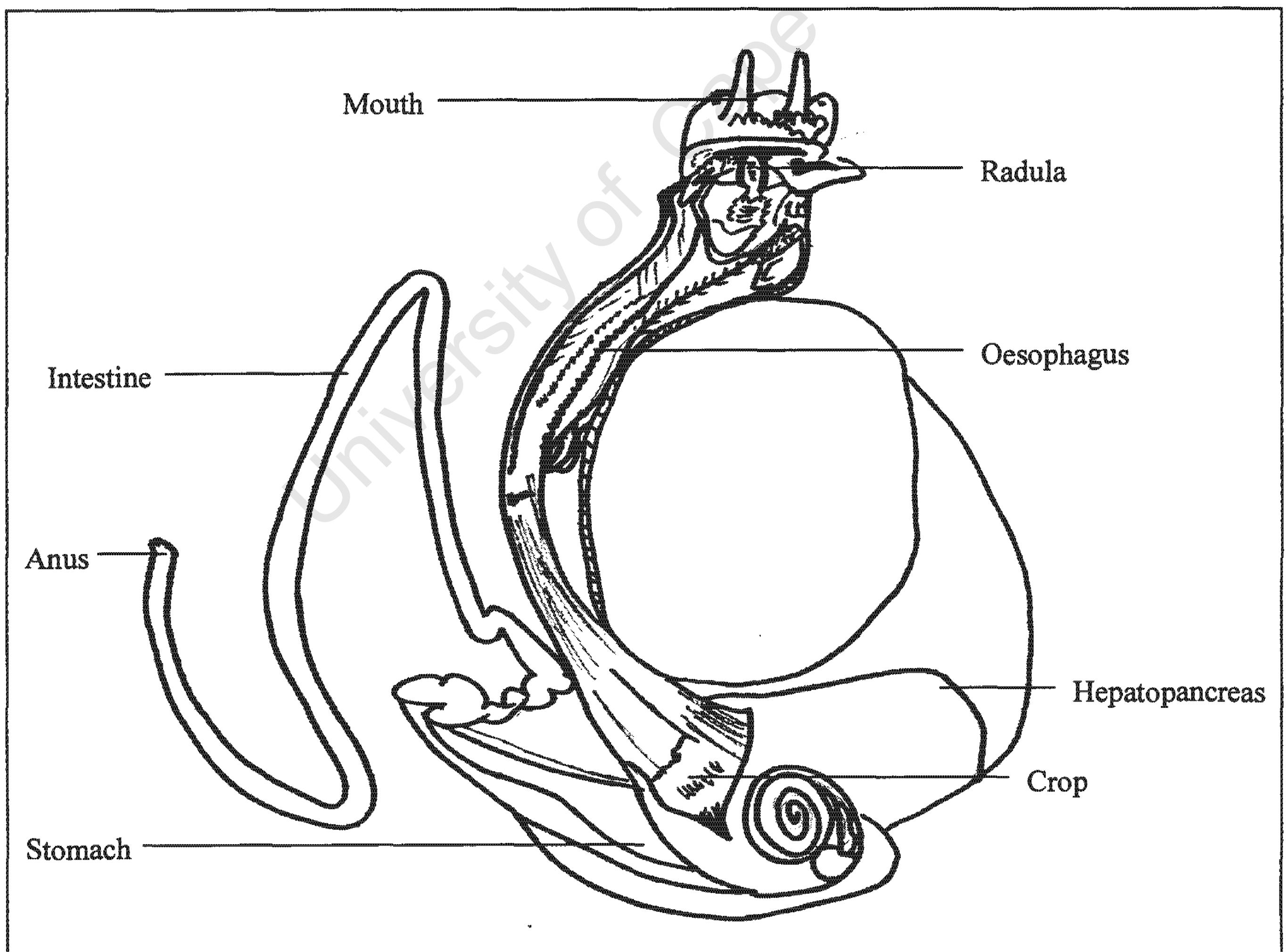

Figure 3. Schematic representation of the abalone digestive tract. Adapted from Ino (1953).

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The largest amount of protein is found in the abalone hepatopancreas (Serviere-Zaragoza *et al.*, 1997, Picos-García *et al.*, 2000) followed by the crop-stomach content, and intestinal and rectal fluid. Protein in the crop-stomach content, intestine, and rectal extracts is derived from food, tissue debris and secretions, whereas protein in the hepatopancreas originates from the tissue itself. Alginate lyase, CMCase, laminarinase, agarase and carrageenase activity have been detected in the hepatopancreas of abalone (Tsujino and Saito, 1962, Nakada and Sweeny, 1967, Erasmus *et al.*, 1997), which has been shown to be sterile in *H. midae* (Erasmus, 1996). Lipases, proteases and glucoside hydrolases have also been described in abalone. Proteolytic activity in *Haliotis fulgens* juveniles and adults has been detected in the hepatopancreas, crop-stomach content and intestinal and rectal fluids (Serviere-Zaragoza *et al.*, 1997, Hernández-Santoyo *et al.*, 1998, Picos-García *et al.*, 2000). A number of bacteria that produce a range of enzymes have been isolated from the digestive tract of abalone and it is thought that they may contribute to digestion in the abalone. Erasmus *et al.* (1997) showed that abalone enteric bacteria were able to degrade laminarin, carboxymethylcellulose (CMC), alginate, agarose and carrageenan and suggested that the bacteria resident in the digestive tract of *H. midae* assist in the digestion of complex polysaccharides. Sawabe *et al.* (1995, 1998, 2003) isolated numerous alginolytic bacteria from the digestive tract of the abalone, *H. discus hannai.* Sixty-eight percent of the alginolytic bacteria only degraded polyG blocks and were non-motile fermenters. Abalone alginate lyase has been reported to preferentially attack the polymannuronate (polyM) block (Nakada and Sweeny, 1967). Sawabe *et al.* (1995) speculated that alginate was degraded by the abalone's digestive enzymes, including polyM-specific alginase, into oligosaccharides or smaller units and metabolised. Afterward, the residue, which included a nondegraded fraction, for example, the polyG block, was further degraded and metabolised by intestinal flora.

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Abalone cannot synthesise 10 of the 20 L-amino acids required to assemble proteins, and their growth rate is thus dependent on the quantity and proportion of these amino acids in their diets (Serviere-Zaragoza *et al.*, 1997). The natural diet of abalone changes markedly over the size range that they are cultured, from a predominately diatom/microalgal diet to a macroalgal diet and different nutritional requirements exist for *H. midae* at different stages of growth. Britz and Hecht (1997) showed that larger abalone (7.0–14.0 g, 20 months old) had a higher protein requirement than smaller abalone (0.2–1.0 g, 6 months old) and that diets containing high levels (10 %) of dietry fat produced significantly lower growth rates. Adult *H. midae* feed on kelp which is deficient in many essential nutrients (Simpson, 1994). Alginate is the main component of brown algae and accounts for more than 30 % of the dry weight of the thallus in some algae (Sawabe *et al.*, 1995). Therefore, since the cell walls of brown algae are formed by linkages of alginate and cellulose and

alginate is a nutritionally poor feed source the addition of alginate degrading probiotics may improve the digestion of kelp in the abalone digestive system.

1.5 THE USE OF PROBIOTICS TO IMPROVE THE GROWTH RATE OF AQUACULTURED MARINE SPECIES

Numerous studies have demonstrated the ability of bacteria to improve the growth of cultured marine organisms. Olafsen (2001) postulated that bacteria may be used as food by marine invertebrates and fish which metabolise cell components or micronutrients, such as essential fatty acids, vitamins, minerals or even enzymes. Addition of strain B5 and an *Alteromonas* strain have been shown to consistently increase the growth rate of the rotifer, *Brachionus plicatilis*, an important food organism for cultured marine finfish and crustacean larvae (Douillet, 2000a & b). Ringø *et al.* (1996) showed that *Vibrio pelagius* added to the rearing water of turbot larvae dominated the bacterial flora of the larvae. *Vibrio pelagius* contains eicosapentaenoic acid which is required for growth of turbot larvae and therefore the authors suggest that this bacterial source of the fatty acid may be beneficial to the nutrition of the larvae. The use of commercial probiotics on a shrimp farm in West Java, Indonesia has resulted in increased production costs but higher yields (total production and shrimp size) resulting in increased profits, making probiotic management more cost effective than traditional production procedures (Jory, 1998). *Bacillus* S11 added to shrimp feed in three different forms (fresh cells, fresh cells in normal saline and lyophilised cells) significantly improved the growth rate and survival of the black tiger shrimp, *Penaeus monodon* (Rengpipat *et al.*, 1998). In the sea bass, *Dicentrarchus labrax* the addition of the yeast *Debaryomyces hansenii* HF1 to feed improved gut maturation in larvae and improved survival (Tovar *et al.*, 2002). *Debaryomyces hansenii* HF1 produces spermine and spermidine which have been shown to be involved in differentiation and maturation of the intestinal tract in mammals. Amylase and trypsin activities were also elevated in 27-day old larvae indicating that maturation of secretory functions of the pancreas occured earlier compared to larvae fed the control diet. Addition of strain CA2 to oyster larvae consistently enhanced growth (Douillet and Langdon, 1994) and may be the result of the bacteria providing essential nutrients not present in algae or providing enzymes which could enhance larval digestive processes. Strain CA2 may also remove metabolic substances released by the larvae or the algae, which may adversely affect larval growth. Growth and survival of the scallop larvae *Argopecten purpuratus* was reduced when finely filtered (0.2 μm) seawater was supplied (Riquelme *et al.,* 1997). Bacteria isolated from scallop larvae may have supplied some nutritional elements to the larvae as they improved the growth rate. Abundant

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populations of *Vibrio halioticoli* have been isolated from the gut of *Haliotis diversicolor diversicolor, Haliotis discus discus, Haliotis diversicolor aquatilis* and *Haliotis midae* (Sawabe *et al.*, 2003). These *V. halioticoli* isolates are able to ferment alginate producing acetic acid and formic acid. Acetic acid is an important oxidizable energy source and precursor for protein, sugar and lipid synthesis in ruminants and therefore *V. halioticoli* may play an important role in the abalone gut by converting alginate to acetic acid.

1.6 CONCLUDING REMARKS AND AIM OF THIS STUDY

Abalone are a highly sort after delicacy demanding high prices which has resulted in the establishment of abalone aquaculture in South Africa. One of the challenges in farming abalone is the ability to increase growth rates in order to increase the turnover of product. Cultured abalone take approximately 3-4 years to reach market size. Decreasing this time period would reduce farming costs and increase profit. Another problem facing abalone farmers, as in any agriculture or aquaculture system, is disease. The outbreak of an infection has the potential to spread through an aquaculture system and destroy the entire stock resulting in substantial economic losses. Increasing the amount of cultured abalone exports would substantially benefit South Africa economically by increasing revenue as well as creating more jobs. One job is directly created for every two tons of abalone produced and a second job is indirectly created through associated practices such as kelp harvesting, marketing and research (Stanford, 2004).

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Probiotics have been used for decades in humans, pigs and ruminants amongst others. Recently the use of probiotics has been applied to aquaculture systems including shrimps (Jory, 1998, Riquelme *et al.*, 1997, Rengpipat *et al.*, 1998, Rengpipat *et al.*, 2000, Chythanya *et al.*, 2002, Meunpol *et al.*, 2003), scallops (Riquelme *et al.*, 2000;) and oysters (Douillet and Langdon, 1994, Gibson *et al.*, 1998). Probiotics are used in an attempt to increase growth rate and improve the general nutritional status of the animal and improve disease resistance and the general health of the animal.

The aim of this study was to evaluate *Pseudoalteromonas* sp. strain C4 for use as a probiotic in abalone aquaculture. The most practical option to introduce the probiotic into the digestive system would be via the feed because of the small size and enormous number of individuals which would have to be treated (Roch, 1999). Therefore, experiments to evaluate the viability and stability of probiotic in the feed of choice were initially performed. The ability of strain C4 to improve the growth of farmed abalone would be evaluated by conducting growth trials with abalone fed a kelp diet supplemented with strain C4. The mechanism by which strain C4 improves growth rates would

be evaluated by determining whether strain C4 contributes enzymes to the pool of digestive enzymes available in the digestive tract of *H. midae* or if strain C4 is itself used as a source of nutrients. The ability of strain C4 to stimulate the immune system of *H. midae* and offer protection against disease would be evaluated by measuring immune parameters such as haemocyte numbers, phagocytosis and extracellular superoxide anion production. Abalone would be challenged with the pathogen, *Vibrio anguillarum,* to evaluate any protective characteristics of strain C4 in preventing infection by this bacterium. Identification of a probiotic for use in abalone aquaculture would substantially benefit abalone aquaculture in South Africa as increased growth rates and improved resistance to pathogen infection would reduce costs, increase export quantities and thereby increase profits.

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CHAPTER 2

CHARACTERISATION OF *PSEUDOALTEROMONAS* SP. STRAIN C4

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2.5 DISCUSSION

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2.1 SUMMARY

Numerous bacteria have been isolated from the digestive tract of the abalone *Haliotis midae* (Erasmus, 1996). A bacterium, designated strain C4, was selected for further study based on its ability to hydrolyse alginate. Small subunit ribosomal RNA sequence analysis, together with phenotypic characteristics, suggests that the bacterium is a member of the genus *Pseudoalteromonas* and is possibly a new species.

Growth characteristics and alginate lyase production of strain C4 were investigated in various culture media. The majority of alginate lyase production was extracellular, although intracellular alginate lyase was also detected. Extracellular alginate lyase activity was detected at basal levels during lag and early to mid–exponential phase and maximum enzyme activity was detected in stationary phase. The addition of alginate to the growth media increased alginate lyase activity in stationary phase by 6.25 fold compared to activity in basal media. The addition of glucose to the media did not affect extracellular alginate lyase activity but significantly reduced intracellular enzyme activity. The extracellular alginate lyase was shown to have optimum activity between pH 6 and 6.5, at temperatures between 22°C and 37°C.

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2.2 INTRODUCTION

Many studies have been performed on the interaction between gut microbes and their invertebrate hosts (reviewed by Harris, 1993). Certain bacteria can enhance the digestive efficiency of the host by providing polysaccharolytic enzymes, and in so doing improve the growth rate of the host (El – Shanshoury *et al.*, 1994). This enzyme contribution is especially important when the host feeds on nutritionally poor seaweeds (Vitalis *et al.*, 1988). Bacteria isolated from the gut of *Haliotis midae* by Erasmus (1996) were able to hydrolyse agar, carrageenan, cellulose, laminarin, and alginate and were predominantly members of the genera *Pseudoalteromonas* and *Vibrio*. Erasmus *et al.* (1997) suggest that bacteria resident in the abalone digestive tract may play a role in digestion of complex polysaccharides found in seaweeds. The most popular food source for farmed South African abalone is the kelp *Ecklonia maxima*, the cell wall of which is mainly comprised of alginate. Alginate is a copolymer of (1→4)-linked α-L- guluronic acid and (1→4)-linked β-D-mannuronic acid units (Kitamikado *et al.*, 1992). Alginate can contain blocks of polymannuronate, polyguluronate and random sequences of mannuronic and guluronic residues that may be cleaved by alginate lyases resulting in oligosaccharides (Malissard *et al.*, 1993). Alginate lyase produced by abalone preferentially degrades the polyM block of alginate (Sawabe *et al.*, 1995). Most bacterial alginate lyases prefer the poly G block although preferences have also been demonstrated for polyM blocks (Kitamikado *et al.*, 1992). This allows for the possibility of complete degradation of alginate ingested by abalone as a consequence of the combined activity of abalone and bacterial alginate lyases. Therefore, bacteria isolated from the gastrointestinal tract of *H. midae* that produced alginate lyase were selected for further investigation for use as a potential probiotic. The investigation into a probiotic for abalone aquaculture later focused on strain C4 due to its elevated levels of alginate lyase activity.

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Probiotics need to be accurately identified in order to ensure that they are not potential pathogens and will not be harmful to the host. Accurate identification is also of importance to guarantee proven reliability of the probiotic preparations and to prove conclusively that the contents are safe and efficacious (Reid, 1999). Accurate identification of probiotics also ensures that probiotic containing products will have the specific impact that they were intended to generate. Identification of isolates by classic biochemical methods is not always sensitive enough and is labour intensive (O'Sullivan, 1999; Tannock, 1999). Molecular tools have greatly expanded the ability to reliably identify isolates and accurate typing is easily achieved by sequence analysis of the 16S rRNA gene. Small subunit ribosomal DNA sequencing has been demonstrated to be a powerful tool for identifying and classifying prokaryote organisms (Olsen *et al.*, 1994a) as 16S

rDNA sequences are highly conserved between closely related bacterial species. Nucleotide sequencing has dramatically expanded the rDNA sequence database and more than 108,000 16S rRNA sequences are catalogued in public databases (Cole *et al.*, 2003, http://rdp.cme.msu.edu/index.jsp, updated September 2004). Although 16S rRNA is a powerful tool, Stackebrandt and Goebel (1994) have recommended that it is ultimately the presence or absence of a particular phenotypic characteristic that should be the deciding factor in describing a new species. Small subunit ribosomal DNA sequence analysis of strain C4 as well as determination of phenotypic and physical characteristics would therefore enable the correct and accurate classification of the bacterium.

The objective of this study was to identify and characterise a potential probiotic for use in abalone aquaculture using biochemical methods as well as 16S rRNA gene analysis. It was decided, for practical reasons, to select a single strain and test its potential for use as a probiotic. Alginate lyase activity was evaluated as it was hypothesised that the growth rate of cultured abalone could be increased by providing a means for more efficient digestion of kelp, a major component of which is alginate. Alginate lyase activity was also investigated under specific abiotic conditions to determine whether the enzyme was likely to be active in the abalone digestive tract.

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2.3 MATERIALS AND METHODS

2.3.1 Alginate lyase activity

Alginate lyase activity of abalone enteric bacteria, previously identified as alginolytic (Erasmus, 1996), was quantified. Strains J1, J2, J3, J4, T4, Y5, and C4 were cultured for 16 h in 5 ml of alginate broth (AB, Appendix A1.17) at 22°C with agitation at 120 rpm. The optical density of each culture was determined at 600 nm and cultures were diluted to a standard optical density in 100 mM phosphate buffer, pH 6.7 (Appendix A2.11). One millilitre samples were centrifuged for 5 minutes at 14 300 x g. Harvested cells were resuspended in 1 ml of 100 mM phosphate buffer. Alginate lyase activity was determined in both the culture supernatant and the resuspended cells using the thiobarbituric acid method (Appendix B1) and defined as μg malondialdehyde equivalents released 100 μl^{-1} 30 min^{-1} at 37°C (1 unit).

2.3.2 Biochemical characterisation of strain C4

2.3.2.1 Cell shape and Gram reaction

Strain C4 was tested for Gram reaction by staining (Appendix A2.15) cells using the Hucker modification of the Gram stain (Bailey and Scott, 1966) and viewing them under a light microscope fitted with a 100X oil immersion lens. Bacterial cell shape was also observed.

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2.3.2.2 Motility test

Bacterial motility was determined by microscopic observation of 16–hour bacterial cultures grown in marine broth (MB; Appendix A1.3) at 22°C.

2.3.2.3 Oxidase and catalase test

The presence of cytochrome oxidase and catalase activity was determined using Kovac's reagent and 3% H_2O_2 (Appendix A2.16), respectiviely (Smibert and Krieg, 1981; Bailey and Scott, 1966).

2.3.2.4 Oxidative metabolism of carbon sources

Strain C4 was tested for its ability to metabolise twenty carbon sources aerobically and anaerobically as described by Hugh and Leifson (1953). The media (Appendix A1.7) were modified by increasing the NaCl content to 3% (w/v) as strain C4 is a marine bacterium. The following carbon sources were added to the medium to a concentration of 1% w/v: D(+)–xylose; L(+)–arabinose; D(+)–galactose; D(+)–glucose; D(-)–fructose; lactose; maltose; sucrose; D(+)–trehalose; raffinose; D (+)–cellobiose; dextrin; glycogen; glycerol; adonitol; D (-)–mannitol; mannose; threonine; rhamnose; and sorbitol.

2.3.2.5 Nitrate reductase and denitrification tests

Strain C4 was cultured in defined media (Appendix A1.8) for 16 h at 22°C and tested for its ability to reduce nitrate to nitrite and carry out denitrification, using the standard nitrate reduction and denitrification tests (Smibert and Krieg, 1981).

2.3.2.6 Test for indole production

Strain C4 was grown in 10 ml modified medium containing tryptone (Appendix A1.9) for 48 h at 22°C. The ability to produce indole was tested using the standard method described by Smibert and Krieg (1981).

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2.3.2.7 Test for H_2S production

Strain C4 was inoculated in 5 ml of a defined medium (Appendix A1.10) and its ability to produce H_2S was tested using the standard method described by Smibert and Krieg (1981).

2.3.2.8 Test for carrageenolytic activity

The ability of strain C4 to degrade carrageenan was tested by culturing the bacterium on a 2% carrageenan medium (Appendix 1.11) and examining for zones of hydrolysis after 5 days incubation at 22°C.

2.3.2.9 Test for gelatinase activity

The ability of strain C4 to degrade gelatin was tested by culturing the bacterium on a 12% gelatin medium (Appendix 1.12) and scoring for zones of hydrolysis after 5 days incubation at 22°C.

2.3.2.10 Test for starch hydrolysis

The ability of strain C4 to hydrolyse starch was tested by culturing the bacterium on a 0.2% starch medium (Appendix 1.13). After 5 days incubation at 22°C the plates were flooded with Gram's iodine and hydrolysis was indicated by the presence of clear zones surrounding single colonies.

2.3.2.11 Test for cellulase activity

Strain C4 was grown for 5 days on a carboxymethyl-cellulose (CM-cellulose) medium (Appendix A1.14) at 22°C after which the plates were flooded with Congo Red (Appendix A2.13) and scored for zones of cellulose hydrolysis.

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2.3.2.12 Test for lipase activity

Strain C4 was grown for 5 days on a lipid medium (Appendix A1.15) at 22°C after which the lipolytic activity of the bacterium was assessed by scoring for zones of lipid hydrolysis surrounding single colonies.

2.3.2.13 Test for casein hydrolysis

Strain C4 was tested for its ability to hydrolyse casein by incubating the bacterium for 5 days on a casein media (Appendix A1.16) at 22°C after which the casein hydrolytic activity of the bacterium was assessed by scoring for zones of casein hydrolysis.

2.3.2.14 Determination of culture luminescence

A loopful of strain C4 was streaked onto marine agar (MA, Appendix A1.4) and incubated overnight at 22°C. The plate culture was exposed to an UV light source and observed for luminescence.

2.3.3 Growth at different temperatures

To determine the range of temperatures at which strain C4 grows, 100 ml of basal marine broth (BMB, Appendix A1.1) were inoculated with 100 µl of an overnight culture of strain C4 that had been incubated at 22°C. Cultures were incubated for 16 h at 4, 17, 22, 30, and 37°C with agitation at 120 rpm and the optical density at 600 nm was determined.

2.3.4 Growth at different NaCl concentrations

To determine whether strain C4 required a NaCl base for growth, 100 ml of marine broth (MB, Appendix A1.3) containing either 3%, 1.5% or 0% NaCl was inoculated with 100 µl of an overnight culture of strain C4 that had been incubated at 22°C. Cultures were incubated for 16 h with agitation at 120 rpm and the optical density at 600 nm was determined.

2.3.5 Determination of G + C (mol%) content

Strain C4 genomic DNA was isolated as described by Ausubel *et al.* (1989). The G+C (mol %) was determined using the Ulitzur spectroscopic method (Ulitzur, 1972).

2.3.6 Scanning electron microscopy

A positively charged carbon coated copper grid was placed on the surface of an exponential phase and stationary phase, 15 µl aliquot of strain C4 culture and the negatively charged bacterial cells were allowed to adhere for 10 min. The grid was rinsed in distilled water and stained with a 1% methylamine tungstate solution (Appendix A2.18) for 10 min. The stain was removed with a brief wash in distilled water and the grid was subsequently air–dried (Dykstra, 1993). The sample was viewed on a Zeiss Electron Microscope Leo EM912 (Zeiss).

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2.3.7 Determination of 16S rRNA gene sequence

Strain C4 was cultured in 100 ml MB for 16 h at 22°C. Genomic DNA was extracted as described by Ausubel *et al.* (1989). The DNA was re–dissolved in TE buffer (pH 8) (Appendix A2.7) and quantified spectrophotometrically (Beckman DU–64 spectrophotometer) by measuring the absorbance at 260 nm (Appendix B4).

The 16S rRNA gene was sequenced using a modified PCR strategy described by Weisburg *et al.* (1991). Five overlapping DNA fragments encompassing most of the 16S rRNA gene were amplified using a Hybaid Omnigen thermal cycler. Six PCR primers were designed (F1, R1, F3, R3, F5, R5) which consisted of a nucleotide sequence complementary to one of four highly conserved regions of the bacterial 16S rDNA gene. These highly conserved regions are located between nucleotides 9 and 26, 516 and 535, 1052 and 1073 and 1493 and 1511 on the *Escherichia coli* 16S rRNA gene (GENBANK database, accession number JO1859). Each of these nucleotide sequences was attached to a common 24 bp sequencing primer (Appendix B2). The primers were used in five different amplification reactions that included 10 μM each of the following combinations of forward (F) and reverse (R) primers: reaction 1 – F1 and R1; reaction 2 – F1 and R3; reaction 3 – F3 and R5; reaction 4 – F5 and R5; and reaction 5 – F1 and R5 (Figure 1). Fifty nanograms of C4 genomic DNA was amplified using the cycle profile described in Appendix B2. One hundred microlitre reactions were carried out using 5 units of Red Hot *Taq* polymerase (Applied Biotechnologies), 10 μl 10 x *Taq* polymerase buffer (Applied Biotechnologies), and equimolar amounts of all four dNTPs (6.25 mM each; Boehringer Mannheim). The PCR products (15 μl) were analysed by electrophoresis (Sambrook *et al.*, 1989) on a 1% agarose gel (Appendix B3) to verify their sizes. The amplified products were subsequently purified using a High Pure PCR Purification Kit (Boehringer Mannheim) and re–dissolved in sterile, distilled water. The DNA products were quantified on a 1% agarose gel using known amounts of bacteriophage λ DNA as standards (Appendix B4).

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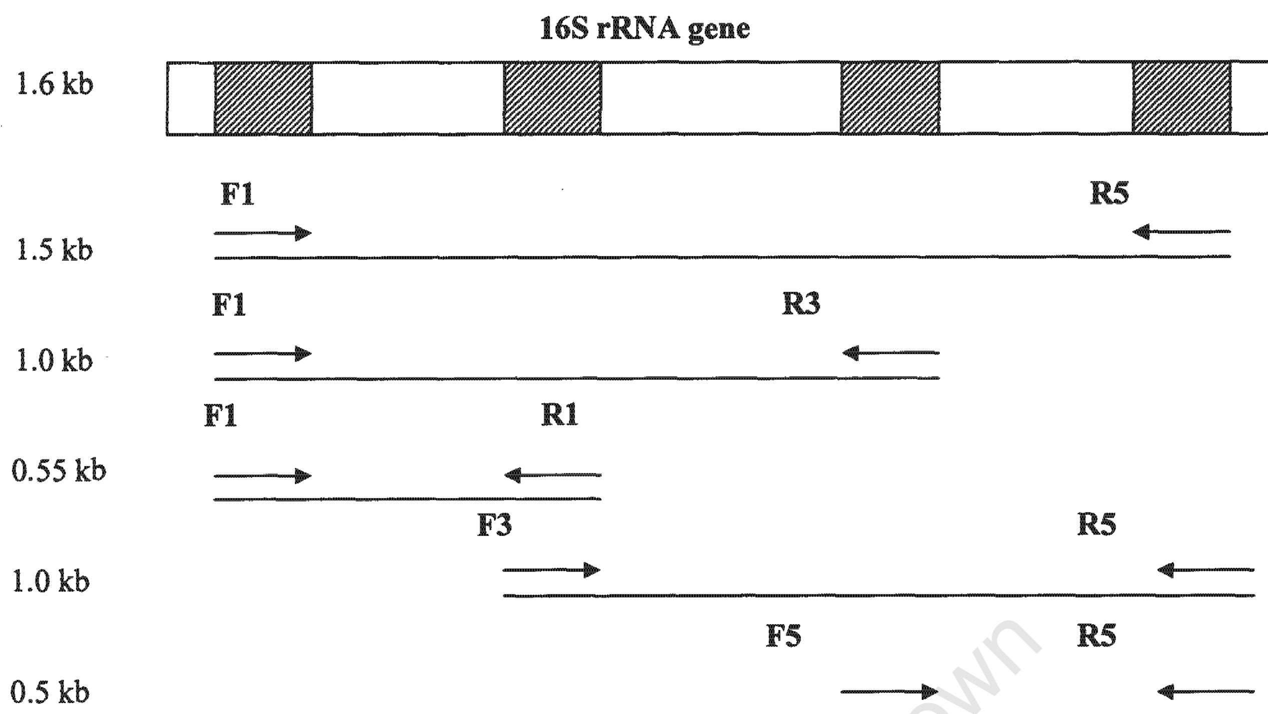

Figure 1. The PCR strategy employed to amplify the 16S rRNA gene of bacterium C4 required five primer pairs (as described in section 2.3.6). Primers F1, F3, and F5 represent the forward primers and primers R1, R3, and R5 represent the reverse primers. The shaded areas represent conserved regions in the 16S rRNA gene that occur in most eubacteria. The sizes depict the length of the PCR products expected.

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The amplified 16S rDNA products were sequenced (Appendix B5) using a Thermosequenase cycle–sequencing kit (Amersham) and an ALFexpress™ DNA sequencer (AM Version 3.01, Pharmacia Biotech).

The 16S rRNA gene fragment sequences were aligned with each other to generate a complete 16S rRNA gene sequence using DNAsis Version 2.1 (Hitachi Software Engineering). A nucleotide homology search of the GenBank database was performed using the program BlastN V. 2.0.4 (Altschul *et al.*, 1997) to determine whether the sequence was homologous to any other 16S rRNA gene sequences in the database.

2.3.8 Phylogenetic tree construction

16S rRNA gene sequences homologous to the strain C4 16S rRNA gene were retrieved from the Genbank database. Sequence data from these strains were aligned and edited using DNAMAN (version 4.13; Lynnon Biosoft). Alignments were exported in clustal format for phylogenetic and molecular evolutionary analysis using MEGA (version 2.1; Kumar *et al.*, 2001).

Both neighbour joining (NJ) and maximum parsimony (MP) trees were constructed to test the level of support for the resulting trees at various levels since NJ is a distance analysis and MP is parsimony based. NJ trees (Saitou and Nei, 1987) were constructed using MEGA. The Kimura 2-parameter (pairwise distances) model (Kimura, 1980) was used for distance estimation. Alignment gaps and sites with missing information were retained initially and were excluded as necessary using the pairwise deletion option as gaps and missing data are not used in computing tree lengths in MEGA. Standard error was estimated by the bootstrap method (Felsenstein, 1985). A thousand replicates were tested with a random starting seed.

Strict consensus parsimony (MP) trees were inferred using MEGA. Parsimony analysis was performed using the close–neighbour–interchange heuristic search. The initial tree was generated by randomly selecting a sequence and adding it to the growing tree on a randomly selected branch (random addition tree option). All nucleotide changes were weighted equally. The reliability of the inferred phylogenetic trees was assessed using the bootstrap test. A thousand replicates were tested with a random starting seed.

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2.3.9 The effect of different carbon sources on the growth rate and alginate lyase productivity of strain C4

Strain C4 was cultured overnight in basal marine broth (BMB, Appendix A1.1) at 22°C with agitation at 120 rpm. After measuring the OD_{600} of the culture, the cells were harvested by centrifugation for 10 min at 11 200 x g, washed twice in synthetic sea salts (SSS, Appendix A2.12) and resuspended in fresh BMB. The bacterial suspension was inoculated into three different media (200 ml of each medium) to obtain a starting OD_{600} of 0.01. Media were designed to determine whether alginate lyase activity is induced or suppressed by particular carbon sources. The different media were BMB, BMB supplemented with 0.2% sodium alginate and marine broth (MB, Appendix A1.3) supplemented with 0.2% sodium alginate. The cultures were incubated at

22°C with agitation (120 rpm) and sampled at hourly intervals (1 ml amounts) over a 12 hour period to measure the culture OD_{600} and alginate lyase activity. Alginate lyase activity was measured (TBA method, Appendix B1) after the cells had been harvested by centrifugation for 5 min at 14 300 x g, the supernatant collected and the cells resuspended in 1 ml of 100 mM phosphate buffer, pH 6.7 (Appendix A2.11). The culture generation time was calculated as the difference in time required for the culture to double its optical density at 600 nm during the exponential growth phase.

2.3.10 Characterisation of strain C4 alginate lyase

An overnight culture of strain C4 was used to inoculate 200 ml of alginate broth (Appendix A1.17) to an OD_{600} of 0.01. Strain C4 was then cultured at 22°C with agitation at 120 rpm for 12 h. The cells were harvested by centrifugation at 11 200 x g for 15 min. The culture supernatant was collected and dialysed overnight against three changes of 100 mM sodium phosphate buffer, pH 6.7 (Appendix A2.11).

2.3.10.1 The effect of temperature on alginate lyase activity

Culture supernatant was diluted in 100 mM sodium phosphate buffer and alginate lyase activity was determined at 4°C, 22°C, 37°C, 42°C, and 55°C by the TBA method (Appendix B1). Alginate lyase activity was defined as μg malondialdehyde equivalents released 100 μl^{-1} 30 min^{-1} (1 unit).

2.3.10.2 The effect of pH on alginate lyase activity

Culture supernatant was diluted in 100 mM sodium phosphate buffer. Alginate lyase activity was determined (TBA method, Appendix B1) by incubating diluted culture supernatant with alginate substrate at pH 3, 6, 6.5, 7, 7.5, 8 and 10 (Appendix A2.20). Alginate lyase activity was defined as μg malondialdehyde equivalents released 100 μl^{-1} 30 min^{-1} at 37°C (1 unit).

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2.3.11 Statistical Analysis

All data are presented as the means of at least three experiments and standard errors. For comparison of two means, paired or unpaired Student's t–tests were used. The limit of significance was $p<0.05$ unless otherwise stated.

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2.4 RESULTS

2.4.1 Alginate lyase activity of different abalone enteric bacterial isolates

Alginate lyase activity from alginolytic enteric bacterial strains isolated from abalone by Erasmus (1996) was quantified. Alginate lyase activity of all the strains was predominately associated with the culture supernatant (Figure 2). Very low levels of activity were observed in the cell fraction. Extracellular alginate lyase activity ranged between 20.27 U (strain Y5) and 56.99 U (strain C4). The highest level of intracellular alginate lyase activity was also observed in strain C4 (11.39 U). Therefore, based on the high levels of alginate lyase activity observed for strain C4, strain C4 was selected for further investigation as a potential probiotic for use in abalone aquaculture.

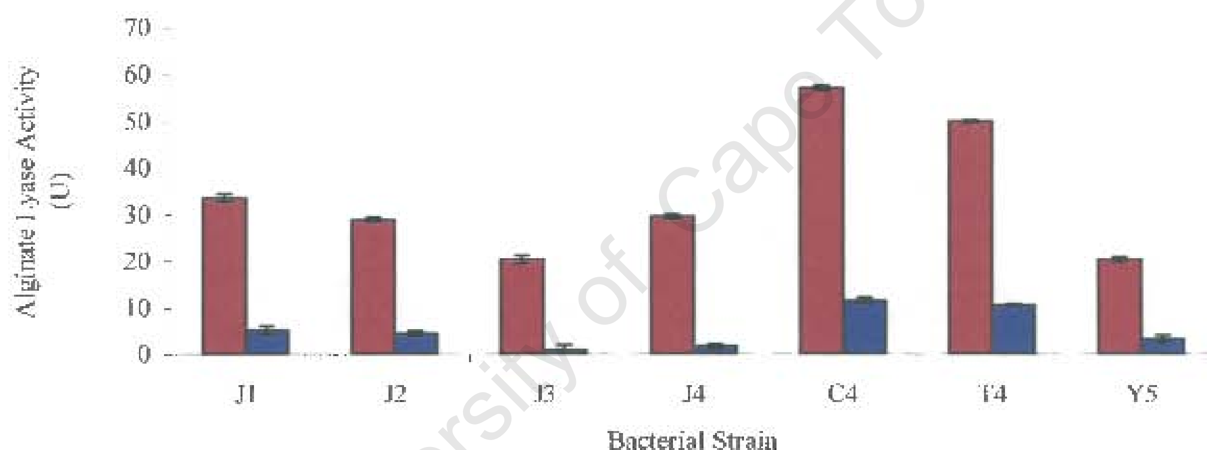

Figure 2 Alginate lyase activity of alginolytic bacterial strains isolated from the abalone gastrointestinal tract. Bars represent mean alginate lyase activity in the culture supernatant (■) and whole cells (■). (U): µg malondialdehyde equivalents released 100 μl^{-1} 30 min^{-1} at 37°C. Error bars represent standard error calculated from three experiments.

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2.4.2 Biochemical characterisation of strain C4

The physical and phenotypic characteristics of strain C4 are summarised in Table 1. The bacterium is a peach coloured, motile, catalase–positive, oxidase–positive, aerobic, Gram–negative, cocci during exponential growth and a cigar–shaped rod during stationary phase. During exponential growth the cells are 1.73 µm (SE ±0.14 µm) in length and 0.97 µm (SE ±0.09

μm) in diameter (Figure 3A) and 1.99 μm (SE ± 0.16 μm) by 0.55 μm (SE ± 0.07 μm) during stationary phase (Figure 3B). The length of the bacterial cell is not significantly different (Student's T-test, $p<0.05$) between the two growth phases, there is however a significant difference in width. A single flagellum is attached to exponentially growing bacteria, while stationary phase cells lack a flagellum (observed floating unattached in the culture medium). Irregular shaped structures, believed to be exopolysaccharide, cover the bacterial cells during stationary phase. The bacterium grows relatively well between temperatures of 17°C (OD_{600} = 3.26) and 30°C (OD_{600} = 3.28), grows poorly at 4°C (OD_{600} = 0.612) and not at 37°C (OD_{600} = 0.07). Strain C4 is unable to grow in the absence of NaCl (OD_{600}=0) and exhibits best growth in media containing 3% NaCl compared to media containing 1.5% NaCl. The bacterium can reduce nitrate to nitrite, carry out denitrification and produces indole but not H_2S. The bacterium readily degrades alginate, CM-cellulose, casein and gelatin but is unable to degrade carrageenan, lipids and starch. It can utilise seven of the 20 carbohydrates tested as a carbon source: D (+)–xylose, L (+)–arabinose, D (+)–galactose, lactose, D (+)-cellobiose, D (-)-mannitol, and D (+)–glucose. However it cannot utilise D (-)-fructose, maltose, sucrose, D (+)-trehalose, raffinose, dextrin, glycogen, glycerol, adonitol, sorbitol, mannose, threonine, and rhamnose. Bacterial colonies are non–luminescent. The G + C content of the DNA is 47.3 ± 1 mol % (Table 1).

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2.4.3 Sequence of the 16S rRNA gene

Alignment of the sequences of the PCR products generated from the five PCR amplification reactions yielded a strain C4 16S rDNA sequence of 1497 bp in length (Figure 4). To determine whether this sequence was homologous to any other known sequences, a sequence–homology search of the GenBank database was performed (Altschul *et al.*, 1997). This search showed that the 1497 bp sequence of strain C4 16S rDNA was very similar to a number of *Pseudoalteromonas* 16S rRNA gene sequences and had 98% similarity to the species listed in Table 2.

Table 1. Summary of the physical and phenotypic characteristics of bacterial strain C4

Characteristic	*Pseudoalteromonas* sp. strain C4
Gram stain	Negative
Cell Shape	Straight rod
Motility	Positive
Polar Flagellum	Positive
Growth in air	Positive
Growth at:	
4°C	Positive
17°C	Positive
22°C	Positive
30°C	Positive
37°C	Negative
Growth at NaCl concentrations of:	
3% (w/v)	Positive
1.5% (w/v)	Positive
0%	Negative
Nitrate reduction	Positive
Denitrification	Positive
Production of:	
Catalase	Positive
Oxidase	Positive
Indole	Positive
H_2S	Negative
Hydrolysis of:	
Alginate, Casein, Gelatin, CM-cellulose	Positive
Starch, Lipids, Carrageenan	Negative
Utilisation of:	
Xylose, Arabinose, Galactose, Lactose, Cellobiose, Mannitol, Glucose	Positive
Fructose, Maltose, Sucrose, Trehalose, Raffinose, Dextrin, Glycogen, Glycerol, Adonitol, Sorbitol, Mannose, Treonine, Rhamnose	Negative
G+C (mol%)	47.3
Colony pigmentation	Peach
Culture luminescence	Negative

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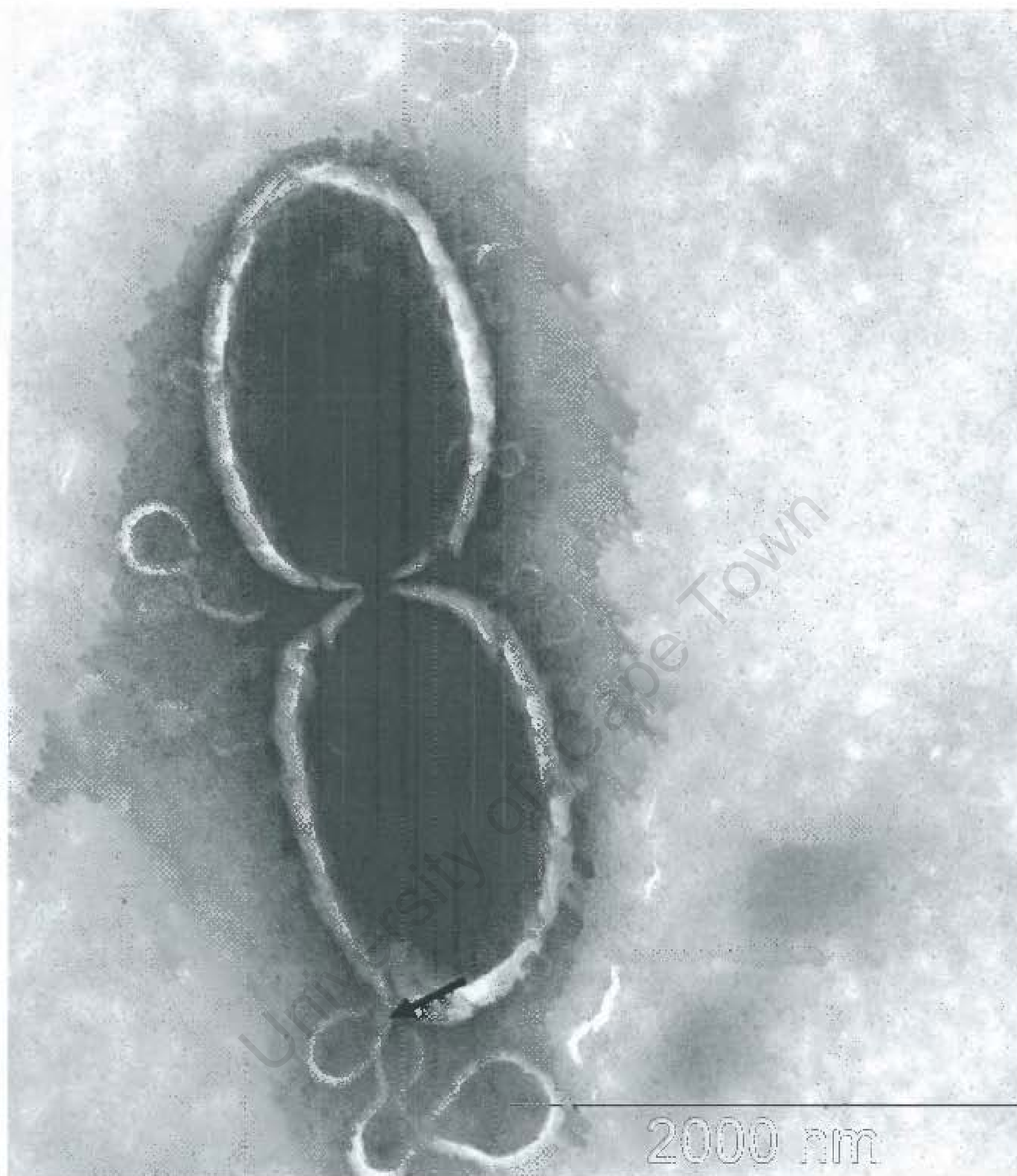

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Figure 3A. Electron micrograph showing the spherical shape of *Pseudoalteromonas* sp. strain C4 cells during exponential phase. The micrograph shows a strain C4 cell dividing. The arrow indicates a single flagellum attached to a cell, flagella are also visible floating unattached in the medium.

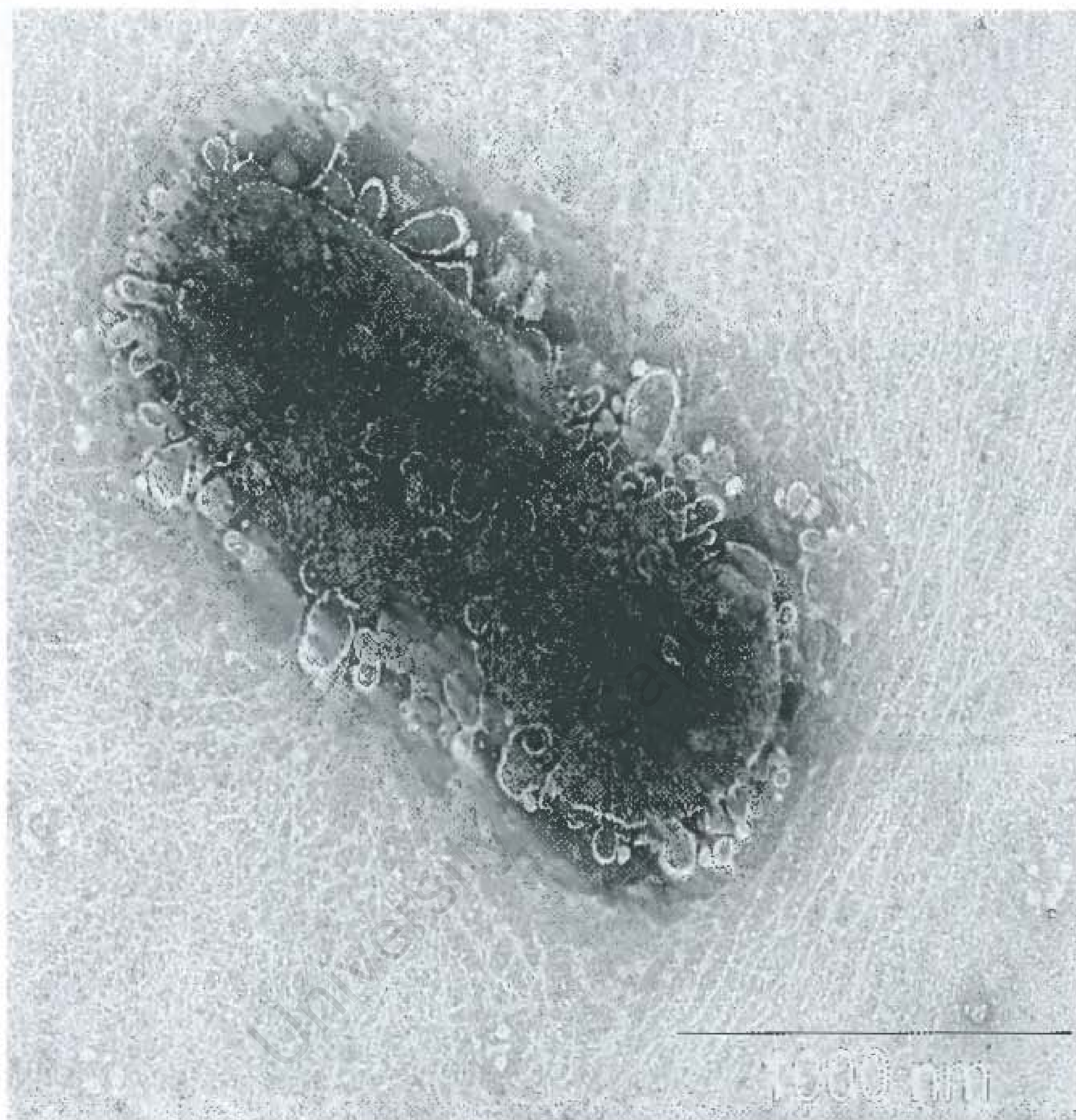

Figure 3B. Electron micrograph showing the cigar-shaped rod shape of *Pseudoalteromonas* sp. strain C4 cells during stationary phase. The flagellum is no longer visible although flagella were seen floating unattached in the media. The irregular shaped structures on the surface of the cell are believed to be the mucoid polysaccharide secreted by strain C4 during stationary phase.

1 **GAGTTTGATC** **CTGGCTCA**GA TTGAACGCTG GCGGCAGGCC TAACACATGC AAGTCGAGCG
61 GTAACAGAGA GTAGCTTGCT ACTTTGCTGA CGAGCGGCGG ACGGGTGAGT AATGCTTGGG
121 AACATGCCTT AAGGTGGGGG ACAACAGTTG GAAACGACTG CTAATACCGC ATGATGTCTA
181 CGGACCAAAG GGGGCTTCGG CTCTCGCCTT TAGATTGGCC CAAGTGGGAT TAGCTAGTTG
241 GTAAGGTAAC GGCTTACCAA GGCAACGATC CCTAGCTGGT TTGAGAGGAT GATCAGCCAC
301 ACTGGAACTG AGACACGGTC CAGACTCTAC GGGAGGCAGC AGTGGGGAAT ATTGCACAAT
361 GGGCGCAAGC CTGATGCAGC CATGCCGCGT GTGTGAAGAA GGCCTTCGGG TTGTAAAGCA
421 CTTTCAGTCA GGAGGAAAGG TTAGTAGTTA ATACCTGCTA GCTGTGACGT TACTGACAGA
481 AGAAGCACCG GCTAACTCCG **TGCCAGCAGC** **CGCGGTAATA** **C**GGAGGGTGC GAGCGTTAAT
541 CGGAATTACT GGGCGTAAAG CGTACGCAGG CGGTTTGTTA AGCGAGATGT GAAAGCCCCG
601 GGCTCAACCT GGGAACTGCA TTTCGAACTG GCAAACTAGA GTGTGATAGA GGGTGGTAGA
661 ATTTCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGAAGGA ATACCGATGG CGAAGGCAGC
721 CACCTGGGTC AACACTGACG CTCATGTACG AAAGCGTGGG GAGCAAACGG GATTAGATAC
781 CCCGGTAGTC CACGCCGTAA ACGATGTCTA CTAGAAGCTC GGGTCCTCGG ACTTGTTTTT
841 CAAAGCTAAC GCATTAAGTA GACCGCCTGG GGAGTACGGC CGCAAGGTTA AAACTCAAAT
901 GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT TAATTCGATG CAACGCGAAG
961 AACCTTACCT ACACTTGACA TACAGAGAAC TTACTAGAGA TAGTTTGGTG CCTTCGGGAA
1021 CTCTGATACA GGTGCT**GCAT** **GGCTGTCGTC** **AGCTCGTG**TT GTGAGATGTT GGGTTAAGTC
1081 CCGCAACGAG CGCAACCCCT ATCCTTAGTT GCTAGCAGGT AATGCTGAGA ACTCTAAGGA
1141 GACTGCCGGT GATAAACCGG AGGAAGGTGG GGACGACGTC AAGTCATCAT GGCCCTTACG
1201 TGTAGGGCTA CACACGTGCT ACAATGGCGC ATACAGAGTG CTGCGAACCT GCGAAGGTAA
1261 GCGAATCACT TAAAGTGCGT CGTAGTCCGG ATTGGAGTCT GCAACTCGAC TCCATGAAGT
1321 CGGAATCGCT AGTAATCGCG TATCAGAATG ACGCGGTGAA TACGTTCCCG GGCCTTGTAC
1381 ACACCGCCCG TCACACCATG GGAGTGGGTT GCTCCAGAAG TAGATAGTCT AACCCTCGGG
1441 AGGACGTTTA CCACGGAGTG ATTCATGACT GGGGTGAA**GT** **CGTAACAAGG** **TAACCGT**

Figure 4. Nucleotide sequence (1497 bp) of the 16S rRNA gene of *Pseudoalteromonas* sp. strain C4. The highly conserved regions of 16S rDNA to which the amplification primers were designed are indicated in bold and underlined. The sequence was submitted to the GENBANK database and assigned the accession number AF280818.

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Table 2. *Pseudoalteromonas* species that showed 98% sequence similarity to *Pseudoalteromonas* sp. strain C4 as obtained from a BLAST search of the GENBANK database with the 1497 bp 16S rDNA sequence of strain C4.

Species	GENBANK accession number
Pseudoalteromonas sp. R30	AF539774
Pseudoalteromonas sp. E30	AF539771
Psychrophilic marine bacterium	AF200216
Pseudoalteromonas gracilis	AF038846
Alteromonas elyakovii	AF082562
Pseudoalteromonas sp. SUR560	AB038036
Pseudoalteromonas sp. KMM3548	AY040229
Pseudoalteromonas sp. ER7	AF155038
Pseudoalteromonas elyakovii	AB000389
Pseudoalteromonas agarovorans	AJ417594

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Fourteen 16S rDNA sequences were aligned with 721 nucleotide sites for genus level analysis. Three 16S rDNA sequences were included from Gram positive bacteria (*Micrococcus luteus, Bacillus cereus and Staphylococcus epidermidis*) and the Gram negative enterobacterium, *E. coli*, as outgroups. The sequences shared 88.5% similarity within the group. Strain C4 was congeneric with the *Pseudoalteromonas* genus as strain C4 clustered with other members of the *Pseudoalteromonas* genus in the same clade in the MP analysis (Figure 5A). This was confirmed by NJ analysis (Figure 5B). Both methods of analysis also resulted in trees with the same topology further supporting the conclusion that strain C4 is a member of the *Pseudoalteromonas* genus by virtue of its similarity with other members of this genus. The bootstrap values in both analyses were suitably high. Based on this data strain C4 was classified as belonging to the genus *Pseudoalteromonas*.

Thirty-eight 16S rDNA sequences from the *Pseudoalteromonas* genus sharing the highest homology with strain C4 and one *Alteromonas* sp. were retrieved from the Genbank database and aligned with 752 nucleotide sites for species level analysis. The *Alteromonas* 16S rDNA sequence was included as an outgroup as it is a closely related taxon to *Pseudoalteromoans* but differs fundamentally from

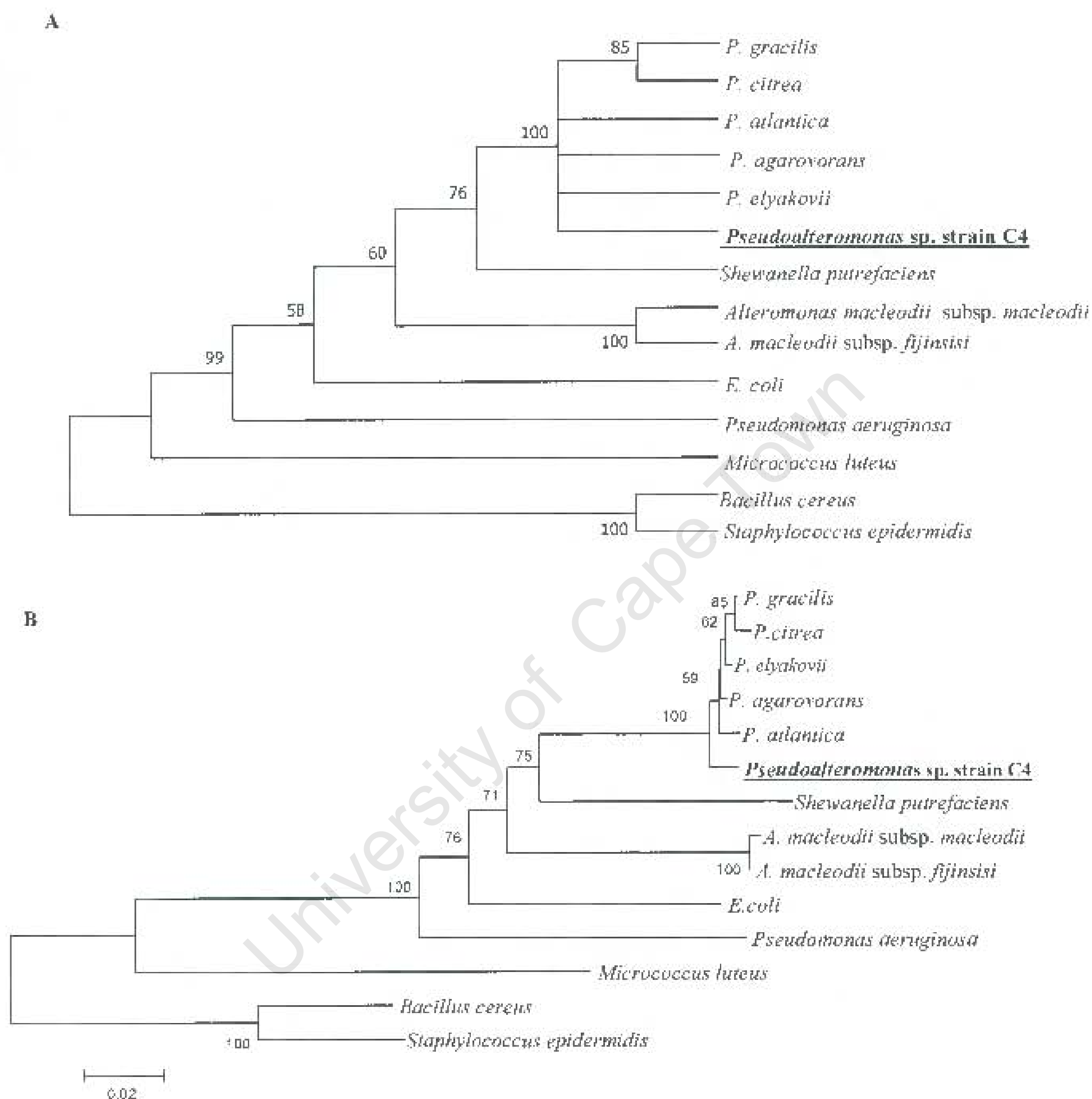

Figure 5. (A) Strict consensus MP tree inferred from 16S rDNA sequence data. Numbers above the branches indicate bootstrap proportions (percentage of 1000 replicates, values below 50 are omitted). (B) Phylogenetic tree inferred from NJ analysis of 16S rRNA gene sequence data. Numbers above the branches indicate bootstrap proportions (percentage of 1000 replicates, values below 50 are omitted). The bar depicts two base substitutions per 100 nucleotide sites.

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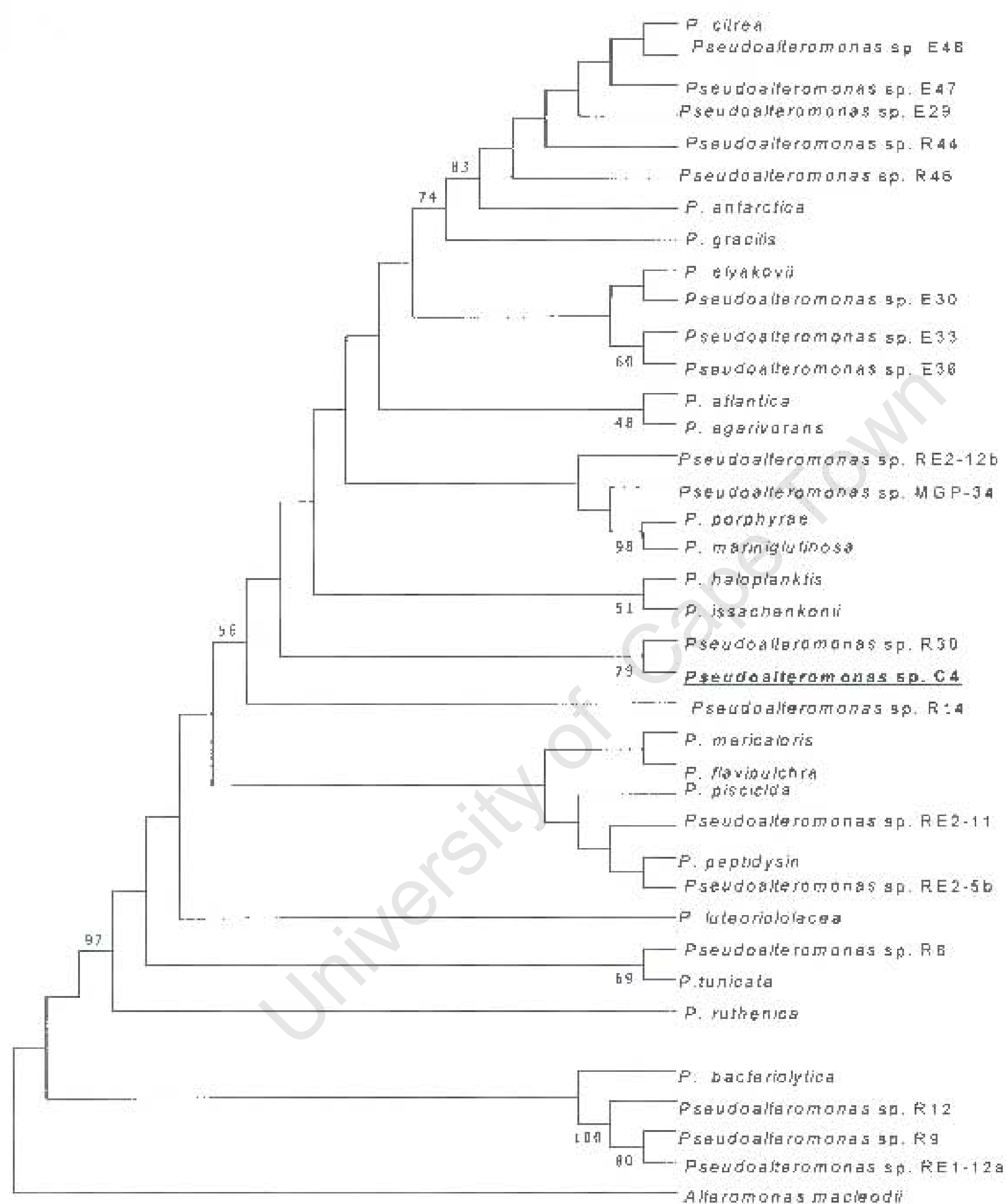

Figure 6. Strict consensus MP tree inferred from 16S rDNA sequences from 38 *Pseudoalteromonas* species and *Alteromonas macleodii* which was the designated out-group. Numbers above the branches indicate bootstrap proportions (percentage of 1000 replicates, values below 40 are omitted).

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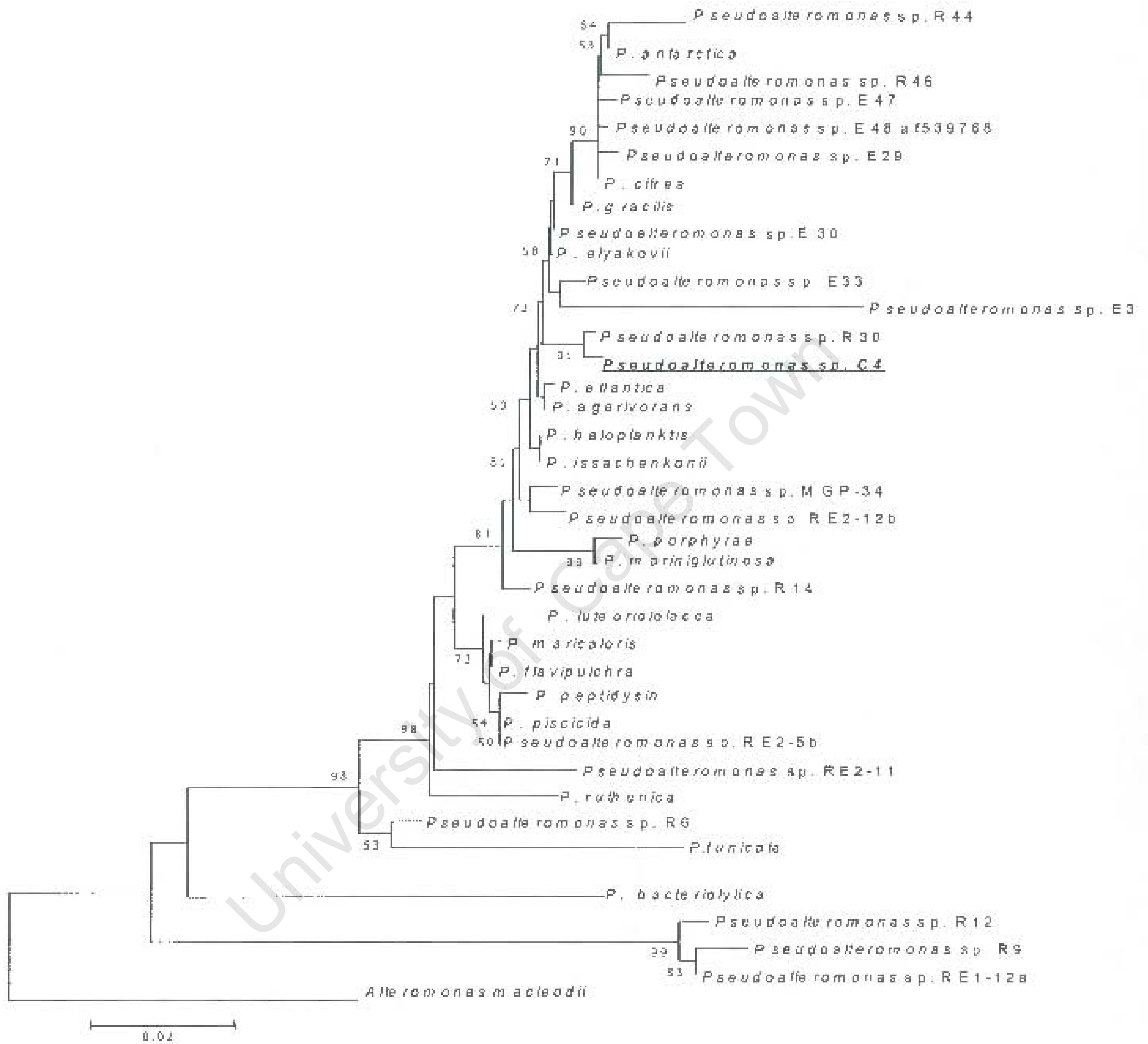

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Figure 7. Phylogenetic tree inferred from NJ analysis of the 16S rDNA sequences from 38 *Pseudoalteromonas* species and *Alteromonas macleodii* which was the designated outgroup. Numbers above the branches indicate bootstrap proportions (percentage of 1000 replicates, values below 50 are omitted). The bar depicts two base substitutions per 100 nucleotide sites.

the pseudoalteromonads. The sequences shared 94.2% homology when aligned with each other. Strain C4 clustered with *Pseudoalteromonas* sp. R30 (Figure 6) and this was confirmed by NJ analysis (Figure 7). Bootstrap values were suitably high for this branch.

2.4.4 Characterisation of the alginolytic phenotype of strain C4

The growth characteristics of strain C4 and its production of alginate lyase were examined in BMB, BMB supplemented with 0.2% sodium alginate and MB supplemented with 0.2% sodium alginate (Figure 8). The final density of the cells was unaffected by the different growth media although the generation times were different. The generation time in BMB (Figure 8A) was 57 min, 36 min in BMB supplemented with sodium alginate (Figure 8B) and 87 min in MB supplemented with sodium alginate (Figure 8C).

Intracellular alginate lyase activity was significantly less compared to extracellular activity in all media tested. Intracellular alginate lyase activity was expressed at basal levels in BMB throughout the growth experiment. Activity increased significantly during mid-exponential phase (6h) in both media supplemented with alginate. During early stationary phase there was a second increase in intracellular alginate lyase activity in media supplemented with alginate. Intracellular alginate lyase activity in media containing glucose (6.93 U) was significantly (Student's t-test, $p<0.05$) lower compared to media lacking glucose (8.06 U).

During lag phase and early to mid log phase, extracellular alginate lyase activity was detected at basal levels similar to that of the intracellular activity. In BMB (Figure 8A) extracellular activity began to increase during late exponential phase (7h) and increased almost 4.5 fold from 0.7 U to 4.0 U by stationary phase. In BMB supplemented with sodium alginate (Figure 8B) extracellular activity began to increase at mid–exponential phase (6h) and increased 25 fold from 0.9 U to 24.39 U by stationary phase. Extracellular activity began to increase in MB supplemented with sodium alginate (Figure 8C) during mid–exponential phase (6h) and also increased 25 fold by stationary phase. In both BMB and MB supplemented with alginate, alginate lyase activity more than doubled at the end of stationary phase. The presence of glucose had no significant effect on the level of extracellular alginate lyase activity, while the addition of alginate to the media resulted in a 6-fold increase in the

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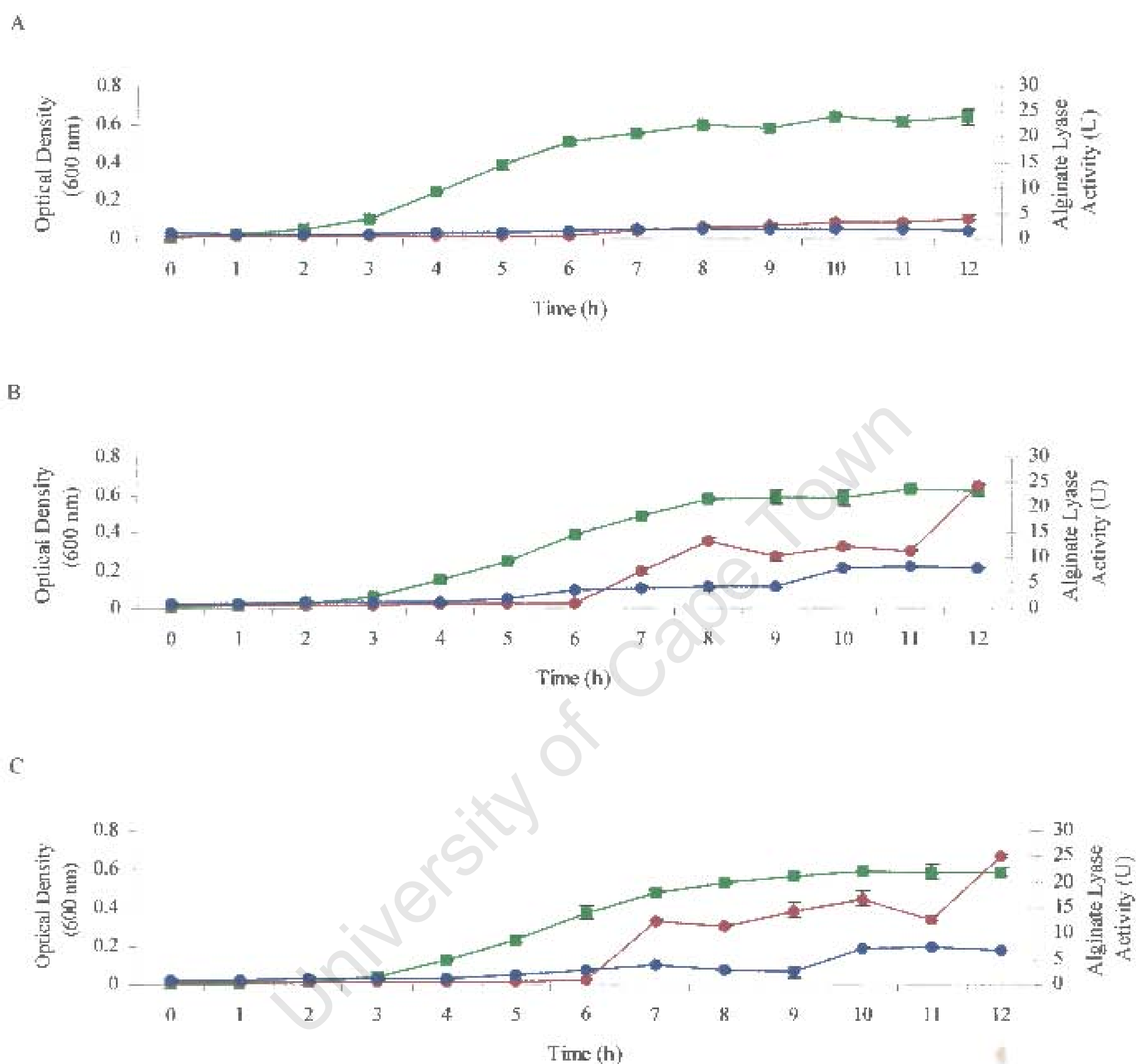

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Figure 8. Growth and alginate lyase activity of strain C4 in different culture media. Curves represent mean optical density at 600 nm ( ), extracellular ( ) and intracellular ( ) alginate lyase activity in (A) BMB; (B) BMB supplemented with 0.2% alginate; and (C) MB supplemented with 0.2% alginate. Error bars represent standard error calculated from three experiments. (U): μg malondialdehyde equivalents released 100 μl^{-1} 30 min^{-1} at 37°C.

level of alginate lyase activity compared to activity in BMB. In media supplemented with alginate, extracellular activity was 3-fold greater in BMB and 3.6-fold greater in MB compared to intracellular activity. In all media tested alginate lyase production reached a maximum level during stationary phase (12h, Figure 8).

2.4.5 Characterisation of strain C4 alginate lyase

The effect of temperature and pH on strain C4 alginate lyase activity was examined to determine the conditions under which the enzyme is most active. The highest levels of alginate lyase activity were observed at 22°C and 37°C (Figure 9A). Alginate lyase activity decreased significantly (Student's t-test, $p<0.05$) at 42°C compared to activity at 22°C and 37°C. No alginate lyase activity was detected at 4°C and at 55°C. Alginate lyase activity was highest under slightly acidic conditions between pH 6 and pH 7 (Figure 9B). At extremely acidic (pH 3) and basic (pH 10) conditions alginate lyase activity was significantly (Student's t-test, $p<0.05$) reduced compared to activity at neutral pH values. Maximum alginate lyase activity was observed at pH 6.5. Optimum conditions for alginate lyase activity are therefore between 22°C and 37°C and at a pH of between 6 - 6.5.

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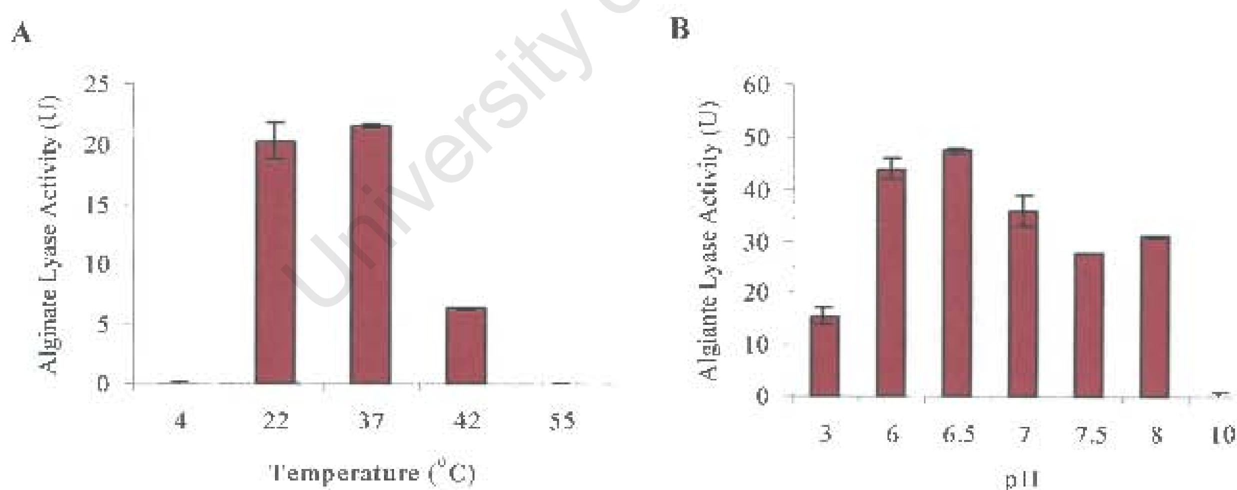

Figure 9. Extracellular alginate lyase activity under different conditions of strain C4 cultured in BMB supplemented with 0.2% sodium alginate. (A) Temperature; (B) and pH. (U): µg malondialdehyde equivalents released 100 μl^{-1}30 min^{-1} at 37°C, except (A) where the temperature is indicated on the x axis. Bars represent mean alginate lyase activity and error bars depict standard errors determined from three experiments.

2.5 DISCUSSION

Erasmus (1996) estimated that there were more than 1 x 10^{10} total bacteria and isolated 3.07 x 10^{8} culturable bacteria from the digestive tract of the South African abalone, *Haliotis midae*. The most common genera were *Vibrio, Alcaligenes, Flavobacteria* and *Pseudomonas*. Two thirds of the bacterial communities isolated from each region of the digestive tract consisted of two to three genera, which is in accordance with previous studies (Dempsey *et al*., 1989). The genera identified were similar to those isolated from other marine invertebrates (Martinez, 1982, De Ridder *et al*., 1985, Dempsey and Kitting, 1987, Musgrove, 1988, Vitalis *et al*., 1988, Douillet, 1993) and it is therefore possible that the enteric bacteria of abalone have similar effects as described in previous studies on marine invertebrates. This may include increasing the growth rate by contributing additional enzymes to aid digestion of seaweed and providing additional carbon and nitrogen sources. Bacteria isolated from the abalone digestive tract were able to hydrolyse agar, carrageenan, cellulose, laminarin and alginate (Erasmus *et al*., 1997) and approximately two thirds of the bacterial isolates could utilize two or three of the substrates tested (Erasmus, 1996).

Strain C4 had previously been reported by Erasmus (1996) as belonging to the genus *Pseudomonas* based on biochemical tests and was shown to hydrolyse alginate, laminarin and cellulose. Since the kelp *Ecklonia maxima* is the most popular feed for farmed abalone in South Africa, strain C4 was identified as a potential probiotic based on its ability to degrade alginate, which is the major component of kelp. 16S rDNA gene sequence analysis and biochemical tests performed during this study revealed that strain C4 belongs to the *Pseudoalteromonas* genus. *Pseudoalteromonas* species are Gram–negative, non–spore forming, straight or curved rods that are 0.2 to 1.5 by 1.8 to 3 µm (Gauthier *et al*., 1995). Most are motile by means of a single unsheathed polar flagellum. The cells are not luminescent, several species produce pigment and all members of this genus are strictly aerobic. They are chemoorganotrophs with respiratory but not fermentative metabolism, oxidase and catalase positive although catalase activity is generally weak and irregular. All species grow at 20°C and require a sodium chloride base for growth. *Pseudoalteromonas* is a genus commonly found in the marine environment in association with marine eukaryotes and includes at least 30 species (Holmström *et al*., 2002) many of which synthesize antibacterial compounds (Lemos *et al*., 1985; Barbieri *et al*., 2001; Hentschel *et al*., 2001). This may benefit *Pseudoalteromonas* cells in their competition with other organisms for nutrients and colonisation of surfaces.

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Phylogenetic analysis of the 16S rDNA gene sequence of strain C4 and other members of the *Pseudoalteromonas* genus revealed that strain C4 is most closely related to *Pseudoalteromonas* sp. strain R30 (Patel *et al.*, 2003). Strain R30 was isolated from natural biofilms on rocks and on the surface of *Enteromorpha* plants on the shore at Devon, England and shares a 98% 16S rDNA sequence similarity with strain C4. Stackebrandt and Goebel (1994) suggested that strains sharing more that 97.5% sequence similarity should be subjected to DNA-DNA hybridisation studies to determine whether the strains are different species. However, DNA-DNA hybridisation results are poorly correlated with degrees of sequence similarity as several groups of organisms have been identified that share almost identical 16S rRNA sequences but have varying values for DNA hybridisation. Sawabe *et al.* (1995) showed that *P. elyakovii* had high DNA–DNA hybridisation values in relation to each other (71.0%-97.4%) but low values in relation to species that clustered close to them in the 16S rDNA tree: *P. espejiana* (29.9%), *P. citrea* (49.5%), *P. carrageenovora* (46.9%) and *P. distincta* (52.4%). When the level of 16S rDNA sequence similarity is greater than 99%, DNA–DNA hybridisation may or may not document the existence of species identity. For example, *Mycobacterium intracellulare* serovar 12 and 19 share 100% 16S rDNA sequence similarity and 88% similarity by DNA-DNA hybridisation and are therefore classified as the same species. However, *Bacillus psychrophilus* and *Bacillus globisporus* share 16S rDNA sequence similarity of 99.8% and only have a 23.5% DNA–DNA hybridisation value (Fox *et al.*, 1992) indicating that they belong to different species. The Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics has suggested that strains sharing a greater than 70% DNA-DNA relatedness be classified as the same species (Stackebrandt and Goebel, 1994). Since a polyphasic approach (biochemical and 16S rDNA analysis) has been employed in identifying strain C4 the informative value of DNA-DNA hybridisation studies was considered minimal. Stackebrandt and Goebel (1994) recommended that it is ultimately the presence or absence of a phenotypic trait that, when comparing similar strains, should be the deciding factor in describing a new species. Since strain R30 is as yet undescribed it is not possible to determine whether *Pseudoalteromonas* sp. strain C4 and *Pseudoalteromonas* sp. strain R30 represent different species. However, a 98% sequence similarity (which is equivalent to 20 nucleotide differences along the length of the 16S rRNA gene) suggests that the strains may belong to different species but, until *Pseudoalteromonas* sp. strain R30 has been described, the relationship between the two species remains unclear.

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Alginate depolymerising enzymes have been isolated from fungi, marine molluscs and seaweeds (Maki *et al.*, 1993). Most alginate lyases have however been purified and characterised from bacteria

(Stevens and Levin, 1977, Linker and Evans, 1984, Dunne and Buckmire, 1985, Caswell, *et al.*, 1989, Muramatsu and Sogi, 1990, Davis, 1992, Gacesa and Goldberg, 1992, Kitamikado *et al.*, 1992, Ertesvåg *et al.*, 1998, Preston *et al.*, 2000, Sawabe *et al.*, 2003). Expression of alginase activity in bacteria is invariably induced by alginate, and one example of catabolite repression has been reported (Gacesa, 1992). Precise localisation of the enzyme has been determined in only a few cases and the results have often proved to be inconclusive. Most alginate lyase producing bacteria have been shown to have both intra- and extracellular alginate lyase activity: *Vibrio* sp. (Davis, 1992), *Pseudomonas* sp. OS–ALG–9 (Maki *et al.*, 1993), *Pseudomonas* sp. strain F6 (Muramatsu and Sogi, 1990), *Klebsiella pneumoniae* (Caswell *et al.*, 1989, Gacesa and Goldberg, 1992) and *P. aeruginosa* (Dunne and Buckmire, 1985). *Vibrio harveyii* AL–128, *Vibrio alginolyticus* (Kitamikado *et al.*, 1992) and *Alginovibrio aquatilis* (Stevens and Levin, 1977) have been reported to only produce extracellular alginate lyases. Alginate lyase activity in *Pseudoalteromonas* sp. strain C4 was shown to be predominantly extracellular although significant amounts of intracellular activity were also detected. Maximum production was achieved during stationary phase and production was induced by the addition of alginate to the growth media (25 U in BMB supplemented with alginate compared to 4 U in BMB). The addition of glucose to the media did not result in catabolite repression. BMB and MB supplemented with alginate both resulted in strain C4 producing a maximum amount of alginate lyase (25 U) during stationary phase. The onset of alginate lyase production in MB containing alginate was not delayed compared to BMB containing alginate.

Most alginate lyases isolated from bacteria have been shown to have optimum activity at neutral to slightly basic conditions. *Pseudomonas* sp. strain F6 showed maximum activity at pH 7.5 (Muramatsu and Sogi, 1990); *Azotobacter vinelandii*, pH 8.1–8.4 (Ertesvåg *et al.*, 1998); *P. aeruginosa* pH 6.2 and 20–40°C (Linker and Evans, 1984); *V. harveyi*, pH 7.8 and *V. alginolyticus*, pH 8.2 (Kitamikado *et al.*, 1992); *Azotobacter vinelandii* phage at pH 7.7 and *Pseudomonas syringae* pv. syringae at pH 7.0 and 42°C (Preston *et al.*, 2000). *Bacillus circulans* (Hansen *et al.*, 1984) showed optimum activity at the slightly acidic pH of 5.8. The extracellular alginate lyase of *Pseudoalteromonas* sp. strain C4 characterised in this study also showed optimum activity under slightly acidic conditions. Relatively high levels of activity were however also detected under slightly basic conditions. Optimum activity under slightly acidic conditions would allow for alginate metabolism in the crop and intestine of *H. midae* where the pH ranges between 5.3 and 5.7 and 6.0 and 6.4 respectively (Serviere–Zaragoza *et al.*, 1997, Harris *et al.*, 1998). Optimum activity at 22°C,

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and growth of strain C4 between 17°C and 30°C, favours strain C4 as a probiotic for abalone aquaculture as sea water temperatures range between 11.5°C and 21.2°C on the west and south coasts of South Africa throughout the year (Bolton, 1986).

The identification of strain C4 as belonging to the *Pseudoalteromonas* genus and its ability to produce an extracellular alginate lyase resulted in its selection for further study as a potential probiotic for use in abalone aquaculture. Members of the *Pseudoalteromonas* genus are generally not regarded as pathogens for marine invertebrates and the production of an extracellular alginate lyase allows for the hypothesis that the presence of strain C4 in the abalone digestive tract would increase the pool of digestive enzymes available to the abalone to digest its feed.

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CHAPTER 3

THE ROLE OF *PSEUDOALTEROMONAS* SP. STRAIN C4 IN NUTRITION AND GROWTH OF THE ABALONE, *HALIOTIS MIDAE*

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3.1 SUMMARY

The addition of *Pseudolateromonas* sp. strain C4 to artificial kelp feed resulted in the detection of alginate lyase activity in the feed and an increase in the amount of alginate degradation products compared to standard feed. Alginate lyase activity in the digestive system of the abalone, *Haliotis midae*, was greater in abalone fed kelp supplemented with strain C4 compared to abalone fed unsupplemented kelp. Gnotobiotic abalone and abalone not treated with antibiotics were shown to incorporate ^{14}C–labelled strain C4 proteins into their tissue although more incorporation was observed in gnotobiotic abalone. Although most of the radiolabelled bacterial protein was incorporated into the hepatopancreas, incorporation was also observed in the gills, foot, adductor muscle and intestine. Abalone fed kelp supplemented with strain C4 showed an increased growth rate compared to abalone fed standard kelp feed both under laboratory and farm conditions. Gnotobiotic abalone showed a significant decrease in growth rate compared to abalone that had not been treated with antibiotics when fed an unsupplemented kelp diet, indicating the importance of gastrointestinal microflora in abalone growth.

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3.2 INTRODUCTION

A major problem facing abalone farmers is the slow growth rate of abalone. The growth rate of abalone in natural populations is low, taking up to 8 years to reach maturity and 12–13 years to reach the legal harvesting size of 11.43 cm shell width. Although the growth rate under aquaculture conditions is much faster, abalone still require about 4 - 5 years to reach market size of 80 mm. The natural diet of abalone is the kelp *Ecklonia maxima*, and because it is readily available in South Africa it is often used as feed on abalone farms. However, *E. maxima* does not produce very high growth rates in farmed abalone (Simpson, 1994). Maximal growth rate is achieved by maximising protein deposition by the abalone (Britz and Hecht, 1997). Protein must be readily digestible and provide essential amino acids in the correct proportions. Insufficient amounts of dietary protein have resulted in lower growth rates in fish (Smith, 1989) and animals have had to use non protein energy sources, usually carbohydrates and lipids, to meet their energetic requirements. Seaweeds are rich in carbohydrates but poor in nitrogen, amino acids and proteins. *E. maxima* has been shown to have a protein content of 8.13% (% dry weight), an energy content of 11.77 $kJ.g^{-1}$ and ash content (% dry weight) of 25.44% (Simpson, 1994). Simpson (1994) found a significant positive correlation between protein and energy intake to both shell length and body weight growth rates. Allen and Kilgore (1975) found that ten amino acids (threonine, valine, methionine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine and arginine) were essential to *Haliotis rufescens* and needed to be supplied in the diet. It is generally assumed that the same amino acids are essential to different species of abalone, although essential amino acids have only been determined for *H. rufescens*. Simpson (1994) found the essential amino acids methionine, tyrosine, phenylalanine and arginine to be limiting in *E. maxima*.

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Recent research to improve growth rates of farmed abalone has focused on the development of artificial diets (Britz and Hecht, 1997; Knauer *et al.,* 1996; Simpson, 1994), as feed represents the major component of operational costs (Britz *et al.*, 1997, Go'mez-Montesa *et al.*, 2003). The effect of particle size (Sales and Britz, 2002b), energy:protein ratios (Britz and Hecht, 1997, Go'mez-Montesa *et al.*, 2003) and nutrient digestibility (Sales and Britz, 2002a) on feeding efficiency and growth rates of abalone have been investigated. However, it has been suggested that enteric bacteria may be involved in the nutrition of marine invertebrates (Prim and Lawrence, 1975, Muir *et al.*, 1986, Vitalis *et al.*, 1988, Erasmus *et al.*, 1997). This is especially important where less than optimally nutritious seaweeds or other plants are eaten, where foods are particularly indigestible or where a variety of seaweeds are consumed (Vitalis *et al.*, 1988). El–Shanshoury *et al.* (1994)

suggested that bacteria may enhance the digestive efficiency of a host by supplying polysaccharolytic enzymes and consequently improve the growth rate of the host. Bacteria isolated from the gut of the sea hare, *Aplysia juliana* (Vitalis *et al.*, 1988), sea urchins (Prim and Lawrence, 1975), the minke whale (Olsen *et al.*, 1994b) and abalone (Knauer *et al.*, 1996, Erasmus *et al.*, 1997) have been shown to produce enzymes capable of hydrolysing complex polysaccharides present in the host's food. Bacteria are also capable of releasing glucose from carbohydrate storage materials such as maltose and starch, and structural material such as carrageenan (Prim and Lawrence, 1975; Vitalis *et al.*, 1988). Bacterially mediated breakdown products of seaweeds, whether glucose or amino acids (Vitalis *et al.*, 1988), would be expected to be readily absorbed and used by an animal in its own metabolism. Vitalis *et al.* (1988) suggested several possible sources of amino acids for host animals due to bacterial activity: (1) normal extracellular digestion of algal protein by bacteria; (2) synthesis of amino acids by bacteria and the release into the gut lumen; (3) digestion of bacteria by other bacteria; (4) autolysis of bacteria and (5) digestion of bacteria by the host animal's own digestive enzymes.

Numerous studies have demonstrated that bacteria may also provide the host animal with carbon and nitrogen. Muir *et al.* (1986) suggested that bacteria may be potentially rich sources of carbon and nitrogen for their hosts as well as supplying essential micronutrients and coenzymes. Bacteria in upwelling water are able to supply 30% of the hourly carbon demand of the mussel *Choromytilus meridionalis* (Muir *et al.*, 1986) and organic carbon used by hydrothermal vent animals was shown to be indirectly derived from photosynthetic food sources (Herry *et al.*, 1989). Symbiotic bacteria in the gills of the clam, *Loripes lucinalis*, are able to fix carbon that is then translocated into the gills, foot and mantle. Herbivores feeding on low protein material must process large quantities of food to meet their protein requirements. The sea urchin *Strongylocentrotus droebachiensis* exhibited nitrogenase activity associated with the gastrointestinal tract (Seiderer *et al*, 1984). Guerinot and Patriquin (1981) showed that nitrogen–fixing bacteria in the gut of the sea urchin fixed nitrogen that was then incorporated into host tissue and Newell and Field (1983) suggested that utilisation of bacteria associated with kelp detritus could contribute as much as 69% of the nitrogen requirement of the consumer community as a whole. Anthozoan species have been shown to utilise bacteria as a food source (Sorokin, 1973 and references therein). The giant sea anemone, *Stoichactis giganteum*, digests coelenteric bacteria as soon as they reach a threshold level suggesting that part of their energy requirements may be derived from incorporating bacterial carbon into host tissue (Herndl *et al.*, 1985). The removal of gut bacteria would limit the rate of protein synthesis and metabolism and thus limit growth rate as this also results in the removal of a potential source of amino acids and glucose (Vitalis *et al.*, 1988).

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Abalone tissue contains high levels of glycogen, galactose and free amino acids (phosphoarginine, alanine and taurine), which are involved in energy storage and metabolism, but low levels of fat (Knauer *et al.*, 1996, Britz and Hecht, 1997). Macroalgae consumed by abalone generally contain high levels of storage carbohydrates but low levels of fat and protein. Erasmus *et al.* (1997) and V. Coyne and T. Andlid (personal communication) suggested that enteric bacteria may play an integral role in abalone nutrition by hydrolysing complex polysaccharide components of macroalgae to simple polymers and smaller units which are rapidly assimilated by abalone. Erasmus *et al.* (1997) showed that abalone enteric bacteria produced enzymes able to degrade agar, carrageenan, laminarin and alginate and that 70–90% of enzyme activity was extracellular suggesting that bacterial enzymes are secreted into the lumen of the gut where they are able to hydrolyse complex algal polysaccharides.

The objectives of this study were to determine the potential of the alginolytic bacterium *Pseudoalteromonas* sp. strain C4 to improve the growth rate of farmed abalone and to elucidate a possible mechanism for increased growth rates associated with farmed abalone fed a diet supplemented with *Pseudoalteromonas* sp. strain C4.

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3.3 MATERIALS AND METHODS

3.3.1 Alginate Lyase Activity and Alginate Degradation Products in Kelp Feed

Kelp cakes were prepared as described in Appendix A1.5, either including strain C4 or without the addition of strain C4. Segments from different cakes were excised and weighed five days after the feed had been prepared. Each piece was homogenised in an equal volume (w/v) of 100 mM phosphate buffer, pH 6.7 (Appendix A2.11). Alginate lyase activity and alginate degradation products were quantified using the TBA assay (Appendix B1). A unit of alginate lyase activity is defined as μg malondialdehyde equivalents released g $feed^{-1}$ 30 min^{-1} at 37°C (1 Unit) and degradation products are expressed as μg malondialdehyde equivalents present g $feed^{-1}$.

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3.3.2 Alginate Lyase Activity in the Abalone Digestive Tract

Thirty abalone were placed into each of four tanks with a tank volume of 98 L and a constant water flow rate of 5.5 L/min. Abalone in two of the tanks were fed standard kelp cakes, whereas abalone in the remaining two tanks were fed kelp cakes supplemented with strain C4 over a period of 7 days. At 0, 1, 3 and 7 days after feeding of strain C4 supplemented kelp cakes had been initiated, three abalone were sacrificed from each tank and the digestive tract was removed. During the next 7 day period all abalone were fed standard kelp cakes. Three abalone were sacrificed from each tank at 1, 3 and 7 days after the strain C4 supplemented diet had been halted and the oesophagus, crop, stomach and intestine removed (Appendix C3). Each of the tissue samples was weighed and homogenised in 0.1 M citric acid/0.2 M phosphate buffer, pH 5.2 (Appendix A2.8) at a volume of 2 ml/ g tissue. The samples were dialysed for 48 h against 0.1 M citric acid/0.2 M phosphate buffer, pH 5.2 at 4°C with two changes of buffer. The protein concentration in each sample was determined using the Bradford Assay (Appendix B6) and alginate lyase activity was determined using the TBA assay, for each sample. Alginate lyase activity was defined as μg malondialdehyde equivalents released μg $protein^{-1}$ 30 min^{-1} at 37°C (1 unit).

3.3.3 Incorporation of *Pseudoalteromonas* sp. strain C4 proteins in abalone tissue

3.3.3.1 Radiolabelling of strain C4 proteins

Strain C4 was cultured overnight at 22°C in 100 ml of yeast extract (YE)/peptone broth (Appendix A1.18). The overnight culture was used to inoculate 25 ml of YE/peptone broth to an optical density of 0.1 at 600 nm. The culture was incubated for 1 h at 22°C before the addition of 100 μCi of ^{14}C–sodium acetate (55.0 mCi/mmol, Amersham). The culture was incubated for 16 h at 22°C, cells were harvested by centrifugation for 10 min at 7 800 x g and washed twice with synthetic sea salts (SSS, Appendix A2.12). Cells were resuspended in 1 ml of SSS, divided into 70 μl aliquots and stored at –20°C.

3.3.3.2 Experimental set-up

Four 24 L glass tanks were washed with ethanol and allowed to dry. Three tanks (Figure 1, Tanks 1–3) were filled with 17 L of autoclaved sterilised seawater and one tank with unsterilised seawater (Figure 1, Tank 4). Water was allowed to recirculate through a carbon-filter system.

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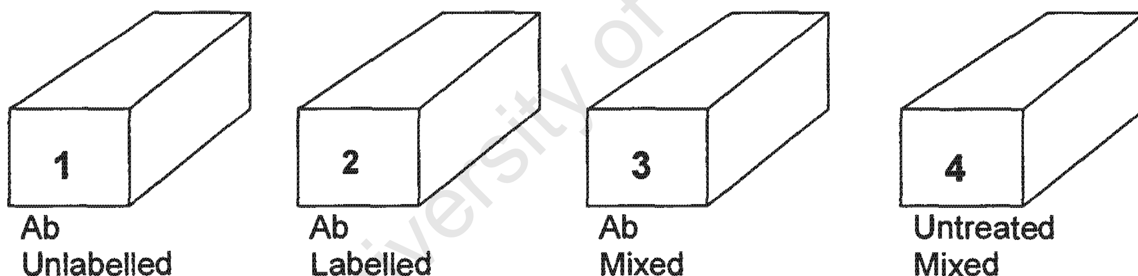

Figure 1. Schematic diagram of experimental set up of tanks and treatments used for investigating the incorporation of strain C4 proteins into abalone tissue. Ab = antibiotic treated (gnotobiotic abalone); Untreated = no antibiotic treatment (untreated abalone). Unlabelled/Labelled = abalone inoculated with either unlabelled or ^{14}C-labelled strain C4. Mixed = abalone inoculated with either ^{14}C-labelled or unlabelled strain C4 and kept in the same tank.

Five abalone were placed in tanks 1 and 2 and ten abalone were placed in tanks 3 and 4. Abalone were starved for 2 days and then fed kelp cakes. Abalone in tanks 1, 2 and 3 were fed kelp cakes containing antibiotics (chloramphenical 250 μg/ml; cefotaxine 500 μg/ml; ampicillin 250 μg/ml,

Appendix A2.1) and abalone in tank 4 were fed kelp cakes lacking antibiotics for 4 days. Each day the water was removed and fresh seawater added. After two days of feeding antibiotic kelp cakes, the tanks were cleaned, fresh antibiotic kelp cakes were added and antibiotics were added to the water in tanks 1, 2 and 3 (ampicillin, 100 mg/l; chloramphenicol, 100 mg/l; cefotaxime, 160 mg/l, Appendix A2.1). Fresh antibiotics were added for two consecutive days. The effectiveness of antibiotic treatment was evaluated as described in Appendix B7. After antibiotic treatment all abalone were fed kelp cakes for the remainder of the experiment.

3.3.3.3 Inoculation of abalone and determination of protein incorporation

Abalone were anaesthetised in 6% (w/v) $MgSO_4$ (Appendix A2.9) in seawater. Five abalone from tanks 2, 3 and 4 (Figure 1) were inoculated orally with 5 µl of labelled strain C4. The remaining five abalone in tanks 3 and 4 and the five abalone in tank 1 were inoculated with 5 µl of unlabeled strain C4 (control abalone). After inoculation abalone were placed upside down for 5 min, control abalone (inoculated with unlabelled strain C4) were marked with Pratley® putty, and all abalone were returned to their respective tanks. Abalone inoculated with ^{14}C-labelled strain C4 and unlabelled strain C4 were mixed in tanks 3 and 4 to determine whether ^{14}C-labelled protein could be transferred between abalone and possibly determine how this transfer occurs. Abalone were re-inoculated with either radiolabelled strain C4 or unlabelled strain C4 (control abalone) each day for 5 consecutive days, using the procedure described above. The amount of radiolabel (CPM/5 µl) was determined each day by counting 5 µl of labelled cells in a Beckman scintillation counter to quantify the amount of ^{14}C inoculated per day.

After 5 days all abalone were sacrificed and the gills, foot, adductor muscle, stomach, hepatopancreas and intestine were removed (Appendix C1 and Appendix C3). Each sample was weighed and incubated with 1 ml Soluene–350 (Packard) /100 mg wet tissue for 48 h at 55°C to solubilise the tissue (Muir *et al.*, 1986). Five hundred microlitres of each sample was added to 5 ml scintillation fluid (Beckman), bleached with 500 µl 30% H_2O_2 and a few drops of glacial acetic acid. The mixture was incubated in the dark for 2 h to reduce chemiluminescence and the radioactivity counts were determined in a Beckman scintillation counter. The data was expressed as DPM/g wet tissue. At the time of sacrifice, a sample of water and a sample of faeces was removed from each tank and the amount of radioactivity associated with each sample was determined.

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3.3.4 Abalone Growth Trials

3.3.4.1 Laboratory growth trials

Laboratory based growth trials were carried out at the Marine and Coastal Management Research Aquarium, Sea Point, Cape Town. Four 24 L glass tanks were used with a constant water flow rate of 860 ml/min. Two of the tanks were kept relatively sterile (Figure 2, Tanks 1 and 2) by circulating 0.2 µM filtered sea water and air as well as ethanol cleaning all equipment used to take samples. Abalone in the other two tanks were not treated with antibiotics and supplied with 10 µM filtered sea water and unfiltered air (Figure 2, Tanks 3 and 4). Forty abalone, measuring approximately 25.1 mm each, were placed in each tank.

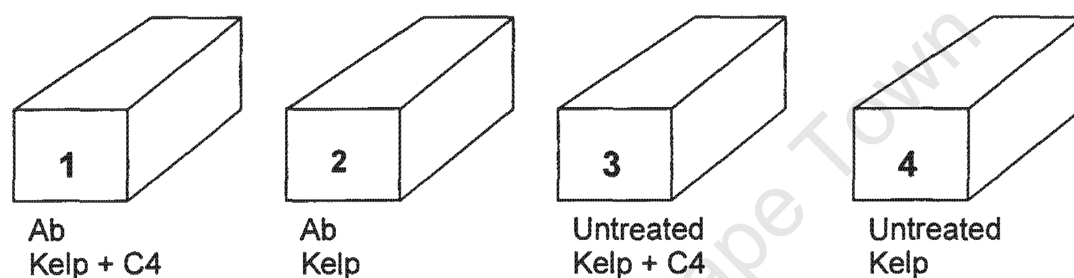

Figure 2. Schematic diagram of experimental set up of tanks and treatments used for laboratory based growth trials. Ab = antibiotic treated (gnotobiotic abalone); Untreated = no antibiotic treatment (untreated abalone); Kelp = abalone fed standard kelp cakes; Kelp + C4 = abalone fed kelp cakes supplemented with strain C4.

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Abalone were initially starved for two days after which abalone in tanks 1 and 2 were fed kelp cakes containing antibiotics (Appendix A1.5 and Appendix A2.1) for four days. After antibiotic kelp cakes had been fed for two days the water supply was turned off for 24 h and antibiotics were added to the water (ampicillin, 1 ml/L and chloramphenicol, 1.25 ml/L). This was repeated for two 24 h periods during which the abalone were fed kelp cakes containing antibiotics. After 24 hours the sea water was again replaced with fresh 0.2 µm filtered sea water and the water was allowed to circulate again. Tanks 3 and 4 were untreated. The effectiveness of antibiotic treatment was evaluated as described in Appendix B7. Abalone in tanks 1 and 3 were fed kelp supplemented with strain C4 (Appendix A1.5) and abalone in tanks 2 and 4 were fed standard kelp cakes after the antibiotic treatment was completed. Tanks were cleaned every 3 days by siphoning out the sea water. Abalone were fed 50 g of kelp cakes per tank every second day and abalone shell length was measured every month over an 8 month period and the lengths recorded.

3.3.4.2 Farm based growth trials

Farm based growth trials were carried out at HIK Abalone Farm in Hermanus, Western Cape. Approximately 1500 abalone, measuring approximately 16.74 mm and weighing 1.03 g, were placed in each of four tanks with a volume of 144 L. Abalone in two of the tanks were fed kelp supplemented with strain C4 and abalone in the other two tanks were fed standard kelp cakes. Abalone were fed approximately 600 g of kelp cakes/tank every second day. Thirty abalone were randomly selected for weight and shell length measurements every month over an 8 month period. At the end of this period abalone were moved from the weanary to raceway tanks for grow out and fed regular *Ecklonia maxima*. Shell length measurements and weights were recorded for another 3 months.

3.3.5 Statistical Analysis

All data are presented as the means of at least two experiments and standard errors. For comparison of two means, paired or unpaired Student's t–tests were used. Two-factor analysis of variance (ANOVA), followed by a multiple comparison test (Tukey) was used to determine the significant differences in alginate lyase activity in the digestive tract due to time and type of feed variables. To meet the normality assumption of ANOVA, some data were $\log_{10}$ transformed. The limit of significance was $p<0.05$ unless otherwise stated.

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3.4 RESULTS

3.4.1 Alginate lyase Activity and Digestion Products in Kelp Feed

The presence of alginate lyase activity and alginate degradation products was compared between a standard kelp feed and kelp feed supplemented with strain C4. Alginate lyase activity was 3 fold greater in feed that had been supplemented with strain C4 compared to the standard kelp feed (Figure 3A). The amount of hydrolysed alginate was 4 fold greater in the supplemented feed compared to the standard feed (Figure 3B).

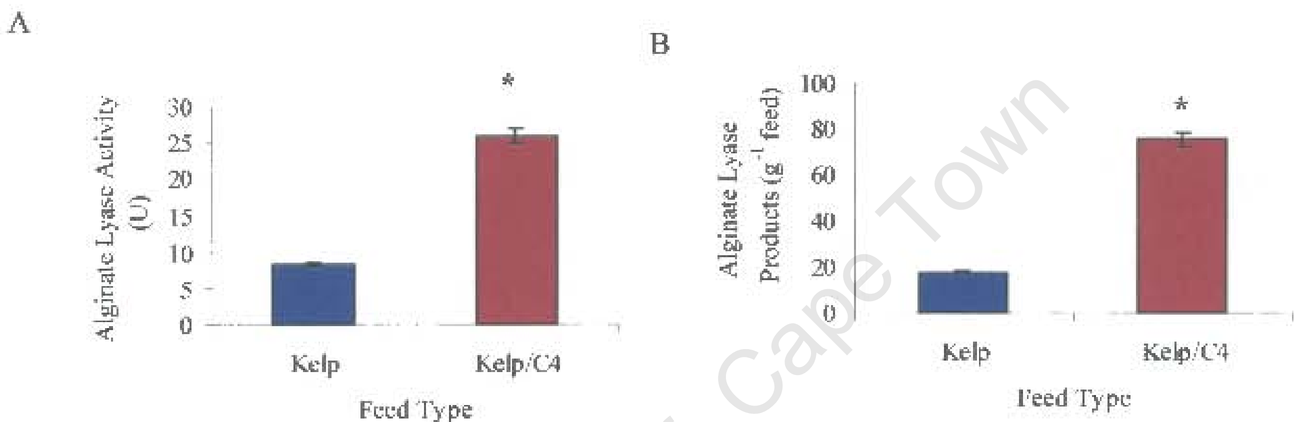

Figure 3. (A) Alginate lyase activity and (B) alginate degradation products in kelp feed (■) and kelp feed supplemented with strain C4 (■). Bars represent the average alginate lyase activity or amount of degradation products present g^{-1} feed. Error bars depict standard error, determined from three experiments. Asterisk (*) indicates significant difference (Student's t-test, $p<0.05$) compared to control.

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3.4.2 Alginate Lyase Activity in the Abalone Digestive Tract

The presence of alginate lyase activity in the digestive tract was compared between abalone fed a standard kelp diet and a kelp diet supplemented with strain C4 to determine whether strain C4 contributes to the pool of digestive enzymes present in the abalone digestive system. The amount of alginate lyase activity in the digestive tract of abalone fed a kelp diet supplemented with strain C4 was significantly greater compared to abalone fed a standard kelp diet (Figure 4). There was no significant difference between the two groups at day 0 and day 1. At day 3 and day 7, *in situ* alginate lyase activity was significantly greater (Two-way ANOVA, Tukey's test $p<0.05$) in abalone fed a kelp diet supplemented with strain C4.

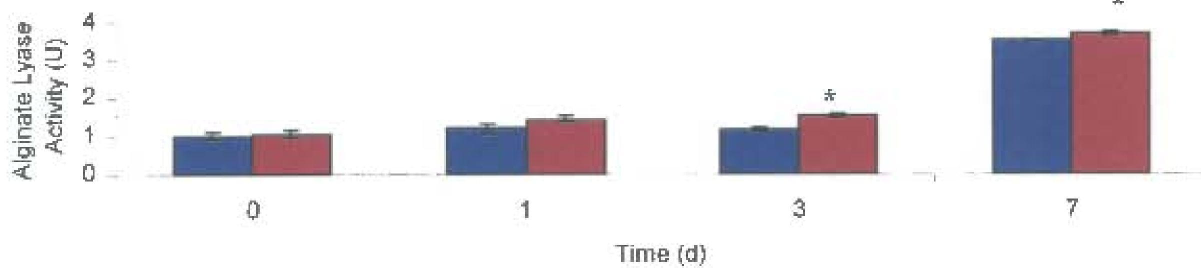

Figure 4. Alginate lyase activity in the digestive tract of abalone fed either a standard kelp diet ( ) or a kelp diet supplemented with strain C4 ( ). Bars represent the average alginate lyase activity and error bars depict standard error, determined from six experiments. Asterisk (*) indicates significant difference (Two-way ANOVA, Tukey's test $p<0.05$) compared to control.

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Alginate lyase activity was determined in the entire gastrointestinal (GI) tract as it was assumed that over the 7 day period food containing strain C4 would be continuously moving through the GI tract and if strain C4 contributed any alginate lyase activity this activity would also be distributed throughout the GI tract. After the seventh day, feeding the strain C4 supplemented kelp diet was halted and all abalone were fed the standard kelp diet. Alginate lyase activity was then determined in individual sections of the GI tract as it was thought that activity associated with strain C4 would only be found in sections of the GI tract in which strain C4 was present either by colonising that section of the GI tract or through transient movement through the GI tract with the feed. Alginate lyase activity in the oesophagus, crop, stomach and intestine was determined over a second 7 day period and compared to abalone fed the standard kelp diet throughout the experiment (Figure 5). There was no difference in alginate lyase activity in the oesophagus between the two groups after the strain C4 supplemented kelp diet had ended (Figure 5A). At day 3, the alginate lyase activity in the crop (Figure 5B) and stomach (Figure 5C) was significantly higher (Two-way ANOVA, Tukey's test, $p<0.1$, Appendix D) in abalone previously fed a strain C4 supplemented diet compared to abalone fed a kelp diet. However at day 7 there was no difference in alginate lyase activity in the crop and stomach between abalone that had previously been fed a kelp diet supplemented with strain C4 and those continually fed a kelp diet. At day 1 alginate lyase activity was significantly greater (Two-way ANOVA, Tukey's test, $p<0.1$) in the intestine of abalone previously fed kelp supplemented with strain C4 compared to abalone fed only kelp (Figure 5D). The highest amount of alginate lyase activity was observed in the stomach, followed by the crop, in both groups of

abalone. Alginate lyase activity was greatly reduced at day 7 in the crop, stomach and intestine in both groups of abalone.

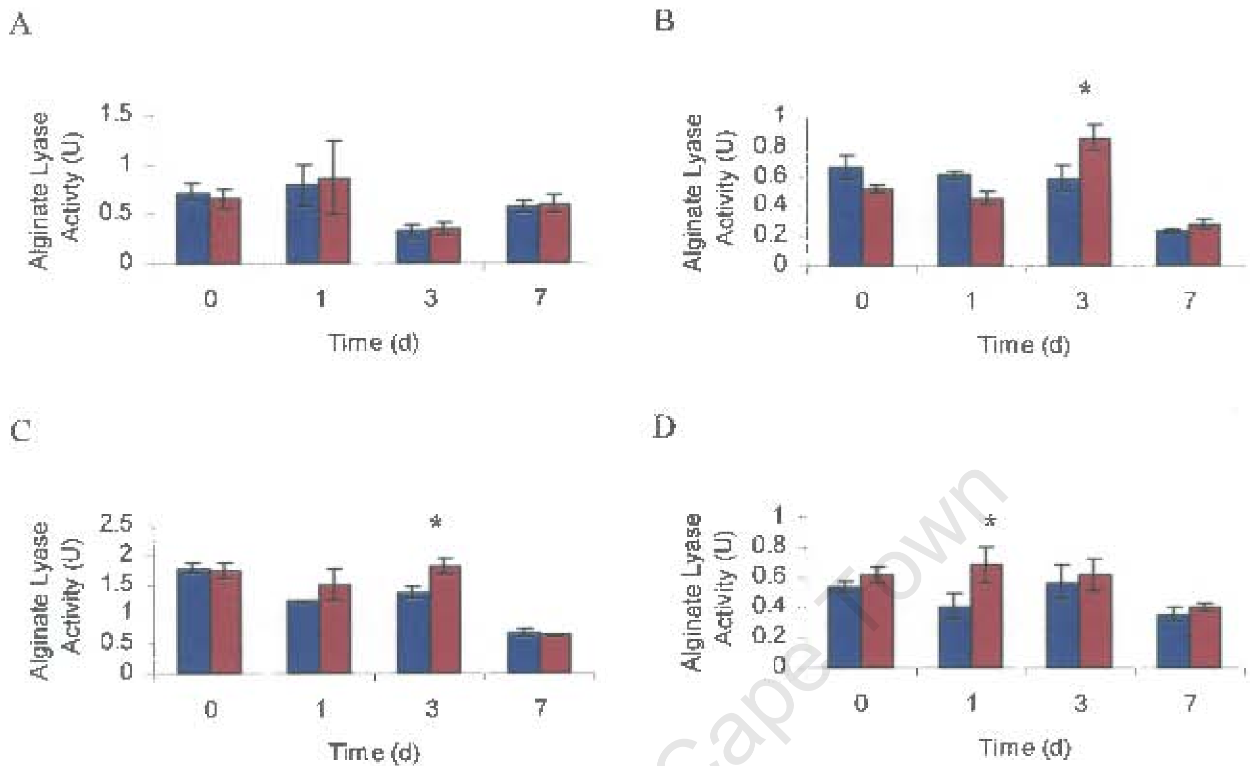

Figure 5. Alginate lyase activity in the (A) oesophagus, (B) crop, (C) stomach and (D) intestine of abalone fed either a kelp diet (■) or a strain C4 supplemented kelp diet (■). Bars represent the average alginate lyase activity and error bars depict standard error, determined from three experiments. Asterisk (*) indicates significant difference (Two-way ANOVA, Tukey's test $p<0.1$) in activity between abalone fed a strain C4 supplemented kelp diet and those fed a standard kelp diet at each time point.

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3.4.3 Incorporation of ^{14}C- Labelled Proteins from strain C4 in Abalone Tissue

Strain C4 was radioactively labelled with ^{14}C-sodium acetate. Acetate reacts with oxaloacetate in the presence of acetyl CoA to produce citrate (Edwards and Hassall, 1971). This is the first step in the tricarboxylic acid cycle that is responsible for the synthesis of amino acids. Therefore, labelling strain C4 with ^{14}C-acetate would allow for labelling of many amino acids that are then used in protein synthesis. Abalone inoculated with ^{14}C-labelled strain C4 were inoculated with a total of $2.9\text{x}10^5$ cpm (SE = $9.4\text{X}10^2$) over 5 days. The total amount of radioactive counts in unlabelled strain C4 inoculated into each abalone was $1.2\text{x}10^3$ cpm (SE = 82.6). The amount of

radioactive counts incorporated into abalone tissue was determined. Gnotobiotic abalone that were inoculated with ^{14}C–labelled strain C4 and kept separate from abalone inoculated with unlabelled strain C4 showed significantly (Student's t-test, $p<0.05$) higher incorporation compared to control animals in all tissues except the stomach (Figure 6 and 7A). When gnotobiotic abalone that had been inoculated with labelled strain C4 were placed in a tank containing gnotobiotic abalone that had been inoculated with unlabelled strain C4 no difference was detected in the amounts of ^{14}C labelled protein incorporated by the two groups of abalone (Figure 6 and 7B). The same observation was observed in abalone not treated with antibiotics (Figure 6 and 7C). In all abalone, the highest level of ^{14}C incorporation was detected in the hepatopancreas (Figure 6). At the time of sacrifice both water and faecal samples were removed from each of the tanks. Although a significant amount of ^{14}C was detected in faecal samples isolated from the tanks, water sampled from the tanks showed background levels of ^{14}C (Table 1).

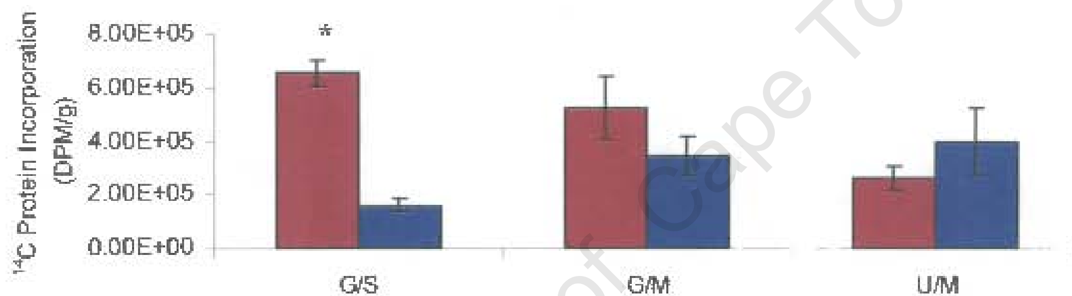

Figure 6. Incorporation of ^{14}C labelled strain C4 protein into the abalone hepatopancreas. Abalone were inoculated with either labelled (■) or unlabelled (■) strain C4. G/S = Gnotobiotic abalone that were inoculated with either labelled or unlabelled strain C4 and kept in separate tanks, G/M = Gnotobiotic abalone that were inoculated with either labelled or unlabelled strain C4 and kept in the same tank, U/M = Abalone not treated with antibiotics, inoculated with either labelled or unlabelled strain C4 and maintained in the same tank. Bars represent the average ^{14}C protein incorporation per gram tissue (wet wt.) and error bars depict standard error, determined from two experiments (n=5/experiment). Asterisk (*) indicates significant difference ($p<0.05$) compared to control.

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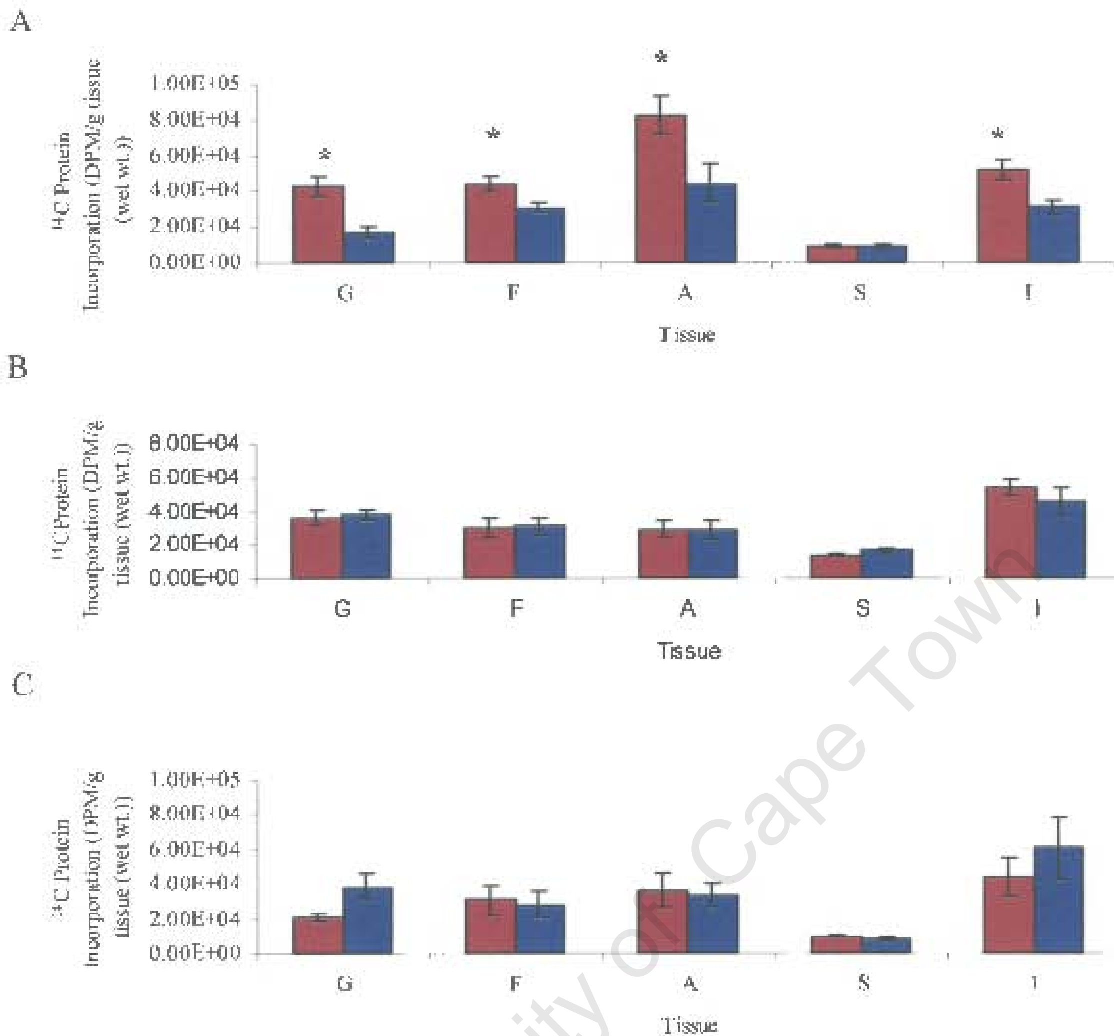

Figure 7. Incorporation of ^{14}C-labelled strain C4 protein into abalone tissue. (A) Gnotobiotic abalone that were inoculated with ^{14}C-labelled strain C4 (■) and kept in separate tanks to gnotobiotic abalone that were inoculated with unlabelled strain C4 (■), (B) Gnotobiotic abalone that were inoculated either with ^{14}C-labelled or unlabelled strain C4 and kept in the same tank, (C) Abalone not treated with antibiotics, inoculated with either ^{14}C-labelled or unlabelled strain C4 and maintained in the same tank. G = gills; F = foot; A = adductor muscle; S = stomach; I = intestine. Bars represent the average ^{14}C protein incorporation and error bars depict standard error, determined from two experiments (n=5/experiment). Asterisk (*) indicates significant difference (p<0.05) compared to control.

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Table 1. Amount of ^{14}C detected in water and faecal samples sampled from tanks 1 = Gnotobiotic abalone that were inoculated with unlabelled strain C4, 2 = Gnotobiotic abalone that were inoculated with labelled strain C4, 3 = Gnotobiotic abalone that were inoculated with either labelled or unlabelled strain C4, 4 = Abalone not treated with antibiotics and inoculated with either labelled or unlabelled strain C4.

Tank	Water (CPM/500 ml)	Faeces (CPM/g)
1	2.43E+02	8.65E+03
2	2.78E+02	1.29E+04
3	2.21E+02	7.83E+03
4	2.69E+02	4.11E+04

Similar amounts of ^{14}C were detected in the faecal samples from gnotobiotic abalone that had been inoculated with unlabelled strain C4 (Tank 1, Table 1) and the gnotobiotic mixed group of abalone inoculated with either ^{14}C-labelled strain C4 or unlabelled strain C4 (Tank 3, Table 1). Slightly greater levels of ^{14}C were detected in samples from gnotobiotic abalone that had been inoculated with labelled strain C4 (Tank 2, Table 1) while the highest level of radioactivity was detected in faecal samples from abalone that were not treated with antibiotics and inoculated with either labelled or unlabelled strain C4 and maintained in the same tank (Tank 4, Table 1).

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3.4.4 Abalone Growth Trials

Laboratory based growth trials clearly indicated that the addition of strain C4 to the abalone diet greatly increased the growth rate compared to abalone fed a standard kelp diet (Figure 8). Gnotobiotic abalone fed a kelp diet lacking strain C4 showed the poorest growth rate (4.2 µm/day). The growth rate of abalone not treated with antibiotics and fed a kelp diet lacking strain C4 (7.9 µm/day) was significantly higher than the gnotobiotic abalone fed kelp cakes. The growth rate of abalone not treated with antibiotics and fed a kelp diet supplemented with strain C4 (19.2 µm/day) was significantly higher than abalone not treated with antibiotics and fed a kelp diet lacking strain C4. There was no significant difference (Student's t-test, $p<0.05$) in growth rate between gnotobiotic abalone fed a kelp diet supplemented with strain C4 (19.1 µm/day) and abalone not treated with antibiotics and fed the same diet (19.2 µm/day).

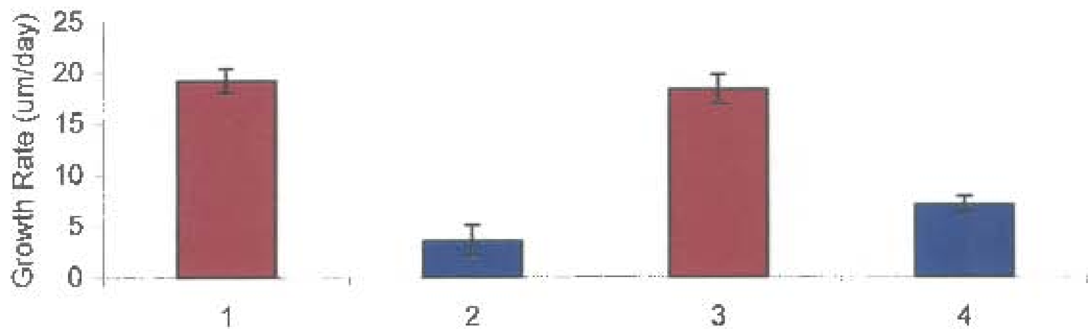

Figure 8. Growth rate (shell length, µm/day) of gnotobiotic abalone fed either (1) kelp supplemented with strain C4 or (2) a standard kelp diet, or (3) abalone not treated with antibiotics fed either kelp supplemented with strain C4 or (4) a standard kelp diet. Bars represent average growth rate/day/abalone over an eight month period, error bars depict standard errors calculated from three different sets of measurements.

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Farm based growth trials on weanery sized abalone (16 mm, 1.0 g) also indicated that the addition of strain C4 to abalone feed improved the growth rate of these animals (Figure 9). The addition of strain C4 to the feed increased the growth rate in terms of length by 16.1 µm/day (Figure 9A) and of weight by 3.6 µg/day (Figure 9B). Twenty days after abalone were moved to the raceways and fed regular *Ecklonia maxima*, the shell length growth rate decreased significantly (Student's t-test, $p<0.05$), whereas growth rate based on weight increased in both groups of abalone. The growth rate in terms of both length and weight was still significantly higher in abalone previously fed a kelp diet supplemented with strain C4. Fifty-six days after abalone were moved to the raceway and fed regular *Ecklonia maxima*, the shell length growth rate had decreased further but there was no significant difference in the weight growth rate compared to the growth rate measured 20 d after being moved to the raceway in abalone previously fed a kelp diet supplemented with strain C4. The shell length and weight growth rate of abalone fed a standard kelp diet continued to increase after 56 days in the raceway. Even though the growth rate of abalone continuously fed a standard kelp diet was increasing, the growth rate of abalone previously fed a strain C4 supplemented diet was still significantly greater at 56 days after the supplemented diet had been halted.

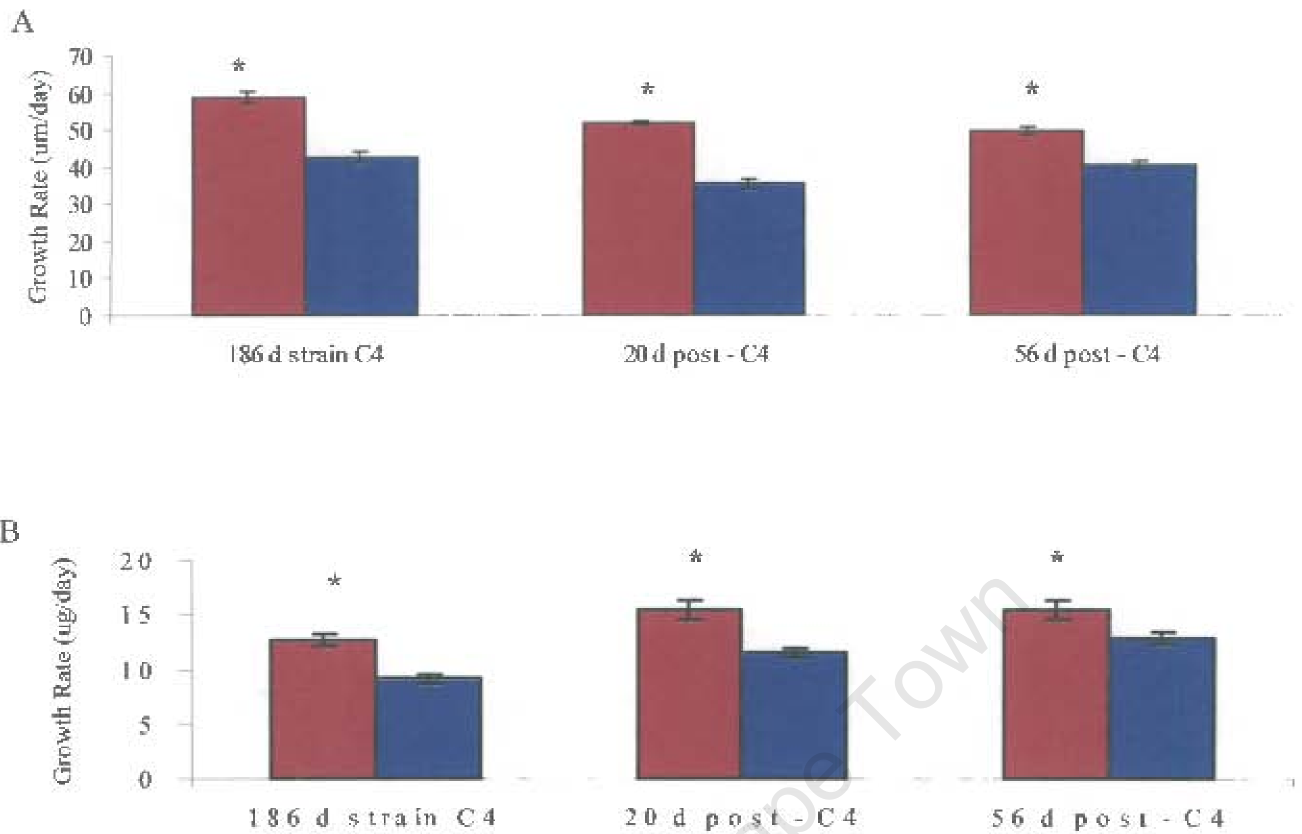

Figure 9. Eight month growth trial of abalone initially fed kelp supplemented with strain C4 (■) or kelp lacking strain C4 (■). After five months, feeding kelp supplemented with strain C4 was stopped and all abalone were fed regular *E. maxima* lacking strain C4. Bars represent average growth rate/day/abalone with respect to length (A), and with respect to weight (B), error bars depict standard error calculated from 12 different sets of measurements. Asterisk (*) indicates significant difference ($p<0.05$) compared to control.

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3.5 DISCUSSION

Aquacultured abalone in South Africa are typically fed the nutrient poor kelp *Ecklonia maxima* and take approximately four years to reach the 80 mm market size. This study investigates the possibility of supplementing the kelp diet of *H. midae* with the potential probiotic *Pseudoalteromonas* sp. strain C4 with the aim of improving the growth rate of farmed abalone. An increase in growth rate may be achieved through a number of mechanisms: (1) increasing nutrients available to the abalone for absorption in the gastrointestinal tract, (2) increasing the pool of digestive enzymes in the digestive system and (3) use of added bacteria as an additional nutrient source.

The increase in alginate lyase activity in kelp supplemented with strain C4 resulted in an increase in alginate degradation products in the kelp feed. A diet consisting of kelp supplemented with strain C4 would therefore make more nutrients, specifically alginate degradation products, immediately available for absorption in the abalone gastrointestinal tract. Vitalis *et al* (1988) showed that incubation of gut bacteria from the sea hare with the alga *Ulva fasciata* increased the amount of several essential amino acids and some non–essential amino acids, indicating a possible nutritional contribution of the gut bacteria to their host. Pre-degradation of alginate may also provide a more easily digestible substrate for the abalone digestive enzymes thereby decreasing the amount of energy required for digestion and increasing energy available for growth. Forro (1987) suggested that partial digestion of polysaccharides like alginate may occur in kelp debris and abalone might find this decomposing material easier to digest and that the bacteria themselves could contribute protein supplies. Prim and Lawrence (1975) suggest that bacterial and fungal enzymes are probably more important in extra–organismal digestion of plant detritus, particularly of plant cell walls. This would remove the cell wall barrier to digestive enzyme action on plant cell contents and produce a bacterial population which itself would be susceptible to utilisation by the host.

An increase in the amount and diversity of digestive enzymes in the gastrointestinal tract may improve the nutritional status of abalone by improving the efficiency of digestion and thereby increasing the growth rate of abalone. An increase in the amount of enzymes available could increase the rate of digestion, while increasing the diversity of enzymes available may allow for more complete digestion of the nutrient source. The amount of alginate lyase activity in the gastrointestinal tract was compared between abalone fed a standard kelp diet and those fed a kelp diet supplemented with strain C4 to determine whether strain C4 contributes to the pool of digestive enzymes and is thereby able to improve the nutritional status of the abalone. Abalone fed kelp

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supplemented with strain C4 showed a significant increase in alginate lyase activity in the digestive tract after 3 days compared to abalone fed a standard kelp diet. This result suggests that strain C4 may contribute to the pool of polysaccharolytic enzymes available in the digestive tract for the digestion of complex algal polysaccharides that are ingested by abalone. Marine deposit-feeders have been shown to use between 5% and 30% of organic matter from sediments. However, the burrowing sea urchin, *Echinocardium cordatum*, increases the yield of ingested sediment by harbouring gut living fermenting microorganisms that digest food particles anterior to the gastrointestinal system (Thorsen, 1998). These microbial fermenters produce short–chained fatty acids including acetate and propionate that can be utilised by the host. Prim and Lawrence (1975) suggested that bacterial enzymes are important in digestion in the digestive tract of the regular echinoid *Lytechinus variegates* when food is retained in the gut for long periods of time. Bacteria in the foregut of the minke whale have been shown to digest fish and pelagic crustaceans (consisting mostly of proteins and lipids, but also polysaccharides) fermentatively, releasing volatile fatty acids (Olsen *et al.*, 1994b). Sawabe *et al.* (1995) reported that the gut bacteria of two sea urchins, *Strongylocentrotus intermedius* and *Strongylocentrotus nudus*, and the abalone *H. discus hannai* degrade alginate and speculated that they may therefore contribute to the nutritional requirements of their host. Alginolytic bacteria isolated from abalone were shown to preferentially degrade the polyG block of alginic acid whereas abalone alginate lyase preferentially degrades the polyM block. Sawabe *et al.* (1995) therefore speculated that alginate was first degraded by the abalone's digestive enzymes and then further degraded by intestinal bacteria. *Vibrio halioticoli* isolated from the abalone digestive tract potentially contributes to host nutrition in addition to supporting alginate degradation (Sawabe *et al.*, 1998, Sawabe *et al.*, 2003). Degradation of alginate by *V. halioticoli* produces acetic acid which has been shown to be absorbed by gut tissue and metabolised as an oxidative energy source, and used as a precursor for protein, sugar, and lipid synthesis. The increase in alginate lyase activity observed in the digestive tract of abalone fed a kelp diet supplemented with strain C4 may allow for partial digestion of kelp by bacterial enzymes and then further digestion of the kelp by abalone digestive enzymes. Alginate lyase activity in the oesophagus of abalone fed either a standard kelp diet or a kelp diet supplemented with strain C4 remained the same after both groups of abalone were subsequently fed a standard kelp diet. One day after the strain C4 supplemented diet was stopped, alginate lyase activity was greater in the intestine of abalone previously fed the supplemented diet compared to those fed the standard diet. Similar results were observed in the crop and stomach three days after the supplemented diet was stopped. These results suggest that strain C4 is present in the crop and stomach of *H. midae* up to 3 days after strain C4 has been introduced into the gastrointestinal tract and that strain C4 supplies additional enzymes, specifically alginate lyase, to aid digestion. The increased activity in the

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intestine at day 1 and in the crop and stomach at day 3 suggests that strain C4 is retained longer in the crop and stomach compared to the oesophagus and intestine. If strain C4 were simply moving through the gastrointestinal tract with the food as part of the normal digestion process it would be expected that alginate lyase activity would be observed for longer periods in the intestine compared to the crop and stomach. These results therefore suggest that strain C4 may, at best, be transiently present in the crop and stomach. However, the ability of strain C4 to colonise the digestive tract of *H. midae* needs to be studied in greater detail. The significant decrease in alginate lyase activity in both the crop and stomach 7 days after the strain C4 supplemented kelp diet was halted may be the result of environmental factors such as changes in water temperature or quality. It is interesting that most of the alginate lyase activity was detected in the crop and that increased activity was still observed at 3 days after the strain C4 supplemented diet was halted since strain C4 was originally isolated from the crop of *H. midae* (Erasmus *et al.*, 1997). Proteolytic enzyme activity in the crop-stomach content of *H. fulgens* was shown to occur between pH 6 and 8 (Seviere–Zaragoza *et al.*, 1997). However, proteoliytic enzyme activity peaks in *H. fulgens* (Seviere–Zaragoza *et al.*, 1997) occurred at different pHs depending on the region of the digestive tract being investigated. Activity in the hepatopancreas increased between pH 2 and 5 and crop-stomach content activity increased between pH 2 and 7. Protease activity in the intestinal and rectal fluids was detected between pH 7 and 11. The pH of the crop of *H. midae* is 5.6 (Erasmus *et al.*, 1997) and the optimum activity of alginate lyase also occurs at slightly acidic conditions (pH 6.5, Chapter 2).

Since *Ecklonia maxima* is a poor source of protein and does not provide all the essential amino acids required by the abalone for growth (Simpson, 1994), it was hypothesised that strain C4 may provide an additional source of protein which could improve the nutritional value of kelp feed and consequently improve the growth rate of farmed abalone. Abalone were inoculated with ^{14}C-labelled strain C4 and incorporation of the label into abalone tissue was monitored. Gnotobiotic abalone inoculated with ^{14}C–labelled strain C4 exhibited significantly greater amounts of protein incorporation in the gills, foot, adductor muscle, intestine and hepatopancreas compared to abalone that were inoculated with unlabelled strain C4. The majority of protein was incorporated in the hepatopancreas. Seviere Zaragoza *et al.* (1997) reported that protein is produced in the hepatopancreas, secreted into the digestive fluid and transported into the lumen of the gut where the proteins may be involved in enzymatic digestion of food. Therefore, protein found in the crop, stomach, intestine and rectal extracts are derived from food, tissue debris and secretions whereas protein found in the hapatopancreas is produced by the abalone. This may explain the large amount of protein incorporated in the abalone hepatopancreas. Shimizu *et al.* (2003) reported that alginate lyase is produced in the hepatopancreas of the abalone *Haliotis discus hannai* and the amount of

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soluble proteins have also been shown to be more abundant in the hepatopancreas of the abalone *H. fulgens* (Serviere–Zaragoza *et al.*, 1997) and the squid *Illex argentinus* (Picos–García *et al.*, 2000) compared to the crop-stomach, intestine and rectum. Muir *et al.* (1986) showed that when the mussel, *Choromytilus meridionalis*, was exposed to ^{14}C–labelled bacteria most of the label was incorporated in the digestive gland, stomach and gut tissue, with low amounts in the gonads and adductor muscle.

When abalone inoculated with labelled strain C4 were kept together with abalone inoculated with unlabelled strain C4 there was no difference in the amount of protein incorporated in any of the tissues analysed. Since only background counts were found in the water and a significant amount of counts were detected in the faeces, it was postulated that abalone inoculated with unlabelled strain C4 were able to incorporate labelled proteins that they ingested via the faeces. The greatest incorporation of radioactively labelled protein was detected in the faeces of abalone that had not been treated with antibiotics. This may be due to competition between bacteria resident in the digestive tract and the inoculated strain C4 for attachment sites in the abalone digestive tract. Fewer counts were detected in faeces from gnotobiotic abalone that were inoculated with labelled strain C4 and kept together with control abalone compared to ^{14}C-labelled strain C4 inoculated gnotobiotic abalone that were maintained separately. A possible reason for this could be that gnotobiotic abalone inoculated with unlabelled strain C4 ingested labelled bacteria excreted by abalone inoculated with ^{14}C-labelled strain C4 thereby decreasing the counts in the faeces. Counts detected in faeces from the mixed gnotobiotic abalone are less than those detected in the faeces of gnotobiotic abalone inoculated with unlabelled strain C4 suggesting that these are background counts and that all of the labelled protein is incorporated by abalone inoculated with either labelled or unlabelled strain C4 in the mixed tank. The amount of ^{14} C detected in the faeces of abalone inoculated with unlabelled strain C4 was higher compared to the counts detected in the water and this may indicate that the background level of ^{14}C naturally present in abalone is higher than the background level detected in sea water. Colonisation studies will determine whether label detected in the gills, foot, adductor muscle, stomach and intestine are due to the presence of strain C4 bacterial cells in these organs or whether the bacteria are digested and themselves used as a nutrient source. However, it is unlikely that bacteria orally ingested by abalone will travel through the digestive tract and then colonise the foot and adductor muscles and far more likely that proteins obtained from digested bacterial cells are used by abalone to build muscle tissue. Since the hepatopancreas is sterile (Erasmus, 1996) it can be assumed that the protein incorporated in the hepatopancreas is due to digestion of bacteria in the gut and translocation of bacterial proteins to the hepatopancreas. Herry *et al.* (1989) reported lysis of bacterial cells in the clam *Loripes lucinalis*,

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indicating that the clam not only benefits from sulphide detoxification and translocation of the carbohydrate product of bacterial metabolism, but also from direct digestion of bacterial cell material. Lysozyme–like enzymes in the style of *Choromytilus meridionalis* are capable of lysing approximately 57% of the free–living bacteria in the water column adjacent to kelp beds, which could meet the estimated nitrogen requirements of the mussels (Seiderer *et al.*, 1984). Lysozyme–like enzymes have also been found in *M. edulis, Modiolus modiolus, Chlamys opercularis* and *Mya arenaria* (McHenery *et al.*, 1979) and it has been suggested that these enzymes play a primary role in the utilisation of bacteria. McHenery *et al.* (1985 a&b) later reported the presence of lysozyme in about 30 different bivalve species and showed how bivalves processed radiolabelled material from bacteria.

Partial digestion of kelp feed, increased alginate lyase activity in the digestive tract and incorporation of strain C4 proteins in the abalone hepatopancreas suggests a possible role for strain C4 in the nutrition of *H. midae* and the consequent improvement of growth rates. This hypothesis was tested by comparing the growth rate of *H. midae* fed a standard kelp diet to that of animals fed a kelp diet supplemented with strain C4. Growth experiments indicated that addition of strain C4 to abalone food significantly increased the growth rate of the abalone both under laboratory and farm conditions. The extremely poor growth of gnotobiotic abalone confirms the importance of the presence of enteric bacteria with regard to abalone growth. Vitalis *et al.* (1988) showed that when antibiotics were added to the sea hare, *Aplysia juliana*, the growth rate was significantly reduced in animals maintained on three different diets. Weanery sized abalone previously fed kelp supplemented with strain C4 continued to exhibit a greater growth rate compared to abalone only fed kelp after abalone were moved to raceways for grow out. This result suggests that either strain C4 is able to colonise the abalone digestive system or that the effects of strain C4 are long lasting (up to 2 months post-treatment). However, colonisation studies will be necessary in order to draw any concrete conclusions from these results. Extrapolation of the increased growth rate over the entire grow-out phase (4–5 years) suggests a reduction of 17 months in the time required for abalone to reach market size. This would mean greater productivity and reduced costs. However, the ability of strain C4 to increase the growth rate of farmed abalone has only been established for weanery (pre-grow out) sized abalone and growth rate experiments will need to be performed on larger abalone to determine the effect of strain C4 during the grow out phase.

Most research on improving the growth rate of farmed abalone has focused on the development of artificial diets. These results indicate that addition of strain C4 to a kelp diet can significantly increase the growth rate of farmed abalone. Based on the results of this study it is probable that

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strain C4 improves the growth rate of abalone by increasing the amount of alginate lyase, and possibly other extracellular enzymes such as proteases, cellulases and gelatinases (Erasmus, 1996; Chapter 2), required for the digestion of kelp and thereby making more nutrients readily available for absorption by the abalone. Addition of strain C4 to the feed also immediately increases the amount of nutrients available to the abalone for absorption since kelp alginate is pre-degraded by the presence of strain C4 in the feed. In addition to providing additional enzymes and making nutrients more readily available, strain C4 is itself used as a protein source by the abalone.

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CHAPTER 4

PSEUDOALTEROMONAS SP. STRAIN C4 STIMULATION OF THE IMMUNE SYSTEM AND INCREASED RESISTANCE OF *H. MIDAE* TO PATHOGEN INFECTION

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4.5 DISCUSSION

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4.1 SUMMARY

The potential of *Pseudoalteromonas* sp. strain C4 to stimulate the immune system of the abalone, *H. midae* and improve resistance to bacterial infection was evaluated. Strain C4 was shown to inhibit the *in vitro* growth of the pathogens *V. alginolyticus* and *V. anguillarum* by 42.7% and 32.9% respectively. In addition, strain C4 decreased the *in vitro* ability of *V. anguillarum* to adhere to mucus isolated from the oesophagus of abalone. However, strain C4 had no effect on the adhesion capabilities of *V. alginolyticus* to mucus isolated from the oesophagus, intestine or stomach of abalone.

The ability of strain C4 to stimulate the immune system of *H. midae* was evaluated by measuring percentage phagocytosis and extracellular superoxide anion production by haemocytes. Abalone fed kelp supplemented with strain C4 showed an increase in the number of phagocytic haemocytes and the production of extracellular superoxide anions compared to a control group of abalone fed a standard kelp diet. When challenged with the abalone pathogen *V. anguillarum,* the number of *V. anguillarum* cells isolated from infected abalone haemolymph was lower in abalone fed a strain C4 supplemented kelp diet compared to those fed a standard kelp diet. Abalone fed a standard diet showed higher mortality rates and more pronounced disease symptoms compared to those fed a strain C4 supplemented diet.

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4.2 INTRODUCTION

Invertebrates do not possess an inducible immune response with the high degree of specificity and memory that is observed in the vertebrate immune response (Pipe *et al.*, 1997) since they lack immunoglobulin, T-cell receptors and major histocompatibility complex high diversity molecules (Arala-Chaves and Sequeria, 2000). They are however capable of cellular recognition and cytotoxic reactions against foreign cells. Mollusc defence mechanisms involve a humoral response that comprises antibacterial activity, agglutination and clotting factors (Lacoste *et al.*, 2002) produced by the haemocytes (Bachère *et al.*, 1995, Roch, 1999) and cellular responses such as phagocytosis and oxidative killing through the production of superoxide anions by haemocytes (Rengpipat *et al.*, 2000). Upon infection by a pathogen, the host animal initiates an immune response in an attempt to eliminate or segregate the pathogen. Haemocyte migration is stimulated upon contact with a pathogen and is followed by phagocytosis and intracellular degradation of the pathogen by means of lytic enzymes or the production of reactive oxygen intermediates (Coles *et al.*, 1995, Roch, 1999). Phagocytic haemocytes are the primary, non–specific defence mechanism in invertebrates against invasion of pathogenic organisms. The generation of reactive oxygen species by haemocytes of molluscs has been widely demonstrated suggesting that this may represent a generalised phenomenon among molluscs. Reactive oxygen species production has been reported in four snail species, *Biomphalaria glabrata, Lymnaea stagnalis, Planorbarius corneus,* and *Helix aspersa* (Dikkeboom *et al.*, 1988), the bivalves *Patinopecten yessoensis* (Nakamura *et al.*, 1985), *Mytilus edulis* (Pipe, 1992) and *Crassostrea gigas* (Lacoste *et al.*, 2002) and in the abalone, *Haliotis tuberculata* (Malham *et al.*, 2003), *H. rufescans* and *H. cracherodii* (Martello and Tjeerdema, 2001). The production of reactive oxygen species was however not observed in the clam *Mercenaria mercenaria* (Cheng, 1976).

Disease is an increasingly important factor that impacts on the economic success of abalone aquaculture (Mialhe *et al.*, 1995a & b). Disease can be caused by a number of factors including pathogenic bacteria, viruses, toxic algal blooms, environmental pollution and stress as a result of sorting, grading and transportation associated with the aquaculture process (Bachère *et al.*, 1995, Mialhe *et al.*, 1995b, Lacoste *et al.*, 2002, Malham *et al.*, 2003). The susceptibility of abalone to disease depends in part on the general immunostatus of individual animals. The ability to mount a quick, effective defence response upon infection reduces the infectivity of pathogenic organisms. Susceptibility to pathogen infection is increased when the host immune system is suppressed which raises the possibility of improving resistance by stimulating the innate immune response (Miles *et al.*, 2001). Substances containing alginate, lysophospholipids, which act as biosurfactants to

improve feed conversion in mammal and poultry farming, and vitamins C, E and trace quantities of the B vitamins have been shown to stimulate the immune response in the striped snakehead, *Channa striata* (Miles *et al.*, 2001). Immunostimulated fish had reduced mortalities and showed higher anti-*Aphanomyces invadans* antibody concentrations after infection with *A. invadans* even though the incidence of infection was not reduced. β-glucan has been shown to stimulate the production of superoxide anions by *Penaeus monodon* haemocytes (Song and Hsieh, 1994) and the fungicide propiconazole induced an increase in respiratory burst in *Panaeus vannamei* haemocytes between 1 and 6 days of exposure to the fungicide. Brewer's yeast and its derivative products such as cell walls have been shown to have immunostimulating properties when orally administered to fish and shrimp with their feed (Suphantharika *et al.*, 2003). Brewer's yeast ß-glucan preparations added to the shrimp diet resulted in a significant increase in phenoloxidase activity in *P. monodon* haemocytes both *in vitro* and *in vivo*. Research by Rengpipat *et al.* (2000) has shown that boosting the immune system of *P. monodon* increases the animal's ability to respond more rapidly to pathogenic infection. Thus, by boosting the immune system of farmed abalone, it may be possible to improve their general health and ability to resist disease thereby reducing economic losses caused by mortalities.

The use of probiotics and other immunostimulants for controlling disease in aquaculture is more desirable and environmentally friendly compared to the use of antibiotics and chemicals. The use of chemotherapeutic agents has led to the occurrence of resistant bacteria in fish (Nikoskelainen *et al.*, 2001). Probiotics may provide an alternative way to reduce the use of antibiotics in aquaculture and simultaneously avoid the development of antibiotic–resistant bacteria. Interaction with mucus is the first step in bacterial adhesion to the intestinal mucosa and other mucosal surfaces (Kirjavainen *et al.*, 1998). This may lead to competitive exclusion of pathogens (Jory, 1998) by probiotic microorganisms blocking adhesion receptors, competition for nutrients, and production of antimicrobial substances (Gibson *et al.*, 1998, Skjermo and Vadstein, 1999, Chythanya *et al.*, 2002). The result may be blockage of ports of entry for pathogens. Other mechanisms by which probiotics may affect pathogens include binding of bacterial toxins, modulation of the immune system, and stabilisation of a normal gut microflora.

Many methods of testing immunostimulants have been reported (Miles *et al.*, 2001). Potential immunostimulants may be injected or added to feed, and the results may be evaluated by examination of immune parameters or challenge with a pathogen. Immune parameters such as number of circulating haemocytes, phagocytosis rates and reactive oxygen species production are often used as tools to investigate the immunostatus of cultured mollusc stocks (Oubella *et al.*, 1993,

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Coles *et al.*, 1995, Martello *et al.*, 2000, Ordas *et al.*, 2000, Lacoste *et al.*, 2002, Malham *et al.*, 2003, Cheng *et al.*, 2004a,b & c). Phagocytic haemocytes have also been used to determine shrimp health and for evaluation of crustacean cellular immune responses (Rengpipat *et al.*, 2000).

The objectives of this study were to investigate whether *Pseudoalteromonas* sp. strain C4 could stimulate *H. midae* immunity by stimulating the number of circulating haemocytes, phagocytic activity and the production of superoxide anions and to determine whether the addition of strain C4 to abalone diets increased resistance of *H. midae* to pathogen infection and possibly establish how an increase in resistance was achieved.

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4.3 MATERIALS AND METHODS

4.3.1 *In vitro* growth inhibition of bacterial pathogens of *H. midae* by strain C4

Three approaches were employed to determine whether strain C4 was able to inhibit the growth of pathogenic bacteria *in vitro*. Two methods involved growth on solid media and a third method investigated inhibition in liquid media. The abalone pathogens *Vibrio alginolyticus* strain 7157 and *Vibrio anguillarum* strain 1989 were isolated from diseased abalone at Onderstepoort Veterinary Research Centre by Dr A. Mouton and were maintained on MA solid media at 22°C.

Pseudoalteromonas sp. strain C4 and the two pathogenic bacteria, *Vibrio alginolyticus* and *Vibrio anguillarum,* were cultured overnight in 10 ml of MB (Appendix A1.3) at 22°C with constant agitation at 120 rpm. Cultures were centrifuged at 14 300 x g and harvested cells washed twice in PBS (Appendix A2.10). The cells were resuspended in 1 ml of fresh MB. The culture supernatant of strain C4 was filter sterilised using a 0.22 μm Millipore filter. One hundred microlitres of each pathogen was spread plated on MA (Appendix A1.4) solid media in triplicate and the Petri dishes were allowed to dry. Three wells were made in the MA solid media. Fifty microlitres of sterile MB, filter sterilised supernatant and strain C4 cell suspension was added to each of the wells. The Petri dishes were incubated for three days at 22°C after which they were examined for zones of inhibition.

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Strain C4 was streaked across MA solid media and each of the three pathogens were streaked perpendicular to strain C4. The Petri dishes were incubated for three days at 22°C and scored for zones of inhibition.

Strain C4 and each of the three pathogens were cultured for two days in 5 ml MB at 22°C with agitation at 120 rpm. The cultures were centrifuged for 5 min at 14 300 x g to concentrate the cells, washed twice with PBS and then resuspended in PBS to an optical density of 0.5 at 600 nm. One hundred microlitres of a pathogenic strain and strain C4 were added to 5 ml of MB and incubated for three days at 22°C with constant shaking at 120 rpm. One hundred microlitres of each pathogen and 100 μl of PBS lacking strain C4 were added separately to MB as experimental controls. Each of the cultures were diluted in SSS (Appendix A2.12) and a 100 μl aliquot of each was plated onto TCBS solid media (Appendix A1.6). The Petri dishes were incubated for two days at 22°C after

which the number of bacteria were determined. Percentage inhibition was calculated as follows: (cfu/ml in the presence of strain C4 / cfu/ml in the presence of PBS) x 100.

4.3.2 Exclusion of bacterial pathogens of abalone by strain C4

Twelve abalone, weighing approximately 100 g each, were dissected (Appendix C3) and mucus extracted from the oesophagus, intestine and stomach. Each of the dissected digestive tract sections were placed in a sterile Petri dish and the mucus gently scraped off the membranes using a scalpel. Mucus samples from four abalone were pooled. Five millilitres of HH buffer, pH 7.4 (Appendix A2.2) was added to each mucus sample. The samples were centrifuged for 15 min at 12 000 x g at 4°C and then for 15 min at 27 000 x g at 4°C. The supernatant fractions were collected and the concentration of protein in each fraction was determined using the Bradford method (Appendix B6). Samples were diluted to a protein concentration of 0.5 mg/ml in HH buffer, pH 7.4.

Overnight cultures of *Vibrio alginolyticus* and *Vibrio anguillarum* were used to inoculate 10 ml MB. Two hundred microlitres of ^{3}H thymidine (20 µl/ml (final concentration); 70 – 86 Ci/mmol) were added to the cultures and incubated overnight at 22°C with constant shaking. Cultures were centrifuged for 10 min at 7 800 x g at 4°C, washed twice with PBS and resuspended in 10 ml PBS. The optical density was determined at 600 nm and adjusted to 0.25. One hundred microlitres of the adjusted culture was added to 2 ml of scintillation fluid and the DPM/100 µl was determined in a Beckman scintillation counter. *Pseudolateromonas* sp. strain C4 was inoculated into 100 ml of MB and incubated with constant shaking overnight at 22°C. The culture was centrifuged for 10 min at 11 200 x g at 4°C, washed twice with PBS and resuspended in 100 ml of PBS. The optical density was determined at 600 nm and adjusted to 0.25.

One hundred microlitres of each mucus sample was added to microtitre wells (Nunc Maxisorp) and incubated for 15–20 h at 4°C. Wells were washed twice with 200 µl of HH buffer, pH 7.4 and 100 µl of strain C4 (A_{600} = 0.25) was added to each well. Microtitre plates were incubated for 1 h at 22°C to allow strain C4 to adhere to the mucus and washed twice with HH buffer, pH 7.4. One hundred microlitres of ^{3}H thymidine labelled *V. alginolyticus* (126156.4 DPM) and *V. anguillarum* (119191.9 DPM) was added to each well and incubated for 1 h at 22°C. The wells were washed twice with 200 µl HH buffer. Two hundred and fifty microlitres of 1% SDS/0.1 M NaOH (Appendix A2.3) was added to each well and incubated for 1 h at 60°C. The cell lysate was removed and added to 2 ml of scintillation fluid. The DPM was determined using a Beckman

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scintillation counter and the percentage of pathogenic bacterial cells that adhered to the mucus samples was calculated as follows: (DPM cell lysate/initial DPM added) x 100.

4.3.3 Haemolymph collection and determination of haemocyte concentration

Five hundred microlitres of haemolymph was extracted from the pedal sinus (Appendix C2) of four abalone using 2 ml syringes and 26G x ½" needles. The extracted haemolymph was pooled and 100 µl of each pooled haemolymph sample was fixed with 200 µl of Alsevers solution (Appendix A2.22.1). Each sample was fixed in duplicate. The number of haemocytes was counted using a haemocytometer and each sample was counted twice. For the measurement of immune parameters, the haemocyte concentration was adjusted to 10^6 cells/ml with modified HBSS containing 3 µg EGTA/100 ml buffer (Appendix A2.22.4).

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4.3.4 Phagocytosis Assay

Vibrio anguillarum was cultured overnight in 100 ml MB at 22°C. Cells were fixed in 10% formaldehyde (Appendix A2.22.3) and then centrifuged for 10 min at 11 200 x g (Malham *et al.*, 2003). Cells were washed twice using PBS and resuspended in 10 ml 0.1 M $NaHCO_3$, pH 9.0 (Appendix A2.22.2) containing 0.1 mg/ml fluorescein 5–isothiocyanate isomer 1 (FITC, Sigma). Cells were incubated in the dark for 1 h at 25°C, centrifuged for 10 min at 7 800 x g and resuspended in 10 ml PBS. The concentration of bacteria was adjusted to 1 x 10^8 bacteria/ml by counting the bacterial cells using a haemocytometer and diluting the bacterial cells in PBS. Cells were stored at –20°C.

Glass microscope slides were immersed in 50% acetic acid (Appendix A2.22.5) for 24 h, rinsed with tap water and then allowed to dry. A 1 x 1 cm square was made with silicon at one end of the slide prior to use. One hundred microlitres of haemolymph containing 10^6 haemocytes/ml in modified HBSS was placed inside the silicon square on the glass slide. Slides were incubated in a dark, moist chamber for 20 min. At the end of the incubation period 100 µl of FITC labelled *V. anguillarum* was added to the haemocytes, giving a ratio of 100:1 bacteria:haemocyte. Slides were incubated for a further 30 min after which they were rinsed with modified HBSS buffer (Appendix A2.22.4) and then covered with 100 µl of ethidium bromide (10 mg ethidium bromide/ml PBS, Appendix A2.22.6). The ethidium bromide was removed after 1 min at 22°C. The slides were rinsed with modified HBSS, the remaining liquid was carefully removed and a cover slip was placed over the slide. The number of phagocytic haemocytes was determined using a 488 nm

emission filter on a Zeiss microscope. The percentage of phagocytosing haemocytes was determined from a total of 200 haemocytes (% phagocytosis = (phagocytosing haemocytes/total haemocytes counted) x 100). Each sample was assayed in duplicate and each duplicate was counted twice.

4.3.5 Measurement of intracellular superoxide anion production by *H. midae* haemocytes

Two hundred microlitres of haemolymph (1 x 10^6 haemocytes/ml) was added to 200 μl nitroblue tetrazodium (NBT, Sigma) (Appendix A2.22.7) in triplicate, and incubated in the dark (Malham *et al.*, 2003) for 4 h. Controls consisted of haemocytes and superoxide dismutase (SOD, Sigma) at 300 U/ml, and NBT-SOD with and without haemocytes. Samples were centrifuged for 10 min at 120 x g, the supernatant discarded and pellet resuspended in 200 μl modified HBSS. Pellets were washed twice in modified HBSS and then fixed for 10 min at 22°C using 200 μl 100 % methanol (Saarchem). Samples were centrifuged for 10 min at 300 x g, the supernatant was decanted and the cells were air dried. The cells were rinsed three times in 50% methanol (Appendix A2.22.9) prior to the addition of 240 μl of 2 M KOH (Appendix A2.22.8) and 280 μl of dimethylsulphoxide (DMSO, Sigma). Each sample was vortexed, the supernatant removed and the optical density of the supernatant was determined at 620 nm. The presence of basal levels of intracellular superoxide anions in abalone haemolymph was expressed as OD values/10^6 haemocytes/ml. Since the production of intracellular superoxide anions is activated by phagocytosis, intracellular superoxide anion production was also measured in the presence of a stimulant. Yeast strain AY1, previously isolated from the *H. midae* digestive tract (T. Andlid, per. comm.), was cultured for 16 h in 100 ml of YPD liquid media (Appendix A1.19) at 22°C with agitation at 120 rpm. Cells were harvested by centrifugation for 10 min at 11 200 x g and resuspended in PBS (Appendix A2.10) and the optical density was adjusted to 1.0 at 600 nm. Cells were disrupted by sonication at 4°C at 95 W for 5 min with 20 s cooling intervals (VirSonic Digital 475 Cell Disruptor) and cell debris was harvested by centrifugation for 10 min at 7 800 x g. The cell debris was resuspended in an equal volume of PBS. Two hundred microlitres of the sonicated yeast cell suspension was added to 200 μl haemolymph (1 x 10^6 haemocytes/ml) and 200 μl of NBT and the assay was performed as described above.

4.3.6 Effect of strain C4 on the immunostatus of *H. midae*

Thirty abalone were placed into each of four tanks with a volume of 98 L sea water and a flow rate of 5.5 L/min. Abalone were allowed to acclimatise for two weeks during which all abalone were

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fed kelp cakes (Appendix A1.5). After the acclimatisation period (t = 0) abalone in two of the tanks were fed kelp cakes supplemented with strain C4 (Appendix A1.5), while the abalone in the remaining two tanks continued to be fed kelp cakes. At t = 0, 0.25,1, 2, 4, 7 and 14 days, blood was extracted and pooled from four abalone in each tank as described in section 4.3.3. The number of haemocytes, percentage phagocytosis and internal respiratory burst was determined as described in sections 4.3.3 – 4.3.5.

4.3.7 Effect of strain C4 on *H. midae* infected with *V. anguillarum*

Thirty abalone were placed into each of six baskets (Figure 1). Two baskets were placed into each of three tanks with a volume of 98 L sea water with a flow rate of 5.5 L/min. Abalone in four baskets were fed kelp cakes for two weeks, while abalone in the other two baskets were fed kelp cakes supplemented with strain C4. After two weeks, the standard kelp diet was changed to a strain C4 supplemented diet in two of the baskets. At this point (t = 0) half the abalone from each of the feeding strategies were injected with 200 µl of *Vibrio anguillarum* at a cell titre of 1 x 10^{10} cfu/ml between the anterior left lobe of the mantle and the dorsal surface of the foot (Appendix C1). The remaining animals were injected with 200 µl synthetic sea salts (SSS, Appendix A2.12) and served as a control. At t = 0, 1, 2, 4, 7 and 10 days post infection 500 µl of haemolymph was extracted from the pedal sinus of four abalone from each basket and pooled. Haemocytes were counted, percentage phagocytosis and number of bacteria per 10^6 haemocytes was determined as described in sections 4.3.3-4.3.5. Disease symptoms and accumulative mortalities were also recorded.

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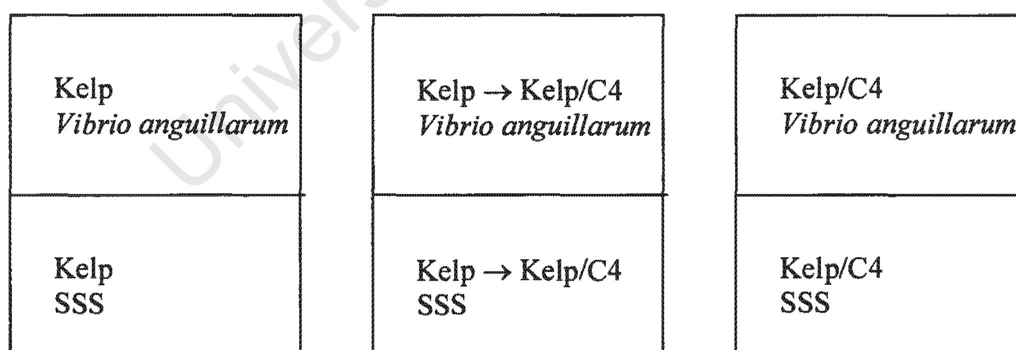

Figure 1. Schematic diagram of experimental set up of tanks and treatments used for investigating the effect of strain C4 on *H. midae* infected with *Vibrio anguillarum*. Kelp and Kelp/C4 indicate the feeding strategy of abalone in that basket. *Vibrio anguillarum* and SSS (synthetic sea salts) refer to the media with which the abalone were injected.

4.3.8 Statistical Analysis

All data are presented as the means of at least two experiments and standard errors. For comparison of two means, paired or unpaired Student's t–tests were used. Two-factor analysis of variance (ANOVA), followed by a multiple comparison test (Tukey) was used to determine the significant differences among immune parameters due to time and type of feed variables. To meet the normality assumption of ANOVA, some data were $\log_{10}$ transformed. The limit of significance was $p<0.05$ unless otherwise stated.

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4.4 RESULTS

4.4.1 *In vitro* growth inhibition of bacterial pathogens of abalone by strain C4

The ability of strain C4 to inhibit the growth of *V. alginolyticus and V. anguillarum* was tested by inoculating strain C4 and one of each pathogen on MA solid media. After 3 days incubation at 22°C no zones of inhibition were observed. Growth inhibition of pathogens was also tested by incubating each of the pathogens on MA solid media in the presence of strain C4 in liquid culture and the strain C4 culture supernatant. Neither strain C4 nor the spent culture medium inhibited pathogen growth. The ability of strain C4 to inhibit the growth of *V. alginolyticus* and *V. anguillarum* was determined by co-culture in liquid media. During co–culture in MB strain C4 significantly (Student's t-test, $p < 0.05$) inhibited the growth of *V. alginolyticus* by 42.7% and *V. anguillarum* by 32.9% (Table 1).

Table 1. Growth inhibtion of *V. alginolyticus* and *V. anguillarum* when co–cultured with strain C4 in marine broth. Standard error was determined from two experiments.

	Culturable bacteria (cfu/ml ± SE)		Inhibition (%)
	+ strain C4	+ PBS	
V. alginolyticus	$2.39 \times 10^9 \pm 2.31 \times 10^8$	$4.17 \times 10^9 \pm 6.71 \times 10^8$	42.7
V. anguillarum	$3.02 \times 10^9 \pm 2.57 \times 10^8$	$4.50 \times 10^9 \pm 3.61 \times 10^8$	32.9

4.4.2 *In vitro* exclusion of bacterial pathogens of abalone by strain C4

The effect of strain C4 on the capability of bacterial pathogens to adhere to mucus isolated from the gastrointestinal (GI) tract of abalone was investigated. Strain C4 had no effect on the ability of the pathogen *V. alginolyticus* to adhere to any of the mucus samples obtained from different regions of the digestive tract (Figure 2A). Strain C4 significantly (Student's t-test, $p<0.005$) reduced the ability of the pathogen *V. anguillarum* to adhere to *H. midae* oesophageal mucus (Figure 2B). The ability of *V. anguillarum* to adhere to mucus isolated from the oesophagus was reduced from 0.2% in the absence of strain C4 to 0.1% in the presence of strain C4. The adhesion ability of *V. anguillarum* to stomach or intestinal mucus was not effected by the presence of strain C4.

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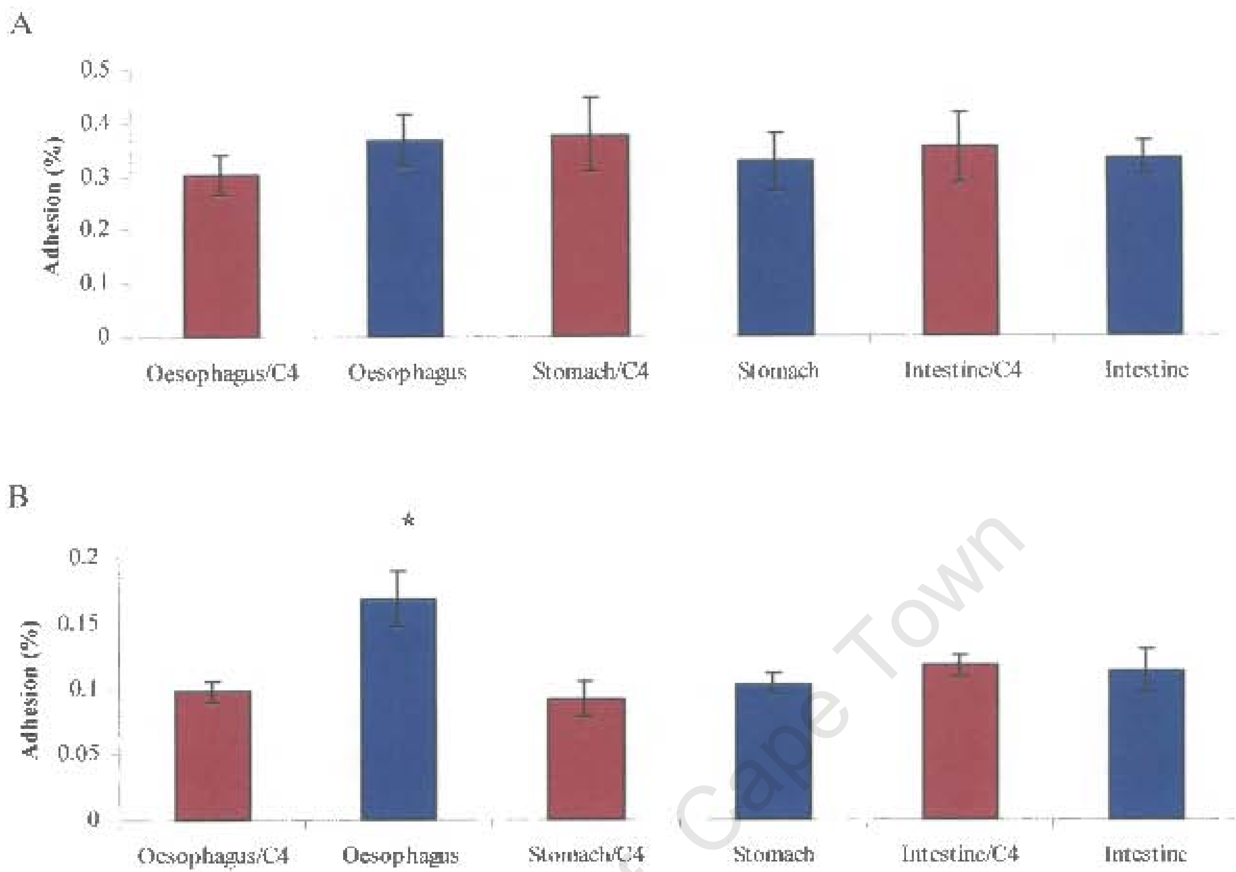

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Figure 2. Adhesion of the abalone pathogens (A) *V. alginolyticus* and (B) *V. anguillarum* to mucus isolated from different regions of the abalone digestive tract in the presence (■) and absence (■) of strain C4. Bars represent the average percentage adhesion and error bars depict standard error, determined from three experiments. Asterisk (*) indicates significant difference ($p<0.05$) compared to control.

4.4.3 The effect of strain C4 on the immunostatus of *H. midae*

The effect of the addition of strain C4 to abalone feed on the immunostatus of the abalone was determined by measuring a number of immune parameters.

The supplementation of kelp feed with strain C4 had no significant (Two-way ANOVA, Appendix D) effect on the number of circulating haemocytes (Figure 3A) or the intracellular superoxide anion production (Figure 3C) compared to abalone fed a standard kelp diet. There was no significant

change in either of these parameters over the 14 day period in either abalone fed a standard kelp diet or abalone fed a kelp diet supplemented with strain C4.

The percentage of phagocytic haemocytes increased significantly (Two-way ANOVA, Appendix D, Tukey's test $p<0.05$) after day 1 in abalone fed a kelp diet supplemented with strain C4 (Figure 3B). This significant difference was observed between haemocytes from abalone fed either a kelp diet or a kelp diet supplemented with strain C4 at all time points tested for the remainder of the experimental period. There was no significant change in the phagocytic activity of haemocytes from abalone fed a standard kelp diet over the 14 day period. The initial increase in phagocytic haemocytes observed in abalone fed a kelp diet supplemented with strain C4 remained at an elevated level for the remainder of the experimental period.

Since the production of superoxide anion is stimulated by phagocytosis, sonicated AY1 yeast cells were used as a stimulant in determining the production of superoxide anions from the haemocytes. When feeding of the different diets was initiated, intracellular superoxide anion production was significantly greater in the group of abalone to be fed the unsupplemented kelp diet (Day 0, Figure 3D). However, this is a reflection of the initial immune status of the abalone and not a reflection of the effects of the different feeding strategies. *H. midae* fed a kelp diet supplemented with strain C4 showed an increased ability to produced superoxide anion at day 0.25, day 1 and day 4 compared to *H. midae* fed a standard kelp diet (Figure 3D). At day 2 and day 7 there was no significant difference between the two groups of abalone. Although the production of superoxide anions varied greatly over the 14 day period, increases and decreases from one sampling point to the next followed the same trend in abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4 and may be the result of environmental factors. In both groups of abalone there was an initial decrease in intracellular superoxide anion production after 6 hours followed by an increase at day 1. Superoxide anion production continued to increase in abalone fed a standard kelp diet at day 2 whereas there was no change in abalone fed a kelp diet supplemented with strain C4. At day 4 there was another dramatic decrease in superoxide anion production, followed by an increase at day 7 in abalone fed a standard kelp diet and a further increase in both groups of abalone at day 14. At the end of the experimental period the production of superoxide anions was significantly greater in abalone fed a standard kelp diet compared to abalone fed a kelp diet supplemented with strain C4.

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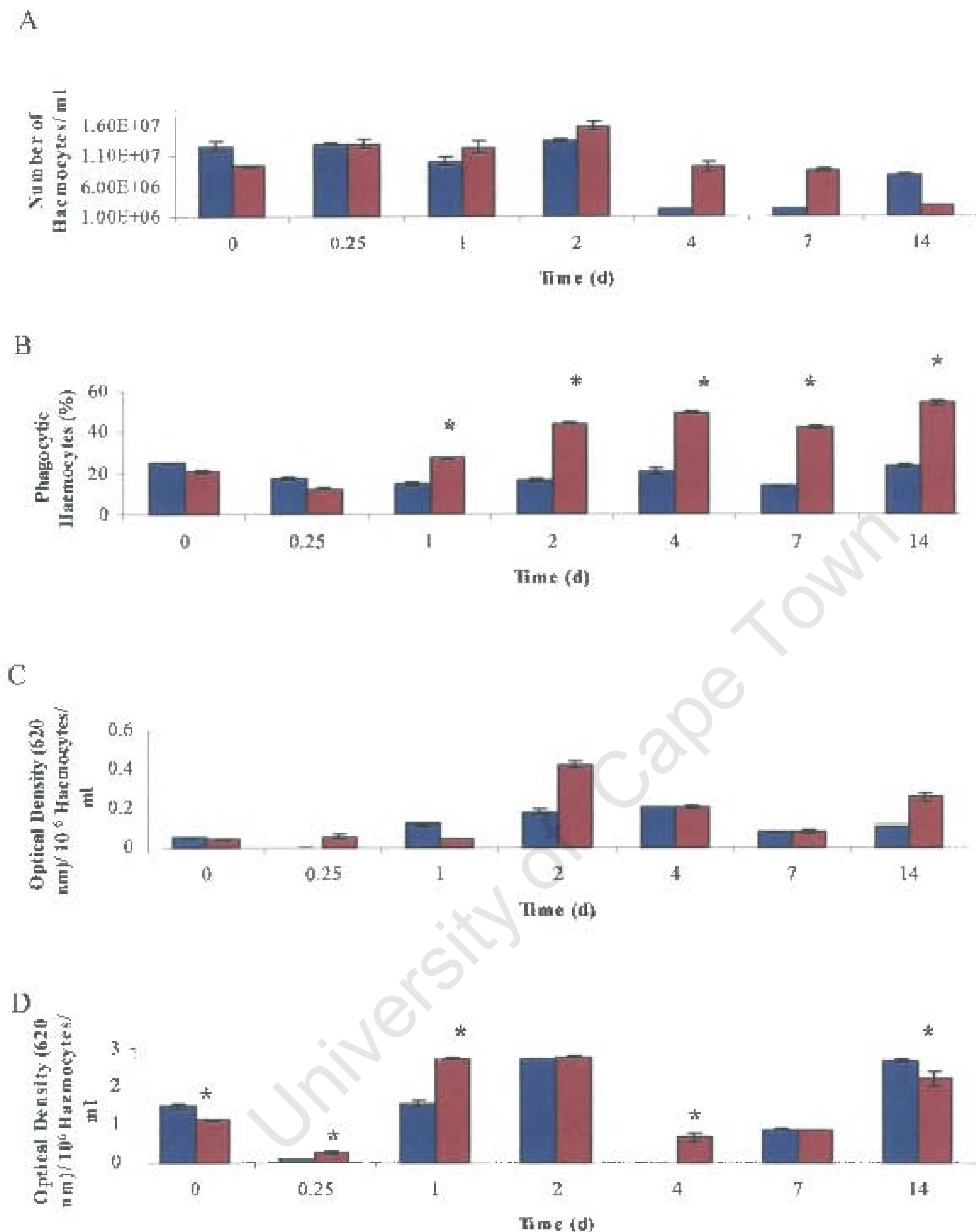

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Figure 3. Effect of strain C4 on the immune parameters of kelp-fed (■) abalone compared to abalone fed kelp supplemented with strain C4 (■). Bars represent (A) Number of haemocytes; (B) percentage phagocytic haemocytes; (C) intracellular superoxide anion production and (D) intracellular superoxide production in the presence of a stimulant. Error bars depict standard errors, determined from four experiments. Asterisk (*) indicates a significant ($p<0.05$) difference between abalone fed a kelp diet supplemented with strain C4 and standard kelp diet at that sample point.

4.4.4 The response of *H. midae* fed strain C4 supplemented kelp to *V. anguillarum* infection

The effect of a strain C4 supplemented kelp diet on the ability of *H. midae* to respond to infection by the abalone bacterial pathogen, *V. anguillarum*, was evaluated by measuring the response of various immune parameters and recording accumulative mortalities. *Vibrio anguillarum* was selected as a test pathogen because of strain C4's *in vitro* capability to reduce the adherence of *V. anguillarum* to mucus isolated from the oesophagus of *H. midae* and strain C4's ability to inhibit the growth of *V. anguillarum* during co-culture in liquid medium.

At day 1 post *V. anguillarum* infection a significant decrease was observed in all immune parameters tested in both abalone fed a kelp diet supplemented with strain C4 and abalone fed a standard diet. At day 2 post *V. anguillarum* infection abalone fed a kelp diet supplemented with strain C4 had significantly (Two-way ANOVA, Appendix D, Tukey test $p<0.05$) higher numbers of haemocytes compared to those fed a standard kelp diet (Figure 4A). At 2 days post *V. anguillarum* infection there was a significant decrease in the number of haemocytes in abalone fed a standard kelp diet. The number of haemocytes was unchanged in abalone fed a kelp diet supplemented with strain C4 until 7 days post infection. At 10 days post infection there was a significant increase in the number of haemocytes in both groups of abalone.

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The percentage of phagocytic haemocytes was significantly (Two-way ANOVA, Appendix D, Tukey test, $p<0.05$) greater in abalone fed a kelp diet supplemented with strain C4 at day 1 compared to abalone fed a standard kelp diet (Figure 4B). At day 2 however, the percentage of phagocytic haemocytes in abalone fed a standard kelp diet increased to levels equal to that of abalone fed a kelp diet supplemented with strain C4 and no difference was observed between the two groups for the remainder of the experimental period. There was no change in the percentage of phagocytic haemocytes during the 10 days post infection in abalone fed a kelp diet supplemented with strain C4.

Significant differences were observed in the production of superoxide anions between abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4 (Figure 4C). At day 1 and day 7 post infection superoxide anion production was significantly (Two-way ANOVA, Appendix D, Tukeys test, $p<0.05$) greater in abalone fed a kelp diet supplemented with strain C4. At days 2, 4 and 10 the production of superoxide anion was greater in abalone fed a standard kelp

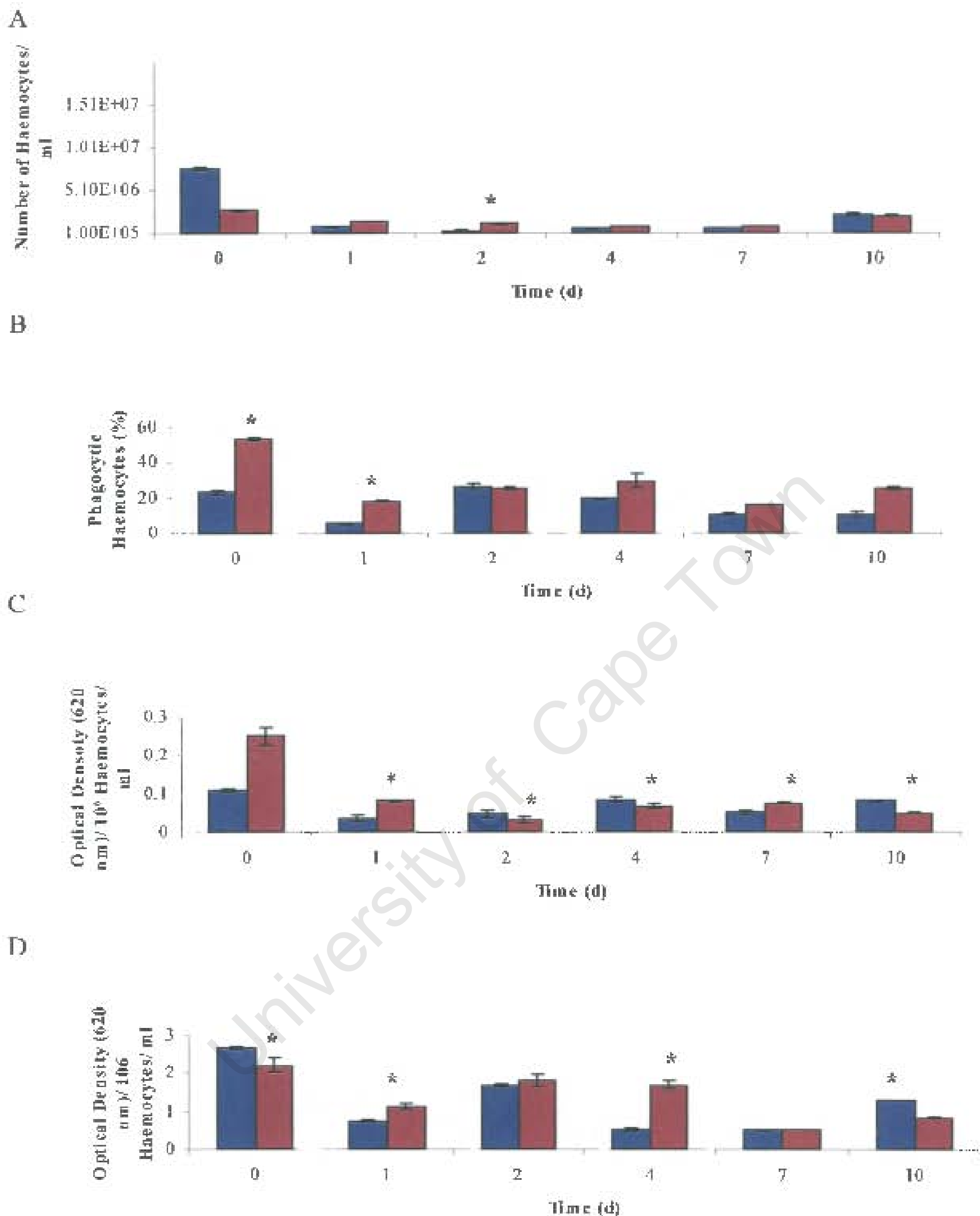

Figure 4. Comparison of abalone fed a standard kelp diet (■) and abalone fed a kelp diet supplemented with strain C4 (■) challenged with the pathogen *V. anguillarum*. Bars represent (A) number of haemocytes, (B) percentage of phagocytic haemocytes, (C) intracellular superoxide production and (D) intracellular superoxide production in the presence of stimulant. Error bars depict standard errors determined from four experiments. Asterisk (*) indicates a significant (p<0.05) difference between a kelp diet supplemented with strain C4 and standard kelp diet at that sample point.

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diet compared to abalone fed a kelp diet supplemented with strain C4. In abalone fed a kelp diet supplemented with strain C4 there was a decrease in superoxide anion production at 2 days post infection, followed by an increase at 4 days post infection. There was no change at day 7, there was however a decrease in superoxide anion production at 10 days post infection in abalone fed a kelp diet supplemented with strain C4. There was no significant change in superoxide anion production in abalone fed a standard kelp diet until 4 days post infection, at which point production increased. This increase was followed by a decrease at 7 days post infection followed by another increase at 10 days post infection.

The addition of stimulant indicated that abalone fed a kelp diet supplemented with strain C4 had greater potential to produce superoxide anions at day 1 and day 4 post infection. At day 1 post infection, superoxide anion production was significantly (Two-way ANOVA, Appendix D, Tukey test, $p<0.05$) greater in abalone fed a kelp diet supplemented with strain C4. There was no difference in stimulated superoxide anion production in abalone fed a standard kelp diet or abalone fed a kelp diet supplemented with strain C4 at 2 and 7 days post infection. At 2 days post infection there was an increase in the production of superoxide anion production in both groups of abalone. Production remained unchanged in abalone fed a kelp diet supplemented with strain C4 but decreased in abalone fed a standard kelp diet at 4 days post infection. At 7 days post infection there was a dramatic decrease in superoxide anion production in abalone fed a kelp diet supplemented with strain C4 and no change was observed in abalone fed a standard kelp diet. At 10 days post infection there was a significant increase in superoxide anion production in both groups of abalone, however production was greater in abalone fed a standard kelp diet.

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Haemolymph isolated from abalone fed a standard kelp diet and infected with *V. anguillarum* contained significantly (Student's t-test, $p<0.05$) higher numbers of *V. anguillarum* at days 1, 2 and 4 post infection compared to abalone fed a kelp diet supplemented with strain C4 (Figure 5). The highest number of *V. anguillarum* in the haemolymph was observed at 1 day post infection in both groups of abalone, however abalone fed a standard kelp diet had significantly higher numbers of *V. anguillarum* (124 cfu/10^6 haemocytes) compared to abalone fed a kelp diet supplemented with strain C4 (53 cfu/10^6 haemocytes). At day 7 there was no significant difference between the numbers of *V. anguillarum* in the haemolymph from abalone fed a kelp diet supplemented with strain C4 and abalone fed a standard kelp diet. However, abalone fed a kelp diet supplemented with strain C4 exhibited no disease symptoms at this time point.

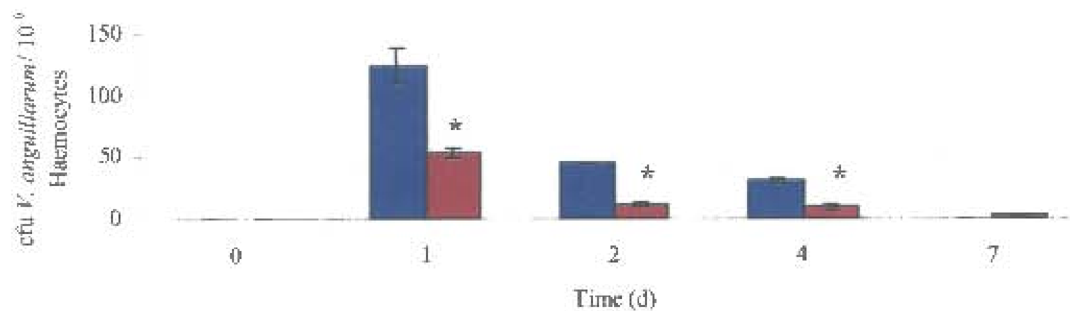

Figure 5. Number of *V. anguillarum* cells isolated from 10^6 haemocytes of abalone fed either a kelp diet (■) or a kelp diet supplemented with strain C4 (■). Error bars depict standard errors derived from four experiments. Asterisk (*) indicates a significant difference between abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4 at that sampling point.

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Abalone fed a kelp diet supplemented with strain C4 for two weeks prior to infection with *V. anguillarum* showed significantly (Student's t-test) lower cumulative mortalities (1.7) compared to abalone fed a standard kelp diet (7.6) and abalone fed a kelp diet supplemented with strain C4 only from the time of infection (6.3) (Table 2). There was no significant difference in mortalities between abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4 from the time of infection.

All abalone fed either a standard kelp diet or a kelp diet supplemented with strain C4 from the time of infection showed disease symptoms (inability to adhere to substrate, blistering on foot muscle). Abalone fed strain C4 supplemented kelp two weeks prior to infection that showed disease symptoms all recovered by day 4 post infection, while abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4 from the time of infection still showed symptoms of disease at the end of the experimental period.

Table 2. Cumulative mortalities in abalone fed a kelp diet supplemented with strain C4 and abalone fed a standard kelp diet. Percentage mortality was calculated by dividing the number of deceased abalone by the total number of abalone in that basket. Values in brackets represent standard errors calculated from four experiments. Thirty abalone were used for each treatment in each experiment. Asterisk (*) indicates a significant difference (Students T-test, $p<0.05$) compared to abalone fed a standard kelp diet.

	Number of Mortalities ± SE	% Mortality ± SE
Standard Kelp Diet	7.6 (±1.5)	25.6 (± 5.1)
Kelp Diet Supplemented with Strain C4 (Pre-fed)	1.7 (± 1.5)*	5.6 (± 5.1)*
Kelp Diet Supplemented with Strain C4 (Fed at time of infection)	6.3 (± 1.5)	21.1 (± 5.1)

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4.5 DISCUSSION

The possibility of pathogen infection is a constant threat to the economic success of abalone aquaculture. In recent years there has been growing interest in the use of probiotics in aquaculture as a means of stimulating immunity with the aim of decreasing the farmed animal's susceptibility to pathogen infection. A *Vibrio* and *Bacillus* species isolated from *Penaeus vannamei* were reported to have immunostimulating effects in the shrimp (Gullian *et al.*, 2004). Rainbow trout, *Oncorhynchus mykiss*, immune parameters were enhanced by using the probiotic bacterium *Lactobacillus rhamnosus* (Nikoskelainen *et al.*, 2003). *Penaeus monodon* treated with *Bacillus* S11 showed significant increases in immune functions as well as greater survival rates after exposure to *Vibrio harveyii* compared to untreated shrimp (Rengpipat *et al.*, 2000). The use of probiotics to stimulate the immune system of molluscs has received little research interest. An enhanced and more rapid immune response is expected to decrease the host animal's susceptibility to pathogen infection. The validity of this hypothesis in relation to immune responses in *H. midae* was tested in this study.

Potential probiotics for immunostimulation are usually selected on their *in vitro* ability to produce antimicrobial compounds that inhibit *in vitro* growth of pathogens (Gatesoupe, 1999; Jacobsen *et al.*, 1999; Gullian *et al.*, 2004; Vine *et al.*, 2004). Strain C4 did not inhibit the growth of any of the abalone bacterial pathogens tested. However, when *V. anguillarum* and *V. alginolyticus* were cultured in the presence of strain C4, strain C4 was able to significantly retard the growth of the pathogens. This may either be the result of differences in growth rates between strain C4 and the pathogens or more efficient use of the available nutrients by strain C4. In the event of pathogen infection, it could be expected that, assuming strain C4 is present in large enough numbers, strain C4 would be able to minimise pathogen proliferation in the gastrointestinal (GI) tract of *H. midae*. Pathogens also establish an infection by adhering to the gastrointestinal epithelium or mucous present in the GI tract. Although indigenous microorganisms normally prevent pathogens from establishing themselves in the GI tract, farming practices sometimes disrupt the indigenous microorganism communities, making the host animal more susceptible to pathogen infection. Rengpipat *et al.* (2000) suggested that residency of *Bacillus* S11 in the shrimp gut further protected *P. monodon* against pathogenic bacterial infection by competitive exclusion. Addition of strain C4 could theoretically block adhesion sites potentially available for use by pathogens. *In vitro* addition of strain C4 to mucus isolated from the oesophagus of *H. midae* reduced the ability of *V. anguillarum* to adhere to the mucus. Strain C4 had no effect on the adhesion of *V. anguillarum* to mucous isolated from the stomach or the intestine or the adhesion of *V. alginolyticus* to mucus

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isolated from the oesophagus, intestine or stomach. It should however be noted that the adhesion capability of *V. anguillarum* was significantly less compared to that of *V. alginolyticus* and that this naturally poor adhesion to mucus isolated from the abalone digestive tract may play a role in the ability of strain C4 to reduce this adhesion capability even further. While *in vitro* tests are useful in selecting the most promising probiotics, *in vivo* conditions are usually very different and the ability of bacteria to act as probiotics *in vivo* needs to be evaluated. Strain C4 may be able to reduce the pathogenicity of these and other bacterial pathogens *in vivo* by other methods, including stimulating the immune system.

Supplementing the diet of *H. midae* with strain C4 had significant effects on the immune parameters tested. There was no significant change in the total haemocyte count (THC) or the production of superoxide anions between abalone fed a standard kelp diet and those fed a kelp diet supplemented with strain C4. Stimulation of phagocytosis by *V. anguillarum* and superoxide anion production by yeast AY1 resulted in significant differences between the two groups of abalone. The percentage of phagocytic haemocytes was significantly greater in abalone fed a strain C4 supplemented diet between 1 and 14 days of feeding the strain C4 supplemented kelp. There was no significant difference in the percentage of phagocytic haemocytes from abalone fed a standard kelp diet over this time period. The stimulated production of superoxide anions was greater at 6 h, 1 d and 4 d after feeding of the strain C4 supplemented diet was initiated but was slightly less at 14 days. This reduction in superoxide anion production may be the result of external factors such as water temperature, water quality or aeration. The stimulated production of superoxide anions fluctuated greatly over the 14 day period. However, increases and decreases in superoxide anion production followed the same pattern in abalone fed a standard kelp diet and abalone fed a kelp diet supplemented with strain C4. These fluctuations could therefore be the result of external factors as mentioned previously. Ordas *et al.* (2000) reported that changes in environmental conditions also alter the number of circulating haemocytes. Although there was no significant difference in THC, it is possible that a differential haemocyte count would show an increase in the proportion of granular haemocytes. This would explain the increase in superoxide anion production and phagocytosis as granular haemocytes are involved in these functions. Rengipipat *et al.* (2000) reported a significant increase in phagocytic haemocytes after the shrimp, *Panaeus monodon* had been treated with the probiotic bacterium *Bacillus* S11. They suggested that the cell wall of the bacterium might elicit an immune response in shrimps by acting on granulocytes and thereby increasing phagocytic activity. As a result, the phagocytic haemocytes engulf foreign particles more aggressively. The results reported in this study suggest that strain C4 may act in a similar manner. Surface antigens or strain C4 metabolites may act as immunogens for abalone haemocytes.

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Addition of a stimulant increased the production of superoxide anions. Le Gall *et al.* (1991) also reported an increase in production of reactive oxygen intermediates, *in vitro*, by stimulation of phagocytosis with zymosan, a *Saccharomyces cerevisiae* cell wall extract. It must still be established whether the increase in superoxide anion production is the result of decreased superoxide dismutase activity as many molluscs have been found to produce antioxidants that protect against oxidative stress (Martello and Tjeerdema, 2001). Reactive oxygen intermediate scavengers neutralise or package potentially reactive oxygen intermediates and therefore an increase in production may be the result of an increase in the generation of superoxide anions or suppression of inhibitory antioxidant enzymes. Constitutive production of superoxide anions and continuous stimulation of immune capabilities would result in high energetic costs to the animal and could be detrimental. Therefore, increasing the potential for a more rapid and aggressive immune response is the most effective means of decreasing susceptibility to pathogen invasion. The ability of haemocytes of abalone fed a strain C4 supplemented diet to more effectively phagocytose foreign particles and generally produce increased amounts of superoxide anions when stimulated indicates that in the event of a pathogen attack, abalone fed a strain C4 supplemented diet would be able to respond more rapidly and effectively compared to abalone fed a standard kelp diet. The addition of strain C4 to abalone diets therefore acts to prime the abalone immune system that it may respond quickly and efficiently to pathogen infection as opposed to a continuous stimulation of abalone haemocytes to produce superoxide anions, which is energetically expensive. This hypothesis was tested by challenging the abalone with *V. anguillarum*.

The number of circulating haemocytes, phagocytic haemocytes and the production of intracellular superoxide anion production in abalone fed a standard kelp diet and a kelp diet supplemented with strain C4 were significantly reduced after infection with the pathogen *V. anguillarum* compared to pre-infection immune status (Figure 3, day 14). Recent studies have revealed that noradrenaline, the principal chatecholamine released during stress, exerts an inhibitory effect on oyster immune functions such as haemocyte phagocytosis and reactive oxygen species production (Lacoste *et al.*, 2002). Stress causes a reduction in oyster haemocyte migratory and phagocytic activity and inhibits reactive oxygen species production. The initial decrease in all immune parameters tested was significantly greater in abalone fed a standard kelp diet compared to abalone fed a kelp diet supplemented with strain C4. The significantly greater percentage phagocytosis and intracellular superoxide anion production observed 1 d after infection in abalone fed a kelp diet supplemented with strain C4 possibly contributed to an increase in resistance to the pathogen *V. anguillarum* as these abalone showed increased survival rates compared to abalone fed the standard kelp diet. Phagocytic haemocytes are the primary, non-specific defence mechanism against invasion of

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pathogenic organisms in invertebrates (Rengpipat *et al.*, 2000). Phagocytosis also results in the production of superoxide anions that are involved in bacterial killing. The increased phagocytic activity and superoxide anion production observed 1 d after infection in abalone fed a strain C4 supplemented diet possibly resulted in the significant decrease in *V. anguillarum* cells isolated from the haemolymph (Figure 5). Decreasing the bacterial load would result in less severe infection by the pathogen and increased survival rates. Supplementing the kelp diet with strain C4 allows the abalone to respond more rapidly and more aggressively to pathogen infection. An alternative explanation may be that the immune status of abalone is related to their nutritional status and therefore since abalone fed a strain C4 supplemented diet exhibit an increased growth rate, these abalone may be generally more healthy and have excess nutrients available initiating an immune response. Rengpipat *et al.* (2000) showed that *Penaeus monodon* growth and survival was greater in shrimp treated with the probiotic bacterium, *Bacillus* S11. *Vibrio harveyi* infection of *P. monodon* decreased the total haemocyte count in both probiotic treated and non-treated shrimp, with a significant decrease in the probiotic treatment. Similar rapid and marked reduction in circulating haemocyte numbers has been reported in freshwater crayfish (*Astacus astacus*) and shore crabs (*Carcinus maenas*), which indicated initiation of cellular defence reactions (Smith and Söderhäll, 1983). Carpet shell clams naturally infected with *Perkinsus atlanticus* showed a decrease in the rate of phagocytosis (Ordas *et al.*, 2000) as was the case with oysters infected with *Perkinsus marinus* (La Peyre *et al.*, 1995). Infected clams also showed a trend of decreasing numbers of circulating haemocytes. *In vitro* experiments have shown that *P. marinus* extracellular products inhibit haemocyte motility that is closely related to the phagocytic activity. The reduction in superoxide anion production observed in *H. midae* fed either a standard kelp diet or a kelp diet supplemented with strain C4 after *V. anguillarum* infection compared to pre-infection production could be the result of a decrease in phagocytic haemocytes as superoxide anion production is activated during phagocytosis. The production of superoxide anions is significantly greater in abalone fed a standard kelp diet compared to abalone fed a strain C4 supplemented kelp diet at 2 and 4 days post-infection and this may be the result of the abalone attempting to clear the haemolymph of the relatively high concentration of *V. anguillarum* still present. The stimulated production of superoxide anions is greater for abalone fed a kelp diet supplemented with strain C4 at 1 and 4 days post-infection indicating that the potential for these abalone haemocytes to fight bacterial infection is still greater during an infection compared to abalone fed a standard kelp diet. At 4 days post-infection there is a dramatic decrease in stimulated superoxide anion production in abalone fed a standard kelp diet that is not observed in abalone fed the kelp diet supplemented with strain C4 suggesting that the addition of strain C4 to the abalone diet increases the immunocompetence of the abalone in the event of bacterial infection. Both superoxide anion production and stimulated production are significantly

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higher in abalone fed a standard kelp diet at 10 days post-infection. At this time point abalone fed the standard diet still showed disease symptoms whereas abalone fed the kelp diet supplemented with strain C4 had all recovered by 4 days post-infection. This may explain the increased superoxide anion production at 10 days post-infection as abalone fed the standard diet are still fighting infection whereas abalone fed the supplemented diet have successfully eliminated the infection.

The increase in survival rates observed in abalone fed a strain C4 supplemented diet two weeks prior to infection confirms that the addition of strain C4 to abalone diets increases resistance to pathogen infection. When strain C4 was introduced into the abalone diet at the same point at which pathogen infection was initiated, no increase in resistance was observed indicating that strain C4 needs to be present prior to infection to increase resistance.

Extensive variability in immune functions exists in invertebrate populations due to the polygenic nature of each population (Pipe *et al.*, 1995) and this is considered a major difficulty when trying to assess overall immunocompetence. The red abalone *H. rufescens* showed a decrease in the chemiluminescent (CL) response at both high and low salinity in the presence of PCP. The black abalone, *H. cracherodii* showed an increase in the CL response at both low and high salinity in the presence of PCP (Martello and Tjeerdema, 2001). The results indicated that even closely related species may have different immune mechanisms and, in this particular situation, varying concentrations of reactive oxygen intermediate scavengers that are active under different conditions. The oyster, *C. gigas*, lacks lysozyme while its close relative *C. virginica* has high concentrations of the enzyme, again reflecting differences in immune mechanisms in even closely related species (Martello and Tjeerdema, 2001). Mollusc immunology has largely been studied in oysters and mussels and more knowledge is required regarding abalone immunology in order to conclusively report the effects of immune stimulation and increased resistance. The results obtained in this study indicate that the addition of strain C4 to abalone feed is capable of priming the immune response so that upon infection, *H. midae* is able to respond more rapidly and more aggressively, resulting in decreased susceptibility to the pathogen. This decreased susceptibility may be the result of increased phagocytic activity and superoxide anion production as well as retardation of pathogen proliferation and competitive exclusion of the pathogen from adhesion sites in the gastrointestinal tract. The use of strain C4 in abalone aquaculture may however also be more or less effective against other pathogens depending on their virulence and the ability of strain C4 to act against other pathogens.

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CHAPTER 5
GENERAL DISCUSSION

Abalone aquaculture is a relatively new industry in South Africa. Two of the major problems facing abalone farmers are the slow growth rate of abalone and the susceptibility of abalone to disease. The objective of this study was to identify a bacterium that could be used as a probiotic in abalone aquaculture to increase the growth rate of farmed abalone and reduce the susceptibility of abalone to pathogenic infections.

A number of bacteria were isolated from the digestive tract of *Haliotis midae* and characterised for their ability to degrade the complex polysaccharides found in seaweeds (Erasmus, 1996). The bacterial isolate strain C4 was selected from these isolates based on its ability to degrade alginate, a major component of *Ecklonia maxima*. Biochemical tests and 16S rDNA sequence analysis identified strain C4 as belonging to the *Pseudoalteromonas* genus. Strain C4 is most closely related to *Pseudoalteromonas* sp. strain R30 displaying 98% rDNA sequence similarity. Strain R30 was isolated from the surface of *Enteromorpha* plants on the shore at Devon, England. Strain C4 is believed to be a novel species, however this result is inconclusive since DNA-DNA hybridisation studies are outstanding. Strain C4 alginate lyase activity is inducible in the presence of alginate and maximum activity occurs between 22°C and 37°C and under slightly acidic conditions. Based on these characteristics, conditions in the digestive tract of *H. midae* are therefore favourable for *Pseudoalteromonas* sp. strain C4 alginate lyase activity, particularly in the crop region where conditions are slightly acidic (Harris *et al.*, 1998, Serviere-Zaragoza *et al.*, 1997).

The ability of strain C4 to improve the growth rate of *H. midae* was investigated by incorporating strain C4 into a kelp-based feed and conducting growth trials in the laboratory and on an abalone farm. This study has clearly shown that incorporation of strain C4 into the abalone's diet results in a significant increase in the growth rate of weanery sized abalone. Extrapolation of this increased growth rate over the grow-out period of farmed abalone equates to a 17 month reduction in the time for abalone to reach market size. However, abalone have different nutritional requirements throughout their life-cycle and strain C4 may be beneficial for weanary sized abalone but larger abalone may have different requirements. Therefore growth trials need to be extended to larger abalone to determine whether this effect is observed throughout the grow-out phase of farmed abalone. Since strain C4 produces an alginate lyase and *H. midae* is fed

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predominantly kelp, it was hypothesised that strain C4 may contribute additional alginate lyase in the abalone digestive tract to assist in digestion. This study revealed that alginate lyase activity is increased in the digestive tract of abalone fed a strain C4 supplemented diet compared to abalone fed a standard diet. Elevated levels of activity were still observed in the intestine 1 day after the supplemented diet was halted and in the stomach and crop 3 days after feeding was halted compared to abalone fed an unsupplemented diet throughout the experiment. Seven days after the strain C4 supplemented diet was halted there was no difference in the amount of alginate lyase activity between abalone previously fed the supplemented and standard diets in any region of the digestive tract. These results suggest that strain C4 probably does not colonise the digestive tract, but rather, is present transiently and that the crop-stomach region is a more favourable environment for strain C4. Interestingly, strain C4 was initially isolated from the crop of *H. midae* (Erasmus, 1996). However, additional studies need to be conducted in order to establish whether strain C4 is able to colonise the digestive tract of *H. midae* or whether it is only transiently present. Colonisation studies will aid the development of a probiotic feeding regime. Strain C4 may either need to be continuously fed to farmed abalone or need only be fed at certain intervals depending on the length of time the bacterium remains in the abalone digestive tract. Experiments to determine the colonisation abilities of strain C4 may include raising antibodies against strain C4 surface antigens and using these antibodies in *in situ* western hybridisation studies. Cross reactivity of strain C4 specific antibodies to digestive tract extracts of abalone may be monitored for several days after feeding of the strain C4 supplemented diet had been halted. A positive result would indicate the presence of strain C4. Alternatively, it may be possible to clone the alginate lyase gene from strain C4 and use it to design a probe for use in *in situ* Southern hybridisation studies. Cloning the alginate lyase gene from strain C4 would also allow the construction of an alginate lyase mutant C4 strain through homologous recombination. The mutant strain could be used in growth trials, as described in this study, to confirm the role of the strain C4 alginate lyase in increasing the growth rate of abalone fed a diet supplemented with strain C4. The role of strain C4 alginate lyase in increasing the growth rate of farmed abalone may be further analysed by purifying and further characterising the enzyme. It would be interesting to determine whether strain C4 alginate lyase has a preference for polyM or poly G blocks of alginate, since alginate lyase isolated from abalone preferentially degrades polyM blocks of alginate. Strain C4 was also shown to provide an additional protein source to the abalone diet. This is an important finding since *E. maxima* is a nutritionally poor protein source. The majority of strain C4 protein was incorporated in the hepatopancreas, from which enzymes are secreted into the crop and stomach where they are used in digestion. This phenomenon was only observed in gnotobiotic abalone. An increased amount of ^{14}C-label was detected in the

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faeces of abalone not treated with antibiotics. This suggests that other microbes present in the digestive tract of *H. midae* reduce the number of niches available to strain C4 thereby decreasing the amount of strain C4 protein incorporated into abalone tissue. Furthermore, it is probable that abalone would also incorporate protein provided by other microbes present in the digestive tract, thereby further diluting the amount of radiolabelled strain C4 protein incorporated.

The potential of strain C4 to protect *H. midae* against bacterial infection was initially investigated *in vitro*. Strain C4 does not appear to produce any antimicrobial substances that inhibit the growth of either *V. anguillarum* or *V. alginolyticus*. However, strain C4 reduced the growth of both of these bacterial strains when co-cultured in liquid medium. This suggests that strain C4 competes well with these pathogens for nutrient sources. Strain C4 also reduced the *in vitro* adhesion of *V. anguillarum* to mucus isolated from the oesophagus of *H. midae* suggesting that strain C4 may also out compete pathogens for adhesion sites in the digestive tract of *H. midae*. Supplementing the abalone diet with strain C4 resulted in an increase in phagocytic activity of the haemocytes and increased intracellular superoxide anion production by haemocytes stimulated with yeast AY1. Upon challenge with the bacterial pathogen *V. anguillarum*, abalone fed a strain C4 supplemented diet initially showed increased levels of intracellular superoxide anion production and greater phagocytic activity by the haemocytes. The number of *V. anguillarum* cells isolated from haemolymph of infected abalone was also significantly less in abalone fed the supplemented diet. Abalone mortality was significantly reduced from 26% in abalone fed a standard diet to 6% in abalone fed a strain C4 supplemented diet.

The data collected in this study suggests that strain C4 is a good candidate for use as a probiotic in abalone aquaculture. Strain C4 significantly increased the growth rate of weanery sized abalone under farming conditions. The increase in growth rate is believed to be the result of additional enzymes, particularly alginate lyase, and an additional protein source contributed by strain C4. Strain C4 reduced mortalities in abalone infected with the bacterial pathogen *V. anguillarum*. It is hypothesised that increased survival is a result of strain C4 out competing the pathogen for both nutrients and adhesion sites in the abalone gastrointestinal tract as well as the stimulation of the immune system of abalone by strain C4, particularly phagocytic activity and intracellular superoxide anion production. The more rapid immune response to infection and efficient clearing of *V. anguillarum* from the haemolymph observed in abalone fed a strain C4 supplemented diet compared to abalone fed a standard diet probably also plays a role in reducing the number of mortalities.

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This study has provided a basis for selecting and using probiotics in abalone aquaculture and strain C4 has proven to be a successful probiotic. However, additional factors need to be investigated. At present the greatest obstacle is developing a method for introducing strain C4 into the digestive tract of abalone on a commercial scale, as the diet used in this study is impractical for farming purposes. Firstly, the culture of strain C4 on an industrial scale needs to be investigated. These studies will include the development of a culture medium that is both affordable and allows for high biomass production of strain C4. Secondly, strain C4 needs to be incorporated into a commercial feed source. This may include incorporation into an artificial feed or inoculation of harvested kelp with strain C4, possibly by spraying liquid strain C4 cultures onto kelp fronds. The viability and number of strain C4 colony forming units will need to be established for whichever method is selected.

Finally, a probiotic 'cocktail' containing a number of different strains may further improve results. Additional strains, possibly those producing enzymes capable of degrading other constituents found in abalone feed or those that produce antimicrobial compounds, may be investigated for their ability to increase the growth rate and stimulate the immune system of farmed abalone.

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APPENDIX A
MEDIA AND SOLUTIONS
CONTENTS

A1 Media

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A1.11 Media for carrageenase activity test
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A1.13 Media for amylase activity test
A1.14 Media for cellulase activity test
A1.15 Media for lipase activity test
A1.16 Media for casein hydrolysis test
A1.17 Alginate Broth (AB)
A1.18 YE/Peptone Broth
A1.19 YPD Broth

A2 Solutions

A2.1 Antibiotic solutions for kelp cakes and tank water
A2.2 HH buffer
A2.3 1% SDS/0.1M NaOH
A2.4 Ethidium Bromide
A2.5 0.5M EDTA
A2.6 1M Tris-HCl
A2.7 Tris-EDTA (TE) buffer
A2.8 Citric acid/phosphate buffer for dialysis of abalone tissue

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A2.9 Magnesium sulphate for anaesthetising abalone

A2.10 Phosphate buffered saline (PBS)

A2.11 Phosphate buffer

A2.12 Synthetic sea salts (SSS)

A2.13 Solution for cellulase activity test

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A2.17 Solutions for agarose gel electrophoresis

A2.18 Solution for electron microscopy

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A2.20 Solutions for determining optimum alginate lyase activity

A2.21 Solution for Bradford method of protein quantification

A2.22 Solutions for abalone immunology assays

A2.23 Solutions for cycle-sequencing

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All media were autoclaved at 121°C for 20 min prior to use, unless otherwise specified.
Water used for making solutions, media and diluting buffers was purified using a Milli-RO Plus (Millipore) water purification system.

A1 Media

A1.1 Basal Marine Broth (BMB)

NaCl (Saarchem)	30.0 g
$MgCl_2.6H_2O$ (Saarchem)	2.3 g
KCl (Saarchem)	0.3 g
Casamino acids (Difco)	5.0 g
Yeast extract (Biolab)	1.0 g
Water to	1 L

A1.2 Basal Marine Agar (BMA)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Casamino acids	5.0 g
Yeast extract	1.0 g
Agar (Biolab)	20.0 g
Water to	1 L

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A1.3 Marine Broth (MB)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Casamino acids	5.0 g
Yeast extract	1.0 g
Glucose (Saarchem)	2.0 g
Water to	1 L

A1.4 Marine Agar (MA)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Casamino acids	5.0 g
Yeast extract	1.0 g
Glucose	2.0 g
Agar	20.0 g
Water to	1 L

A1.5 Kelp Cakes

Dried Kelp (Kelpak)	300 g
Agar	15 g
SSS (Appendix A2.12)	1 L

Allow dried kelp to swell overnight in SSS. For kelp cakes supplemented with strain C4, harvest 4 l of strain C4 cultured in MB. Resuspend in 100 ml SSS (approximately 3 x 10^{10} cfu/ml) and add to autoclaved, cooled kelp cake mixture. This gives approximately 2.4 x 10^{10} cfu/g. After 1 week at room temperature approximately 1.92 x 10^{10} cfu/g. Antibiotics were added to kelp cakes as described in section A2.1.

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A1.6 TCBS Agar

TCBS Agar (Difco)	89 g
Water to	1 L

Boil to dissolve completely. Do not autoclave.

A1.7 Media for carbohydrate utilization test

Peptone (Difco)	2.0 g
NaCl	30.0 g
K_2HPO_4 (Saarchem)	0.3 g
1% Bromophenol blue (w/v) (Merck)	3.0 ml
water to	1 L

The pH is adjusted to 7.1 before adding bromophenol blue. The carbohydrate to be added is sterilized separately and added to give a final concentration of 1%. The media is then tubed to a depth of about 4 cm.

A1.8 Media for nitrate reduction and denitrification test

D-glucose (Saarchem)	0.20 g
Casamino acids	0.50 g
Yeast extract	0.10 g
NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
KNO_3 (Saarchem)	0.10 g
Agar	0.17 g
Water to	100 ml

The media was decanted into 5 ml aliquots in standard containers and autoclaved.

A1.9 Media for indole production test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Tryptone (Biolab)	1.00 g
Water to	100 ml

The media was decanted into 10 ml aliquots in standard containers and autoclaved.

A1.10 Media for H_2S production test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Peptone (Difco)	1.00 g
Water to	100 ml

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A1.11 Media for carrageenase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Casamino acids	0.50 g
Yeast extract	0.10 g
D-glucose	0.20 g
Carrageenan (Sigma)	2.00 g
Water to	100 ml

A1.12 Media for gelatinase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Gelatin (Merck)	12.00 g
Water to	100 ml

A1.13 Media for amylase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Starch (BDH)	0.20 g
Agar	1.50 g
Water to	100 ml

A1.14 Media for cellulase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
CM-Cellulose (Sigma)	0.10 g
Agar	0.80 g
Water to	100 ml

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A1.15 Media for lipase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Agar	2.00 g
Water to	100 ml
Melted butter (Floro)	10.00 g

A1.16 Media for casein hydrolysis test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Skim milk powder (Elite)	10.00 g
Agar	2.00 g
Water to	100 ml

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A1.17 Alginate Broth (AB)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Casamino acids	5.0 g
Yeast extract	1.0 g
Sodium alginate (Merck)	2.0 g
Water to	1 L

A1.18 YE/Peptone Broth

Peptone	0.5 g
Yeast extract	0.1 g
SSS to	100 ml

A1.19 YPD Broth

Peptone	2.0 g
Yeast extract	1.0 g
Glucose	2.0 g
SSS to	100 ml

A2 Solutions

A2.1 Antibiotic solutions in growth media, kelp cakes and tank water

Ampicillin (Sigma)(100 mg/ml)

Dissolve 2 g in 20 ml water. Filter sterilise and store aliquots at 4°C.

Dilute 1:1000 tank water for final concentration of 100 μg/ml

Dilute 2.5:1000 into kelp cakes for a final concentration of 250 μg/ml

Chloramphenical (Roche) (80 mg/ml)

Dissolve 1.6 g in 20 ml EtOH. Store aliquots at -20°C.

Dilute 3.13:1000 into kelp cakes for a final concentration of 250 μg/ml

Dilute 1.25:1000 into tank water for a final concentration of 100 μg/ml

Cefotaxine (Claforan®, Roussel) (250 mg/ml)

Dissolve 5 g in 20 ml water. Filter sterilise and store aliquots at -20°C

Dilute 2:1000 into kelp cakes for a final concentration of 500 μg/ml

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A2.2 HH Buffer

NaCl	8.00 g
KCl	0.40 g
$CaCl_2.H_2O$	0.05 g
KH_2PO_4	0.35 g
$MgSO_4.7H_2O$	0.20 g
HEPES (Sigma)	2.60 g
Water to	1 L

Dissolve in 800 ml water and adjust pH to 7.4 with 1 M NaOH. Make up to 1 l with water.

A2.3 1% SDS/0.1 M NaOH

10% SDS (Saarchem)	1 ml
1 M NaOH (Saarchem)	1 ml
water to	10 ml

A2.4 10 mg/ml Ethidium Bromide

Ethidium bromide (Sigma)	0.1 g
Water to	10 ml

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

A2.5 0.5 M EDTA

EDTA (Saarchem)	93.05 g
NaOH	10.00 g
Water to	500 ml

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

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A2.6 1 M Tris-HCl

Tris (Roche)	12.1 g
Water to	100 ml

Dissolve the Tris in 80 ml water, adjust pH to required level with concentrated HCl and make up to a final volume of 100 ml.

A2.7 TE Buffer (Tris-EDTA)

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

A2.8 Citric acid/Phosphate buffer for dialysis of abalone tissue

Citric acid (Merck)	21.01 g
0.2 M NaH_2PO_4 (Merck)	250 ml
0.2 M Na_2HPO_4 (Merck)	250 ml
water	500 ml

A2.9 6% $MgSO_4$ for anaesthetising abalone

$MgSO_4$ (Saarchem)	60 g
Water to	1 L

A2.10 10X Phosphate buffered saline (PBS)

NaCl	87.0 g
Na_2HPO_4 (Merck)	22.5 g
KH_2PO_4 (Saarchem)	2.0 g
Water to	1 L

Dissolve NaCl, Na_2PO_4 and KH_2PO_4 in 900 ml water. Adjust pH to 7.4 and make up to 1 L with water. For 1XPBS dilute 1:10 with distilled water.

A2.11 0.1 M Phosphate buffer

$Na_2HPO_4.2H_2O$	16.59 g
$NaH_2PO_4.H_2O$	0.94 g
Water to	1 L

Dissolve NaCl, $Na_2HPO_4.2H_2O$ and K_2HPO_4 in 800 ml water and adjust pH to 6.7. Make up to a final volume of 1 L.

A2.12 Synthetic sea salts (SSS)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
Water to	1 L

A2.13 Solution for cellulase activity

Congo Red

Congo Red (BDH)	0.10 g
Sterile water	100 ml

A2.14 Solution for lipase activity test

Saturated Copper sulphate

Copper sulphate (Saarchem)	
Water	100 ml

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A2.15 Solutions for Gram-staining procedure

A2.15.1 Crystal violet

Crystal violet (Merck)	2.0 g
Absoloute EtOH (Merck)	10 ml
Ammonium oxalate (BDH)	4.0 g
Water to	500 ml

A2.15.2 Gram iodine

Iodine (Saarchem)	1.0 g
Potassium iodide (Saarchem)	2.0 g
Water	245 ml
5% Sodium bicarbinate (Saarchem)	60 ml

A2.15.3 Acetone solution

Absoloute EtOH	250 ml
Acetone (Saarchem)	250 ml

A2.15.4 Counter stain

Safranin (Merck)	0.5 g
Absoloute EtOH	100 ml

A2.16 Solutions for catalase and oxidase tests

A2.16.1 Kovac's reagant

Tetramethyl-ρ-phenylene-diamine (Merck)	0.5 g
Water to	50 ml
α-napthol (Saarchem)	0.5 g
absoloute EtOH to	50 ml

Dissolve the tetramethyl-ρ-phenylene-diamine in water and α-napthol in EtOH. Combine the two in equal ratio. Freshly prepared. Do not autoclave.

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A2.16.2 3% Hydrogen peroxide solution

30% Hydrogen peroxide (w/v) (Saarchem)	1 ml
Sterile water to	10 ml

Freshly prepared. Do not autoclave.

A2.17 Solution for agarase gel electrophoresis

A2.17.1 50X TAE (Tris-acetate buffer)

Tris	242.0 g
Glacial acetic acid (Saarchem)	57.1 ml
0.5 M EDTA	100 ml
water to	1 L

A2.17.2 6X Tracking dye

Bromophenol blue	0.25 g
Sucrose (Saarchem)	40.0 g
EDTA (0.5 M, pH 8)	4.0 ml
Water to	100 ml

A2.18 Solutions for electron microscopy (EM)

1% Methylamine tungstate

Methylaminie tungstate (Trevor Sewell, EM Unit, UCT)	0.5 g
Water to	50 ml

Filter sterilise before use. Store at 4°C.

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A2.19 Solutions for Thiobarbituric acid assay for alginate lyase activity

A2.19.1 Alginate Substrate

Sodium alginate (Sigma)	0.4 g
Phosphate buffer (pH 6.7)	100 ml

A2.19.2 Periodate

Potassium m-periodate (Sigma)	0.115 g
98% H_2SO_4 (Merck)	0.25 ml
water to	20 ml

Freshly prepared. Do not autoclave.

A2.19.3 Sodium Arsenate

Sodium arsenate (Saarchem)	0.7 g
32% HCl (Merck)	2 ml
water to	35 ml

Do not autoclave.

A2.19.4 Thiobarbituric Acid (TBA)

TBA (Sigma-Aldrich)	0.3 g
Water to	100 ml

Add TBA to 80 ml water and heat to dissolve. Allow to cool to room temperature and adjust pH to 2.3 with HCl. Make up to a final volume of 100 ml with water. Do not autoclave.

A2.20 Solutions for determining optimum alginate lyase activity

A2.20.1 100 mM Phosphate buffer at various pH's

(A) 1 M monobasic sodium phosphate solution

NaH_2PO_4 (Saarchem)	27.8 g
Water to	1 L

(B) 1 M dibasic sodium phosphate solution

$Na_2HPO_4.7H_2O$ (Saarchem)	53.65 g
Water to	1 L

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Combine x ml of A with y ml of B, diluted with water to a total of 10 ml

pH	X (ml)	Y (ml)
5.8	9.2	0.8
6.0	8.8	1.2
6.4	7.5	2.5
7.0	4.2	5.8
7.4	2.3	7.7
8.0	0.7	9.3

A2.20.3 Alginate substrate

Sodium alginate	0.4 g
Phosphate buffer at appropriate pH	100 ml

A2.21 Solutions for determining protein concentration using Bradford assays

A2.21.1 Bovine serum albumin (BSA) (1 mg/ml)

BSA (Sigma)	0.01 g
Sterile water	10 ml

Do not autoclave. Aliquot and store at -20°C.

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A2.21.2 0.15 M NaCl

NaCl	0.88 g
Water to	100 ml

A2.21.3 Coomasie brilliant blue solution

Coomasie brilliant blue G250 (Saarchem)	100 mg
Absoloute EtOH	50 ml
Phosphoric acid (Saarchem)	100 ml
Water to	1 L

In a 1 L volumetric flask dissolve Coomasie brilliant blue G250 in EtOH. Add 85% phosphoric acid. Bring volume to 1 L with water. Filter through Whatman no 1 filter paper. Do not autoclave. Store at 4°C.

A2.222 Solutions of immunology assays

A2.22.1 Alsevers solution

D-glucose	10.4 g
Sodium citrate (Saarchem)	4.0 g
EDTA	1.68 g
NaCl	11.2 g
Formaldehyde (Merck)	60 ml
Water to	500 ml

Dissolve in 400 ml water, adjust pH to 7.5 and make up to final volume of 500 ml. Store at 4°C up to 1 month.

A2.22.2 0.1 M $NaHCO_3$ (pH 9)

$NaHCO_3$ (Saarchem)	1.06 g
Water to	100 ml

Dissolve the sodium carbonate in 80 ml of distilled water. Adjust the pH to 9 using 1 M NaOH and then adjust the volume to 100 ml with distilled water.

A2.22.3 10% formaldehyde

Formaldehyde	100 ml
Water	900 ml

A2.22.4 Modified Hank's Buffered Salt Solution (MHBSS)

D-glucose	10.4 g
NaCl	11.2 g
KCl	0.41 g
KH_2PO_4 (Merck)	0.1 g
$CaCl_2.2H_2O$ (Merck)	0.47 g
$MgCl_2.6\ H_2O$ (Saarchem)	2.79 g
$MgSO_4.7\ H_2O$ (Saarchem)	3.22 g
EGTA (Sigma)	0.015 g
Water to	500 ml

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A2.22.5 50% acetic acid

Acetic acid (Merck)	500 ml
Water	500 ml

A2.22.6 Ethidium bromide (10 mg/ml)

Ethidium bromide	0.2 g
PBS	20 ml

A2.22.7 Nitorblue Tetrazodium (NBT)

NaCl	0.2 g
NBT (Sima)	0.04 g
1 M Tris-HCl (pH 7.6) to	20 ml

A2.22.8 2 M KOH

KOH (Saarchem)	11.22 g
Water to	100 ml

A2.22.9 50% Methanol

Methanol (Saarchem)	50 ml
Water	50 ml

A2.23 Solutions for cycle-sequencing

A2.23.1 10X Tris-Borate-EDTA buffer (TBE)

Tris	108 g
Boric acid (Saarchem)	36 g
0.5 M EDTA	20 ml
water to	1 L

A2.23.2 10% Ammonium Persulphate

Ammonium persulphate (Pharmacia)	1 g
Water to	10 ml

Do not autoclave. Aliquot into eppendorf tubes and store at -20°C.

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APPENDIX B
STANDARD METHODS
CONTENTS

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B1 Thiobarbiturc acid assay for alginate lyase activity

(Preiss and Ashwell, 1962; Weissbach and Hurwitz, 1959)

In triplicate, 100 µl of culture supernatant or resuspended bacterial cells was added to 300 µl of alginate substrate (Appendix A2.19.1) and incubated at 37°C for 30 minutes. One hundred microlitres of freshly-prepared periodate (Appendix A2.19.2) was added to each reaction and incubated at 22°C for 20 min. The reaction was stopped by adding 80 µl of sodium arsenate (Appendix A2.19.3) and incubating at 22°C for 2 min, after which 800 µl of thiobarbituric acid (Appendix A2.19.4) was added. The reaction was incubated in a boiling water bath for 10 min, the tubes subsequently cooled to 22°C and the optical density determined at 550 nm. Alginate lyase activity (alginolytic units: U) was expressed as µg malondialdehyde equivalents released per 100 µl per 30 min, where the concentration of malondialdehyde was determined from a standard curve of optical density at 550 nm versus amount of malondialdehyde (1,1,3,3 tetraethoxypropane, Sigma).

B2 PCR of 16S rDNA fragment from *Pseudoalteromonas* sp. strain C4

B2.1 Primers used for strain C4 16S rDNA amplification

F1 5' CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCI TGG CTC AG 3'
F2 5' CGC CAG GGT TTT CCC AGT CAC GAC GCC AGC AGC CGC GGT AAT AC 3'
F3 5' CGC CAG GGT TTT CCC AGT CAC GAC GCA TGG ITG TCG TCA GCT CGT 3'
R1 5' CAG GAA ACA GCT ATG ACG TAT TAC CGC GGC TGC TGG CAC 3'
R2 5' CAG GAA ACA GCT ATG ACC ACG AGC TGA CGA CAI CCA TG 3'
R3 5' CAG GAA ACA GCT ATG ACA CGG ITA CCT TGT TAC GAC TT 3'

I = A, C, G or T

Underlined sequence represents sequence complementary to the primers used for cycle-sequencing. Non-underlined sequence represents sequence complementary to highly conserved regions of the 16S rRNA gene.

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B2.2 PCR protocol

Prepare a master mix as follows:

Reagents (Roche)	Volume (µl)
C4 genomic DNA (50 ng/µl)	30
PCR buffer (10X)	60
Taq polymerase (5U/µl)	6
dNTP's (25 mM)	6
Sterile water	438
Total	540

Aliquots (90 µl) were sub divided into 5 PCR eppendorf tubes. Five microlitres of each of the following primers were added to the 5 PCR tubes:

Tube number	Primer (10 µM)
1	F1 and R5
2	F1 and R3
3	F1 and R1
4	F3 and R5
5	F5 and R5

PCR cycle profile was setup as follows:

Temperature (°C)	Time (s)	Cycles
96	120	1
96	45	
51	30	
72	90	25
96	45	
51	30	
72	180	1

The PCR was performed in a thermal cycler with a heated lid (Hybaid). The PCR reactions were purified with QIAquick PCR Purification Kit Protocol (Qiagen).

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B3 Agarose gel electrophoresis

(Ausubel *et al.*, 1989 unit 2.5)

Melt agarose (1% w/v) in 1X TAE (Appendix A2.17.1) by heating in microwave and swirling to ensure even mixing. Add ethidium bromide (Appendix A2.4) to a final concentration of 0.5 µg/ml. Cool the melted agarose to 55°C before pouring onto the gel platform. Seal the gel-casting platform with masking tape if it is open at the ends. Pour in the melted agarose and insert the gel comb, ensuring that no bubbles are trapped underneath the comb. After the gel has hardened, remove the tape from the casting platform and withdraw the gel comb. Place the gel-casting platform containing the set gel in an electrophoresis tank. Add sufficient 1X TAE to cover the gel. Load DNA samples into the wells of the gel. Attach leads so that DNA migrates into the gel toward the anode. Run the gel at 8 to 10 V/cm until the dye in the loading buffer reaches the end of the gel.

B4 Quantification of DNA samples

(Coyne *et al.*, 1996)

B4.1 <u>Spectrophotometric quantification of DNA</u>

Determine the absorbance of DNA at 260 nm. The absorbance at 260 nm allows the calculation of the concentration of the DNA since 1 OD unit at 260 nm is equivalent to 50 µg/ml for double stranded DNA.

B4.2 <u>Ethidium bromide fluorescent quantification of DNA</u>

Prepare three λ DNA standards with known concentrations: 5 ng/10 µl, 10 ng/10µl and 20 ng/10 µl. Load 10 µl from each standard with 2.5 µl gel tracking dye (Appendix A2.17.2) into the wells of a 1% TAE agarose gel. Prepare several dilutions of the DNA sample of unknown concentration in 10 µl. Add 2.5 µl tracking dye and load next to the standards on the agarose gel. Electrophorese the samples at 100 V for 5 min. Visualise the DNA bands using a 254 nm UV transilluminator. Determine the concentration of the DNA sample by comparing the intensity of the DNA band to that of the standards. If 10 µl of a 1/10 dilution of the DNA sample is loaded and it corresponds to intensity equivalent to that of the 10 ng standard, the DNA sample will have a concentration of 10 ng/µl.

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B5 Automated sequencing

All the primers were labelled with Cy5-Far Red fluoroscein (Pharmacia)

B5.1 PCR primers for cycle-sequencing

Forward primer (16S rDNA): 5' CGC CAG GGT TTT CCC AGT CAC GAC 3'

Reverse primer (16S rDNA): 5' CAG GAA ACA GCT ATG AC 3'

B5.2 Cycle-sequencing protocol

(All reagents were in the Thermosequenase Cycle-Sequencing Kit, Amersham Life Sciences)

Ten microlitres of A, C, G or T termination mix was added to 0.5 µl PCR tubes. In separate microfuge tubes, 1µg of DNA was diluted to 22 µl in sterile water containing 2.1 pmol of cycle-sequencing primers. The DNA mix (5.1 µl) was aliquoted into each termination mix tube, mixed and placed in a thermal cycler with a heated lit (Hybaid). DNA products were amplified as follows:

Temperature (°C)	Time (s)	Cycles
93	300	1
93	30	
55	30	
70	60	30
93	30	
55	30	
72	300	1

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After amplification, the tubes were placed on ice and 4 µl of Stop solution was added to each tube prior to sequencing the DNA using an automated sequencer (ALFexpress™, AM Version 3.01, Pharmacia Biotech).

B5.3 Preparing the sequencing gel

Urea (Pharmacia)	19 g
1.5X TBE buffer (Appendix A2.23.1)	7.5 ml
50% Long Ranger gel solution (Pharmacia)	0.5 ml
ultrapure water made up to	50 ml

The above was gently stirred in a 100 ml Erlenmeyer flask covered with parafilm, for 30 min on a magnetic stirrer. The solution was filter-sterilised through a 0.45 μm vinyl filter (Millex, Millipore). Prior to pouring the gel the following was added:

10% (w/v) ammonium persulphate (Appendix A2.23.2)	250 μl
TEMED (Sigma)	25 μl

The above solution was mixed very well in a fumehood and rapidly syringed between the two glass plates of the ALFexpress™ gel apparatus. The acrylamide was left to polymerise for 2 h.

B5.4 Running of sequencing gel

The DNA samples to be sequenced were heat-denatured at 96°C for 5 min and 4 μl of each sample was loaded into the wells. The gel was run in 0.6% TBE buffer. The ALFexpress™ was run with the following settings:

1000 V
60 mA
25 W
55°C
2 s sample time
720 min running time

The data was processed with the Pharmacia software package AM version 3.02, which controlled and evaluated the sequence data generated by the ALFexpress™ automated sequencer.

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B6 Bradford protein assay for protein quantification

(Ausubel *et al.*, 1989 unit 10.1)

Aliquot, in duplicate, the following amounts of BSA (Appendix A2.21.1) and 0.15 M NaCl (Appendix A2.21.2) into eppendorf tubes.

Tube	BSA	NaCl
1	2.5 µl (2.5 µg/ml)	97.5 µl
2	5 µl (5 µg/ml)	95 µl
3	10 µl (10 µg/ml)	90 µl
4	15 µl (15 µg/ml)	85 µl
5	20 µl (20 µg/ml)	80 µl

Add 100 µl of protein sample with unknown concentration (in duplicate) to an eppendorf tube. Add 1 ml of Coomassie Brilliant Blue (Appendix A2.21.3) to the standard and sample tubes. Vortex for 5 seconds. Allow the tubes to stand at room temperature for 5 min. Determine the optical density at 595 nm of all the samples and plot a standard curve of optical density at 595 nm versus protein concentration, using the standards. Use the curve to determine the protein concentration of the sample.

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B7 Determination of the effectiveness of antibiotic treatment of abalone

After antibiotic treatment was completed an abalone from each tank was sacrificed. The gastrointestinal tract was removed from the sacrificed abalone and homogenised in 1 ml 0.1 M phosphate buffer, pH 6.7 (Appendix A2.11). The homogenate was serially diluted in 0.1 M phosphate buffer, pH 6.7 and 100 µl aliquots of each dilution were spread plated on MA solid media (Appendix A1.4). The Petri dishes were incubated for 2 days at 22°C after which the cfu/ml was determined. The homogenate of abalone treated with antibiotics contained 3.7×10^1 cfu/ml (SE $= 3 \times 10^1$) compared to 2.7×10^4 cfu/ml (SE $= 1.2 \times 10^4$) for abalone not treated with antibiotics.

APPENDIX C

ABALONE DIAGRAMS

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C1 Dorsal view of abalone with the shell removed

C2 Longitudinal section of buccal cavity

C3 General dissection of the alimentary canal.

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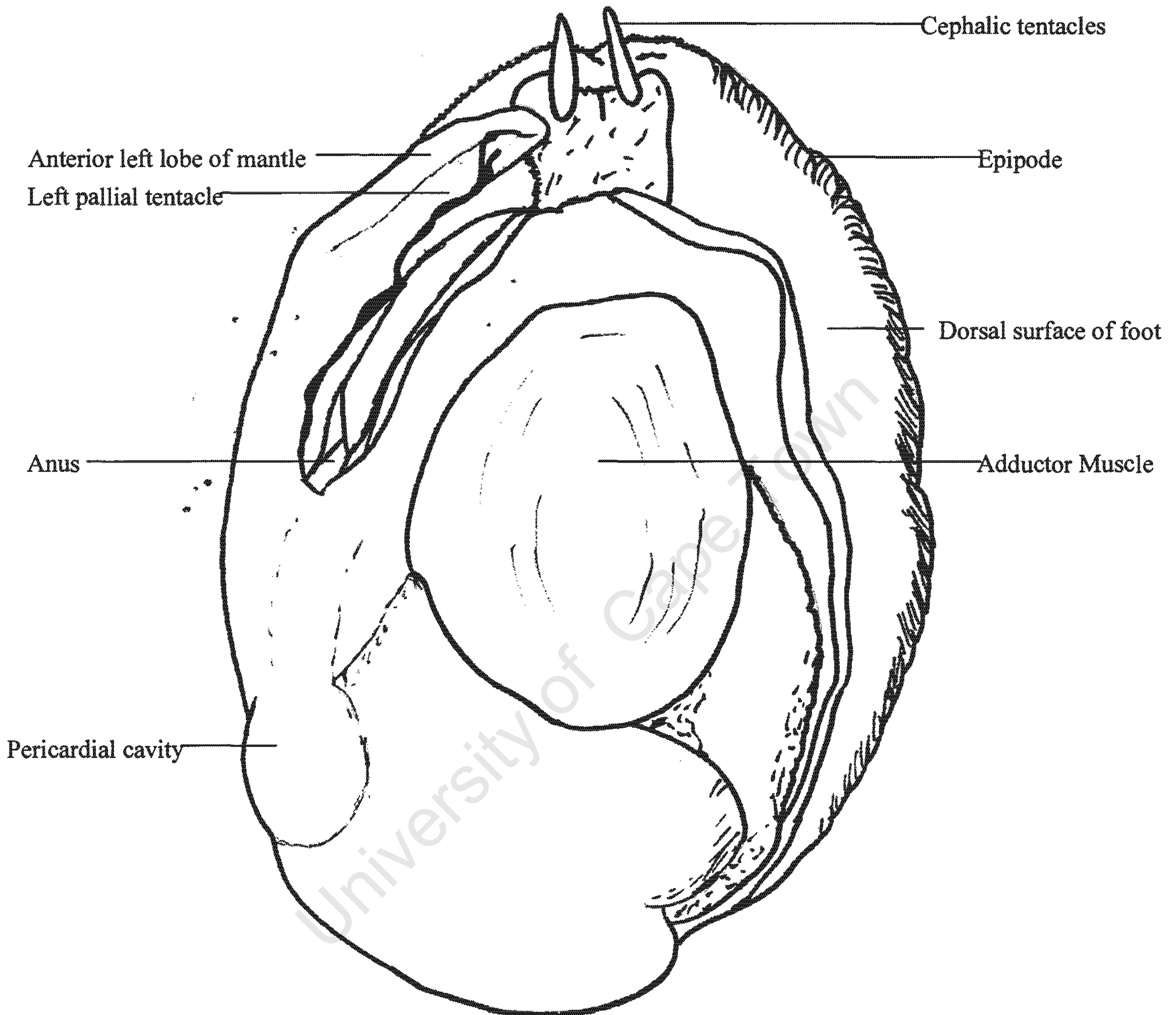
APPENDIX C1



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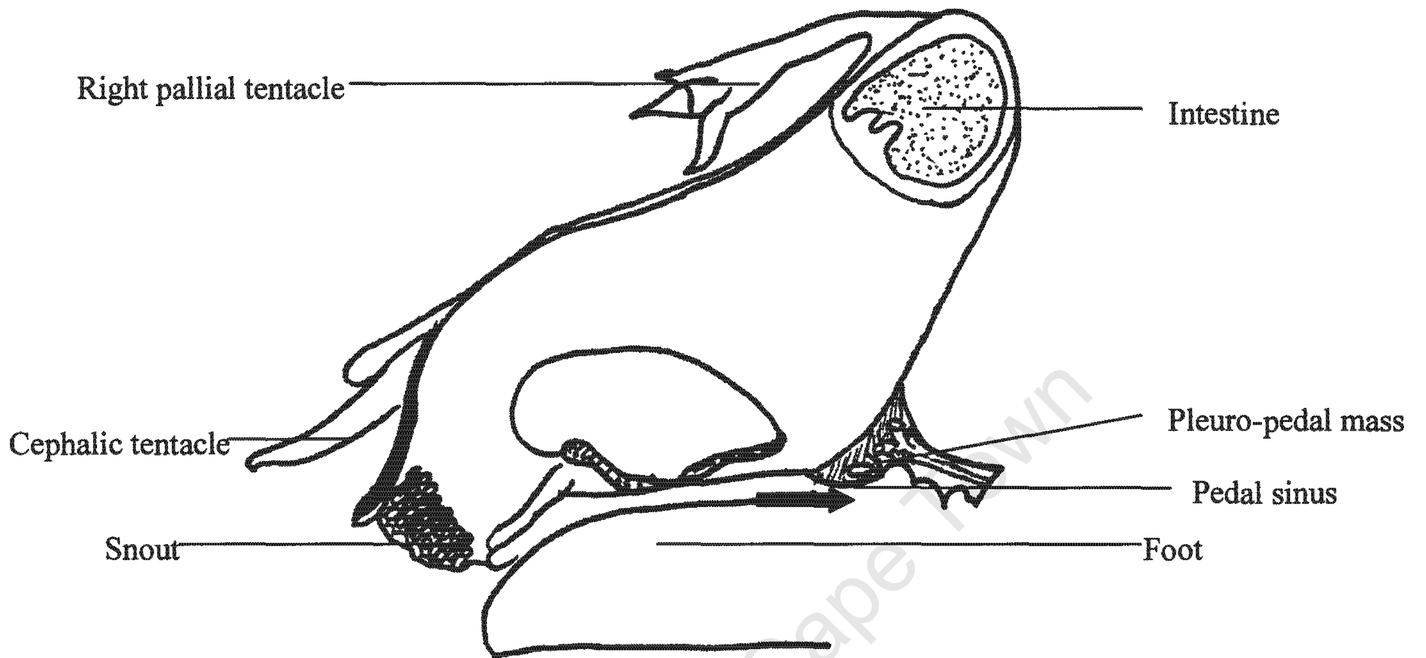
Dorsal view of abalone with the shell removed. The arrow indicates the site of infection with *Vibrio angulillarum*. Adapted from Ino (1953).

APPENDIX C2



Longitudinal section of the buccal cavity. Haemolymph was extracted from the pedal sinus. Arrow indicates where syringe was inserted to extract haemolymph. Adapted from Ino (1953).

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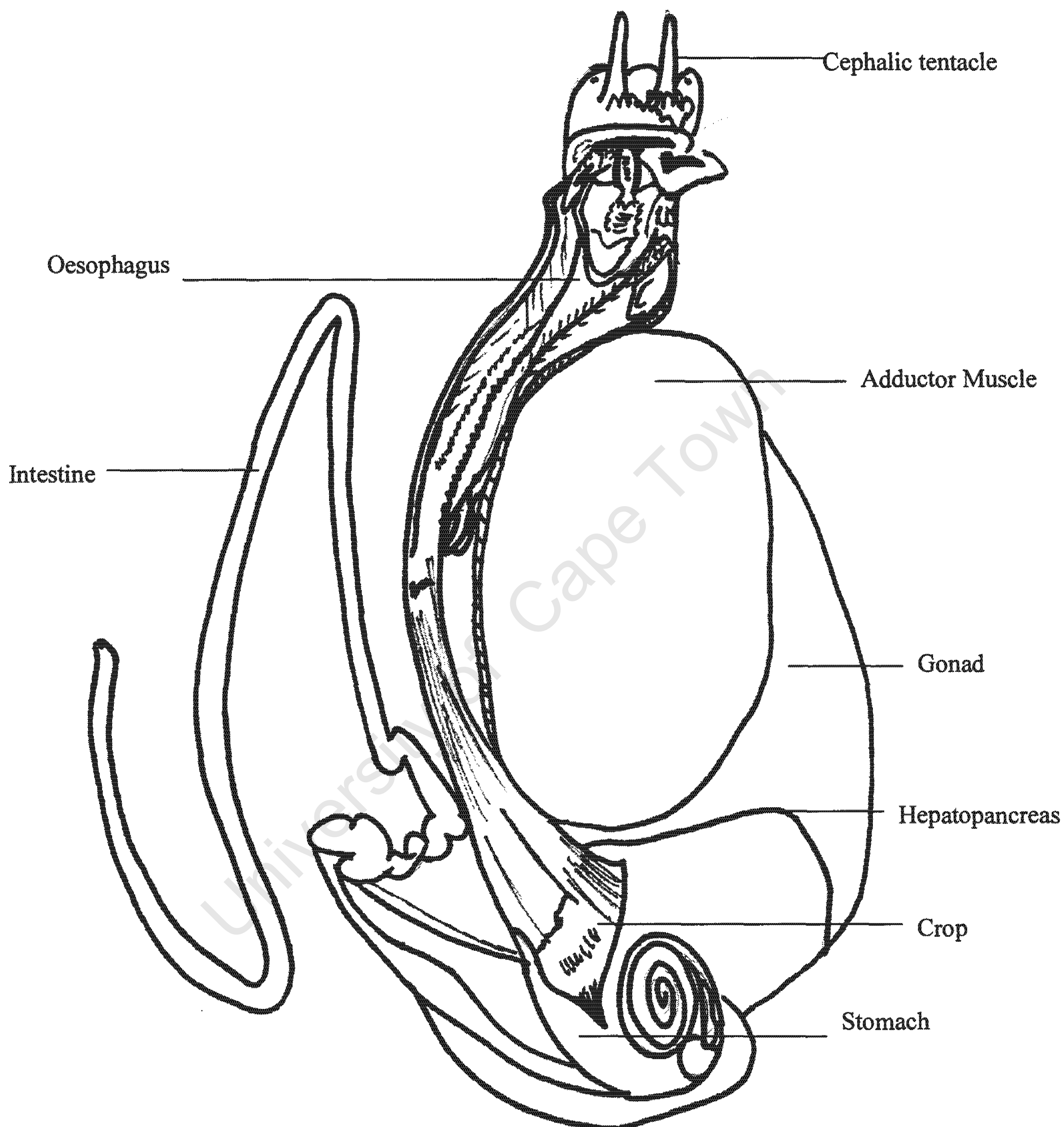
APPENDIX C3



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General dissection of the alimentary canal. The buccal cavity, oesophagus and stomach have been cut along the mid-dorsal line. Adapted from Ino (1953).

APPENDIX D
STATISTICAL DATA

Table D1 Statistical data for alginate lyase activity in the gastrointestinal tract of abalone fed different diets

Table D2 Statistical data for alginate lyase activity in different regions of the gastrointestinal tract in abalone fed a kelp diet supplemented with strain C4

Table D3 Statistical data for alginate lyase activity in different regions of the gastrointestinal tract in abalone fed a standard kelp diet

Table D4 Statistical data for immune parameters in abalone fed different diets

Table D5 Statistical data for immune parameters in abalone fed different diets and infected with *Vibrio anguillarum*

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The measurements of each sample were arranged in a contingency table as follows:

		Variable B				
		Situation 1	Situation 2	Situation 3	Situation 4	Total for Row
Variable A	Situation A	Sample 1 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 2 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 3 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 4 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	n_t $\sum x_t$ $\sum x_t^2$
	Situation B	Sample 5 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 6 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 7 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	Sample 8 n x s s^2 $\sum x$ $(\sum x)^2$ $\sum x^2$	n_t $\sum x_t$ $\sum x_t^2$
	Total for Column	n_t $\sum x_t$ $\sum x_t^2$	n_t $\sum x_t$ $\sum x_t^2$	n_t $\sum x_t$ $\sum x_t^2$	n_t $\sum x_t$ $\sum x_t^2$	n_T $\sum x_T$ $\sum x_T^2$

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The mean, standard deviation, the sum of the data were all calculated. The sums of the sample size (n), experimental values (x) and the square of the sum of the experimental values were calculated for each row and column. The procedure for computing ANOVA was then followed according to Fowler and Cohen (1990).

Table D1. F values for the effects of time, type of feed and the interaction between time and feed on alginate lyase activity in the gastrointestinal tract of *H. midae.*

	F(Var Feed)	*F(Var Time)*	*F(Var Interaction)*
Oesophagus	0.12	3.98	2.89
Crop	21.41	7.34	6.93
Stomach	1.58	15.17	1.08
Intestine	6.11	4.06	1.5

The null hypothesis that the variable does not have a significant effect on the data is rejected if the F value exceeds the tabulated F values at specific levels of significance. The tabulated F values at p=0.05 is 4.26 and at p=0.01 is 7.82. If the F values indicate a significant difference the Tukey Test is used to establish which means are different.

Table D2. F values for the effects of time, region of the gastrointestinal tract and the interaction between time and region on alginate lyase activity in abalone fed a kelp diet supplemented with strain C4.

F(Var GI Region)	*F(Var Time)*	*F(Var Interaction)*
87.5	20.63	15.31

The tabulated F value at p=0.01 is 6.99 therefore both time and region of the gastrointestinal tract have an effect on alginate lyase activity and there is an interaction between time and region of GI tract on alginate lyase activity.

Table D3. F values for the effects of time, region of the gastrointestinal tract and the interaction between time and region on alginate lyase activity in abalone fed a standard kelp diet.

F(Var GI Region)	*F(Var Time)*	*F(Var Interaction)*
7.34	2.03	0.69

The tabulated F value at p=0.01 is 6.99 therefore only the region of the GI tract has an effect on alginate lyase activity in abalone fed a standard kelp diet.

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Table D4. F values for the effects of time, type of feed and the interaction between time and feed on the immune parameters of *H. midae*.

	F(Var Feed)	*F(Var Time)*	*F(Var Interaction)*
Blood Counts	3.60	1.85	0.56
Phagocytosis	69.54	10.58	10.35
Respiratory Burst	0.34	2.52	5.46
Stimulated Respiratory Burst	142.50	200.25	420.00

The tabulated F value at p=0.05 is 5.99 for the blood counts and phagocytosis and is 233.99 for the respiratory burst and the stimulated respiratory burst. Therefore there is no significant effect on blood counts or respiratory burst by either time or type of feed. Both time and type of feed have a significant effect on phagocytosis and the interaction between time and type of feed significantly affects the stimulated respiratory burst.

Table D5. F values for the effects of time, type of feed and the interaction between time and feed on the immune parameters of *H. midae* infected with *Vibrio anguillarum*.

	F(Var Feed)	*F(Var Time)*	*F(Var Interaction)*
Blood Counts	92.95	42.56	17.16
Phagocytosis	192.62	63.26	19.94
Respiratory Burst	35.45	80.13	35.45
Stimulated Respirstory Burst	132.00	88.80	31.20

The tabulated F value for blood counts, phagocytosis and the stimulated respiratory burst at p=0.01 is 13.75 and the F value for the respiratory burst at p=0.01 is 8.47. Therefore both time and the type of feed have a significant effect on the immune parameters tested and there is an interaction between time and feed on blood counts, phagocytosis, the respiratory burst and the stimulated respiratory burst.

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