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ISOLATION AND CHARACTERIZATION OF A β(1-4) AGARASE OF AN EPIPHYTIC BACTERIAL PATHOGEN, *PSEUDOALTEROMONAS GRACILIS* B9, OF THE RED ALGA, *GRACILARIA GRACILIS*.

by

Declan Cosmo Schroeder

University of Cape Town

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

Cape Town
January 2001

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Declan Cosmo Schroeder
January, 2001

Department of Molecular and Cellular Biology, University of Cape Town,
Private Bag, Rondebosch, 7701, South Africa

ABSTRACT

The red alga, *Gracilaria*, has been an economically important seaweed for South Africa since the 1950's. A number of collapses experienced by the natural *G. gracilis* population in Saldanha Bay over the last decade made investors very cautious in expanding the industry by establishing commercial scale farms at Saldanha Bay. Thus, the future of mariculture of *G. gracilis* at Saldanha Bay depends on understanding and preventing *Gracilaria* collapses at Saldanha Bay. Jaffray and Coyne (1996) developed an *in situ* assay to positively identify putative bacterial pathogens of this macroalga. Of the epiphytic bacteria tested, a positive correlation between the agarolytic phenotype and bacterial pathogenicity was discovered. The aim of this study was to isolate the gene(s) that encode this agarolytic activity from an epiphytic bacterial pathogen of *G. gracilis* from Saldanha Bay. Upon characterization of the enzyme(s), the enzyme(s) would be used as a tool to elucidate the virulence mechanism, and thus, strategy employed by the bacterial in eliciting disease in *G. gracilis*.

Several different epiphytic bacterial isolates were isolated from the thallus surface of the agarophyte *G. gracilis* from Saldanha Bay. The bacterium B9 was selected for further characterization due to its ability to degrade agar. The bacterium B9 was shown to cause disease in *G. gracilis*, i.e. an *in vitro* assay positively identified the isolate B9 as a putative bacterial pathogen of *G. gracilis*. The 16S rRNA gene sequence analysis together with the comparative physical and phenotypic characteristics of the bacterium B9 with respect to related bacteria, suggested that it was a new member of the genus *Pseudoalteromonas*. The bacterium was designated *Pseudoalteromonas gracilis* B9.

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The growth characteristics of strain B9 and its production of agarolytic activity were tested in different media. It was consistently observed that production of the extracellular β-agarase is comparatively delayed in media containing a reducing sugar such as glucose or galactose. Although the production of extracellular β-agarase was delayed in the presence of a reducing sugar, higher agarolytic activity was observed in growth media supplemented with a reducing sugar than in non-supplemented media. Omission of agar in the basal media resulted in a 4.2 fold reduction of agarase production. Alternatively, substitution of agar with agarose saw a 2.5 fold reduction of agarolytic activity. Thus, *P. gracilis* B9 agarase production is also regulated by substrate composition. Agarase production was generally first detected during the early stationary phase of growth, reaching maximal levels during the late stationary phase of *P. gracilis* B9 growth.

A *P. gracilis* B9 genomic library was screened for agarolytic activity. Two *E. coli* transformants containing plasmids designated as pDA1 and pDA3, respectively, were positively identified as having agarolytic activity. Restriction endonuclease maps of pDA1 and pDA3 were constructed. The similarity shared between pDA1 and pDA3 was also confirmed with Southern hybridization. The agarolytic-encoding region was sub-cloned from pDA1 into the pBluescript KS cloning vector. The subsequent plasmid, pDA012, was sequenced and analyzed. A BLAST search in GENBANK database showed that the ORF located in the agarolytic region had 85% identity to the β-agarase (*dag*A) from *Pseudoalteromonas atlantica* ATCC 19262^T (or IAM 12927^T) at the amino acid level. Therefore, it was concluded that the 873 bp ORF encoded a putative β-agarase, and was designated *aag*A. AagA is a new member to the family 16 glycoside hydrolases. The putative promoter region of *aag*A was determined and Northern hybridization analysis showed that the mRNA transcript of *aag*A is 1.2 kb in length.

A novel β(1-4) agarase, which hydrolyzes the β(1-4) linkages of agarose to yield predominately neoagarotetraose was isolated from both the wild-type bacterium, *P. gracilis* B9, and an *E. coli* JM109 (pDA16) transformant. *In vitro* transcription/translation of recombinant plasmids encoding an agarolytic gene from *P. gracilis* B9, gave a single band (M_r, 31,500) on SDS-PAGE. The extracellular agarases were purified 35-fold and 22-fold from the wild-type *P. gracilis* B9 and *E. coli* JM109 (pDA016) transformant by using gel filtration chromatography and a combination of gel filtration and ion-exchange chromotography, respectively. Both purified proteins gave a single band (M_r, 30,000) following SDS-PAGE. The optimum temperature and

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pH for both enzymes was 50°C and pH 7, respectively. However, their stability profiles at 37°C were different. Thin-layer chromatography of the digestion products produced by both proteins showed that both enzymes have the same substrate specificity; i.e. they both hydrolyze the β-1,4 linkages of agarose to yield predominately neoagarotetraose, with saccharides the size of neoagarotetraose and smaller not subject to hydrolysis.

Polyclonal antibodies against the β-agarase purified from *E. coli* JM109 transformed with pDA16 were generated in a rabbit. Western hybridization confirmed that the cloned agarase was the extracellular β-agarase of *P. gracilis* B9. The anti-AagA antibodies cross-hybridized only to the 30 kDa band in the extracellular extract of *P. gracilis* B9, confirming that under the defined growth conditions, AagA is the only β-agarase secreted into the growth medium. The observed relationship between disease symptoms and the agarolytic phenotype of *P. gracilis* B9 was confirmed. Comparison of the infected alga to the unbleached thalli of healthy *G. gracilis*, showed a weakening of the cell structure in the former plants. Immuno-gold labeled antibodies localized the agarase *in situ* to the cell walls of bleached *G. gracilis*. The more severe the symptoms of bleaching, the greater the degradation of the cell walls was observed. Thus, the weakening observed in the cell structure was attributed to degradation of the mucilaginous component of the cell wall of the bleached thalli.

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ABBREVIATIONS

α	alpha
β	beta
Δ	delta
γ	gamma
λ	lambda
σ	sigma
ι	iota
κ	kappa
μ	micro
μg	microgram(s)
μl	microliter(s)
μm	micrometer(s)
μM	micromolar
$	US dollars
°E	degrees east
°S	degrees south
°C	degrees Celsius
%	percentage
‰	parts per thousand
A	adenine
Amp^r	ampicillin resistant
ATP	adenosine triphosphate
BA	Basal agar
BM	Basal media
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CHB	church hybridization buffer
Ci	Curie
cm	centimeter(s)
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
d	day(s)
dATP	deoxy-adenine 5'-triphosphate
dCTP	deoxy-cytosine 5'-triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxy-guanine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
dNTP	deoxy-ribonucleoside triphosphates (dATP, dCTP, dTTP and dGTP)
dry wt	dry weight
DTT	dithiothreitol
dTTP	deoxy-thymine 5'-triphosphate

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EDTA	ethylenediaminetetra-acetic acid
g	gram(s)
G	guanine
Glu	glutamic acid
h	hour(s)
ha	hectare(s)
k	kilo
K	thousand
kb	kilobase(s)
kcal	kilocalories
kDa	kilodalton(s)
kg	kilogram(s)
km	kilometer(s)
Km^r	kanomycin resistant
l	Liter(s)
LA	Luria agar
LB	Luria broth
m	meter(s)
M	molar
M	molar
mA	milli-Amperes
MCS	multiple cloning site (pBluescript KS)
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter(s)
mM	millimolar
mol	mole(s)
MOPS	(3-[N-morpholino]propane-sulfonic acid)
M_r	relative molecular mass
mRNA	messenger RNA
ng	nanogram(s)
nm	nanometer(s)
O/N	overnight
OD	optical density
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
per. comm.	Personal communication
PIPES	piperazine-*N*-*N*'-bis(2-ethanesulfonic acid)

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rDNA	ribosomal DNA
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second(s)
SDS	sodium dodecyl sulphate
sp.	species
SRU	Seaweed Research Unit of Marine and Coastal Management
SSC	sodium chloride tri-sodium citrate buffer
SSW	sterile seawater
STE	sodium chloride tris-EDTA buffer
T	thymine
^{T}	type strain
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane
U	unit(s)
US / USA	United States of America
UV	ultraviolet
V	volts
v	volume
W	Watt(s)
w	weight
WBA(B)	wash buffer A or B

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CHAPTER 1

GENERAL INTRODUCTION

CONTENTS

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1.1 The biology of the genus *Gracilaria*

Gracilaria is classified as a red alga, but has different colour manifestations (Figure 1A). It can be black, red, green or yellow. The thalli in this genus are terete, compressed, or flattened to foliose (Figure 1B) (Oliveira and Plastino, 1994). Simon (1977) eloquently describes the local species of *Gracilaria* found abundantly at Saldanha Bay as ramifying, stringy streamers, while the thalli look like branching, reddish-brown, bootlaces (Figure 1A(i)).

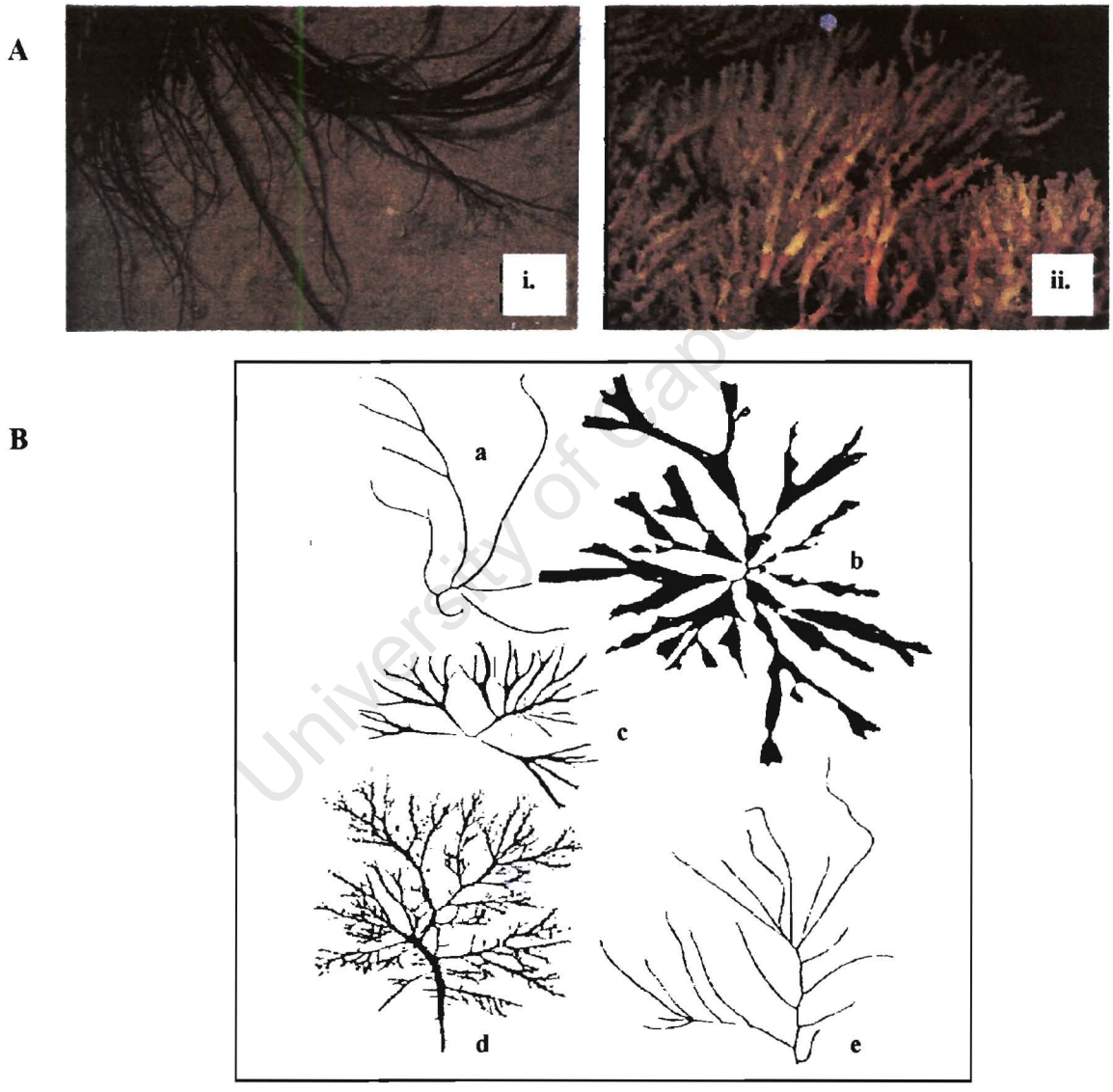

Figure 1 A) (i): *G. gracilis* with dark red terete thalli; (ii): *G. aculeata* with yellow-brown tubular thalli (Branch *et al.*, 1999). B) Basic frond morphology in *Gracilaria*; (a): *G. gracillis*; (b): *G. mammillaris*; (c): *G. foliifera*; (d): *G. cervicornis*; (e): *G. tenuifrons* (Oliveira and Plastino, 1994).

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1.1.1 The taxonomic position of *Gracilaria*

Garbary and Gabrielson (1990) published a historical account of the classification of red algae (Rhodophyta). Since the end of the nineteenth century, a major reclassification of this phylum of algae has been observed.

> "Many new taxa have been recognized at higher taxonomic ranks, and some traditional groups have been discarded or are undergoing major revision. New characters are being applied to classically defined groups, and the circumscriptions of these taxa are being modified." (Garbary and Gabrielson, 1990).

Over the last two decades, sophistication of technology has allowed scientists to explore and re-explore unstudied or poorly known groups. Garbary and Gabrielson (1990) believe that sufficient model systems are available so that taxonomic and evolutionary problems can be examined using a variety of ultrastructural, chemotaxonomic, developmental, and genetic approaches.

The taxonomy of the genus of interest in this study, *Gracilaria* Greville, is a good example of how various tools were employed by a variety of researchers. *Gracilaria* belongs to the family Gracilariaceae first established by Kylin in 1930. In 1932, Kylin shifted the family Gracilariaceae to the order Gigartinales (Oliviera and Plastino, 1994). However, Fredericq and Hommersand (1989) proposed that Gracilariaceae be separated from Gigartinales to form a new order, Gracilariales, in the same class, Florideophyceae. Gracilariales constitutes one of the largest orders in the Rhodophyta phylum, accounting for 42 families, 170 genera and over 1120 species (Murano, 1995). Gracilariaceae is well defined in terms of vegetative and reproductive anatomy (Fredericq and Hommersand, 1989). However, the taxonomic concepts within it have been unstable for years. This is attributed to the difficulty in delimiting the species in the large genus, *Gracilaria* Greville (Bird, 1995). A number of recent reviews deal with developments within the taxonomy of the family, Gracilariaceae (Bird and Rice, 1990; Oliveira and Plastino, 1994; and Bird, 1995). The most recent systematic compilation of genera in Gracilariaceae included *Gracilaria* Greville, *Gracilariopsis* Dawson, *Hydropuntia* Montagne, *Curdiea* Harvey, *Melanthalia* Montagne and the parasites *Gracilariphila* Wilson *et* Setchell *in* Wilson and *Congracilaria* Yamamoto (Bird, 1995).

The genus *Gracilaria* consists of 110 currently recognized species (Oliveira and Plastino, 1994) out of nearly 300 specific names proposed since the late 1700's (Bird, 1995). A number of

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molecular tools have aided and are aiding in the delineation of different *Gracilaria* species (Bhattacharya *et al.*, 1990; Bird and Rice, 1990; Bird *et al.*, 1990; Scholfield *et al.*, 1991; Bird *et al.*, 1994; Freshwater *et al.*, 1994; Goff *et al.*, 1994; Ragan *et al.*, 1994; and González *et al.*, 1996). The type species of *Gracilaria* Greville is *G. verrucosa* (Hudson) Papenfuss with its type locality in south England (Oliveira and Plastino, 1994). However, Steentoft *et al.* (1995) re-classified this British type species, which had also been previously referred to as *G. confervoides* (Stackhouse) Greville in Italy and southern Africa, and *G. verrucosa* (Hudson) Papenfuss in China, Japan and Argentina (Bird and Kain, 1995), as *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham. Hence the full current taxonomic classification of this important cosmopolitan seaweed is as follows:

DIVISION:	Rhodophyta
CLASS:	Florideophyceae
ORDER:	Gracilariales
FAMILY:	Gracilariaceae
GENUS:	*Gracilaria*
SPECIES:	*gracilis*

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1.1.2 The habitat characteristics of *Gracilaria*

The genus *Gracilaria* is widely distributed around the world. It occupies a variety of habitats, both in tropical and temperate latitudes. It has been noted that in temperate latitudes, some species can reach a density of between 1 to 7 kg m^{-2}. Such areas include the northern Adriatic Sea, Norway, the Atlantic coast of Canada, the east coast of U.S.A, California, Chile, Argentina, New Zealand and the west coast of southern Africa. In tropical latitudes, plant densities do not usually exceed 2 kg m^{-2}. Hence climatic conditions affect the ultimate densities reached by the seaweed (Santelices and Doty, 1989). Kain and Destombe (1995) found that in temperate regions growth is fastest and biomass highest in late summer, while biomass peaks mainly in the winter in the tropics.

Large amounts of *Gracilaria* are found on intertidal or shallow-subtidal, wave sheltered, horizontal or slightly inclined plane surfaces where there is unconsolidated, generally non-carbonate, sandy to muddy sediment. *Gracilaria* usually encounters and survives frequent fresh-water dilutions, high fertilizer regimes, very low water motion, as well as high temperatures and

burial in sediment. A number of species have been shown to be eurythermal and euryhaline (Santelices and Doty, 1989). Engledow and Bolton (1992) performed culture studies on *Gracilaria gracilis* from Saldanha Bay, South Africa (section 1.2) to ascertain the optimum range of environmental factors for growth. They showed that the plants grew well at 15-25°C (25°C optimal). The seaweed was shown to be euryhaline and grew well between 9 and 45‰ salinity (31‰ optimal). An irradiance of 80 μmol m^{-2} s^{-1} was sufficient for maximal growth.

Major accumulations of *Gracilaria* frequently occur as free-floating populations or as combinations of free-floating and temporarily attached thalli. They may become attached to a variety of surfaces such as other algae, mussels, shells, small stones etc., or simply become partially buried in soft sediments due to the dynamics of sandy bottoms. The attached populations usually show a three-phase life cycle (section 1.1.3), while the free-floating thalli are usually sterile (Isaac and Molteno, 1952; and Santelices and Doty, 1989).

1.1.3 The Life history of *Gracilaria*

Oliveira and Plastino (1994) and Kain and Destombe (1995) compiled reviews addressing the area under discussion, i.e. the life history of *Gracilaria*. Unless otherwise cited, this section is a compilation gained from both reviews.

The basic life history of *Gracilaria* is of the three-phase *Polysiphonia* type, i.e. they have morphologically identical diploid tetrasporophyte and haploid gameteophyte phases, the latter consisting of equal numbers of male and female plants (Figure 2). Of the *Gracilaria* species investigated, most have been shown to have a chromosome number of 24, although a few have been observed to have 32 (Cole, 1990). The carpogonial branch is fertilized *in situ* on the female gametophyte and the zygote develops as a third phase producing a diploid cystocarp entirely dependent on the female gametophyte. The female gametophytes with these cystocarp structures are called carposporophytes. The spermatia are released from spermatangial crypts on the male gametophyte. The resulting carpospores, the product of a single fusion of gametes, are numerous and genetically identical. Each spore can develop into a diploid tetrasporophyte in which reproduction involves meiosis, resulting in haploid genetically variable tetraspores. These tetraspores can develop into male and female gametophytes. This life history has been completed in culture, taking 5-12 months, in several *Gracilaria* species.

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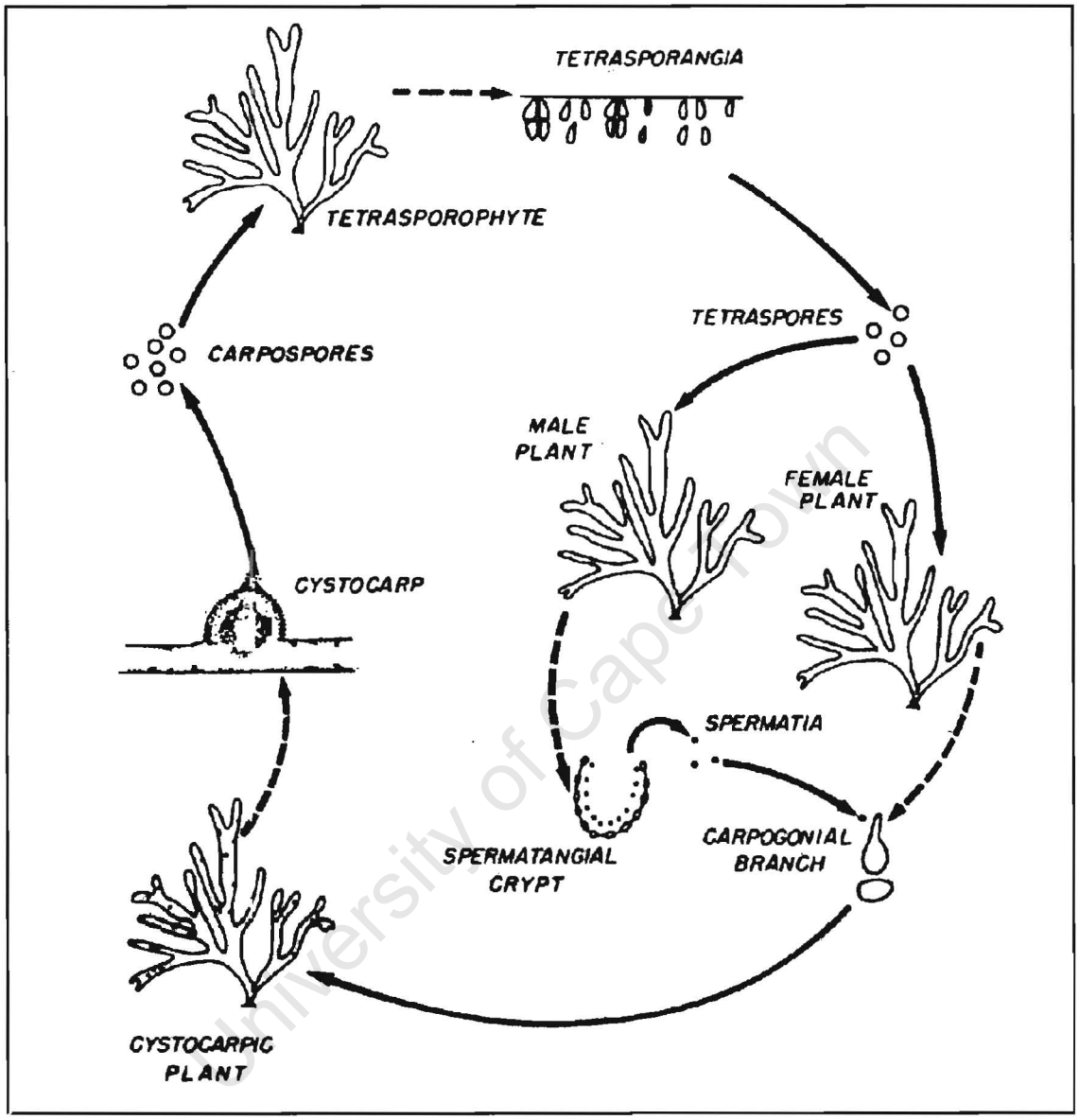

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Figure 2 A diagram of a *Polysiphonia*-type life history as reported in most species of *Gracilaria* (Oliveira and Plastino, 1994)

In natural populations of *Gracilaria* this afore-mentioned life cycle is not as unambiguous. Plants have been known to bear both gametangia and tetrasporangia. Explanations include the *in situ* germination of tetraspores, the coalescence of spores or developing discs, mitotic recombination during cell division in the mature diploid thallus, various mutations and initial failure of cell wall development of tetrasporangia. Polyploids can be produced from plants with diploid gametangia. Diploid juvenile plants have been observed to survive better than haploid plants. Female gametophytes have appeared to be more abundant than male gametophytes.

While the diploid and haploid phases are usually equal in abundance, the substratum plays an important role in balancing the phases. Plants that grow on a soft bottom are invariably diploid and grow vegetatively, since soft substrata are not conducive for spore development. The sterile populations can grow indefinitely and can be vegetatively propagated merely by fragmentation (Santelices and Doty, 1989). Hard substrata, however, are suitable for spore settlement and consequently, the alternating life cycle can be completed, allowing a mixture of phases in the population. Unattached populations were observed to be sterile. At high latitudes reproduction peaks in late summer whereas in the tropics it may remain high all year round.

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1.1.4 The cell structure and cell wall ultrastructure of *Gracilaria gracilis*

The following section deals firstly with the cell structure and secondly with the ultastructure of the cell wall of *Gracilaria gracilis* in particular. The general cellular features within the genus *Gracilaria* are similar, but for clarity I will focus mainly on *G. gracilis*, which is the species of interest in this thesis. Only the vegetative form of *G. gracilis* is discussed since the South African population of *Gracilaria* at Saldanha Bay is sterile and grows by vegetative propagation (section 1.2).

A cross-section through a mature vegetative thallus reveals two to three layers of small cortical cells. No distinct boundary separates these outer cortical cells from the large inner medullary cells (Figure 3A). The cortical cells are highly pigmented and the medullary cells are highly vacuolated and thick walled (Fredericq and Hommersand, 1989).

The cell wall, as defined by Craigie (1990), includes those polymeric materials originating through the metabolic activities of the alga and lying exterior to the plasmalemmal membrane.

The outer wall of the *G. gracilis* thallus has a three-layered structure, with the inner layer (the layer directly in contact with the plasmalemmal membrane) composed of roughly parallel, compact and circumferentially arranged microfibrils (Figure 3B). The inner layer (il) is covered by a thick middle layer (ml), which is composed of scarce microfibrils and abundant amorphous mucilage (Figure 3B). Finally, a thin electron-dense cuticle (cu) composed of sulphated polysaccharides covers the thallus surface (Figure 3B). The intercellular matrix (im) between the internal cells showed a loose fibrillar network, which is thought to support the amorphous mucilage (Figure 3C). The inner layer of the internal cells has a loosely arranged microfibrillar network (Figure 3C) (Mariani *et al.*, 1990).

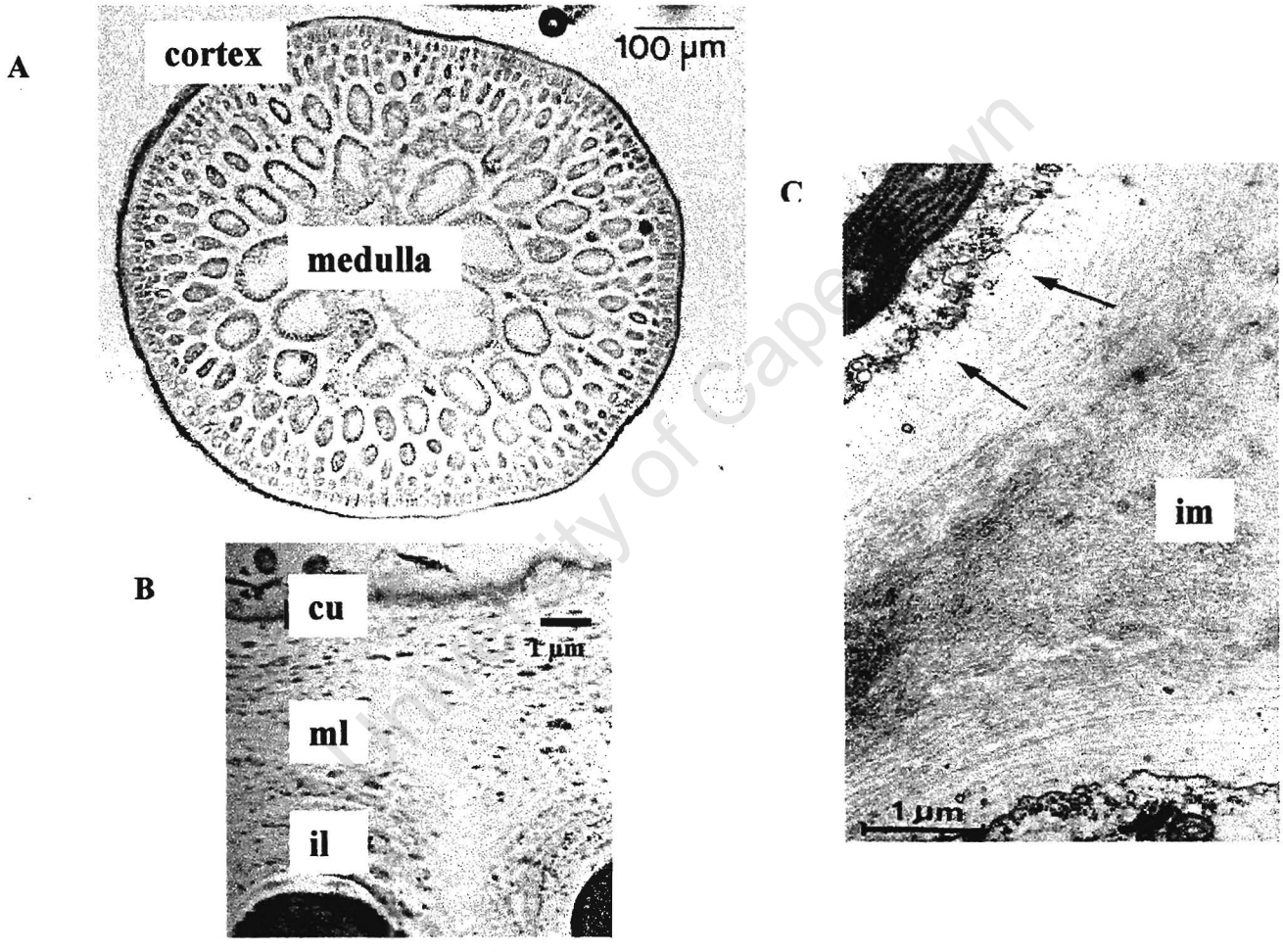

Figure 3 A) A cross-section through a mature vegetative thallus (Fredericq and Hommersand, 1989). B) A cross-section through the surface cells. In the outer layer of the thallus, the inner layer (il) is covered by a thick middle layer (ml) with a thin electron-dense cuticle (cu) (adapted from Mariani *et al.*, 1990). C) The intercellular region also contains a microfibrillar network within an amorphous mucilage, while the inner layer has a loosely arranged microfibrillar network (arrows) (Mariani *et al.*, 1990).

The two components, the fibrillar and the mucilaginous component, of the cell wall of *G. gracilis*, represent the polysaccharide constituent of the cell wall. Polysaccharides, proteoglycans, peptides, proteins, lipids and associated inorganic elements are recognized as constituents of the cell wall (Craigie, 1990). The fibrillar component is regarded as a non- or para-crystalline matrix in which the mucilaginous component is embedded (Mackie and Preston, 1974). The fibrillar part is composed mainly of a long glucose chain that results in a cellulosic network with xylose and mannose appearing as lateral chains along the cellulosic backbone. The mucilaginous component consists of agar (Bellanger *et al.*, 1990). Relatively little is known of the other constituents of algal cell walls (Craigie, 1990).

The cellulosic network, being rigid in nature, plays an important role in the structure and hence strength of the thallus. The mucilaginous agar component, due to its flexibility, has a subtler structural role. Agar is a common name for a family of polysaccharides obtained from some red algae known as agarophytes (Armisen, 1995). The first proposed step of the agar biosynthetic pathway is considered to be the formation of a regular backbone, consisting of 3-linked β-D-galactose and 4-linked α-L-galactose units, termed agaran (Figure 4A). The second is the sulphation of several hydroxyls together with other substitutions that may also be introduced at this stage. The final step is the enzymatic elimination of the sulphate from position 6 of a few 4-linked α-galactose residues to give 3,6-anhydro-α-galactose residues; termed agarose (Figure 4B) (Knutsen *et al.*, 1994). The other substitutions may be O-methyl-ether and/or pyruvic acid ketal groups (Rees, 1972; and Duckworth and Yaphe, 1971).

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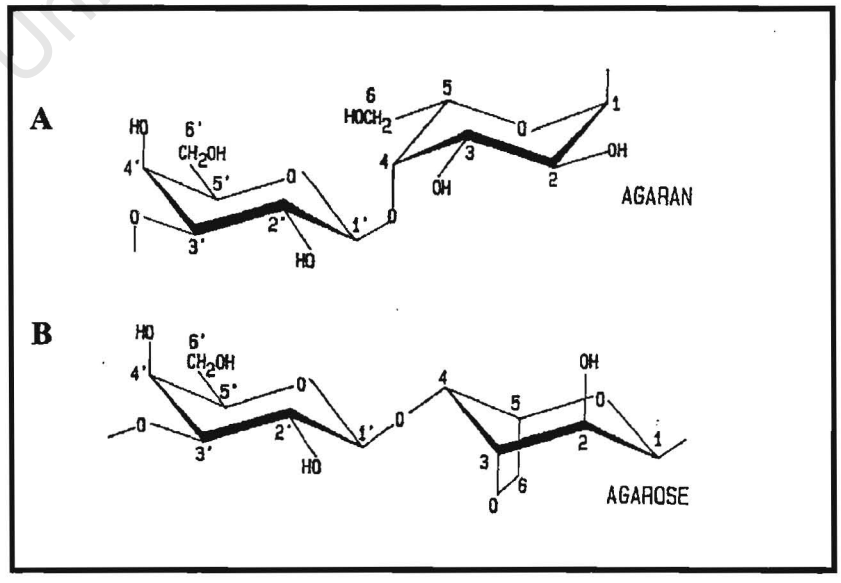

Figure 4 The repeating backbone structures that constitute agar polymers (Knutsen *et al.*, 1994).

The agaran form of agar has never been isolated from natural sources (Knutsen *et al.* 1994). Izumi (1972) characterized the chemical heterogeneity of agar from *G. gracilis* as various intermolecular forms between the two extreme types of macromolecules, that is, a non-sulphated, highly methylated galactan and a non-methylated, highly sulphated galactan. Christiaen *et al.* (1987) showed that *G. gracilis* cultivated under growth promoting conditions consists mainly of charged polymers, while agarose becomes the major compound when the seaweed is cultivated under poor growth conditions, i.e. formation of 3,6 anhydrogalactose from the sulphated galactose. Thus, within a seasonal cycle of growth, a continuous turnover of agar polymers in the cell wall of *G. gracilis* can occur. The changes in agar composition play an important structural function. In the case of high concentrations of agarose precursors, the network structure of the polysaccharide gel in the matrix is low, allowing for cell wall expansion, i.e. short thinner fragments at higher temperatures as a consequence of high growth rates during summer. In contrast, when the agar has a high content of agarose, the three-dimensional structure of the molecule forms a rigid cell wall, i.e. thick thallus observed at low temperatures due to slow growth rates during winter. The strong gel obtained under winter conditions may contribute to the physical resistance of plants growing under adverse environmental conditions (Christiaen *et al.*, 1987).

The overall sulphate content of *Gracilaria* agars is generally less than 10% (w/w). The presence of 6-*O*-methyl-D-galactose is widespread in *Gracilaria*, while methylation of the 3,6-anhydro-L-galactose residue is less extensive. However, in a few *Gracilaria* species including *G. gracilis*, an unusual type of methylation was proposed to occur. Single 4-O-methyl-α-L-galactose residues are (1-6) linked as a branch to the β-D-galactose residue of the main agar chain. Pyruvic acid is not frequently detected in agars from *Gracilaria* and its content is generally below 1.0% (w/w) (Murano, 1995). These charged polymers are also thought to play a minor role in controlling ion exchange between the medium and cytoplasm (Christiaen *et al.*, 1987; and Mariani *et al.*, 1990). The degree of sulphation, methylation and pyruvylation of the agarose also affects commercial interest in the agar produced from *Gracilaria* (Murano, 1995).

1.1.5 The various uses of *Gracilaria*

People have been using seaweeds or their extracts for thousands of years. However, westerners do not usually use seaweed as food, as do Orientals, but use seaweed extracts such as agar in

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their food preparations (Abbott, 1996). Abbott divides the uses of seaweed in general into two areas, "ethnic uses" and "industrial uses".

1.1.5.1 Ethnic uses

Species of the genus *Gracilaria* are used in the Philippines as a salad called gulamon; to which fresh tomatoes, onions and ginger are added. The Japanese pour boiling water over it, and then serve it with vinegar sauce. The Hawaiians chop up the seaweed and add coarse salt, while the Tahitians grate fresh coconut or coconut cream over the seaweed (Abbott, 1996). *Gracilaria* is not only for human consumption. For example, a percentage of the tons of *Gracilaria* produced per year is marketed for feeding maricultured abalone (McHugh, 1984 and 1991).

1.1.5.2 Industrial uses

A publication by Chapman and Chapman (1980) describes the extensive uses of agar in a number of industries. Agar can be used as a lubricant of tungsten wire for electrical lamps. It has been used as a substitute for gelatine in the photographic industry for making plates and films. Agar can also be employed in the finishing processes of leather manufacture, in order to impart a gloss and stiffness. It is sometimes a constituent of high-grade adhesives, and is used in the manufacture of plywood.

In western countries, it has an important use in beer brewing and in the manufacture of wines and coffee, where the agar is used as a clarifying agent. It has been used as a base in shoe-stains, shaving soaps, cosmetics and hand lotions. In war times, it was used in connection with wound dressings since it assists in blood clotting. In dentistry it can be employed for making impressions of gums for manufacturing false plates. Agar has also been employed in the making of moulds required by those who model plaster of Paris and for the casting of artificial legs. Other uses are as a raw material in making linoleum; artificial leathers and silks; as an insulating material against sound and heat; as an ingredient in water-based paints and in the manufacture of storage batteries for submarines (Chapman and Chapman, 1980). Many of the afore-mentioned applications of agar are not always in use. Either other gelling agents like alginate or carrageenan can be used as a substitute or the industry no longer requires its use.

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In cooking, agar is invaluable for thickening soups and sauces, whilst considerable quantities are used for making fruit jellies. It is widely employed in both Europe and America as a thickening agent in the manufacture of malted milks, jelly, candies and pastries. Agar functions as a stabilizer in the preparation of ice-creams, sherbets and cheeses. It has also been used in the manufacture of cream cheeses and in making icings, custards and mayonnaise. "Imperishable milk" was made in the USA by separating the cream from the solids, which was then emulsified with agar. This process removed most of the material upon which bacteria grow and so kept the milk fresh. Agar is largely indigestible, and thus does not modify the nutritive value of the foods in which it is incorporated. It is also useful as roughage. It is sometimes used in place of pectin for making jellies, jams, marmalade and preserves. In China, traveling mobile canteens sell faintly scented agar jelly as a dessert. In Japan, it is often cooked with rice to form "agar rice" (Chapman and Chapman, 1980). The 'strip' and 'square' form of agar, known as Kanten, produced from *Gelidium* is very popular in Japan. Many substitutes for Kanten have been developed such as Ogo produced from *Gracilaria*, but its quality is not as good as Kanten. However this Japanese 'natural agar' can sometimes also be prepared from a combination of different seaweeds. Agar extracted from *Gracilaria* is also used in the meat and fish canning industries (Isaac, 1942; and Abbott, 1996). Agar also has a very valuable laxative action. In China and Japan it was used for gastrointestinal disorders. In 1955 the USA Dispensatory list had it listed as a laxative (Chapman and Chapman, 1980).

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In the above described food industry, agar is usually divided into two grade categories: food-grade and 'sugar reactive' grade. The 'sugar reactive' agar is so called due to the increase in gel strength due to the addition of sugar (Armisen, 1995). Currently, *Gracilaria* species represent the most important source of food and 'sugar reactive' agars (Murano, 1995). The additional advantage of using seaweed extracts such as agar, as opposed to non-seaweed derived hydrocolloids in the food industry, is that there are no documented cases of people being allergic to seaweeds, their extracts or their derivatives (Abbott, 1996).

Another grade of agar not yet mentioned is the bacteriological grade. In 1882, Dr Koch formally announced the use of agar as a new solid culture medium for microorganisms. It is still the media of choice for general microbiological growth and identification (Renn, 1990). However, the bacteriological agars are usually prepared from *Gelidium* and *Pterocladia* because their agars can maintain fairly critical gelling and melting points, which are necessary for the preparation of

media. For example, *Gelidium* agars have gelling temperatures in the range of 34-38°C, while *Gracilaria* agars have gelling temperatures between 40-52°C. The difference in temperatures at which the agar solution gels is mainly due to the methoxyl groups in *Gracilaria* agars (Murano, 1995; and Abbott, 1996).

A particular derivative of agar called 'agarose', is a 'purified agar' whose chemical structure is similar to that of agarose as defined earlier in section 1.1.4 except that it is usually not as pure. It is generally prepared from *Gelidium*, *Pterocladia* and sometimes from *Gracilaria* depending on the species and degree of sulphation and pyruvylation of the agar. The degree of sulphation affects the gelling strength of the agarose, while the degree of pyruvylation affects the electrical properties of the gel and, in particular, the electroendosmosis of the agarose (Knutsen *et al.*, 1994; Murano, 1995; and Abbott, 1996). Almost all reported applications of agarose could be characterized as biotechnology-oriented. They fall into five main categories: electrophoresis, immunology, microorganism culture, chromatography, and immobilized systems technology (Renn, 1990).

The significance of agar in the food industry can be seen from the revenue generated in the USA during 1993 (Figure 5). *Gracilaria* production that accounts for most of the food grade agar is dominated by Chile, with about 50% of the world market. Almost half of that *Gracilaria* came from introduced cultivated stocks (McHugh, 1991). Other nations such as Japan, China, South Africa, Philippines, Indonesia, Namibia, Argentina, Brazil, USA etc. also contribute to the commercial availability of *Gracilaria* either from wild-stocks or cultivated stocks or both (McHugh, 1991; and Armisen, 1995).

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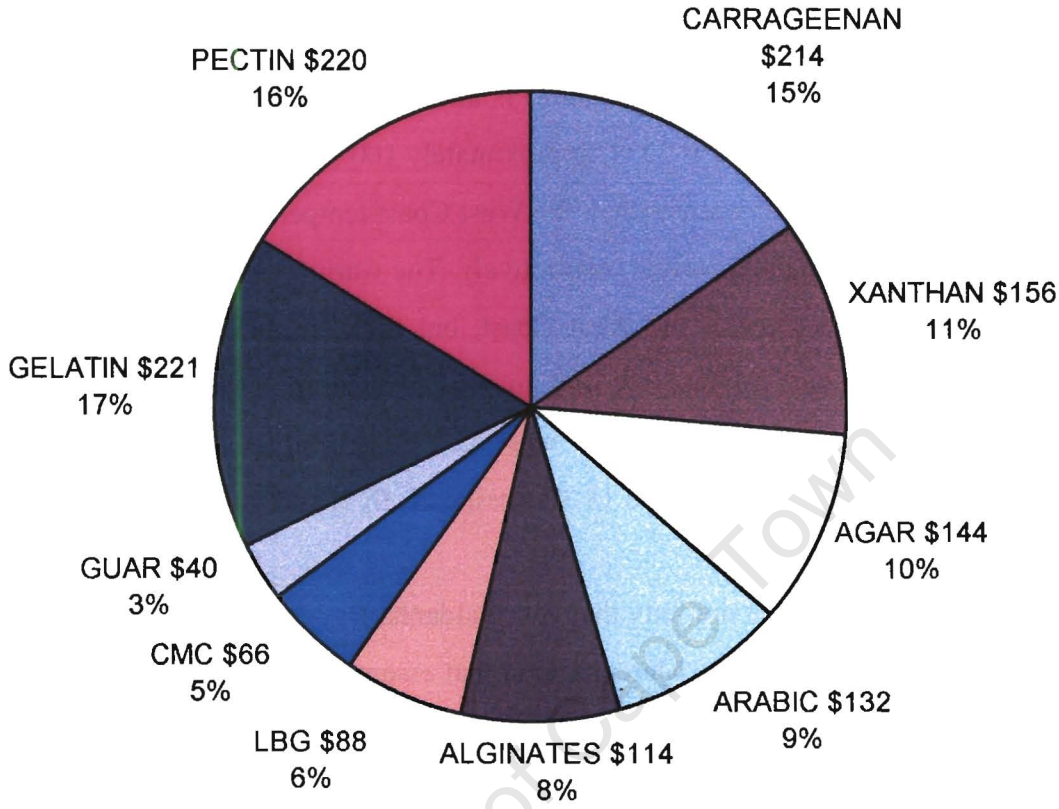

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Figure 5 Revenues (in million of US dollars) from different food hydrocolloids. A total of US$ 1500 million was generated in 1993. CMC: carboxymethyl cellulose, LBG: locust bean gum (Bixler, 1996).

1.2 The history of *Gracilaria gracilis* in the Saldanha-Langebaan area

1.2.1 Location

Saldanha Bay (including Langebaan Lagoon) is an almost land-locked embayment on the west coast of South Africa, about 18°E 33°S, approximately 100 km north of Cape Town (Figure 6A). East Coast temperatures are warmer than the West Coast temperatures due to the warm Agulhas current and the cold Benguela current, respectively. The warmer waters are poorer in phosphates and nitrates than the waters of the West Coast, but they are more saline (Isaac, 1942). The temperature at the mouth of the Bay was monitored over the 1973-1982 period. The mean temperature recorded over this period was 15.4°C, while the minimum and maximum temperatures recorded was 13.1°C and 18.3°C, respectively (Bolton, 1986).

Since the construction of the ore-jetty that split Saldanha Bay into a Small and Big Bay, and the construction of the breakwater to Marcus Island that essentially enclosed Small Bay (Figure 6A), the environmental dynamics changed drastically at Saldanha Bay, especially in Small Bay. In Small Bay during spring and summer (September–March) southerly winds cause upwelling on the adjacent open coast and cold bottom water is advected into the bay, so that stratification develops. This stratification is at its strongest mid summer and thus a strong thermocline occurs where a warm water layer (17-20°C) overlays a cold layer (10-12°C) (Figure 6B). The cold water from the Benguela current is high in nitrate (5-20 μM) while the warmer stratified upper water becomes poor in nitrates (~0 μM). This thermocline was shown to shift between 5 and 10 m every 6-7 days. During autumn and winter (April-August), northerly winds in combination with low air temperatures destratify the bay and thus the water column is well mixed at temperatures between 12-14°C (Anderson *et al.*, 1996b). Stratification of Saldanha Bay had an enormous impact on both the natural and cultivated populations of *Gracilaria* in the bay (section 1.2.2.2).

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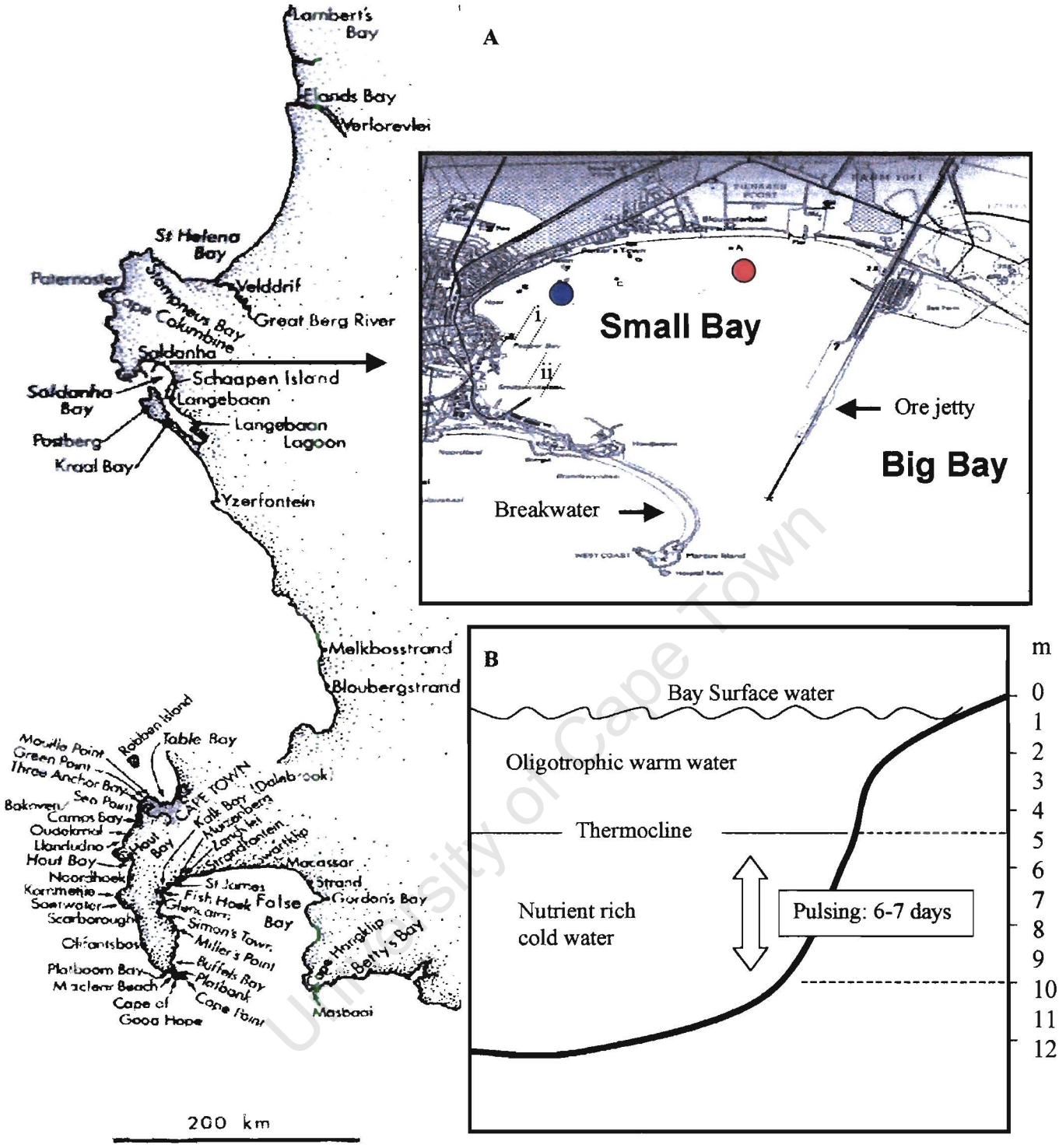

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Figure 6 A) West coast of South Africa. Saldanha Bay is highlighted. i) and ii): positions of the two fish-factory waste plumes (adapted from Stegenda *et al.*, 1997). ●: site of raft near factory (i). ●site of raft away from factory (i).
B) Schematic diagram of summer stratification in Small Bay, Saldanha. Depth in meters (m) is shown (adapted from Anderson *et al.*, 1996b).

1.2.2 Population fluctuations of natural *Gracilaria*

Simons' assessment of the natural populations in the Saldanha-Langebaan area was that Langebaan Lagoon and Saldanha Bay are independent systems and that the source of the beach-casts originated from the respective *Gracilaria* populations occurring in each location (Anderson *et al.*, 1989).

1.2.2.1 Langebaan Lagoon Area

The history of *Gracilaria* in the Saldanha-Langebaan area goes back centuries. In a review of the Algal Flora of Saldanha Bay by Simons in 1977, we read that Thunberg collected the seaweed as far back as 1772. Simons wrote,

> "if any plant typifies Saldanha Bay it is this one. It is because it is present there in phenomenally enormous quantities. *Gracilaria verrucosa* (= *G. gracilis*) is, to all intents and purposes, the only algal macrophyte in the lagoon; it occupies a significant area of the entire bay".

Other records reviewed by Simons such as Barton's records (1893; 1896), Tyson's collections (1913), Isaac's records (1937; 1956), the University of Cape Town's records (1955), Day's records (1959), and his own records (1960, 1976) all bear testimony to the abundance of the seaweed.

A survey made by Isaac in 1942 indicated which species with potential economic value occur on the shores of South Africa in terms of their geographical distribution and abundance. Of the agarophytes, *G. gracilis* was considered the most economically important since it was found to occur in considerable quantity in the protected waters of the lagoon near Langebaan. It was reported in 1936 that this alga was washed up in such large amounts that it formed an extensive bank a few feet across and three to four feet high for a distance of about two miles (Isaac, 1942).

In 1952, Isaac and Molteno reported that the seaweed occurred naturally both as free-floating in the water and weighed down by sand or entangled with mussels on the sandy floor banks of the lagoon. However, the free-floating form comprised the majority of the biomass. The sterile vegetatively reproducing form of the seaweed was mostly observed, although a few fertile forms have been reported (Isaac and Molteno, 1952).

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A survey of the *Gracilaria* population at Langebaan Lagoon, conducted by Isaac (1956), revealed that the *Gracilaria* was found most abundantly in sandy rather than muddy bottoms at depths varying between 0 and 3 m below low water spring tides, although plants were found as deep as 9 m. He also charted the "attached" rather than the loose drifting plants in Langebaan lagoon (Anderson *et al.*, 1989). Simons (1977) indicated that the "attached" plants had expanded their area of cover in the lagoon since Isaac's 1956 survey.

Christie (1981) reported highest biomass and production of *Gracilaria* in the Langebaan area at the entrance of the lagoon, where favorable temperatures and particularly nutrient conditions prevail. Approximately 342,7 ha of seaweed (equivalent to 2130 tons (dry wt)) was observed in the southern section during December. He estimated an annual production of 22 500 tons (dry wt) could occur based on photosynthetic production measurements. However, this calculation did not take decay, grazers and self-shading into account (Anderson *et al.*, 1989).

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1.2.2.2 Small Bay, Saldanha Bay

Simons (1977) noticed that the northern end of Saldanha Bay (now referred to as Small Bay), where sandbanks similar to those of the lagoon are absent, seems to contain *Gracilaria* floating just above the bottom. Isaac (1956) also reported the existence of these beds of *Gracilaria* that were actively growing 2-6 m below the water surface. These beds were believed to be the main source of the extensive *Gracilaria* beach-casts, especially between the months of November and March, on the northern shores of Small Bay.

In 1974, the seaweed population collapsed. The collapse was believed to be due to dredging and marine construction operations (section 1.2.1) (Anderson *et al.*, 1989), since biomass accumulation depends on biological as well as abiotic factors such as illumination, temperature, salinity, water movement, substratum and nutrients (Pizarro, 1986). These factors were severely affected during the construction of the ore-jetty and breakwater. The *Gracilaria* recovered in 1987 with a yield of 170 tons (dry wt) but this was far short of the 1000 tons (dry wt) recovered annually before construction work began in the Bay (Anderson *et al.*, 1989).

The resource at Saldanha Bay collapsed again when beach-casts ceased abruptly after December 1988. By October 1989 the patchy *Gracilaria* beds that occurred between the depths of 3-9 m

had disappeared, leaving only occasional single strands of plant material protruding from the sediment. Sea urchins and keyhole limpets were abundant at depths greater than 4 m. They were also associated with large filter feeding ascidians. In grazer-exclusion experiments, the sea urchins and limpets consumed more than 10% of transplanted seaweed in 24 h. In shallow water (1-2 m) fish consumed about 80% of the transplanted seaweed overnight. The grazers were believed to be responsible for the reduction in *Gracilaria* during 1989, but the reason for the initial collapse that occurred at the end of 1988 remained unclear. It was postulated that the unusually large beach-cast during July of that year might have led to an imbalance in the system, allowing the effects of grazers to outweigh production by the plants. In February 1992, extensive beds of *Gracilaria* at depths of 3-9 m re-established themselves (Anderson *et al.*, 1992).

In 1993, the yield of beach-cast material improved and 377,9 tons (dry wt) were collected. In August 1993, *Ulva lactuca* occurred for the first time in Small Bay, contaminating the *Gracilaria* beach-casts and rendering the harvest useless. Two fish processing factories that discharge ammonium rich waste into Small Bay were shown to play an important role in the occurrence of the *Ulva* (section 1.2.4). Figure 6A shows the locality of the waste plumes produced by the factories (Anderson *et al.*, 1996b).

The natural population of *Gracilaria* collapsed again during October-December 1996. The reason for the collapse could not simply be explained by low nitrogen conditions, herbivores or poor weather conditions. Disease was postulated as a possible cause of the collapse (Anderson *et al.*, 1999).

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1.2.3 The South African *Gracilaria* export industry

Initially, the *Gracilaria* beach-casts caused a public nuisance as they rotted and authorities spent large amounts of money removing the refuse. However, during World War II (1939-1945) it became known that the seaweed was a rich source of agar. Attitudes rapidly changed and export of the seaweed commenced (Simon, 1977).

As a consequence of World War II when agar from Japan became unavailable, South Africa produced 20 tons (dry wt) of agar locally in 1951, almost all being *Gracilaria* collected at Saldanha Bay. Ninety percent of this was used in the meat-canning industry. The local

production ceased shortly afterwards for economic reasons, but resumed in the 1960's with two factories functioning until 1974. When the supply of beach-cast collapsed, ostensibly as a result of dredging and the construction of a large ore-loading jetty at Saldanha Bay, the factories were closed (Anderson *et al.*, 1989). The *Gracilaria* recovered in 1987 with a yield of 170 tons (dry wt) but this was far short of the 1000 tons (dry wt) recovered annually before construction work began in Saldanha Bay. Due to the large swells in July, unusually large beach-casts were collected and thus a high tonnage was recorded in 1988. The seaweed destined for Japan was to be used in the production of "natural agar". This seaweed produces a high quality agar and hence commands a high price (Rotmann, 1990). Between 1984 and 1993, the South African *Gracilaria* industry exported an average (per annum) of 698 tons of *Gracilaria* to Japan, which was equivalent to an average of US$ 1 million (Armisen, 1995). The industry employs between 60 and 100 people in the collecting and exporting of the seaweed (Anderson *et al.*, 1996b).

Since 1997, average yields of 400 tons (dry wt) of *Gracilaria* from beach-casts are being obtained (Anderson per. comm.). However, it was the belief of many since the days when Southern Africa supplied the international markets of the world with 4% red algae and 0.7% of agar, that this could be doubled if agar could be extracted at Saldanha Bay and if farming was introduced into Saldanha Bay (Anderson *et al.*, 1989). Additionally, the natural population is very unstable and cultivation seems the only viable option that can be implemented in order to sustain the *Gracilaria* industry at Saldanha Bay (Anderson *et al.*, 1992). The Seaweed Research Unit of Marine and Coastal Management (SRU) embarked upon a number of pilot studies over the last decade to determine the feasibility of phycoculture at Saldanha Bay.

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1.2.4 Population fluctuations of cultivated *Gracilaria*

Of the farming methods tested by the SRU, rope rafts were found to generate consistently high yields. Over a two-month growth experiment, the *Gracilaria* grew 10% per day in the first month while during the second month, a growth rate of 5% per day was obtained. A third month of growth was not feasible since epiphytes and grazers negatively affected the crop. It has been estimated that a net weight of 16 kg m^{-2} can be produced on the rafts (Anderson *et al.*, 1992).

Over the period May 1993 to February 1994, rafts were placed in the proximity of (blue dot) and away (red dot) from the plumes of factory (i) (Figure 6A). The rafts near the plumes of factory (i)

had its *Gracilaria* population replaced by an *Ulva* population. In comparison, the rafts placed away from the factory plumes generated good healthy *Gracilaria* populations with no *Ulva* contamination, except at depths greater than 3 m. The best growth was observed at a depth of 5.5 m, which is consistent with natural populations where maximum densities occurs at depths between 7 and 8 m. The fish factories discharge their waste between January and August. In the winter months, the ammonium rich fish waste is mixed thoroughly in Small Bay and thus becomes diluted. However, the discharge of fish waste becomes concentrated in the upper layer of warm water in early spring. *Ulva* has a high surface/volume ratio, takes up nitrogen rapidly, but has a low nitrogen storage capacity. By contrast, *Gracilaria* has a low surface/volume ratio, takes up nitrogen at a slower rate than *Ulva* when the nitrogen levels are high, but can use low ambient nitrogen, and stores nitrogen longer than *Ulva* does. Hence, the conditions experienced between August 1993 and February 1994 in the upper stratified layer (section 1.2.1) of Small Bay led to *Ulva* out-competing the raft cultivated *Gracilaria* (Anderson *et al.*, 1996b).

In March 1994, rope cultivated *Gracilaria* collapsed at a depth less than 0.5 m below the water surface. Persistent high temperatures (21°C) from late-February to early March, and strong southerly winds caused strong stratification of Small Bay. Christie (1981) suggests that nutrients, especially nitrates, are the most important growth-regulating factor of *Gracilaria*, even though *Gracilaria* can utilize low levels of ambient nitrogen. This was thought to be the reason for the collapse since the natural population (below 5 m) was for the most part unaffected. Cultivation of *Gracilaria* near the water surface was also shown to be possible. Growth trials conducted at a depth of 0.2 m out performed the 1.15 m growth trials. Greater surface water motion was thought to cause improved nutrient mixing and thus better growth. However this was limited to autumn and winter since massive die-offs were experienced at those depths during spring and summer (Anderson *et al.*, 1996a).

Gracilaria cultivated at Lüderitz, Namibia generated yields of 45 tons (dry wt) per ha. In Saldanha Bay, over a two-year period, an average growth rate of 5% per day was obtained. This is equivalent to 2.2 kg m^{-2} 30 d^{-1}. This is in turn is equivalent to an annual yield of 39.6 tons (dry wt). This is somewhat lower than the yield of 45 tons (dry wt) per ha of *Gracilaria* generated at Lüderitz. However, if the process was scaled-up, i.e. larger stocking weight could increase the yield by 26%, mariculture of *Gracilaria* would be economically feasible in Saldanha Bay. It is estimated that eleven hectares of cultivated seaweed would be equivalent to the current beach-

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cast yield. In addition, an agar yield of 17% of the dry weight of *Gracilaria* was reported all year round at Saldanha Bay (Anderson *et al.*, 1996a).

Both the natural *Gracilaria* population and rope cultivated *Gracilaria* collapsed during October-December 1996. The cultivated seaweed thrived a few months later, December 1996-February 1997. The weather conditions during these latter months were very similar to those experienced during the collapse, and thus stratification and low nitrogen conditions were not the only major role players in the initial collapse. The rafts located near the high nitrogen site (near plumes of factory (i)) had lower growth rates than the rafts situated away from the factory over the recovery period. The carbon to nitrogen ratio of the seaweed was similar at both sites all year round, suggesting that a sufficient nitrogen supply existed for the algae located away from the factory site. Localized disease at the rafts was not ruled out as a possible cause of the collapse (Anderson *et al.*, 1999).

1.3 The bacterial epiphytes of macroalgae

Macroalgae serve a number of important functions for marine bacteria in the seawater column. Algal thalli provide not only an ecological attachment site for the bacteria, but a rich source of nutrients compared with nutrient-poor seawater. As a result of continuous movement and abrasion, damaged thalli are a constant source of organic compounds in the form of algal structural products such as agar, alginate and carrageenan, as well as algal reserve products such as starch, fats etc. (Rheinheimer, 1984; Mow-Robinson and Rheinheimer, 1985). These algal products are broken down into proteins and sugars, which are utilized by the bacteria. Algal thalli also provide dissolved organic carbon, which is released as a result of photosynthesis (Sieburth, 1969; Brylinsky, 1977; Jensen *et al.*, 1996).

Bacteria associated with marine macroalgae occur in considerably larger numbers than are found free in the seawater column (Laycock, 1974; Mow-Robinson and Rheinheimer, 1985; Austin 1988; Jensen *et al.*, 1996). It has also been shown that their distribution on the macroalgae varies according to the state of thallus decomposition. On *Laminaria* thalli which have decomposing frond tips, the number of bacteria colonizing that area is much higher that those colonizing the middle stem and the meristem area (Laycock, 1974; Mazure and Field, 1980). Cundell *et al.* (1977) examined the apical tips of *Ascophyllum nodosum* and showed that there was a complete

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absence of microorganisms on the apical tips. The authors suggested that the rate of secretion of tannin-like compounds at the apical tips of the thalli was much higher than in the mid-region, hence the absence of bacteria. Jaffray *et al.* (1997) reported a similar observation where no epiphytes were observed near the growth tips of *G. gracilis*. The total lack of bacteria, or low numbers of bacteria, which has been observed on various thallus areas (not only apical tips) may be due to the secretion of a variety of antibacterial compounds produced by algae (Sieburth, 1964a, Sastry and Rao, 1994). Rosell and Srivastava (1987) discovered that a large number of fatty acids were secreted from macroalgae as antimicrobial substances. Small amounts of organic acids and polyphenols, which have some antimicrobial activity, were also detected in algal extracts. Both Booth and Hoppe (1985) and Corre *et al.* (1989) showed that young algal thalli are relatively free of bacteria, as opposed to older thalli which host much higher numbers.

There seems to be seasonality in epiphytic bacterial numbers in that higher bacterial numbers are associated with macroalgae during summer months, when water temperatures are raised (Kong and Chan, 1979). Shiba and Taga (1980) reported that bacterial numbers were also influenced by the physiological condition of the algal tissue. Lemos *et al.* (1985) showed that a large number of epiphytic bacteria, assigned to the *Pseudomonas-Alteromonas* group, on the seaweed *Enteromorpha intestinalis*, exhibited antibiotic activities which affected the growth of other epiphytic bacteria. It is possible that this mechanism has been adopted as a strategy to prevent colonization by competing strains.

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Bacteria, like any other transmissible agent (fungi, nematodes, other algae or viruses), can be infectious (Andrews, 1976). Symptoms of disease are exhibited permanently in the alga. Some infectious diseases of macroalgae may be considered a multi-pathogen phenomenon (Andrews, 1976 and Correa, 1996). Craigie and Correa (1996) have shown that an infectious disease induced in cultivated *Chondrus crispus* could have resulted from the interaction of several organisms including bacteria, fungi, algal endophytes and nematodes.

Andrews (1976) summarized the descriptions of three seaweed diseases attributed to bacterial action. Since 1976, only a few other macroalgal diseases attributed to bacteria have been described. Gall-like malformations found on red algae, *Prionitis decipiens* and *Polyneuropsis stolonifera*, were attributed to bacteria present inside the gall tissue (McBride *et al.*, 1974). Apt (1988) described gall formations due to bacterium on a few *Gracilaria* species. Correa *et al.*

(1993) also reported gall development due to cyanobacterium in the red algae *Iridaca laminarioides*.

'Ice-ice', a white powdery growth observed on a *Eucheuma* species was thought to be caused by bacteria (Uyenco *et al.*, 1981). The same disease identified in *Eucheuma denticulatum* and *Kappaphycus alvarezii* was triggered by bacteria in laboratory experiments (Largo *et al.*, 1995). Agarolytic bacteria belonging to the *Cytophaga*, *Flavobacterium* and *Vibrio* genera were identified as possible infectious agents. In 1992, Lavilla-Pitogo reported symptoms of white to pinkish discolorations known as 'rotten-thallus' syndrome caused by agarolytic bacteria on the tetrasporophytic stage in a *Gracilaria* species. Similarly, an agarolytic bacterium, designated *Cytophaga* sp. LR2, caused disease in the red alga, *Rhodella retriculata* (Toncheva-Panova and Ivanova, 1997).

Whitening of thalli tips progressing to thalli disintergration of *Gracilaria conferta*, known as 'white tip disease', was first reported by Friedlander and Gunkel (1992). Environmental factors such as high temperatures, high plant density and lack of aeration were identified as major contributing factors to this disease. However, several agarolytic bacteria, could also induce these symptoms in healthy seaweed. Weinberger *et al.* (1994) showed that a bacterial strain designated OR-I1, was responsible for the disease symptoms. Weinberger *et al.* (1997) showed that a large number of bacterial isolates were capable of inducing apical necrosis on the macroalga. However, the authors found that under normal conditions a balance is usually struck between bacterial epiphytes and the macroalga. Only when the equilibrium is disturbed, does infection of *G. conferta* by bacteria occur.

A large proportion of epiphytic bacteria have been found to be capable of degrading the algal thalli they colonize in order to liberate utilizable dissolved organic matter. In the North Sea and the Kiel Fjord, as well as Tolo Harbour in Hong Kong, agar-degrading bacteria have been found predominantly in association with red, agar-containing algae, and alginate and cellulose decomposers have been found most commonly where brown and green macroalgae exist, respectively (Kong and Chan, 1979; Rheinheimer, 1984; Mow-Robinson and Rheinheimer, 1985; Reiper Kirchner, 1989). Booth and Hoppe (1985) suggested that bacteria living in association with certain macroalgae might have developed special enzymatic and nutrient uptake characteristics for specific substrates of macroalgal origin.

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Jaffray *et al.* (1997) isolated a large number of bacterial epiphytes from the natural population of *Gracilaria gracilis* collected in February 1994 from Saldanha Bay. Thirty four percent of the bacteria isolated were agarolytic. Jaffray and Coyne (1996) developed an *in situ* assay to detect bacterial pathogens of this macroalga and found that of the bacteria tested, all the pathogenic isolates were agarolytic. Weinberger *et al.* (1999) showed that *G. conferta* responded with an oxidative burst, a rapid increase in respiration and halogenation, when it detected the breakdown products of agar. *Gracilaria* recognized the intermediates of agar degradation as messengers when microbial degradation of its cell wall occurs.

1.3.1 Agarolytic bacteria

Gran first described agar-degrading bacteria in 1902 (Yaphe, 1957). In the last century, a large number of agarolytic bacteria have been identified from a variety of habitats, although mostly from marine environments. Yaphe and co-workers were the first to describe an agar-degrading enzyme system from a marine bacterium (Day and Yaphe, 1975; and Groleau and Yaphe, 1977). Other scientists in the field expanded and defined the enzymes and their specificities involved in this system of agar hydrolysis (Morrice *et al.*, 1983a and b). The agar degrading enzyme system was that of a bacterial isolate obtained from a specimen of *Rhodymenia palmate* collected at Halifax, N.S., Canada in 1952 (Yaphe, 1957). The bacterium was classified as *Pseudomonas atlantica* ATCC 19262^T but was later renamed to *Pseudoalteromonas atlantica* ATCC 19262^T by Gauthier *et al.* (1995). The agar-degrading pathway of *P. atlantica* ATCC 19262^T is described in detail in sub-section 1.3.1.1, while agar-degrading enzymes from other bacteria are discussed in comparison to the *P. atlantica* ATCC 19262^T agarase in sub-section 1.3.1.2.

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1.3.1.1 Agar-degradation pathway of *Pseudoalteromonas atlantica* ATCC 19262^T

The pathway of agar metabolism in this organism (Figure 7) involves the initial cleavage of the agarose (alternating 3-*O*-linked β-D-galactopyranose and 4-*O*-linked 3,6-anhydro-α-L-galactopyranose) moiety of agar by an endo-acting enzyme, β-Agarase I, yielding neoagaro-oligosaccharides limited by the disaccharide, neoagarobiose unit [*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-*O*-β-D-galactose], but with predominance of the tetramer, neoagarotetraose [*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-3,6-anhydro-α-L-galactopyranosyl-(1→3)-D-galactose], as the major end product. β-Agarase I

was shown to be specific for regions containing a minimum of one unsubstituted neoagarobiose unit, hydrolyzing the β(1→4) linkages at the reducing end of this moiety. It was shown to be able to slowly degrade the neoagarohexaose but not the neoagarotetraose. β-Agarase I is the predominant extracellular species acting on solutions of agar and higher neoagaro-oligosaccharides as well as upon agar in gel states. It is postulated that in the wild the bacterium would partially degrade the mucilaginous gel in an agarophyte with the extracellular β-Agarase I. The production of fragments larger than the disaccharide would restrict other marine microorganisms, which although lacking agarases, would be able to metabolize the disaccharide (Morrice *et al.*, 1983a and b; and Belas *et al.*, 1988).

The neoagarotetraose, the major end product of β-Agarase I, is cleaved at its central β(1→4) linkages by a neoagarotetraose hydrolase yielding neoagarobiose. However, this enzyme was also shown to be able to degrade species of oligosaccharides, larger than neoagarotetraose, and hence it was given the name β-Agarase II. It was shown to hydrolyze agar by an endomechanism to produce neoagaro-oligosaccharides, hexasaccharides, tetrasaccharides and neoagrobiose, with neoagarobiose being the limiting and predominant species. However, β-Agarase II preferentially and rapidly degrades the hexa- and tetrasaccharide species from the non-reducing end to produce neoagarobiose units. The enzyme is specific for sequences containing neoagarobiose and an exo-action was observed in these rapid degradations. It was evident that the β-Agarase II would be specific for agar molecules in solution type states. Thus in the wild, the products of β-Agarase I, hexasaccharides and predominantly the tetrasaccharide, would be degraded by the cell bound β-Agarase II in an exo-mechanism, thus providing the bacterium with neoagarobiose (Morrice *et al.*, 1983a and b; Groleau and Yaphe, 1977; and Belas *et al.*, 1988).

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The third and final enzyme in the agar-degrading system of *P. atlantica* is a neoagarobiose hydrolase that cleaves the α(1→3) linkage in neoagarobiose to yield the monomeric sugars D-galactose and 3,6-anhydro-L-galactose. This enzyme is also cell bound and has been localized to the outer region of the cell, on or outside the cytoplasmic membrane in the periplasmic space. The enzyme is specific for neoagarobiose. Higher species such as the tetrasaccharide and the agar polymer itself are not degraded by neoagarobiose hydrolase. It is very specific for the glycone, 3,6-anhydro-L-galactose. The sugars produced by this enzyme are presumed to be transported by the cell across its cytoplasmic membrane to the cytoplasm where they are metabolized (Day and Yaphe, 1975; Groleau and Yaphe, 1977; and Belas *et al.*, 1988).

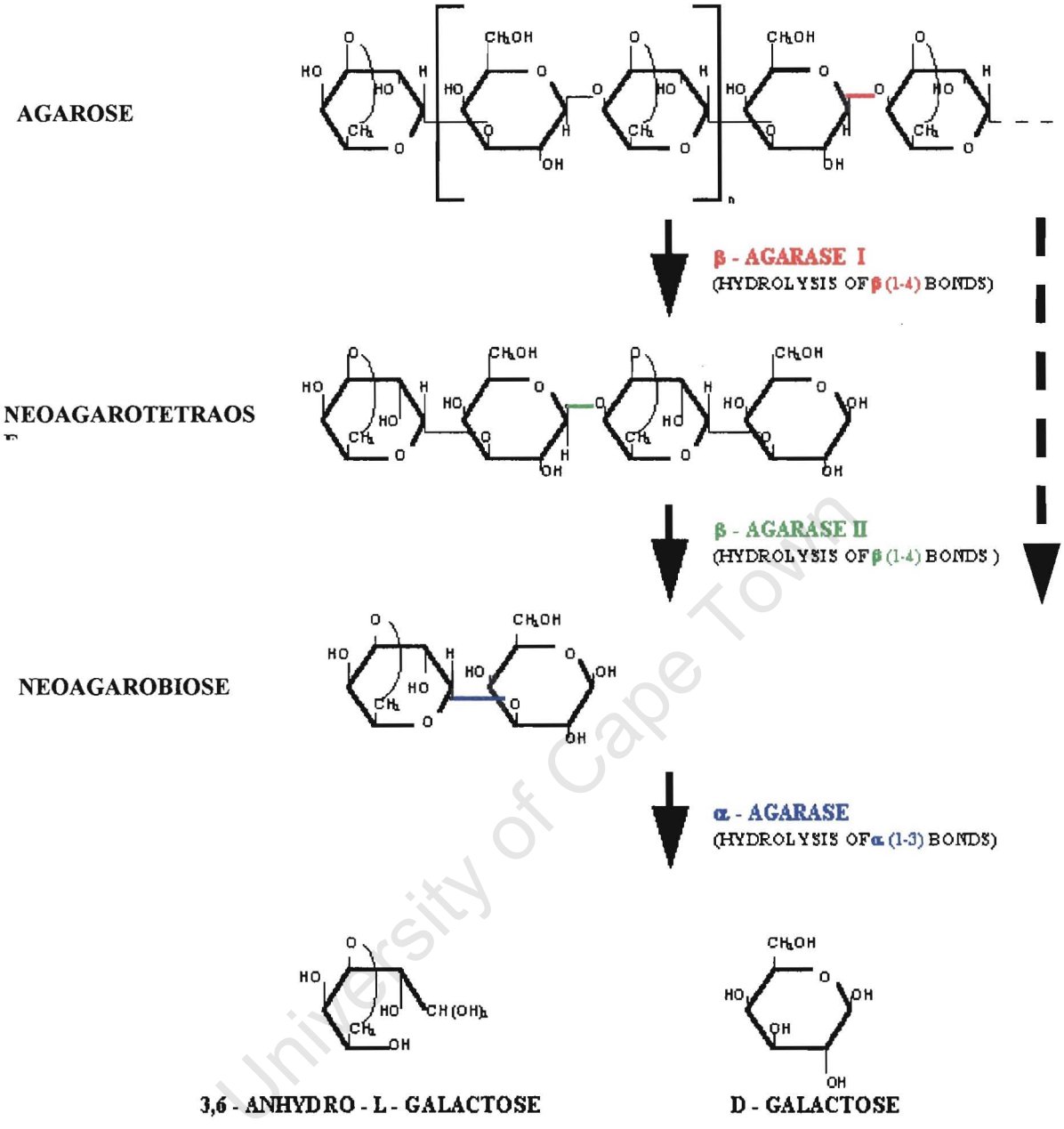

Figure 7 Enzymology of agar digestion in *P. atlantica* ATCC 19262^{T}. The polymer (agarose) is initially cleaved by the extracellular enzyme, β-agarase I (DagA). This enzyme cleaves the polymer at the β(1→4) linkage, producing predominately neoagarotetraose. This tetrameric saccharide is then broken down to neoagarobiose through the action of neoagarotetraose hydrolase, also referred to as β-agarase II. β-agarase II is also capable of digesting oligomeric agar, cleaving it at the β(1→4) linkage and producing neoagarobiose. Neoagarobiose is cleaved at the central α(1→3) linkage by α-neoagarobiose hydrolase to produce the monosaccharides (adapted from Belas *et al.*, 1988).

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The first enzyme in the degradation of agar in the afore-mentioned system, β-Agarase I, was purified by Morrice *et al.* (1983b) from the bacterial growth medium. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated a single protein with a molecular weight of 32 000. Both the crude extracellular extract (Yaphe, 1975) and the purified β-Agarase I (Morrice *et al.*, 1983b) showed optimum activity over a pH range of 5 to 8 with an optimum activity for the crude extract at pH 6 and the purified protein at pH 7. Thus the enzyme is optimally functional at neutral pH's. The enzyme was shown to be stable at temperatures at and below 30°C over a 2 h period. At higher temperatures of 40°C and above, the enzyme is unstable with increasing instability the higher the temperature (Morrice *et al.*, 1983b). The enzyme is specific for agar in neutral or substituted forms such as porphyran and does not act on polysaccharides such as carrageenan (Yaphe, 1957; Morrice *et al.*, 1983a and c). In 1996, the nucleotide sequence of the gene encoding the β-Agarase I, DagA, was submitted to the GENBANK database under the accession M73783. The gene was designated *dag*A.

The second enzyme, β-Agarase II or neoagarotetraose hydrolase, was purified by Morrice *et al.* (1983b) from the soluble fraction of disrupted *P. atlantica* cells. The pH optimum range of 5.5 to 7.5 and the NaCl optimum range of 0.1 to 0.2 M were similar to that obtained by Groleau and Yaphe (1977). The enzyme was specific for agar in neutral or substituted forms such as porphyran (Morrice *et al.*, 1983a). To the best of my knowledge, this enzyme has not been cloned and sequenced to date.

Day and Yaphe (1975) purified the third enzyme, neoagarobiose hydrolase, from the soluble fraction of disrupted *P. atlantica* cells. Optimum activity occurred over a pH range of 7 to 8, indicating a neutral to alkali range of activity. Sucrose density gradient centrifugation indicated that the enzyme has a molecular weight of around 10 000 (Day and Yaphe, 1975).

1.3.1.2 Agarases isolated and characterized from other bacteria

An extracellular enzyme has been isolated from the bacterium *Cytophaga* sp. NCMB 1327. The enzyme degrades both agar and porphyran. The optimum conditions for the enzyme are at a pH of 7.2 and a temperature of 40°C. The enzyme degrades agarose at the β(1→4) linkages in an endo-action to produce predominantly neoagarotetraose but cannot degrade neoagarotetraose or neoagarobiose. It preferentially degrades the neoagarooctaose at its central β(1→4) linkages to

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produce neoagarotetraose as in the case of the β-Agarase I type mechanism, but the production of neoagarohexaose and neoagarobiose can also occur at a much slower rate (Duckworth and Turvey, 1969a, b and c).

Sampietro and Vattuone de Sampietro (1971) characterized the extracellular extract of the soil bacterium *Agarbacterium pastinator* 2AC. Of the polysaccharides tested, only polysaccharides with agar moieties were degraded. The enzymatic action was optimum at pH 7. The β(1→4) linkages were proposed to be cleaved by the enzymatic system present in the extract. Neoagarotetraose and neoagarobiose were the predominant species produced from agar degradation.

Cytophaga flevensis is an agarolytic bacterium isolated from Ijsselmeer, a fresh water lake. The extracellular agarase was purified and shown to have a molecular weight of 26 500. The enzyme was found to be a β-agarase, which hydrolyses agarose by an endo-acting mechanism to produce neoagarotetraose and neoagarobiose. Neoagarooctaose was cleaved to preferentially produce neoagarohexaose and neoagarobiose, thus cleaving the exterior β(1→4) linkages and not the central β(1→4) linkages as in the case of the β-Agarase I type mechanism of *P. atlantica* ATCC 19262^{T}. Optimal conditions for its activity were pH 6.3 and 30°C. The enzyme was specific for agar moieties since other polysaccharides such as the carrageenans were not hydrolyzed. However, this enzyme did not hydrolyze porphyran (van der Meulen and Harder, 1975). Two other enzymes were characterized from *Cytophaga flevensis*. They were both localized to the cytoplasm. The one enzyme was a neoagarotetraose hydrolase, which hydrolyzed the central β(1→4) linkages of the neoagarotetraose to produce neoagarobiose. Optimal conditions for the activity of this enzyme were pH 7.0 and 25°C. The other enzyme, a neoagarobiose hydrolase, hydrolyzed the central α(1→3) linkages of neoagarobiose to produce the monomers. Its optimal conditions for activity were pH 6.75 and 25°C (van der Meulen and Harder, 1976).

Two agarases, agarase I and agarase IIb, were isolated from filtered extracts of an agar-degrading *Pseudomonas*-like bacterium isolated from a sewage treatment plant. Molecular weight determinations indicated agarase I to be a dimer with a combined molecular weight of 210 000. The molecular weight of the monomer agarase IIb was 63 000. Both the enzymes were functional at neutral pH's with optimal activity at pH 6.7, while the optimal temperature was 38°C for agarase I and 43°C for agarase IIb. The agarase IIb was shown to cleave β-linkages in a

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mechanism similar to the β-Agarase I endo-mechanism described for DagA in *P. atlantica* ATCC 19262^T where the enzyme cleaved neoagarooctaose to produce predominantly neoagarotetraose. The agarase I could do the same but could also hydrolyze the substrate to produce neoagarohexaose and neoagarobiose species. The neoagarohexaose is very slowly degraded and hence the neoagarohexaose and neoagarotetraose species predominate upon agarose digestion. Thus this *Pseudomonas*-like bacterium possesses two extracellular agarases that act synergistically to hydrolyze agar to a non-gelling mixture of oligosaccharides (Hofsten and Malmqvist, 1975; and Malmqvist, 1978).

An extracellular agarase gene (*dag*A) was cloned from the Gram positive soil bacterium *Streptomyces coelicolor* A3(2) into the host strain *Streptomyces lividans* 66. The agarase has a molecular weight of 28 000 on SDS-PAGE (Kendall and Cullum, 1984). The sequence of *dag*A predicts a primary translation product of 309 amino acids with a molecular weight of 35 132 (accession number P07883/X05811). Cleavage of the proposed signal sequence would yield a mature protein of 279 amino acids with a molecular weight of 32 239 (Buttner *et al.*, 1987). Bibb *et al.* (1987) also reported the cloning and purification of *dag*A, with the protein having a molecular weight of 29 000. The agarase was active over the pH range of 5 to 8.5, with an optimum activity at pH 7.5. Parro *et al.* (1997) also reported the purification of DagA, with a molecular weight of 32 000 and an activity range of pH 5 to 8 and 25 to 50°C.

Belas *et al.* (1988) cloned another agarolytic enzyme from *Pseudoalteromonas atlantica.* However, their *Pseudoalteromonas atlantica*, strain T6c, was different to the ATCC 19262^T strain. Southern analysis of their cloned agarase gene, *agr*A, showed that it hybridized to DNA from the parent strain (T6c) and also to another *P. atlanica* strain WY but not to DNA from the *P. atlantica* ATCC 19262^T strain. The enzyme was transported across the bacterial cell wall and into the surrounding environment, and consequently, it was considered to have β-Agarase I activity. Site-directed mutagenesis of *agr*A confirmed that the cloned enzyme encoded the primary extracellular agarase of T6c. Belas *et al.* (1989) published the sequence of *agr*A (accession number M22725/P13734), which predicts a primary translation product of 504 amino acids with a molecular weight of 57 486. Morrice *et al.* (1983b) reported the β-Agarase I (DagA) of strain ATCC 19292^T to have a molecular weight of 32 000, thus contrasting the molecular weight predicted for the extracellular agarase (AgrA) from strain T6c and confirming the Southern hybridization data.

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β-agarase was purified from a culture supernatant of a marine bacterium designated *Vibrio* sp. AP-2. The enzyme had a molecular weight of 20 000; a pH optimum of 5.5; was stable over a pH range of 4 to 9 and at temperatures below 45°C. The enzyme has an endo-type cleavage mechanism, which hydrolyzes neoagarotetraose and larger neoagaro-oligosaccharides and agar to give neoagarobiose as the predominant product. The enzyme did not degrade carrageenan (Aoki *et al.*, 1990).

Another four agarolytic enzymes isolated from a *Vibrio* species were described. The bacterium was designated *Vibrio* sp. JT0107. An extracellular agarase of 107 000 molecular weight was purified from the supernatant. The enzyme was found to be an endo-type β-agarase which hydrolyzes the β(1→4) linkages of agarose to yield neoagarotetraose and neoagarobiose. The pH optimum was around 8 and the optimum temperature was 30°C. The enzyme was not capable of degrading sodium alginate or any of the three forms of carrageenan (κ-, ι- and λ-). The enzyme is stable at temperatures below 40°C (Sugano *et al.*, 1993a). This agarase was cloned into *E. coli*, where the mature protein was shown to be composed of 975 amino acids with a molecular weight of 105 271. The agarase gene was designated *aga*A (accession number P48839/D14721), while the protein was referred to as agarase 0107 or AgaA. This enzyme was shown to degrade neoagarotetraose into neoagarobiose, different to the β-Agarase I type action (Sugano *et al.*, 1993b). A second agarase gene, *aga*B (accession number D21202/P48840), was cloned into *E. coli*. AgaB encoded a protein of 955 amino acids and exhibited high homology to AgaA cloned from strain JT0107 (Sugano *et al.*, 1994). A third agarase, agarase 0072, was purified, 39-fold, from the extracellular culture media of *Vibrio* sp. JT0107. Amino acid sequence analysis revealed that the 72 kDa protein (on SDS-PAGE) was a novel bacterial agarase. This agarase hydrolyzed the β(1→4) linkages of agarose to yield neoagarotetraose and neoagarobiose. The optimum pH and temperature of the enzyme were at around pH 8 and 30°C. The enzyme did not degrade sodium alginate or any of the three forms of carrageenan (κ-, ι- and λ-) (Sugano *et al.*, 1995). A fourth enzyme, a novel α-neoagarooligosaccharide hydrolase (α-NAOS hydrolase), was isolated and purified, 383 fold, from the cultured cells of *Vibrio* sp. JT0107. The purified protein gave a single band of 42 kDa on SDS-PAGE. Estimation of the molecular mass by the gel filtration method gave a value of 84 kDa, indicating that the enzyme is dimeric. The enzyme hydrolyzes the α(1→3) linkages of neoagarooligosaccharides to yield agaropentaose, agarotriose, agarobiose, 3,6-anhydro-L-galactose and D-galactose. The optimum temperature and

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pH were 30°C and 7.7, respectively. The enzyme was shown to be stable at temperatures at and below 30°C over a 2 h period (Sugano *et al.*, 1994).

An agarolytic bacterium, *Alteromonas* sp. C-1, was isolated from the Bay of San Vicenta, Chile. The production of agarase was repressed by glucose, with a parallel decrease in bacterial growth. The enzyme was purified and has a molecular weight of 52 000, is salt sensitive, and hydrolyze agar, yielding neoagarotetraose as main product, with an optimum pH of about 6.5 (Leon *et al.*, 1992).

Four agarolytic bacterial strains, belonging to the genus *Microscilla*, were isolated from a tar-ball that drifted ashore at Santa Barbara, U.S.A. The agarases from the four isolates had optimal activities at pH 6.5-6.8, 0.3-0.6 M NaCl and 27-37°C. The molecular masses of the agarases were estimated as 66-68 kDa (Naganuma *et al.*, 1993).

An α-agarase was purified from the culture supernatant of a marine bacterium designated *Alteromonas agarlyticus* GJ1B. This bacterium was capable of liquefying the gel and generating holes, until the colony reaches the bottom of the plate. The enzyme was specific for the α(1→3) linkages present in agarose, producing agarotetraose species as the major end product. This confirmed the findings of Young *et al.* (1978) using the partially purified agarase fraction of bacterium GJ1B. SDS-PAGE indicated a single protein with a molecular weight of 180 000. The enzyme functions as a dimer of approximately 360 kDa. The enzyme is active over a pH range of 6 to 9, with an optimum at pH 7.2. The enzyme is rendered unstable by prolonged treatment at pH's below 6.5, or by temperatures above 45°C, or by removing calcium from the medium. The enzyme was not capable of degrading agarohexaose, agarotetraose or any of the three forms of carrageenan (κ-, ι- and λ-). Upon purification of the α-agarase, β-galactosidase activity was present in all the fractions obtained prior to the final purified α-agarase. The partially purified enzyme hydrolyzed agarose to produce four main products; i.e. agarohexaose, agaropentaose, agarotetraose and agarotriose. The authors proposed a model to describe the mechanism of agar hydrolysis by the extracellularly secreted enzymes of *Alteromonas agarlyticus* GJ1B (Figure 8). Both enzymes require the presence of 3,6 anhydro-L- galactose units at the ends of the reaction products (Potin *et al.*, 1993). In 1999, the nucleotide sequence of the gene encoding the α-agarase was submitted to the GENBANK database under the accession AF121273. The gene was designated *aga*A.

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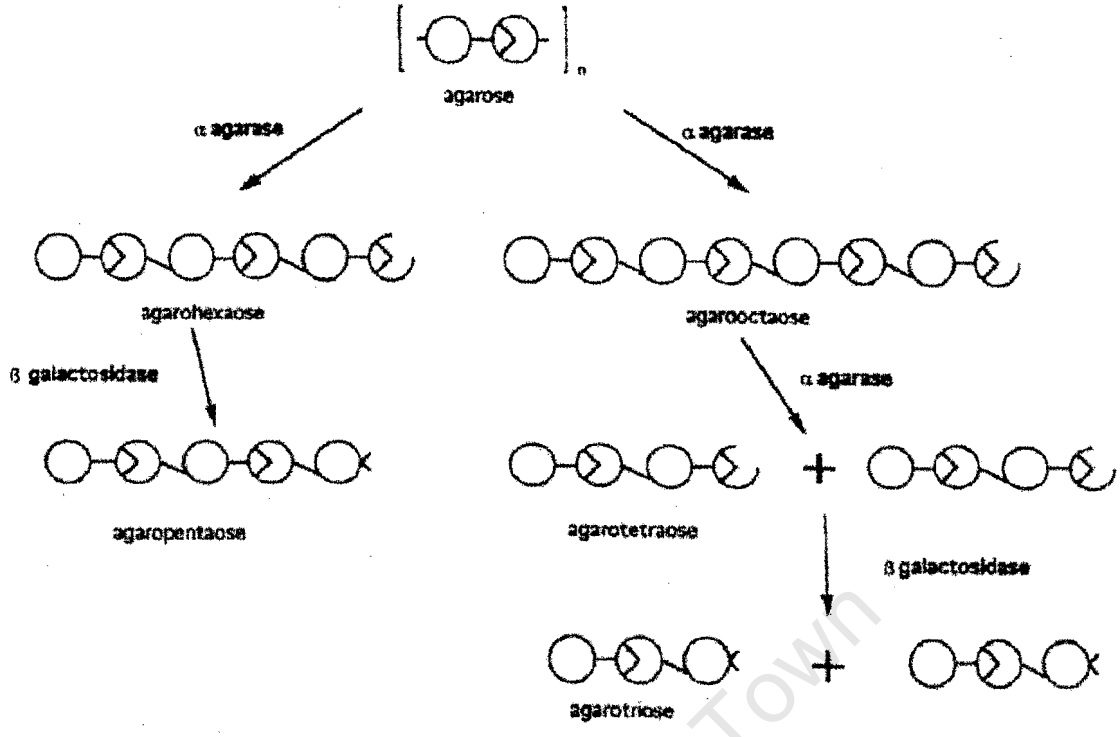

Figure 8 Interpretative scheme for the agarolytic system of *A. agarlyticus* GJ1B. Agaro-oligosaccharides are first formed by the specific cleavage of α(1→3) linkages in agarose. They are then partially degraded by a β-galactosidase acting at the reducing end, yielding oligosaccharides of the agarotriose family, i.e. with an odd number of saccharide units. O: D-galactose; Θ: 3,6 anhydro-L-galactose (Potin *et al.*, 1993).

Two agarolytic enzymes were cloned from a marine bacterium isolated from *Porphyra umbilicalis* from the south sea of Korea. The bacterium was identified as a *Pseudomonas* sp. and named *Pseudomonas* sp. W7. One of the cloned agarases, agarase-pSW1, was localized to the cytoplasmic and periplasmic fractions of the *E. coli* host cell. The other agarase, agarase-pSW3, was found to occur in the cytoplasmic fraction only. SDS-PAGE indicated a single protein of molecular weight 38 000 for agarase-pSW3. The molecular weight of agarase-pSW1 was not reported. Both agarases functioned optimally at neutral pH's, with an optimum at pH 6 and 7 for agarase-pSW1 and agarase-pSW3, respectively. Agarase-pSW1 and agarase-pSW3 showed maximum enzyme activities at 40°C and 30°C, respectively. The enzymes were stable over the temperature range of 20-60°C. The enzymes were identified due to their ability to degrade 0.2% agarose gel. Agarose was also used as a substrate in the enzyme assays. The mechanism of agarose hydrolysis was not determined. An extracellular agarase of 89 000 molecular weight was

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reported by the authors to be secreted by this bacterium, although they failed to clone the gene in this study (Kong *et al.*, 1997). However, an extracellular β-agarase (PjaA) of molecular weight 59 000 was cloned and expressed in *E. coli* from *Pseudomonas* sp. W7. PjaA hydrolyses the β(1→4) linkages of agarose to yield neoagarotetraose as the main product; i.e. member of the β-agarase I enzymes. Optimal enzyme activity was at pH 7.8 and the temperature optimum spanned a broad range of 20-40°C. Enzyme activity was stimulated by NaCl with maximum activity occuring at 0.9 M (Ha *et al.*, 1997). Lee *et al.* (2000) determined the nucleotide sequence of the gene encoding PjaA which was submitted to the GENBANK database under the accession AF153911.

Screening for agar-degrading bacteria from the coastal waters of India yielded two isolates, 10A and LK2. Two agarolytic enzymes from strain LK2, corresponding to molecular masses of about 25 and 28 kDa, were identified (Ghadi *et al.*, 1997).

Two agarolytic fractions, agarase II and agarase IV, were obtained from *Pseudomonas* sp. O-148. Both enzymes were isolated from extracellular material, were found to produce neoagaro-oligosaccharides from agarose and shown to not decompose neoagarotetraose and neoagarobiose. The agarase IV fraction exhibited identical patterns of hydrolysis to the β-Agarase I type enzymes; i.e. cleaves the reducing end of neoagarobiose flanked by the non-reducing side of the neighboring dimer where the non-reduced unit may contain a sulphate on C-6 of the 4-linked α-galactose. The agarase II fraction produced a new type of saccharide unit that had only neoagarobiose units on their non-reducing end and sulphated units internally. This indicated the novelty of agarase II substrate recognition and mechanism of cleavage; i.e. it is not able to cleave when the neighbouring dimer has a sulphated neoagarobiose unit on the 4-linked α-galactose. The distinctions were made when the sulphated, water-soluble component of *Gracilaria* was used as a substrate (Nomura *et al.*, 1998).

Pseudoalteromonas antarctica N-1 was isolated from decomposing algae in Niebla, Chile. An extracellular agarase was purified and shown to possess a molecular weight of 33 000. The enzyme hydrolyzed the β(1→4) linkages of agar, yielding neoagarotetraose and neoagarohexaose as the main products. Its optimal pH for activity was pH 7 and was stable at temperatures up to 30°C (Vera *et al.*, 1998).

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Three kinds of extracellular agarases are produced by the marine bacterium *Vibrio* sp. PO-303. The molecular masses of agarase-a, -b, and -c were estimated to be 87.5, 115, and 57 kDa by SDS-PAGE, respectively. The enzymes had maximal activity at pH 6.5-7.5 and around 38-55°C. Agarase-a hydrolyzed agarose to give neoagarotetraose and neoagarohexaose as predominant products, but could not cleave neoagarotetraose. The main hydrolysis products of agarase-b were neoagarobiose from agarose and neoagarooligosaccharides greater than the dimer. Agarase-c could not cleave neoagarohexaose (Araki *et al.*, 1998).

An intercellular β-agarase was purified from an alkalophilic bacterium, *Alteromonas* sp. E-1. The molecular mass was estimated to be 82 kDa by SDS-PAGE and 180 kDa by gel filtration. The intercellular β-agarase hydrolyzed agarose to give neoagarobiose, neoagarotetraose and neoagarohexaose as end products (Kirimura *et al.*, 1999).

The majority of the bacteria mentioned to date were isolated from the marine environment. Two more examples are the five agarolytic, thermophilic bacterial strains, belonging to the new genus *Alterococcus*, which were isolated from two hot springs in Taiwan (Shieh and Jean, 1998), and an agarolytic, luminous bacterium, *Vibrio harveyi* FLB-17, isolated from coastal waters of Japan (Fukasawa *et al.*, 1987). However, not all were isolated from the marine environment, e.g. the soil bacteria, *A. pastinator* 2AC and *S. coelicolor* A3(2), and the *Pseudomonas*-like bacterium from a seawage treatment plant. In the literature, additional agarolytic bacteria were isolated from other habitats. For example, an agarolytic-fermenting bacterium (strain 16AV) was isolated from an abattoir effluent waste pond (Rees *et al.*, 1994). More examples of agarolytic bacteria from both the marine and non-marine environments are present in literature, but little is known about the agarases produced by these bacteria (e.g. Nikolaeva, *et al.*, 1999). Similarly, a few gene sequences of extracellular agarases isolated from bacteria, not discussed in this section to date, were submitted to the GENBANK database. However, these agarases have yet to be submitted for publication (AF098954/5: *Cytophaga drobachiensis*, AR034164: *Flavobacterium* sp NR19).

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1.4 Concluding remarks and aim of this study

The red alga, *Gracilaria*, is economically important. In this chapter, the importance of this seaweed, especially its polysaccharide extracts (agar and agarose), is seen by the extensive applications that exist in a number of industries. South Africa benefited substantially since the 1950's from the export of *Gracilaria gracilis* that occurs naturally at Saldanha Bay. However, the South African *Gracilaria* industry, which depends solely on the natural *G. gracilis* resource at Saldanha Bay, has experienced a number of setbacks in the last decade or so. Even the pilot studies undertaken by the SRU, whose mandate was to investigate the viability of cultivating *Gracilaria* in Saldanha Bay, experienced a number of setbacks. As outlined in this chapter, these setbacks were due to the major collapse experienced by the natural *G. gracilis* population in Saldanha Bay in 1989, the number of partial die-offs of raft cultivated *G. gracilis* during the summer months in Saldanha Bay post- 1989, and another substantial collapse of both natural and raft cultivated *G. gracilis* during October-December 1996. Subsequently, investors such as Quest International and Taurus Products (Transkei) (Pty) Ltd. are very cautious with respect to establishing commercial scale farms at Saldanha Bay as encouraged by the SRU. Thus, the future of mariculture of *G. gracilis* at Saldanha Bay depends on understanding and preventing *Gracilaria* collapses at Saldanha Bay, as such collapses would be financially devastating.

Jaffray and Coyne (1996) developed an *in situ* assay to identify putative bacterial pathogens of this macroalga. Of the epiphytic bacteria tested, a positive correlation between the agarolytic phenotype and bacterial pathogenicity was discovered. A number of seaweed diseases attributed to bacteria were discussed in this chapter. Other than the afore-mentioned example, four other incidences of disease, 'ice-ice' white powdery disease *Eucheuma* and *Kappaphycus* species; the disease in the red alga, *Rhodella retriculata*; the 'rotten-thallus' syndrome in a *Gracilaria* species; and the 'white-tip disease' of *Gracilaria conferta*, were attributed to agarolytic bacteria (Toncheva-Panova and Ivanova, 1997; Largo *et al.*, 1995; Lavilla-Pitogo, 1992; and Friedlander and Gunkel, 1992). However, the role of the agarases in the virulence mechanism of these bacterial pathogens was only hypothesized.

The aim of this study was to isolate the gene(s) that encode the agarolytic activity associated with an epiphytic bacterial pathogen of *G. gracilis* from Saldanha Bay. Upon characterization of the enzyme(s), the enzyme(s) would be used as a tool to elucidate the virulence mechanism, and

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thus, the strategy employed by the bacterium in eliciting disease in *G. gracilis*. Hopefully, appropriate steps could then be taken to detect and prevent the occurrence of the disease in cultivated *Gracilaria* at Saldanha Bay. Thus, the knowledge gained from this study will be invaluable to the parties interested in large scale mariculture of *G. gracilis* in Saldanha Bay.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *PSEUDOALTEROMONAS GRACILIS* B9

CONTENTS

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2.1 Summary

Several different epiphytic bacterial isolates were isolated from the thallus surface of the agarophyte *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine, *et* Farnham from Saldanha Bay, South Africa. The bacteria were initially classified according to two phenotypic characteristics, i.e. pigmentation of colony and presence or absence of agarolytic activity. The bacterium B9 was selected for further characterization due to its ability to degrade agar. The bacterium was shown to cause disease in *G. gracilis*, i.e. an *in vitro* assay positively identified the isolate B9 as a putative bacterial pathogen of *G. gracilis*. The 16S rRNA gene sequence analysis, together with the comparison of the physical and phenotypic characteristics of the bacterium B9 to that of related bacteria, suggested that it was a new member of the genus *Pseudoalteromonas.* The bacterium was designated *Pseudoalteromonas gracilis* B9.

The growth characteristics of strain B9 and its production of agarolytic activity were tested in different growth media. Agarase production was first detected during the early stationary phase of growth, reaching maximal levels during late stationary phase. It was consistently observed that the production of the extracellular agarase(s) was delayed in media containing a reducing sugar such as glucose or galactose, suggesting that agarase production is regulated in *P. gracilis* B9. Although the production of extracellular agarase(s) were delayed in the presence of a reducing sugar, higher agarolytic activity was observed in growth media supplemented with a reducing sugar than in non-supplemented medium (approximately 1.5 fold more activity). Omission of agar from the basal media resulted in a 4.2 fold reduction of agarase production from 42.46 to 10 U. Alternatively, substitution of agar with agarose saw a 2.5 fold reduction of agarolytic activity from 42,46 to 16,95 U. Thus, *P. gracilis* B9 agarase production is also regulated by substrate composition, i.e. the more substituted the polysaccharide (agar) the greater the activity, while the less substituted (agarose) the lower the activity.

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2.2 Introduction

Macroalgae serve a number of important functions for marine bacteria in the seawater column. They were shown to provide attachment surfaces for bacteria by a number of researchers (Laycock, 1974; Kong and Chan, 1979; Mow-Robinson and Rheinheimer, 1980; Shiba and Taga, 1980; Lemos *et al.*, 1985; Austin, 1988 and Rieper-Kirchner, 1989). Jaffray *et al.* (1997) showed that a large epiphytic population of bacteria exists on the surface of *Gracilaria gracilis*. Macroalgae serve as a good surface for biofilm formation since macroalgae are nutrient-rich surfaces for bacteria in a relatively low nutrient environment such as seawater (Atlas and Bartha, 1987).

The epiphytic bacteria isolated from the surfaces of macroalgae have adapted to their environment by developing mechanisms for exploiting their habitat. As a result of continuous movement and abrasion, damaged thalli are a constant source of organic compounds in the form of algal structural products such as agar, alginate and carrageenan, as well as algal reserve products such as starch, fats etc. (Rheinheimer, 1984; and Mow-Robinson and Rheinheimer, 1985). These algal products are broken down into proteins and sugars, which are utilized by the bacteria. The bacteria encode pathways capable of degrading the polysaccharide(s) that occur within the relevant macroalgae (Mow-Robinson and Rheinheimer, 1985; Rieper-Kirchner, 1989; Kong and Chan, 1979; and Laycock, 1974). Jaffray *et al.* (1997) showed that more than a third of the cultured bacterial epiphytes isolated from *G. gracilis* were agarolytic. This was believed to be significant since these bacteria produced enzymes that can degrade the polymers abundant in *Gracilaria*, i.e. agar being the mucilaginous component of the *Gracilaria* cell wall.

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The existence of thriving populations of *Gracilaria* is indicative of the equilibrium that exists between the macroalga and its epiphytic bacteria. The equilibrium shifts from healthy macroalga towards bleached fragile diseased macroalga during environmental conditions characterized by an increase in water temperature and depletion of seawater nutrients (Jaffray, 1999). This imbalance occurs during the summer months at Saldanha Bay. Jaffray and Coyne (1996) showed that a number of the epiphytic bacteria were putative pathogens of *G. gracilis* since they caused thallus bleaching and breakage, i.e. the symptoms observed during the collapses that occurred at Saldanha Bay (R. Anderson, SRU, pers. comm.).

The first objective of this study was to isolate, identify and characterize a bacterial pathogen of *G. gracilis* from Saldanha Bay. Jaffray and Coyne (1996) developed a reliable assay for detection of putative bacterial pathogens of *G. gracilis*. In addition, Jaffray and Coyne (1996) showed that there is a positive correlation between bacterial pathogenicity towards *G. gracilis* and their agarolytic phenotype. Therefore, an agarolytic epiphytic bacterium of *G. gracilis* was considered a possible candidate for further study and secondly, this afore-mentioned assay was used as a tool for the identification of a putative pathogen of *G. gracilis*.

Bacteria belonging to the genera *Cyanobacterium*, *Cytophaga*, *Flavobacterium*, and *Vibrio*, amongst others, have been identified as agarolytic pathogens of a number of macroalgae (Correa *et al.*, 1993; Largo *et al.*, 1995; Toncheva-Panova and Ivanova, 1997; and Jaffray and Coyne, 1996). In an endeavor to classify a putative bacterial pathogen of *G. gracilis*, a polyphasic approach was employed in this study, i.e. phylogenetic and phenotypic characteristics of the bacterium was determined. The optimal methods for determining phylogenetic relationships are DNA association and 16S rRNA gene sequencing of 1, 000 or more bases (Murray, *et al.* 1990). Carl Woese (1987) in a review in Microbiological Reviews, and later in a mini-review in the Journal of Bacteriology (Oslen *et al.*, 1994), emphasized the usefulness of sequence information in measuring evolutionary relationships. In the 1980's, Carl Woese and colleagues developed the first 16S rRNA gene based trees reflecting the phylogenetic relationships amongst prokaryotes. In addition, sequencing of the 16S rRNA gene is much easier now than it was only a decade ago. As a result, a large database of 16S rDNA sequences know exists. Therefore, 16S rDNA analysis was one of the tools used for the classification of the putative bacterial pathogen of *G. gracilis* isolated in this study.

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In conjunction with the 16S rRNA gene analysis, a variety of physical and phenotypic characteristics of the bacterium were determined. The importance of the physico-chemical characteristics in the classification of a bacterium is illustrated by Stackebrandt and Goebel (1994) who ultimately recommends, "In the end it is the presence or absence of phenotypic coherency among strains that should be the deciding factor about whether to describe species at all".

The second aim of this chapter was to determine when the putative bacterial pathogen of *G. gracilis* produces its agarolytic activity relative to its growth phase and secondly, to determine

whether agarase production is subject to regulation. Since the disease experienced by *G. gracilis* at Saldanha Bay occurs during environmental conditions characterized by an increase in water temperature and depletion in seawater nutrients (Jaffray, 1999), the answers to the above two questions might shed some light on the role the agarolytic trait of the putative bacterial pathogen of *G. gracilis* might have in the disease affecting the seaweed during adverse environmental conditions.

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2.3 Materials and Methods

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

2.3.1 Media and culture conditions

The epiphytic bacteria of *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham were maintained at 22°C in Basal Media broth (BM) (Appendix A.1.1) and aerated on an orbital shaker (Gallenkamp) at 100 rpm. Alternatively, the bacteria were cultured on Basal Agar (BA) plates (Appendix A.1.2).

2.3.2 Isolation of epiphytic bacteria of *G. gracilis*

Bacterial epiphytes were isolated from *G. gracilis* samples as follows: *Gracilaria* thalli, collected from Saldanha Bay in 1993, were subjected to extensive vortexing in sterile seawater (SSW) in order to dislodge a significant amount of epiphytes, which were then diluted and cultured on synthetic growth media (BA) supplemented with amphotericin (10 mg / l) (Appendix A.2.1) to select against fungi. The plates were incubated at room temperature and scored for growth on a daily basis. Colonies were isolated and separated according to pigmentation and the presence or absence of agarolytic activity.

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2.3.3 Assay for bacterial pathogens of *G. gracilis*

Axenic *G. gracilis* was produced and used in a pathogenicity assay as described by Jaffray and Coyne (1996) to confirm whether *P. gracilis* B9 is a putative bacterial pathogen of *G. gracilis* (Appendix B.1).

2.3.4 Phenotypic characterization of *Pseudoalteromonas gracilis* B9

2.3.4.1 Cell shape and Gram reaction

The bacterial cell shape and Gram reaction was tested by staining (Appendix A.2.4) the cells using the Hucker modification and viewing them under a light microscope fitted with a 100 x oil immersion lens (Bailey and Scott, 1966).

2.3.4.2 Motility test

Bacterial motility was determined by microscopic observation of a hanging drop preparation of an exponentially growing bacterial culture grown in BM at 22°C.

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2.3.4.3 Oxidase and catalase tests

The presence of cytochrome oxidase and catalase activity was determined by using Kovac's reagent and 3% H_2O_2 (Appendix A.2.5), respectively (Bailey and Scott, 1966; and Smibert and Krieg, 1981).

2.3.4.4 Aerobic and anaerobic metabolism of carbon sources

The isolate was tested for its ability to metabolize a variety of carbon sources aerobically and anaerobically as described by Hugh and Leifson (1953). The media (Appendix A.1.3) was modified, i.e. the NaCl content was raised to 3% (w/v) and agar was omitted, since strain B9 is a marine bacterium and it can use agar as a carbon source. The carbon sources tested were as follows: D-glucose, D-mannose, D-galactose, D-fructose, sucrose, maltose, cellobiose, melibiose, lactose, D-mannitol, L-threonine, D-ribose and rhamnose.

2.3.4.5 Nitrate reductase and denitrification tests

Strain B9 was cultured in a defined media (Appendix A.1.4) and tested for its ability to reduce nitrate to nitrite and carry out denitrification, using standard nitrate reduction and denitrification tests (Smibert and Krieg, 1981).

2.3.4.6 Test for indole production

Strain B9 that had been cultured in a modified media containing tryptone (Appendix A.1.5) for 48 h at 22°C was tested for indole production using the standard method described by Smibert and Krieg (1981).

2.3.4.7 Test for H_2S production

The standard method described by Smibert and Krieg (1981) was used to determine if strain B9 could produce H_2S in a defined media (Appendix A.1.6).

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2.3.4.8 Test for agarolytic activity

The ability to hydrolyze agar was determined by visual detection of "pitting" of the agar around the bacterial colonies growing on BA plates.

2.3.4.9 Test for carrageenolytic activity

The bacterium was grown on a 2% carrageenan medium (Appendix A.1.7) after which zones of carrageenan hydrolysis were visually monitored around the bacterial colonies after 4 days incubation at 22°C.

2.3.4.10 Test for gelatinase activity

A strain B9 stab culture in 12% gelatinase medium (Appendix A.1.8) was visually monitored for liquefaction of gelatin after several days of incubation at 22°C (Smibert and Krieg, 1981).

2.3.4.11 Test for cellulase activity

The bacterium was grown on a carboxymethyl-cellulose (CM-cellulose) medium (Appendix A.1.9) for 5 days at 22°C after which Congo Red (Appendix A.2.6) was employed to assay for zones of cellulose hydrolysis (Teather and Wood, 1982).

2.3.4.12 Test for starch hydrolysis

The cell free supernatant of an O/N bacterial culture of B9 was tested for its ability to hydrolyze starch using an assay described by Miller (1959). In triplicate, 35 µl of supernatant was added to 15 µl of 0.5% starch (Appendix A.2.7). Two of the reaction mixes were incubated at 30°C for 30 min, while the third was kept on ice and thus served as a control. DNS reagent (150 µl) (Appendix A.2.7) was added to the reaction mixes after the incubation period had elapsed and the resultant mixes were boiled for 5 min. The mixtures were cooled rapidly and 800 µl ultrapure water was added to each reaction mix. The absorbance of the samples was determined at 510 nm with a Spectrophotometer (Beckman).

2.3.5 Determination of the 16S rRNA gene sequence

Pseudoalteromonas gracilis B9 genomic DNA was extracted as described in Appendix B.2. The genomic DNA was used as a template for the isolation of the 16S rDNA via PCR (Weisburg *et al.*, 1991). Five overlapping DNA fragments encompassing the 16S rRNA gene were amplified using a Hybaid Omnigene thermal cycler. Six PCR primers were designed, three forward (F1, F3 and F5) and three reverse (R1, R3 and R5) (Appendix B.3). They were designed such that the 3'end of the primers consisted of a nucleotide sequence that was complementary to conserved regions of the bacterial 16S rDNA. The 5'end of the forward and reverse primers had homology to a 24 bp forward and a 17 bp reverse sequencing primer, respectively (Appendix B.3). The primers were used in amplification reactions in the following combinations: F1 and R5, F1 and R3, F1 and R1, F3 and R5, and F5 and R5 (Figure 3). The PCR was conducted as described in Appendix B.3.

The amplified PCR products (10 µl) were analyzed by agarose gel electrophoresis (Appendix B.4) to verify specificity and sizes. The amplified products were subsequently isolated using

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QIAquick DNA clean-up columns (Qiagen). The 16S rDNA PCR products were sequenced using a Thermosequenase cycle-sequencing kit (Amersham) and an ALFexpress™ automated sequencer (AM version 3.01, Pharmacia Biotech) as described in Appendix B.5. The sequences were assembled using DNASIS version 2.1 (Hitachi Software Engineering). The homology search was carried out using the BLASTN algorithm (Altschul *et al.*, 1989) provided by the Internet service of the National Center for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

2.3.6 Phylogenetic tree construction

The 16S rRNA gene phylogenetic tree for *Proteobacteria* was based on the phylogenetic tree constructed by Woese (1987). The sequences used from Woese (1987) were as follows: α subdivision: *Rhodospirillum rubrum* (D30778), *Agrobacterium tumefaciens* (D14506), *Rhodopseudomonas palustris* (D25312), and *Rhodopseudomonas acidophila* (M34128); β subdivision: *Neisseria gonorrhoeae* (X07714), *Spirillum volutans* (M34131), *Nitrosolobus multiformis* (M96401), *Rhodocyclus gelatinosa* (M60682), and *Rhodocyclus pupureus* (M34132); γ subdivision: *Legionella pneumophila* (X73402), *Pseudomonas aeruginosa* (Z76651), and *Escherichia coli* (AE000452); δ subdivision: *Myxococcus xanthus* (M34114), *Desulfovibrio desulfuricans* (M37316), and *Bdellovibrio stolpii* (M34125). The following four 16S rDNA sequences were added to the analysis: *Shewanella putrefaciens* (X82133), *Alteromonas macleodii* (X85145), *Pseudoalteromonas haloplanktis* (X67024), and bacterium B9. The full 1498 bp sequences equivalent to strain B9 (AF038846, Figure 4), were aligned using the optimal alignment option in DNAMAN version 4.13 (Lynnon BioSoft). The phylogenetic tree was constructed using the various programs in PHYLIP (Phylogeny Inference Package) version 3.57c (Felsenstein, 1995). The genetic distances for the alignment were calculated using the Dnadist program. Phylogenetic inference based on the distance matrix (Neighbor) was applied to the alignment and the tree was visualized and drawn using the TREEVIEW software version 2.1 (Page, 1998).

The 23 *Pseudoalteromonas* 16S rDNA sequences (Table 4) and the two *Alteromonas* 16S rDNA sequences, *Alteromonas macleodii* subsp. *macleodii* (X85145) and *Alteromonas macleodii* subsp. *fijiensis* (X85174), were obtained from the GENBANK database. The stretches of sequences, common to the 1360 bp sequence of strain B9 (Figure 4), were aligned using the

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optimal alignment option in DNAMAN version 4.13 (Lynnon BioSoft). The phylogenetic trees were constructed using the various programs in PHYLIP (Phylogeny Inference Package) version 3.57c (Felsenstein, 1995). The robustness of the alignment was tested with the bootstrapping option (SeqBoot). Genetic distances, applicable for distance matrix phylogenetic inference, were calculated using the Dnadist program in the PHYLIP package. Phylogenetic inferences based on the distance matrix (Neighbor) and parsimony (Dnapars) algorithms were applied to the alignments. In both cases, the best tree or majority rule consensus tree was selected using the consensus program (Consense). The trees was visualized and drawn using the TREEVIEW software version 2.1 (Page, 1998).

2.3.7 Determination of G+C (mol%) content

Pseudoalteromonas gracilis B9 genomic DNA was extracted as described in Appendix B.2. The G+C (mol%) content was determined using the Ulitzur spectroscopic method (Ulitzur, 1972).

2.3.7 Electron microscopy

A carbon coated copper grid was placed on the surface of an exponential phase, 15 µl aliquot of strain B9 culture for 10 min. The grid was stained with a 2% uranyl acetate (pH 5) solution (Appendix A.2.11) for 10 min. The stain was removed with five, 1 min washes of distilled water and the grid was subsequently air-dried (Dykstra, 1993). The sample was viewed on a Zeiss Electron Microscope 109 (Zeiss).

2.3.9 Ferricyanide assay for reducing-sugars

Agarolytic activity was determined using a modified ferricyanide reducing-sugar assay (Appendix B.6).

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2.3.10 The effect of different media on the growth rate and agarase production of the bacterium B9

Strain B9 was cultured in BM at 22°C with agitation at 100 rpm O/N. This culture served as the starter culture for further inoculation into various growth media to determine the effects of nutrients on the growth and agarase production of strain B9. The various media tested were BM, BM supplemented with D-glucose, BM supplemented with D-galactose, BM with the agar component substituted with agarose, and BM lacking agar (Appendix A.1.1). The growth of strain B9 was determined by measuring the OD_{600}. After measuring the OD_{600} of the strain B9 culture, 1 ml aliquots of cell culture were centrifuged (14 000 rpm for 5 min), and the medium supernatant was collected. The supernatant was used to quantitate agarase production as described in Appendix B.6.

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2.4 Results

2.4.1 Isolation of an epiphytic, agarolytic, pathogenic bacterium of *G. gracilis*

The technique used to isolate epiphytic bacteria from *Gracilaria* yielded a total of 9 culturable bacterial isolates (Table 1). The bacterial isolates exhibited a variety of phenotypic variations, i.e. beige (isolate B1); opaque colonies (isolates B2 and B3); yellow colonies (isolates B4, B6 and B7); brown colonies (isolates B8 and B9); and one red colony (isolate B13). Four of the isolates collected, isolates B3, B6, B7 and B9, exhibited agarolytic activity which distinguished them from the isolates with similar color phenotypes. The dark brown pigmenting bacterium B9 produced deep indentations in the agar medium (Figure 1), indicative of the export of enzyme(s) capable of hydrolyzing agar, and consequently, this isolate was considered a possible candidate for further study.

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Table 1 Bacterial isolates from *Gracilaria* collected at Saldanha Bay

Isolate	Colony colour	Agarolytic Activity
B1	Beige	–
B2	Opaque	–
B3	Opaque	+
B4	Bright yellow	–
B6	Yellow with white centre	+
B7	Bright yellow	+
B8	Dark brown with greenish tint	–
B9	Dark brown	+
B13	Red	–

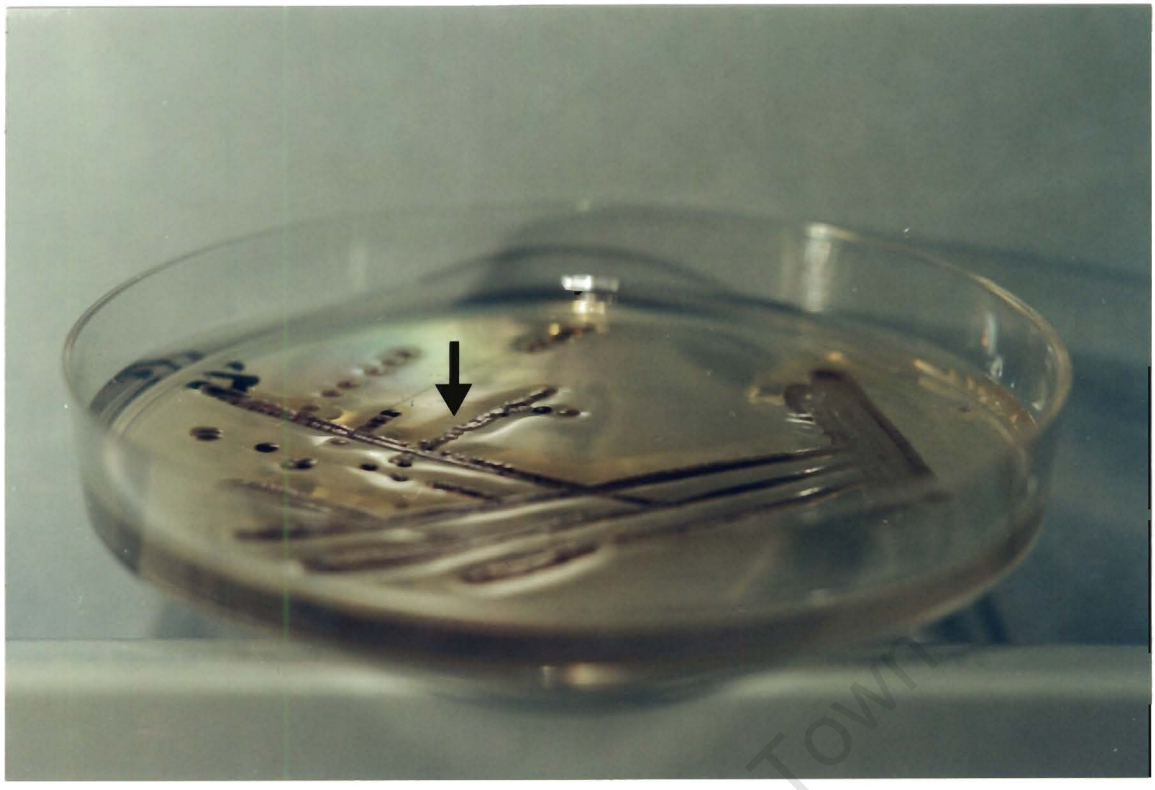

Figure 1 Bacterium B9 cultured on a BA plate. The arrow indicates the zones of agar hydrolysis.

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The pathogenicity assay devised by Jaffray and Coyne (1996) was used to evaluate whether isolate B9 was indeed a pathogen of *Gracilaria*. A typical result obtained with the pathogenicity assay is depicted in Figure 2. The results obtained with this assay were consistent over the 5-day incubation period in three independent experiments. The injected (with sterile seawater) control axenic thallus, which was incubated in BM at either 22°C or 30°C, remained dark and healthy throughout the incubation period (Figure 2A). No bacterial growth was observed from the thallus after the 5-day incubation (Figure 2B). In comparison, bleaching of the axenic thallus following introduction of the bacterium B9 was indicative of the disease symptoms observed in diseased *Gracilaria* (Figure 2A). The symptoms were more severe at the higher incubation temperature. Only the bacterium B9 was re-isolated from the infected thalli, thus fulfilling Koch's postulates (Figure 2B).

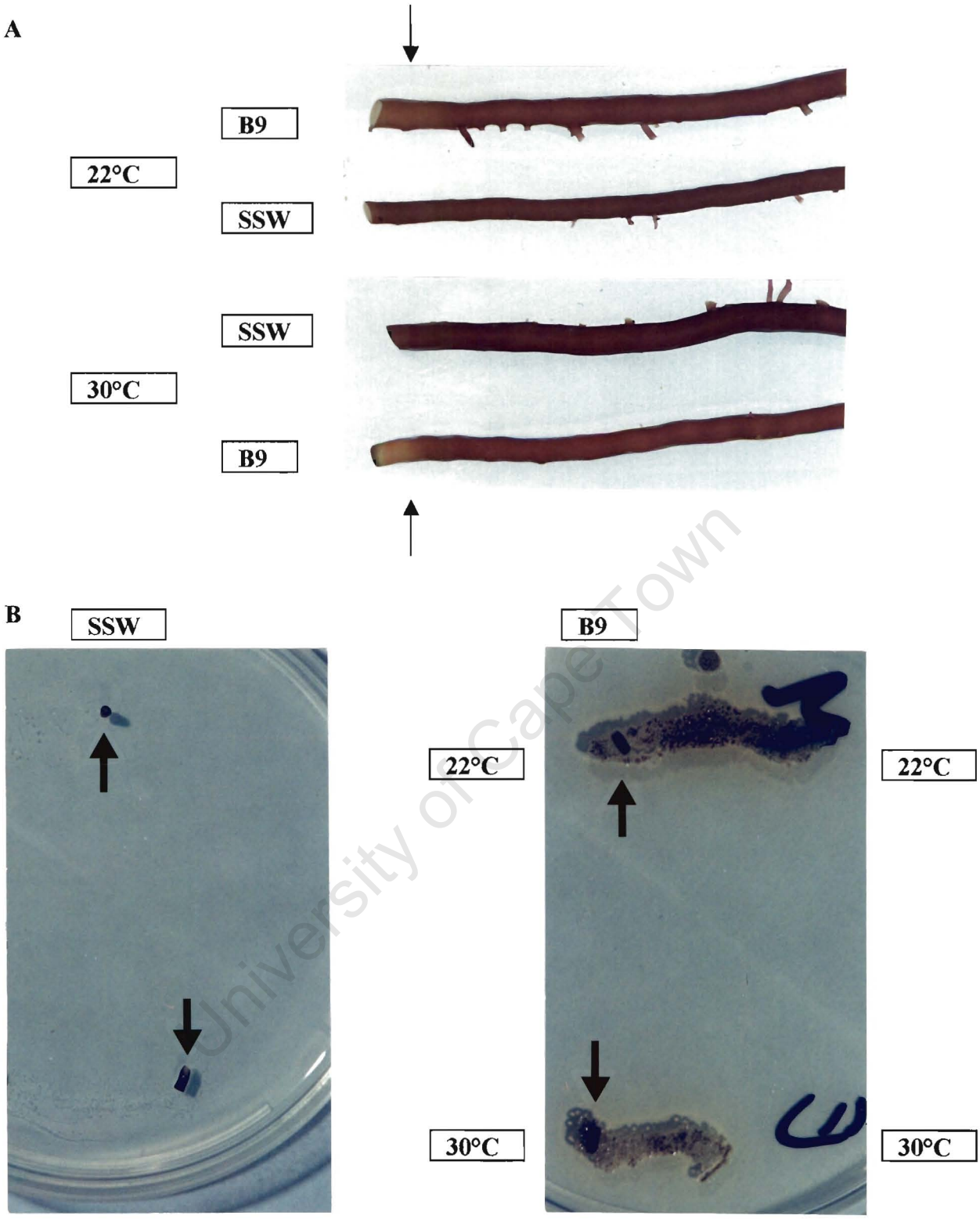

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Figure 2 A) Results of pathogenicity assay showing thallus bleaching caused by the bacterium B9 compared to the SSW injected thallus controls, 5 days after incubation at 22°C and 30°C. Arrows: bleached area.

B) Isolate B9 was recovered from the diseased thallus, while the SSW control was negative for any microbial growth. Arrows: location of thalli on plate.

2.4.2 Classification of *Pseudoalteromonas gracilis* B9

The nucleotide sequences of the products obtained from PCR amplification (Figure 3) of the bacterium B9 16S rRNA gene were assembled, resulting in a full-length sequence of 1498 bp in length (Figure 4). A BLAST search of the GENBANK database revealed that the bacterial B9 16S rDNA sequence showed high identity to a number of *Pseudoalteromonas* 16S rDNA sequences (Table 2). The 16S rDNA phylogenetic analysis clearly showed that bacterium B9 belonged to the γ subclass of the class *Proteobacteria* of the domain *Bacteria* (Figure 5), and branched deeply within the clade belonging to the genus *Pseudoalteromonas* (Figure 6). The bacterium B9 formed a cluster with *Pseudoalteromonas antarctica* N-1, *Pseudoalteromonas citrea* KMM 216, and *Pseudoalteromonas antarctica* $NF3^T$ (Figure 6). This association or cluster exhibited high bootstrap values with both the neighbor-joining and parsimony phylogeny inferences, indicative of a strong association.

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Table 2 The top ten sequence similarities as obtained from a BLAST search of the GENBANK database with the 1498 bp 16S rRNA gene sequence of strain B9.

Species	% sequence similarity	GENBANK accession number
Pseudoalteromonas sp. ER72M2	99	AF155038
Pseudoalteromonas citrea	99	AF082563
Pseudoalteromonas elyakovii	99	AF116188
Pseudoalteromonas elyakovii	99	AF082562
Pseudoalteromonas distincta	99	AF082564
Pseudoalteromonas distincta	99	AF043742
Pseudoalteromonas antarctica	99	X98336
Pseudoalteromonas sp. MB6-05	99	U85860
North Sea bacterium H120	99	AF069667
Pseudoalteromonas sp. IC006	99	U85856

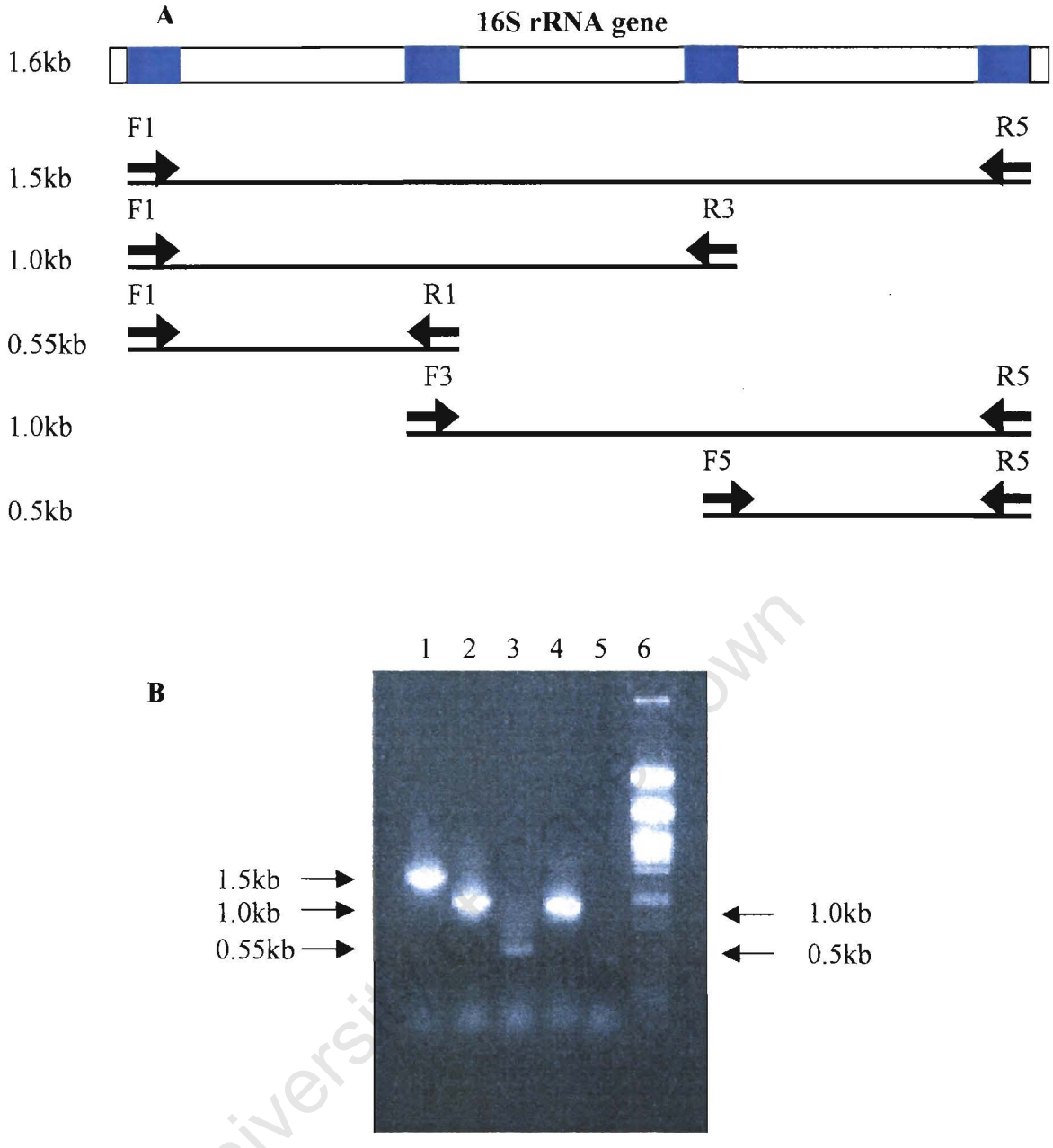

Figure 3 A) Diagram showing the PCR strategy employed to amplify the 16S rRNA gene of the bacterium B9 using five primer pairs as described in section 2.3.5. F1, F3 and F5 represent the forward primers. R1, R3 and R5 represent the reverse primers. The blue shaded areas represent conserved regions in the 16S rRNA gene that occur in most eubacteria. The sizes depict the length of the PCR products.

B) The amplified 16S rDNA PCR products separated on a 1% TAE agarose gel. Lane 1: F1-R5 primer combination, Lane 2: F1-R3 primer combination, Lane 3: F1-R1 primer combination, Lane 4: F3-R5 primer combination, Lane 5: F5-R5 primer combination, Lane 6: lambda DNA digested with *Pst*I.

```
1     GAGTTTGAT CTGGCTCAGA TTGAACGCTG GCGGCAGGCC TAACACATGCA AGTCGAGCGG
61    TAACAGAGA GTAGCTTGCT ACTTTGCTGA CGAGCGGCGG ACGGGTGAGTA ATGCTTGGGA
121   ACATGCCTT GAGGTGGGGG ACAACAGTTG GAAACGACTG CTAATACCGCA TAATGTCTAC
181   GGACCAAAG GGGGGCTTCG GCTCTCGCCT TTAGATTGGC CCAAGTGGGAT TAGCTAGTTG
241   GTGAGGTAA TGGCTCACCA AGGCGACGAT CCCTAGCTGG TTTGAGAGGAT GATCAGCCAC
301   ACTGGGACT GAGACACGGC CCAGACTCCT ACGGGAGGCA GCAGTGGGGAA TATTGCACAA
361   TGGGCGCAA GCCTGATGCA GCCATGCCGC GTGTGTGAAG AAGGCCTTCGG GTTGTAAAGC
421   ACTTTCAGT CAGGAGGAAA GGTTAATAGT TAATACCCGC TAGCTGTGACG TTACTGACAG
481   AAGAAGCAC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TACGGAGGGTG CGAGCGTTAA
541   TCGGAATTA CTGGGCGTAA AGCGTACGCA GGCGGTTTGT TAAGCGAGATG TGAAAGCCCC
601   GGGCTCAAC CTGGGAACTG CATTTCGAAC TGGCAAACTA GAGTGTGATAG AGGGTGGTAG
661   AATTTCAGG TGTAGCGGTG AAATGCGTAG AGATCTGAAG GAATACCGATG GCGAAGGCAG
721   CCACCTGGG TCAACACTGA CGCTCATGTA CGAAAGCGTG GGGAGCAAACG GGATTAGATA
781   CCCCGGTAG TCCACGCCGT AAACGATGTC TACTAGAAGC TCGGAGCCTCG GTTCTGTTTT
841   TCAAAGCTA ACGCATTAAG TAGACCGCCT GGGGAGTACG GCCGCAAGGTT AAAACTCAAA
901   TGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG TTTAATTCGAT GCAACGCGAA
961   GAACCTTAC CTACACTTGA CATACAGAGA ACTTACCAGA GATGGTTTGGT GCCTTCGGGA
021   ACTCTGATA CAGGTGCTGC ATGGCTGTCG TCAGCTCGTG TTGTGAGATGT TGGGTTAAGT
1081  CCCGCAACG AGCGCAACCC CTATCCTTAG TTGCTAGCAG GTAATGCTGAG AACTCTAAGG
1141  AGACTGCCG GTGATAAACC GGAGGAAGGT GGGGACGACG TCAAGTCATCA TGGCCCTTAC
1201  GTGTAGGGC TACACACGTG CTACAATGGC GCATACAGAG TGCTGCGAACC TGCGAAGGTA
1261  AGCGAATCA CTTAAAGTGC GTCGTAGTCC GGATTGGAGT CTGCAACTCGA CTCCATGAAG
1321  TCGGAATCG CTAGTAATCG CGTATCAGAA TGACGCGGTG AATACGTTCCC GGGCCTTGTA
1381  CACACCGCC CGTCACACCA TGGGAGTGGG TTGCTCCAGA AGTAGATAGTC TAACCCTCGG
1441  GAGGACGTT TACCACGGAG TGATTCATGA CTGGGGTGAA GTCGTAACAAG GTACCCGT
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Figure 4 Nucleotide sequence (1498 bp) of 16S rDNA of *P. gracilis* B9. The positions of the highly conserved regions of 16S rDNA to which the amplification primers were designed are shown in blue. The underlined sequence was not used in the phylogenic analysis described in section 2.3.6 (Figure 6). The sequence was submitted to the GENBANK database and assigned the accession number AF038846.

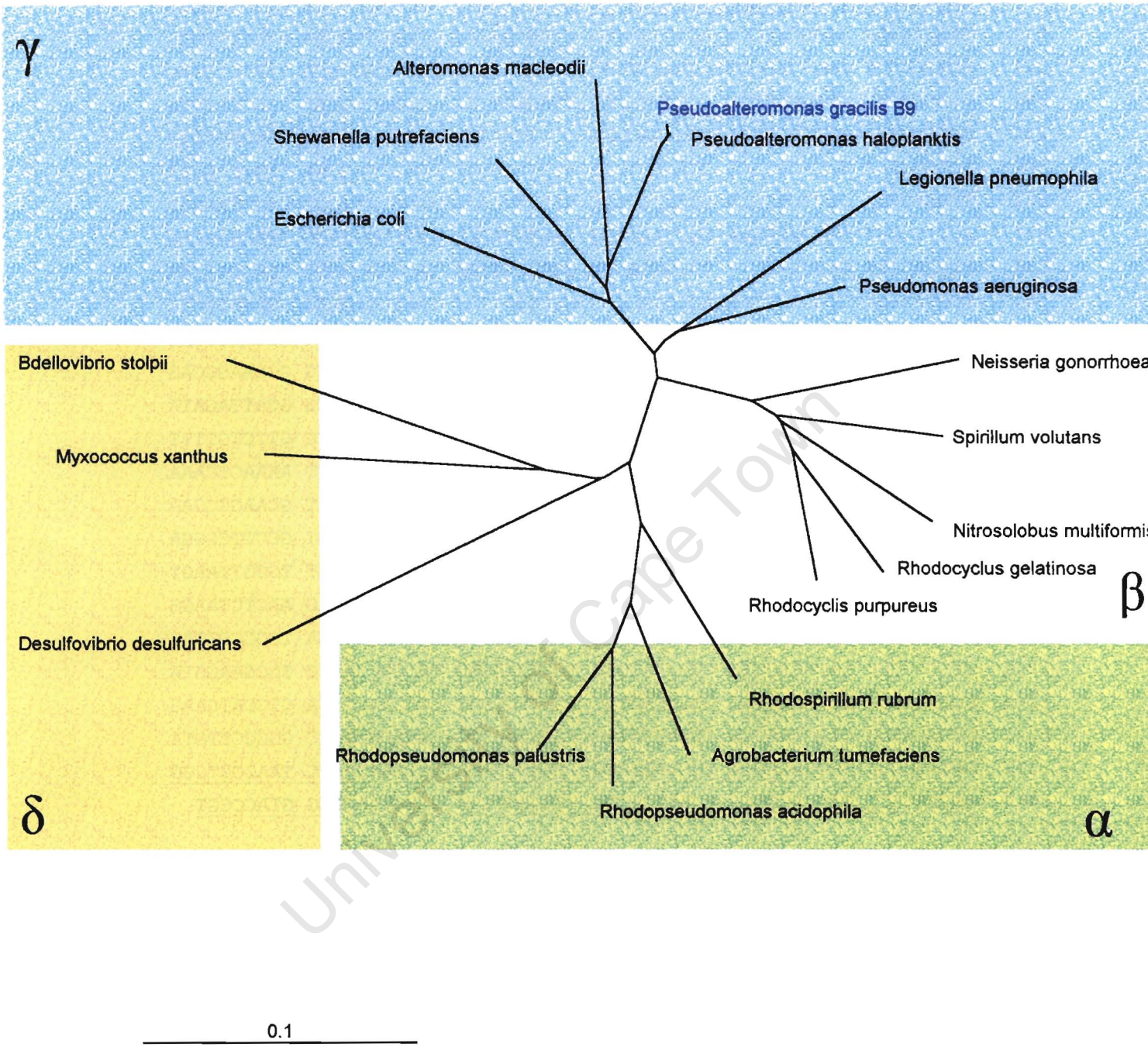

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Figure 5 A phylogenetic tree showing the taxonomic position of *Pseudoalteromonas gracilis* B9 relative to representative members of the *Proteobacteria* (adapted from Woese, 1987). The phylogenetic tree was constructed using Neighbor in PHYLIP version 3.57c (Felsenstein, 1995). The bar depicts 1 base substitution per 10 nucleotide sites.

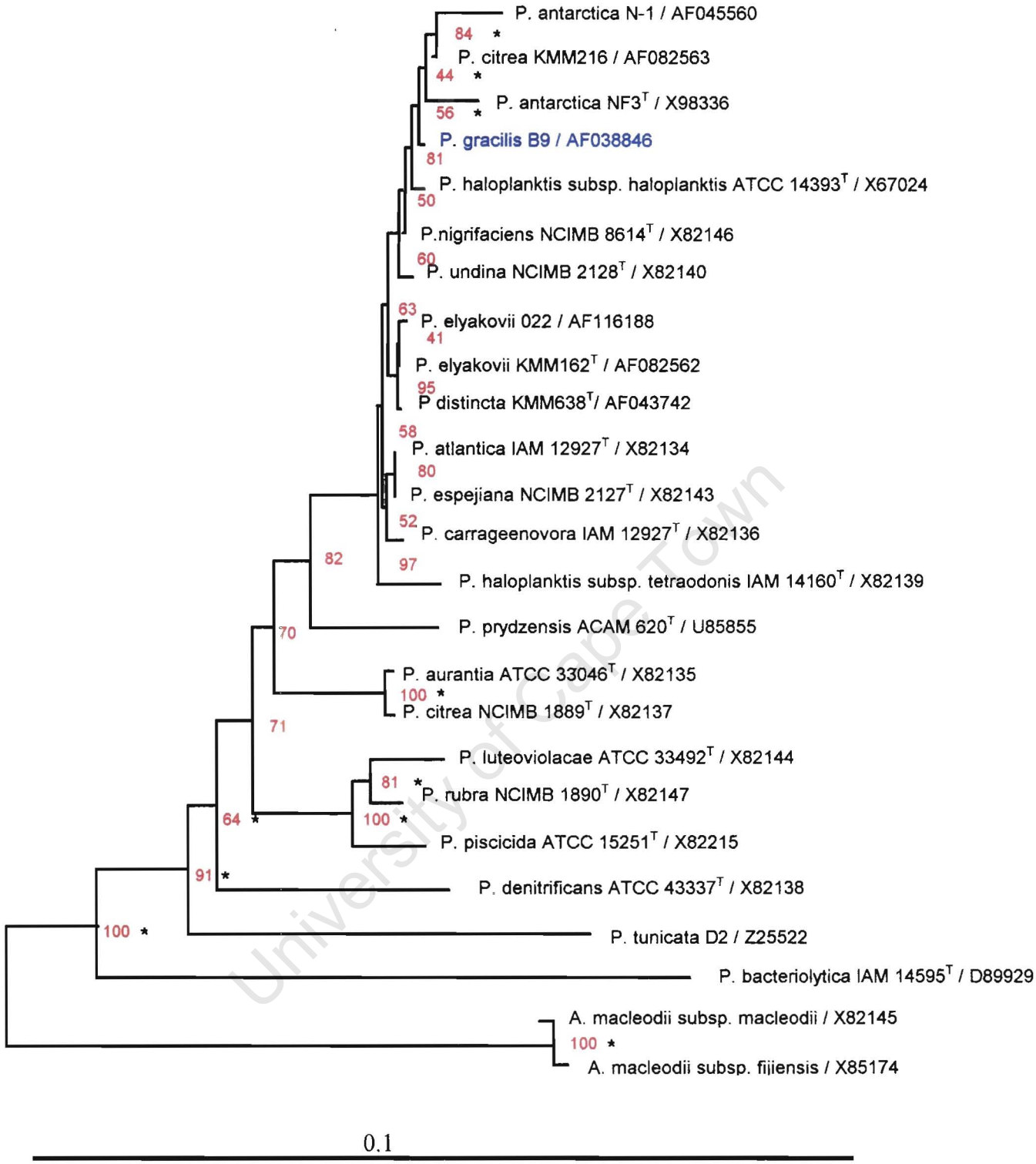

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Figure 6 A phylogenetic inference based on a distance matrix algorithm between *P. gracilis* B9, strains of the genus *Pseudoalteromonas*, and strains of the closely related genus *Alteromonas* (Neighbor, in PHYLIP version 3.57c, Felsenstein, 1995). An alignment of 1360 characters was used. Numbers at nodes indicate bootstrap values retrieved from 100 replicates. *, >60 bootstrap values for parsimony analysis. The bar depicts 1 base substitution per 10 nucleotide sites.

The physical and phenotypic characteristics of the bacterium B9 are summarized in Table 3. The bacterium is a dark brown pigmenting, motile, catalase-positive, oxidase-positive, aerobic, Gram-negative rod with a single polar flagellum of ~5.2 μm in length. Cells are ~0.5 μm in diameter and ~0.9 μm in length (Figure 7). The bacterium is psychrotrophic and grows at temperatures between 4 and 30°C, with 22°C as its optimum. Sodium chloride is essential for growth. It cannot reduce nitrate to nitrite, does not carry out denitrification and does not produce H_2S. It produces indole and degrades agar, gelatin, starch, carrageenan and cellulose. It is positive for the utilization of D-glucose, D-mannose, D-galactose, D-fructose, sucrose, maltose, cellobiose, melibiose, lactose and D-mannitol. Of the carbohydrates tested, all except L-threonine, D-ribose and rhamnose could be utilized by this bacterium. The G+C content of the DNA is 47.3 ±1 mol% (Table 3).

Table 3 Summary of physical and phenotypic characteristics of the bacterial strain B9

Characteristic	*P. gracilis* B9
Gram stain	Negative
Cell shape	straight rod
Motility	+
Polar flagellum	+
Growth in air	+
Na^+ required for growth	+
Growth at:	
4°C	+
22°C	+
35°C	–
Nitrate reduction	–
Denitrification	–
Production of:	
Catalase	+
Oxidase	+
Indole	+
H_2S	–
Hydrolysis of: **Agar, Carrageenan, Gelatin, CM-Cellulose, and Starch**	+
Utilization of: **D-glucose, D-mannose, D-galactose, D-fructose, Sucrose, Maltose, Cellobiose Melibiose, Lactose and D-mannitol**	+
L-threonine, D-ribose, and Rhamnose	–
G+C (mol%)	47.3
Colony pigmentation	dark brown

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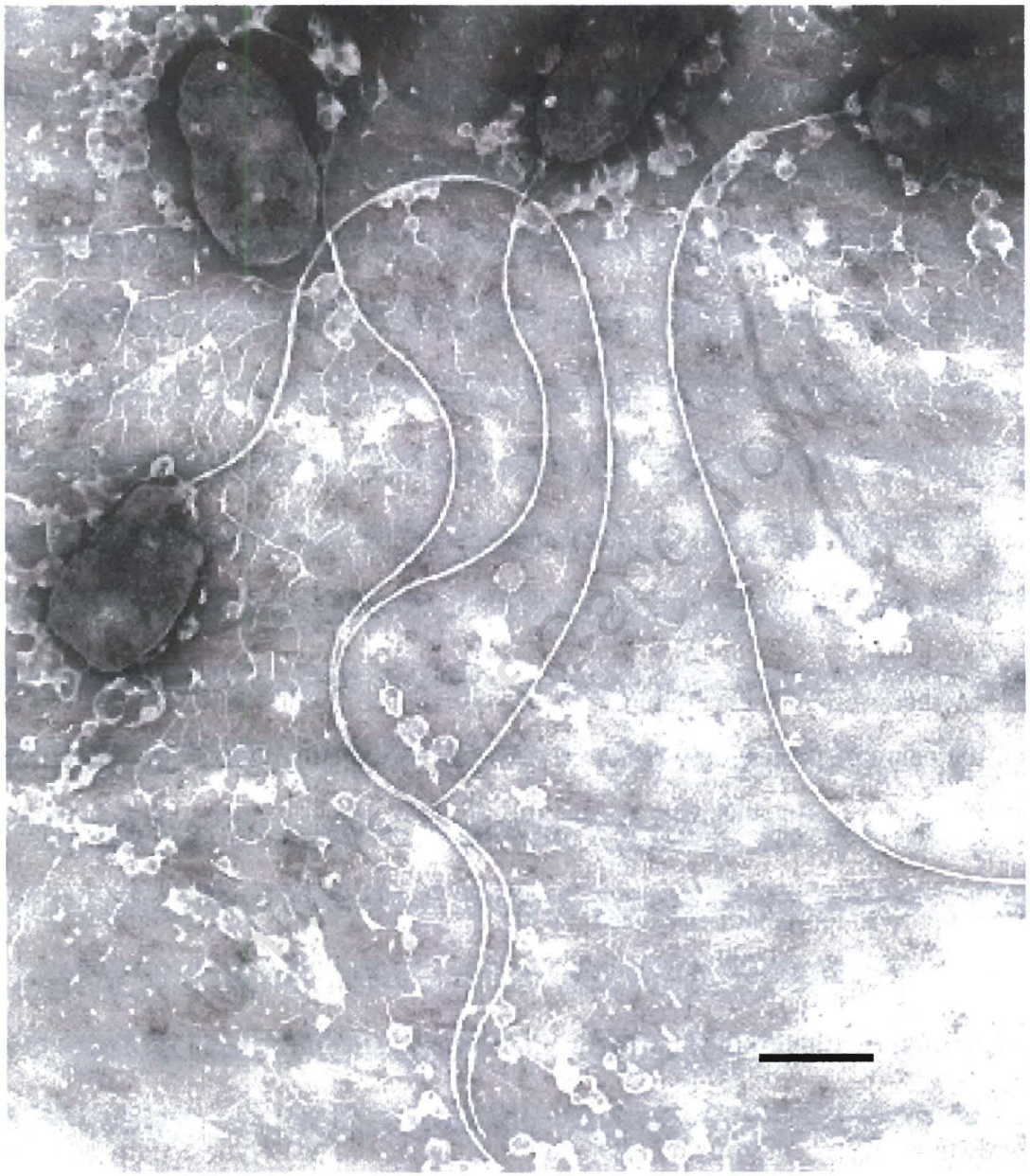

Figure 7 Electron micrograph showing the rod shape of *P. gracilis* B9 cells. The single polar flagellum is clearly visible. Bar: 0.5 μm

The differential characteristics of the species belonging to the genus *Pseudoalteromonas,* whose 16S rDNA sequences have been submitted to the GENBANK database, are shown in Table 4. Amongst the isolates that cluster with strain B9 in the 16S rDNA tree, *P. antarctica* N-1 is the most similar to characteristics exhibited by our isolate. However, *P. antarctica* N-1 differs from B9 in its ability to produce H_2S, degrade alginate, grow at 35°C and its inability to produce indole. Phenotypically, *P. antarctica* N-1 also appears to be different, i.e. non-pigmenting compared to dark brown with respect to B9, despite its ability to secrete melanin into the medium (Vera *et al,* 1998). Of the other members of the genus, *P. atlantica* IAM 12927^T is also very similar to strain B9. However, *P. antarctica* N-1 is even more similar to *P. atlantica* IAM 12927^T since they both produce H_2S, degrade alginate, grow at 35°C and are both unable to produce indole. The other members of this cluster vary in biochemical profile to a greater degree when compared to strain B9. Of the species that have a similar brown pigmented colony phenotype to that of strain B9 (*P. citrea* KMM 216, *P. nigrifaciens* NCIMB 8614^T, and *P. distincta* KMM 638^T), all are negative for the utilization of a variety of carbohydrates that strain B9 can utilize. *P. citrea* KMM 216 is negative for D-glucose, D-mannose, sucrose, maltose, lactose and D-mannitol utilization. *P. nigrifaciens* NCIMB 8614^T is negative for sucrose, maltose and cellobiose, while *P. distincta* KMM 638^T is negative for D-glucose, D-mannose, D-galactose, sucrose, maltose, cellobiose, melibiose and lactose utilization (Table 3). Of the three brown pigmenting species, only *P. citrea* KMM 216 is agarolytic, a characteristic shared with strain B9. However, together with its inability to utilize the afore-mentioned carbohydrates, *P. citrea* KMM 216 is able to grow at temperatures of up to 40°C (Ivanova *et al,* 1998), while strain B9 cannot grow at temperatures exceeding 30°C.

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2.4.3 Characterization of the agarolytic phenotype of strain B9

The growth characteristics of strain B9 and its production of agarolytic activity as determined by the ferricyanide assay were tested in BM and BM supplemented with a reducing sugar, i.e. D-galactose and D-glucose (Figure 8). The growth characteristics of strain B9 was unaffected by the absence or presence of a specific reducing sugar. However, extracellular agarase production was detected earlier in BM lacking a reducing sugar during the early stationary phase of growth (8 h). In BM supplemented with either D-galactose or D-glucose, extracellular agarase production was delayed by 12 and 20h, respectively (Figure 8B and 8C). The levels of agarolytic activity increased 1.5 fold, from 45 to an average of 68 U, during the late stationary phase of

growth in BM supplemented with a reducing sugar when compared to non-supplemented BM (Figure 8).

Substitution of agar with agarose had no affect on the growth characteristics of strain B9 even though a reduction in agarolytic activity from 42,46 to 16,95 U was observed (Figure 9A and 9B). Extracellular agarase production was detected in early stationary phase of growth (8 h). Omission of the agar component of BM resulted in reduced levels of agarase production (never greater than 9.72 U) and the agarolytic activity was only detected during late stationary phase (28 h) (Figure 9C). The growth characteristics of strain B9 were unaffected by the omission of agar when compared to BM with agar. Consistent with all the media tested, maximal agarase production was observed when *P. gracilis* B9 entered late stationary phase.

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Table 4 Differential characteristics of species belonging to the genus *Pseudoalteromonas*

Characteristic	*P. antarctica* N-1	*P. citrea* KMM 216	*P. antarctica* NF3^T	*P. gracilis* B9	*P. haloplanktis* subsp. *haloplanktis* ATCC 14393^T	*P. elyakovii* 022	*P. elyakovii* KMM 162^T	*P. nigrifaciens* NCIMB 8614^T	*P. distincta* KMM 638^T	*P. undina* NCIMB 2128^T	*P. atlantica* IAM 12927^T
Growth at:											
4°C		+	+	+	–	–	+	+	+	–	+
35°C	+	+	–	–	–	+	+	–	+	–	+
G+C (mol%)	40	43.8	42	47.3	42.9	38.7	38.9	42.9	43.8	43.1	41.2
Denitrification	–	–	–	–	–	–	–	–	–	–	–
Production of:											
H_2S	+		–	–							+
Indole	–		–	+							–
Gelatinase	+	+	+	+	+			+		+	+
Lipase		+	–	+	+			+		+	
Chitinase	–	–			–		–	–	–	+	–
Catalase	+	+	+	+	+	+	+		+		+
Hydrolysis of:											
Agar	+	+	–	+	–	–	–	–	–	–	+
Starch	+	+	–	+	–		+	+	–	+	+
Carrageenan		+		+	–	–	–		–		–
Cellulose	+			+	–			–			
Aliginate	+	+		–	–	+	+	–	–	–	+
Utilization of:											
D-glucose	+	–	+	+	+	+	+	+	–	+	+
D-mannose	+	–	+	+	–	+	+	–	–	–	+
D-galactose	+	+	+	+	–	+	+	+	–	–	+
D-fructose	+	+	–	+	+	+	+	+	+	–	+
Sucrose		–	–	+	–	+	–	–	–	+	+
Maltose	+	–	+	+	+	+	–	+	–	+	+
Cellobiose	+		–	+	+		–	–	–	–	+
Melibiose		+	–	+	–	+	+	+	–	–	+
Lactose	+	–	–	+	–	+	+	+	–	–	+
D-mannitol	+	–	+	+	–	+	+	–	+	–	+
L-threonine	–			–	–			–		+	–
Rhamnose			–	–	–			–	–	–	–
Colony pigmentation	–	B	–	B	–	–	–	B	B	–	–
Accession	AF045560	AF082563	X98336	AF038846	X67024	AF116188	AF082562	X82146	AF043742	X82140	X82134
Reference	Vera *et al.*, 1998	Ivanova *et al.*, 1998 & Sawabe *et al.* 2000	Bozal *et al.*, 1997	This study	Baumann *et al.*, 1972	Sawabe *et al.*, 2000	Ivanova *et al.*, 1998 & Sawabe *et al.* 2000	Baumann *et al.*, 1972 & 1984	Sawabe *et al.*, 2000	Chan *et al.*, 1978	Akagawa-Matsushita *et al.*, 1992b

blank: result not available, B: brown, Y: yellow, O: orange, V: violet, R: red, G: green

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Chan *et al.*, 1978	X82143	−	− + + + + + + + + + − +	+ − + −	+ − + +	−	43.1	− −	***P. espejiana* NCIMB 2127[T]**
Akagawa-Matsushita *et al.*, 1992b	X82136	−	− − + + + + + + + + − +	+ + − −	+ − + − +	−	39.5	+ +	***P. carrageenovora* IAM 12927[T]**
Simidu *et al.*, 1990	X82139	−	+ + + + − + + + − − + +	− − −	− + +	−	41	+ +	***P. haloplanktis* subsp. *tetraodonis* IAM 14160[T]**
Buck *et al.*, 1963, Ivanova *et al.*, 1998	X82215	Y/O	− − − + + + + +	+ −		−		+	***P. piscicida* ATCC 15251[T]**
Gauthier, 1976	X82147	R	− − − − − − − + +	− − + −	+ − + + −	−	47.1	+ −	***P. rubra* NCIMB 1890[T]**
Gauthier, 1982	X82144	V	− + − − − − + − − − − +	− + −	− − + + − −	−		+ −	***P. luteoviolacea* ATCC 33492[T]**
Gauthier and Breittmayer, 1979	X82135	O	− − − − + − + − + +	− − −	− + + +	−	38.8	− +	***P. aurantia* ATCC 33046[T]**
Gauthier, 1977	X82137	Y	− − − − − − + − + +	+ −	+ − + + −	−	41.5	− −	***P. citrea* NCMB 1889[T]**
Holmström *et al.*, 1998	Z25522	G	− − − − + − − − + +		+ + −	−	42.2	+ +	***P. tunicata* D2**
Enger *et al.*, 1987	X82138	R	− − + − − − +	+ + −	− + + +	+	37	− +	***P. denitrificans* ATCC 43337[T]**
Bowman, 1998	U85855	−	− − + − − + + − − + +	− + −	+ + + − −	−	38	− +	***P. prydzensis* ACAM 620[T]**
Sawabe *et al.*, 1998	D89929	R	− − − − − + + + + +	− − + −	+ − + +	−	46	+ −	***P. bacteriolytica* IAM 14595[T]**

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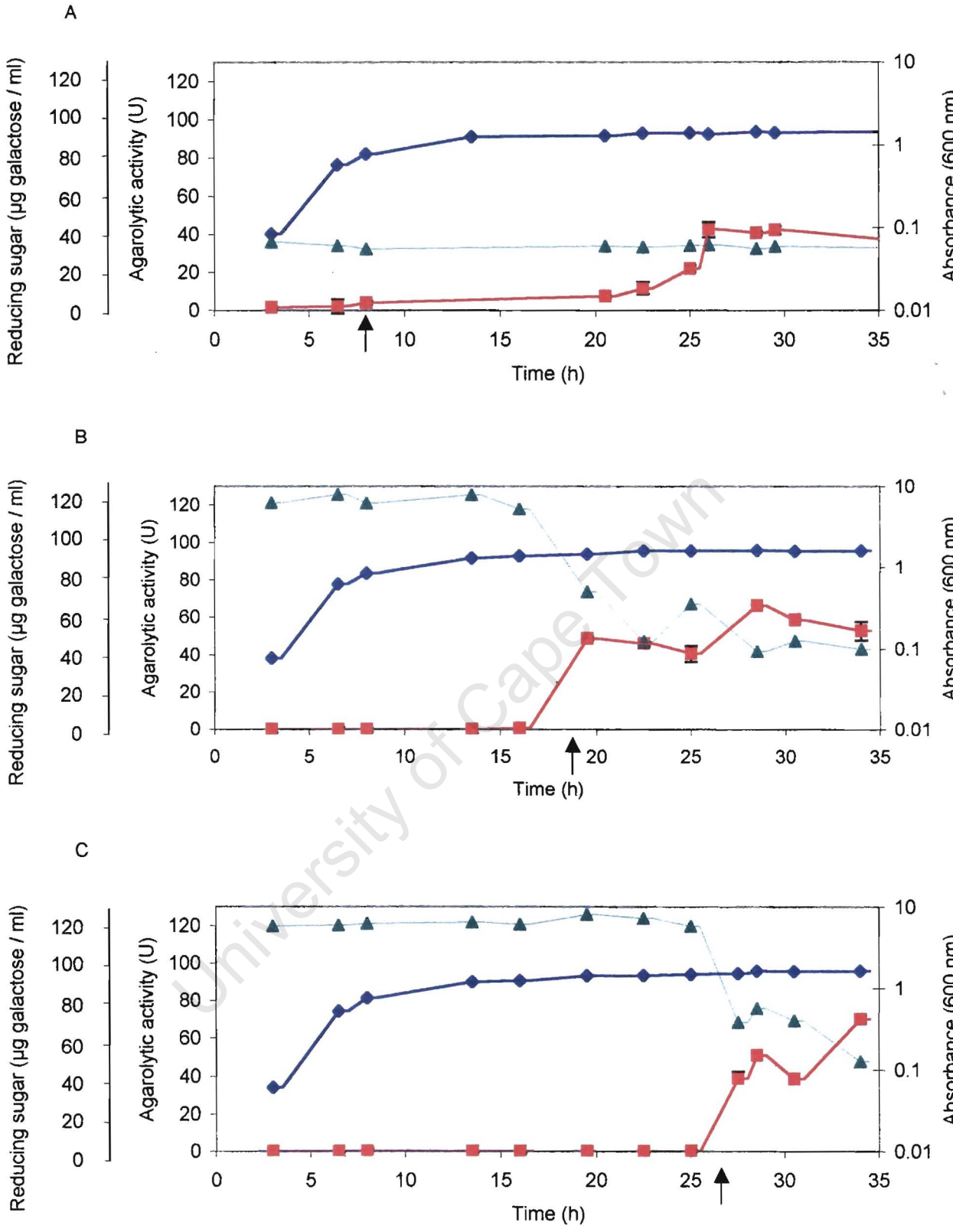

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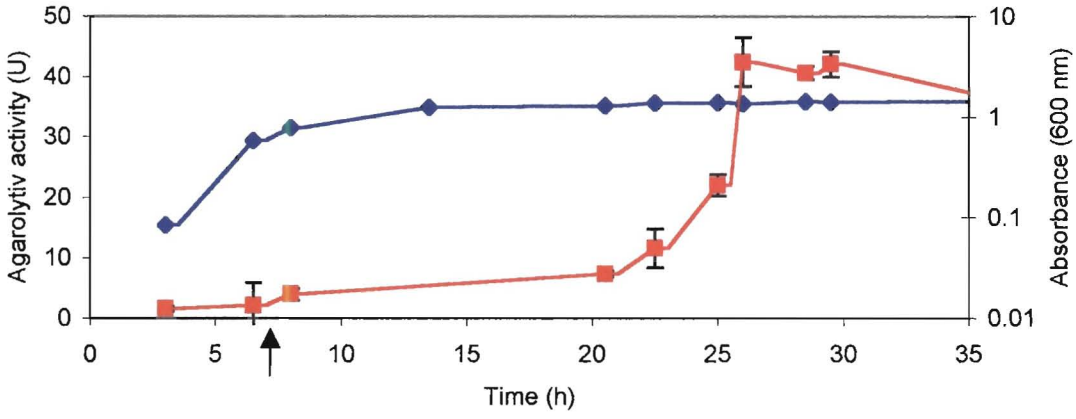
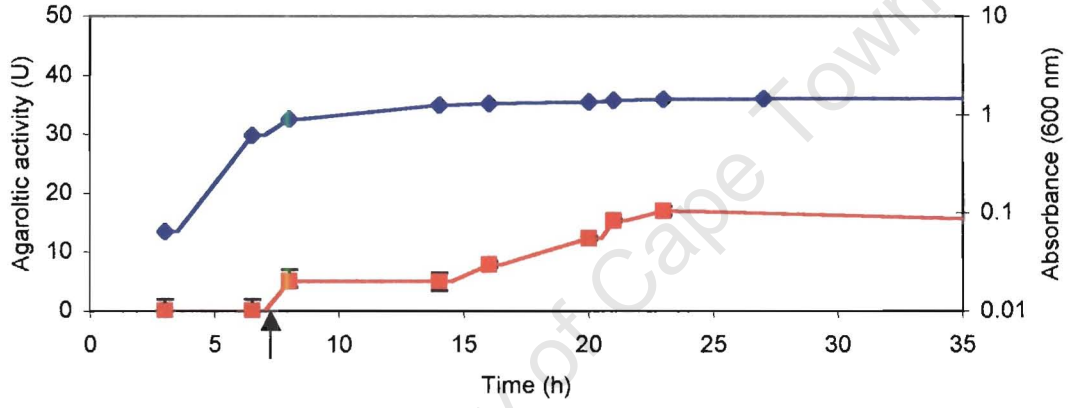
Figure 8 Growth (◆), agarolytic activity (■) and reduced sugar content (▲) of *P. gracilis* B9 cultured in three different media at 22°C. A: BM; B: BM supplemented with 0.2% D-galactose and C: BM supplemented with 0.2% D-glucose. Agarase activity is the mean of 3 values, while the error bars represent the standard deviation of the mean. (U): µg galactose produced/ml/h. Arrow: onset of agarase activity.

A



B



C

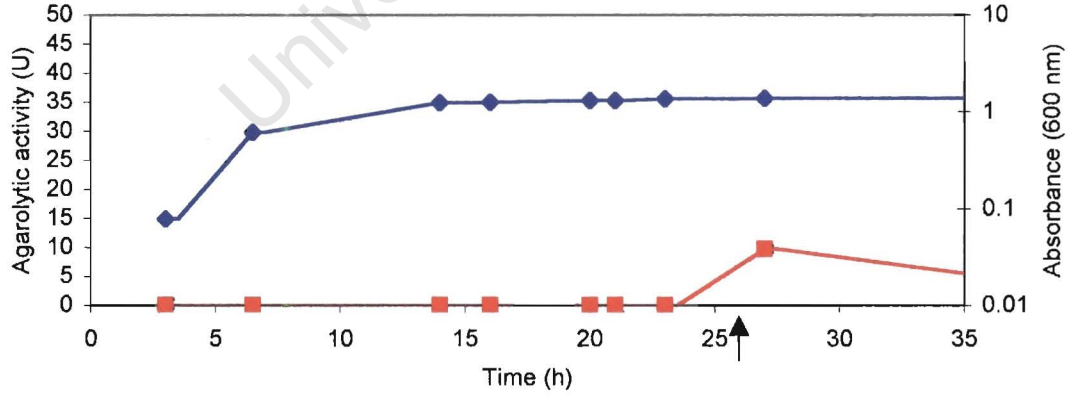


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Figure 9 Growth (◆), and agarolytic activity (■) of *P. gracilis* B9 cultured in three different media at 22°C. A: BM; B: BM where the agar component was substituted with agarose C: BM lacking both agar and agarose. Agarase activity is the mean of 3 values, while the error bars represent the standard deviation of the mean. (U): ug galactose produced/ml/h. Arrow: onset of agarase activity

2.5 Discussion

Jaffray *et al.* (1997) performed a detailed analysis of the epiphytic bacteria that colonize *Gracilaria gracilis* from Saldanha Bay in South Africa and Lüderitz in Namibia. She was able to show that less than 1% of bacteria found on the surface of *G. gracilis* are culturable. Nonetheless, Jaffray *et al.* (1997) isolated 134 culturable bacterial epiphytes from *G. gracilis* from 9 sampling sites. Five of the sampling sites were at Saldanha Bay. Our sampling site was also at Saldanha Bay where 9 isolates were identified. This was similar to the average of 11.6 isolates identified per sampling site at Saldanha Bay by Jaffray (1999).

Jaffray and Coyne (1996) developed a reliable assay for detection of putative bacterial pathogens of *G. gracilis.* The bacterium B9 produced deep indentations, "pitting", of the agar included in the culture media. This phenotype was shown by Jaffray and Coyne (1996) to be an important characteristic of all putative bacterial pathogens of *G. gracilis.* This assay was used to test whether bacterium B9 was a putative pathogen of *G. gracilis.* The isolate B9 fulfilled Koch's postulates, indicating that the bleaching of the thalli could only be due to the bacterium since the bacteria-free controls remained unbleached. The symptoms were more severe at the higher incubation temperature. This could have been due to the increased activity of the agarase(s) produced by the bacterium at the higher temperature (30°C). A significant number of agarases characterized to date have temperature optimum at or above 30°C (section 1.3.1). Thus, from the pathogenicity assay it could be concluded that the bacterium B9 is indeed a putative pathogen of *G. gracilis.*

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Gauthier *et al.* (1995) suggested that the genus *Alteromonas* be reclassified into two genera on the basis of phylogenetic relationships derived from 16S rDNA sequences, thus confirming the earlier rDNA-DNA hybridization data of Van Landschoot and De Ley (1983) and De Vos *et al.* (1989), and the DNA-DNA hybridization data produced by Akagawa-Matsushita *et al.* (1993). The two genera proposed were the original genus *Alteromonas* with *Alteromonas macleodii* subsp. *macleodii* as the type species and the new genus *Pseudoalteromonas* with *Pseudoalteromonas haloplanktis* subsp. *haloplanktis* as the new type species. Bacteria such as *Pseudoalteromonas atlantica* and *Pseudoalteromonas haloplanktis* subsp. *haloplanktis* were previously classified as members of the *Pseudomonas* and *Vibrio* genera, respectively (Yaphe, 1957; and Zobell and Upham, 1944). They were first reclassified as *Alteromonas* species

(Akagawa-Matsuishita *et al.*, 1992a; and Reichelt and Baumann, 1973) and subsequently reclassified as *Pseudoalteromonas* species (Gauthier *et al.*, 1995).

The members of the genus *Pseudoalteromonas* are straight or curved Gram-negative rods, are motile via a single polar flagellum, require seawater base for growth, and commonly inhabit coastal and open water oceans (Gauthier *et al.*, 1995). The 16S rDNA sequence, the physical and phenotypic characteristics of bacterium B9 suggested that it is a member of the *Pseudoalteromonas* genus. Since strain B9 is significantly different to the phenotypic characteristics of the other members of the *Pseudoalteromonas* genus, we propose that bacterium B9 be considered a new species and thus designated *Pseudoalteromonas gracilis* B9. However, although we are aware of the importance of DNA-DNA hybridization in differentiating species, DNA-DNA hybridization data is not absolute in that it has also been shown to be inconclusive in differentiating species (Fox *et al.*, 1992).

Coincidently, an example of this limitation can be seen in the *Pseudoalteromonas* genus. Sawabe *et al.* (2000) showed that the *P. elyakovii* isolates had high DNA-DNA hybridization values in relation to each other (71-97.4%) and low DNA-DNA hybridization values with respect to those isolates, e.g. *P. citrea* KMM 216 (49.5-53.5%), *P. distincta* KMM 638^T (52.4 – 61.2%) etc., that clustered close to them in the 16S rDNA tree. Hence, the isolates were assigned as members of a new species, i.e. *elyakovii*. This is consistent with the current suggested criteria for the classification of bacteria (Wayne *et al.*, 1987; Murray *et al.*, 1990; and Stackebrandt and Goebel, 1994). Similarly, strain KMM 216 was assigned to the species *citrea*, of which *P. citrea* NCIMB 1889^T is the type strain, based on the DNA-DNA hybridization value of 92% (Ivanova *et al.*, 1998). However, based on phenotypic and 16S rDNA phylogeny data, strain KMM 216 does not fall into the description of the species defined by Guathier (1977) as *citrea* (L. adj. *citreus* lemon yellow). Nonetheless, Ivanova *et al.* (1998) classified the isolates as members of the species *citrea* even though Stackebrandt and Goebel (1994) ultimately recommend, "In the end it is the presence or absence of phenotypic coherency among strains that should be the deciding factor about whether to describe species at all".

We are also aware that 16S rDNA sequence data have been shown to have limitations in differentiating strains at the species level (Fox *et al.*, 1992; Akagawa-Matsushita *et al.*, 1993; and Stackebrandt and Goebel, 1994). This also holds true in our case since the bacterium B9 clustered within the 16S rDNA tree (high bootstrap values) with species that have DNA-DNA

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hybridization values that vary between 13.5 to 61.2% (Sawabe *et al.*, 2000). With the absence of DNA-DNA hybridization data due to time constraints, strain B9 is tentatively considered to belong to a novel species based on its uniqueness when comparing its phenotypic characteristics to those of previously described and established species. This approach was considered sufficient for allocating novel species to a number of isolates, especially within the *Pseudoalteromonas* genus; *P. undina* and *P. tunicata* are but two examples (Chan *et al.*, 1978; and Holmström *et al.*, 1998). Hence, strain B9 was designated the species name *gracilis* after the seaweed from which it was isolated, *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham.

Since the original establishment of the genus *Pseudoalteromonas* by Gauthier *et al.* (1995) with 13 species encompassing about 87 strains (including the 12 type strains), this genus has grown to about 20 defined species and an undefined number of unnamed species, totaling over 240 strains. This genus includes members from diverse habitats, i.e. antarctic regions (Bowman *et al.*, 1997; Bozal *et al.*, 1997, etc) to hydrothermal vents (Moyer *et al.*, 1995). They were found associated with a variety of organisms, i.e. seaweeds (this study, Vera *et al.*, 1998; Sawabe *et al.*, 2000, etc), mollusks (Ivanova *et al.*, 1998), sponges (Ivanova *et al.*, 2000), viruses (Kivelä *et al.*, 1999) and phages (Wichels *et al.*, 1998). They are ubiquitous, i.e. from the Sea of Japan to the Atlantic, the Mediterranean to Antarctic. They produce a variety of substances such as hydrolyzing enzymes (this study, Vera *et al.*, 1998; Akagawa-Matsushita *et al.*, 1992b; Tsujibo *et al.*, 1998, etc), novel antibiotics (Gauthier and Breittmayer, 1979; Gauthier 1976, Yoshikawa *et al.*, 1997; etc), algicides (Yoshinaga *et al.*, 1997), organophosphorus acid anhydrolases (DeFrank *et al.*, 1993) and toxins (Simidu *et al.*, 1990). Thus this genus is fast gaining importance due to the ecological and economically important traits of its members.

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In an attempt to understand the importance of the agarolytic trait of *P. gracilis* B9 in the role it plays in pathogenicity of *G. gracilis*, some basic questions were asked. Firstly, relative to the phase of growth of *P. gracilis* B9, when is the agarolytic activity produced and secondly, is the agarase production of *P. gracilis* B9 subject to regulation? *P. gracilis* B9 was cultured in a variety of media in an attempt to answer these questions. In all the media tested, an easily utilizable substrate such as yeast extract was sufficient for bacterial growth. The addition of other carbon sources did not significantly affect the growth rate of the bacterium. However, this behavior is novel amongst agarolytic bacteria characterized to date. The growth of *Alteromonas agarlyticus* GJ1B was reported to be very poor in both enriched media and media lacking agar or

agarose (Potin *et al.*, 1993). A *Pseudomonas*-like bacterium grew better in media supplemented with agar compared to agarose (Hofsten and Malmqvist, 1975). *Cytophaga flevensis* growth improves with higher concentrations of agar (van der Meulen *et al.*, 1974).

Extracellular agarase production occurs in *P. gracilis* B9 during the early stationary phase of growth, with enzyme activity reaching a maximum during the late stationary phase in BM medium. Most of the agarolytic bacteria characterized to date, export the agarase during the exponential phase of growth, with the enzyme reaching a maximum between the late exponential and early stationary phase of growth (Duckworth and Turvey, 1969; van der Meulen *et al.*, 1974; Hofsten and Malmqvist, 1975; Morrice *et al.*, 1983b; Potin *et al.*, 1993; and Ha *et al.*, 1997).

In the medium lacking a reducing sugar, extracellular agarase production was detected in *P. gracilis* B9 during the early stationary phase of growth. In contrast, extracellular agarase production was delayed when the bacterium was cultured in medium supplemented with a reducing sugar (occurring in the late stationary phase). This repression of agarolytic activity by D-galactose is an indication that agarase expression in *P. gracilis* B9 is regulated via end-product repression. D-glucose elicited a similar effect, indicating that agarase expression is also subject to catabolite repression. The agarases of *Alteromonas* sp. C-1, *Streptomyces coelicolor* A3(2) and *Cytophaga flevensis* are also subject to catabolite repression (Leon *et al.*, 1992; Bibb *et al.*, 1987; and van der Meulen *et al.*, 1974). Catabolite repression is thought to occur because sugar monomers are more easily assimilated and metabolized than a complex polymer such as agar. It is known that bacteria produce agarases to breakdown structural products such as agar into sugars, which are utilized by the organism as nutrients (Day and Yaphe, 1975).

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The levels of agarolytic activity increased 1.5 fold during the late stationary phase of growth in media supplemented with a reducing sugar when compared to the non-supplemented media. Even though the growth characteristics appear to be unaffected by the addition of a reducing sugar, it is only once the reducing sugar is metabolized that agarolytic activity is induced. The significance of this increase in agarolytic activity is not known in terms of the biology of the bacterium. Further experimentation would be necessary in which the effect of the presence of reducing sugars on the pathogenicity of the bacterium could be assessed. However, in the media where the agar component was substituted with agarose, a reduction in agarolytic activity occurred. Hence, *P. gracilis* B9 agarase production is also regulated by substrate composition,

i.e. agarose is a less substituted form of agar and does not induce equivalent levels of agarolytic activity. This differential expression of agarolytic activity in response to agar quality was also observed in *Cytophaga flevensis* (van der Meulen *et al.*, 1974).

It seems that the bacterium is able to detect the presence of the agar and agarose polymers since minimal amounts of agarase(s) are excreted by strain B9 in the absence of agar as opposed to when the polymer is present in the media. It could be hypothesized that since agarase production occurs primarily during the stationary phase of growth, the initial low levels of agarase in the medium serves as "scavengers" to detect for the presence or absence of substrate polymers. Hence, it is conceivable that *P. gracilis* B9 would produce the agarase at low levels, detect the polymer (directly or indirectly) and ultimately regulate agarase expression accordingly since enzyme synthesis and export is energetically expensive. Once the polymer is detected, a large amount of enzyme is subsequently produced. However, the bacterium will not expend energy unnecessarily. As mentioned earlier, the bacterium will utilize the less energetically expensive substrates first.

The onset of disease in *G. gracilis* in Saldanha Bay occurs during the summer when high water temperatures and oligotrophic waters predominate (section 1.2). The network structure of the polysaccharide gel in the matrix is low in the cell wall of the thalli, allowing for cell wall expansion, i.e. short thinner fragments at higher temperatures as a consequence of high growth rates during summer (Christiaen *et al.*, 1987). The afore-mentioned network structure is low because of the substituted form of agar. Thus, the higher agarolytic activity observed when *P. gracilis* B9 is grown in the presence of the more substituted form of agar might reflect an adaptation of *P. gracilis* B9 to these conditions. The oligotrophic conditions referred to in Saldanha Bay during summer are usually equated with low nitrogen levels (Anderson *et al.*, 1996b). However, the availability of organic carbon was not determined in the afore-mentioned study. Our study has clearly shown the important effect that carbohydrates have on agarase production.

It could be hypothesized that under the oligotrophic conditions that occur in Saldanha Bay during summer, the bacterium could be subjected to low levels of utilizable, energy inexpensive carbohydrates with polysaccharide agar as the only readily available source of carbon. Since the bacterium is in oligotrophic conditions, it will mostly be in the stationary phase of growth when

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enzyme production occurs. Agarase activity will increase the pool of reducing sugar, which is then metabolized by strain B9, and consequently, enzyme production increases. This could explain the observed decimation of *G. gracilis* at various times in Saldanha Bay (R. Anderson, SRU, pers. comm.). Once the water quality improves and agar is no longer the sole source of carbon available to the bacterium, agarase production will be repressed. Over the following winter and summer seasons, the few left over strands of thalli regenerate vegatatively, thus replenishing the macroalgal populations until conditions are suitable for the infectious process to repeat itself once again.

It could be argued that the results and hypotheses discussed above may not reflect the entire picture as they are based on a one-to-one host-bacterial pathogen interaction. Studies performed on *Chondrus crispis* in Nova Scotia, Canada, concluded that this afore-mentioned association is quite a simplistic approach (Craigie and Correa, 1996; and Correa, 1996). Indeed, it was demonstrated that destruction of *C. crispis* fronds could result from a sequential interaction of several organisms, including bacteria, fungi, algal endophytes and nematodes. Similarly, the role of grazers, i.e. the sea urchins, keyhole limpets, filter feeding ascidians and fish, should not be underestimated in the disease phenomenon experienced by *G. gracilis* at Saldanha Bay (Anderson *et al.*, 1992). In addition, abiotic factors such as temperature, salinity, and nutrients and other biotic factors such as the role of antimicrobial substances produced by macroalgae as a defense mechanism should also not be overlooked (Christie, 1981; Cundell *et al.*, 1977; Sieburth, 1964; Sastry and Rao, 1994; and Rosell and Srivastava, 1987). Future work will have to take a more holistic approach in characterizing and understanding the disease phenomenon experienced by *G. gracilis* at Saldanha Bay. However, the results presented here reaffirm the data presented by Jaffray and Coyne (1996) that pathogenic bacteria, in particular the agarolytic bacteria, play a significant role in the disease of *G. gracilis* at Saldanha Bay.

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CHAPTER 3

CLONING AND SEQUENCING OF THE β-AGARASE GENE FROM *PSUEDOALTEROMONAS GRACILIS* B9

CONTENTS

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3.1 Summary

Pseudoalteromonas gracilis B9 genomic library was screened for agarolytic activity by visual detection of pitting of the agar. Five agar-hydrolyzing *E. coli* clones were obtained. Three of the clones exhibiting agarase activity contained a recombinant plasmid designated pDA1, while the other two clones contained a different plasmid, designated pDA3. Restriction endonuclease maps of pDA1 and pDA3 were constructed. Based on the restriction maps of the two plasmids, a 'common region' was identified. Southern hybridization studies performed on pDA1 and pDA3 confirmed that the cloned DNA harbored on the recombinant plasmids was of *P. gracilis* B9 origin. The similarity shared between pDA1 and pDA3 was also confirmed with Southern hybridization. Subsequent deletion analysis studies performed on pDA1 and pDA3 identified the 'common region' as the location of the agarase gene(s) on the plasmids. The agarolytic-encoding region was sub-cloned from pDA1 into the pBluescript KS cloning vector. The subsequent plasmid, pDA012, was then sequentially deleted from both ends of the inserted DNA fragment and the resultant deletion plasmids were sequenced. The sequences from the various constructs were assembled using the DNASIS software version 2.1 (Hitachi Software Engineering), while the completed assembled sequence was analyzed using DNAMAN version 4.13 (Lynnon BioSoft). A BLAST search of the GENBANK database showed that the ORF located in the agarolytic region of pDA012 had 85% identity to the β-agarase (*dag*A) (M73783) from *Pseudoalteromonas atlantica* ATCC 19262^T (or IAM 12927^T) at the amino acid level. Therefore, it was concluded that the 873 bp ORF encoded a putative β-agarase and was designated *aag*A. AagA is a new member of the family 16 glycoside hydrolases. The putative promoter region of *aag*A was determined and Northern hybridization showed that the mRNA transcript of *aag*A was 1.2 kb in length.

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3.2 Introduction

Agar-decomposing bacteria were first isolated by Gran in 1902 (Yaphe, 1957). Agarolytic enzymes have been reported for several bacterial genera, most of which have been isolated from the marine environment (Chapter 1.3.1). However, a few species have been isolated from other habitats such as soil, seawage and abattoir effluent (Kendall and Cullum, 1984; Sampietro and Vattuone de Sampietro, 1971; Hofsten and Malmqvist, 1975; and Rees *et al.*, 1994). Yaphe and co-workers were the first to describe an agar-degrading enzyme system from a marine bacterium (Day and Yaphe, 1975; and Groleau and Yaphe, 1977). Other scientists in the field defined the enzymes and their specificities (Morrice *et al.*, 1983a and b). The agar degrading enzyme system was that of a bacterial isolate classified as *Pseudoalteromonas atlantica* ATCC 19292^T (IAM 12927^T) by Gauthier *et al.* (1995).

A number of agarases have been characterized since then. A large number of which were isolated from bacteria belonging to the genera *Pseudoalteromonas, Pseudomonas, Cytophaga, Vibrio* and *Streptomyces.* The agarases involved in agar hydrolysis were either cloned and/or purified, and subsequently characterized (section 1.3.1). Despite this, of the enzymes involved in microbial hydrolysis of polysaccharides, the agarolytic enzymes are the poorest understood in terms of their specialized domains (Lee *et al.*, 2000).

The microbial degradation of polysaccharides entails diverse glycoside hydrolases with different specificities and modes of action. More than 800 glycoside hydrolases are grouped into more than 50 families of related amino acid sequences, deduced from the catalytic domain sequences of the cloned genes encoding them (Warren, 1996). In addition, these families were assigned to 'clans' by Couthinho and Henrissat (1999). Of the 10 agarases submitted to the GENBANK database, four were assigned to the family 16 glycoside hydrolases (DagA of *Streptomyces coelicolor* A3(2), AgaA and AgaB of *Cytophaga drobachiensis*, DagA of *Pseudoalteromonas atlantica* ATCC 19262^T (Barbeyron *et al.*, 1994; Barbeyron *et al.*, 1998; and Coutinho and Henrissat, 1999), two were assigned to the family 50 glycoside hydrolases (AgaA and AgaB of *Vibrio* sp. JT0107) (Coutinho and Henrissat, 1999), and one was assigned to the family 86 glycoside hydrolases (AgrA of *Pseudoalteromonas atlantica* T6c) (Coutinho and Henrissat, 1999). The other two β-agarases (PjaA of *Pseudomonas* sp. W7, and an unnamed β-agarase of

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Flavobacterium sp. NR19) and the one α-agarase (AgaA of *Alteromonas agarilytica* GJ1B) have not been assigned, to the best of our knowledge, to a glycoside hydrolase family.

The family 16 glycoside hydrolases contain enzymes with different substrate specificities, suggesting that new specificities were acquired by evolutionary divergence (Warren, 1996). Site directed mutagenesis within the 1,3(4)-β-glucanase gene of *Bacillus licheniformis* showed that Glu^{134} plays an important role in the catalytic domain of this family 16 glycoside hydrolase (Planas *et al.*, 1992). Barbeyron *et al.* (1994) performed multiple sequence alignments with family 16 glycoside hydrolases and found a strong conservation of Glu in relation to Glu^{134} of the 1,3(4)-β-glucanase gene of *B. licheniformis*. In addition, a conserved motif of two Glu residues (of which the afore mentioned Glu is one) separated by 3 or 4 amino acids, depending on whether they are bacterial lichenases and plant xyloglucan endotransglycosylases or galactanases and laminarinases respectively, are characteristic of the catalytic site of family 16 glycoside hydrolases (Barbeyron et al., 1998).

We isolated several different epiphytic bacterial isolates of the agarophyte *G. gracilis* from Saldanha Bay. The bacterium B9, designated *P. gracilis* B9, was selected for further study due to its ability to degrade agar and cause bleaching in *G. gracilis* (Chapter 2). In an endeavor to clone the gene(s) responsible for the agarolytic phenotype, a *P. gracilis* B9 genomic library in *E. coli* HB101 was constructed (Schroeder, 1994). This chapter describes the screening of this genomic library for agarolytic *E. coli* clones. The sequencing and characterization of the plasmids responsible for the agarolytic phenotype in the *E. coli* clones is also described here.

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3.3 Materials and methods

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

3.3.1 Bacterial strains and plasmids

The bacterial strains and plasmids that were used to clone and characterize the β-agarase gene from *P. gracilis* B9 are listed in Table 1.

3.3.2 Media and culture conditions

The *E. coli* strains were either grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA) at 37°C (Appendices A.1.10 and A.1.11). *E. coli* transformants that harbored recombinant pEcoR251 and pBluescript KS plasmids were grown in LB or LA containing 100 μg/ml ampicillin (Appendix A.2.1). *E. coli* transformants that harbored recombinant pEKpllacZ plasmids were grown in LB or LA containing 30 μg/ml kanamycin (Appendix A.2.1). Wild-type *P. gracilis* B9 was maintained at 22°C in either BM or on BA as described in Chapter 2.

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3.3.3 Identification of agarolytic *E. coli* clones

The genomic library was screened for agarolytic activity on LA containing ampicillin by visual detection of pitting of the agar. In order to confirm that the agarolytic phenotype of these clones was plasmid linked, the plasmid DNA from several agarolytic clones was extracted and re-transformed into competent *E. coli* HB101 (Appendix B.7 and B.8). The resulting transformants were screened on LA containing ampicillin. Agarolytic activity was also confirmed by staining the plate with Gran's iodine (Appendix A.2.15). Areas of agar hydrolysis stained lighter than areas where the agar remained intact.

Table 1 Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
E. coli HB101	*supE*44 *hsdS*20 *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	Sambrook et al., (1989)
E. coli JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ(*lac-proAB*) F'(*traD*36 *proAB*' *lacI*^{q} *lacZ*ΔM15)	Sambrook et al., (1989)
P. gracilis B9	Saldanha Bay, South Africa	This work
pBluescript KS	Amp^r, β-galactosidase	Short et al., (1988)
pEcoR251	Amp^r, *Eco*RI endonuclease	Zabeau and Stanley, (1982)
pEKpllacZ	promoterless β-galactosidase, Km^r	Eikmanns *et al.*, 1991
pDA1	pEcoR251 containing ~7 kb *P. gracilis* B9 genomic DNA	This work
pDA10	Derivative of pDA1 containing ~6.5 kb *P. gracilis* B9 genomic DNA	This work
pDA11	Derivative of pDA1 containing ~5 kb *P. gracilis* B9 genomic DNA	This work
pDA12	Derivative of pDA1 containing ~2.6 kb *P. gracilis* B9 genomic DNA	This work
pDA15	Derivative of pDA1 containing ~3 kb *P. gracilis* B9 genomic DNA	This work
pDA16	Derivative of pDA1 containing ~1.3 kb *P. gracilis* B9 genomic DNA	This work
pDA3	pEcoR251 containing ~9 kb *P. gracilis* B9 genomic DNA	This work
pDA31	Derivative of pDA3 containing ~7 kb *P. gracilis* B9 genomic DNA	This work
pDA012	2.75 kb *Hin*dIII-*Xho*I fragment from pDA11 cloned into pBluescript KS	This work
pDA012_A(n)	Various Heinikoff deletion mutants generated from the *Bam*HI site of pDA012	This work
pDA012_K(n)	Various Heinikoff deletion mutants generated from the *Kpn*I site of pDA012	This work
pEKA(n)	Various constructs generated for promoter study in correct orientation in pEKpllacZ	This work
pEK(n)A	Various constructs generated for promoter study in an incorrect orientation in pEKpllacZ	This work
pEKHA9	pEKpllacZ promoter probe vector with *Hin*dIII-*Hin*dIII fragment from pDA012_A9	This work
pEKHA10	pEKpllacZ promoter probe vector with *Hin*dIII-*Hin*dIII fragment from pDA012_A10	This work

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3.3.4 DNA analytical methods

3.3.4.1. Restriction endonuclease analysis of recombinant plasmids

A preliminary digest with three restriction endonucleases, *Eco*RI, *Pst*I, and *Sac*I, was performed on the five agarolytic recombinant plasmids in order to determine whether the *E. coli* recombinants contained similar plasmids (Appendix B.9). The recombinant plasmids were then subjected to a more thorough restriction endonuclease mapping regime in order to determine whether they were similar to pDA1 (Schroeder, 1994). The following restriction endonucleases were used: *Bam*HI, *Bgl*II, *Bst*XI, *Eco*RI, *Hind*III, *Kpn*I, *Mlu*I, *Nco*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sph*I, and *Xho*I. Restriction enzyme digestions were performed as outlined in Appendix B.9. The resulting restriction enzyme fragments were separated on 1% TAE agarose gels (Appendix B.4).

3.3.4.2 Southern hybridization analysis of the recombinant plasmids pDA1 and pDA3

To establish whether the cloned DNA harbored on the recombinant plasmids pDA1 and pDA3 was of *P. gracilis* B9 origin, the 550 bp *Eco*RI (6400)-*Eco*RI (6950) fragment was cut out of pDA1 (Figure 1) by *Eco*RI restriction enzyme digestion as outlined in Appendix B.9 and gel purified (Appendix B.11). The purified DNA fragment was used as a probe against *P. gracilis* B9 genomic DNA. This fragment, based on the restriction enzyme maps of pDA1 and pDA3, lies in a region common to both recombinant plasmids. The *P. gracilis* B9 genomic DNA (Appendix B.2) was digested with the restriction enzymes *Pst*I, *Hind*III and *Sac*I and the resulting fragments were separated on a 0.8% TAE agarose gel. In addition, pDA3 (Appendix B.7) was digested with the restriction enzymes *Eco*RI, *Pst*I and *Sac*I and the resulting fragments were separated on a 1% TAE agarose gel. The Southern hybridization procedure that was followed is described in Appendix B.13.

3.3.4.3 Deletion analysis of the recombinant plasmids pDA1 and pDA3

Deletion analysis of pDA1 and pDA3 was employed in order to identify the regions within the cloned DNA that were responsible for agar hydrolysis in *E. coli* HB101. pDA1 was digested with either *Eco*RI or *Hin*dIII, and pDA3 with *Sal*I and *Xho*I, in order to generate various deletions. Restriction enzyme digestions, agarose gel electrophoresis and ligation procedures were

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performed as described in Appendix B.2, B.4, and B.14, respectively. The resulting constructs were transformed into *E. coli* JM109 (Appendix B.8) and subsequently assayed for their ability to hydrolyze agar (section 3.3.3).

3.3.4.4 Sub-cloning of agarolytic region into pBluescript KS

The agarolytic region of pDA11 was sub-cloned into pBluescript KS for sequencing purposes. The 2.75 kb *Hin*dIII-*Xho*I restriction enzyme fragment from pDA11 was sub-cloned into pBluescript KS. The resultant plasmid was designated pDA012 (Figure 7). The sub-cloning procedures, i.e. restriction enzyme digestions, agarose gel electrophoresis, ligation and *E. coli* JM109 transformation procedures were performed as described in Appendix B.9, B.4, B.14 and B.8, respectively

3.3.4.5 Heinikoff shortening of pDA012

pDA012 was digested with the restriction enzymes *Bst*XI and *Bam*HI to generate the 3' and 5' overhangs, respectively, required by exonuclease III for shortening in the forward direction. Reverse shortenings were obtained by cleaving with *Kpn*I (3' overhang) and *Xho*I (5' overhang). The Heinikoff shortening procedure was carried out as described in Appendix B.15. We identified and isolated sequential deletions in both the forward and the reverse direction. The resultant deletion plasmids were designated pDA012_A(n) and pDA012_K(n), respectively (n: number of the deletion; Figure 8). These sequentially deleted constructs were also subsequently assayed for their ability to hydrolyze agar (section 3.3.3).

3.3.4.6 DNA sequencing of pDA012

Double stranded sequencing of the forward shortened constructs of pDA012 (pDA012_A(n), section 3.3.4.5) was performed by the dideoxynucleotide chain termination method using the Sequenase sequencing kit (Amersham-Pharmacia) and [α-^{35}S]-dATP (Sanger *et al.*, 1977; Appendix B.16). The reverse shortened constructs of pDA012 (pDA012_K(n), section 3.3.4.5) were sequenced using a Thermosequenase cycle-sequencing kit (Amersham) and an

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ALFexpress™ automated sequencer (AM version 3.01, Pharmacia Biotech) as described in Appendix B.5.

Sequence data was analyzed using DNAMAN version 4.13 (Lynnon BioSoft) and DNASIS software version 2.1 (Hitachi Software Engineering). Homology searches with both DNA and protein sequences were carried out using the BLAST algorithm (Altschul *et al.*, 1990) provided by the Internet service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nlm.nih.gov/BLAST/).

3.3.4.7 Promoter analysis of *aag*A

pDA1 was used as a template for the isolation of the promoter region of *aag*A via PCR. Six PCR primers were designed, five forward (A9, A10, A11, A11.5 and A11.7) and one reverse (R) (Appendix B.17). The five regions, namely A9-R, A10-R, A11-R, A11.5-R and A11.7-R, containing 536 bp, 381 bp, 189 bp, 114 bp and 63 bp regions upstream of the start codon (ATG) of *aag*A, respectively, were amplified using a Hybaid Omnigene thermal cycler (Figure 11). The primers were designed so as to amplify the promoter regions that coincided with some of the Heinikoff deletions generated from the *Bam*HI restriction site of pDA012 (section 3.3.4.5). They were also designed with the forward primers having a *Pst*I restriction site and the reverse primer a *Sal*I restriction site for directional cloning into the promoter probe vector, pEKpllacZ. The PCR was set up as described in Appendix B.17.

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The PCR product, A9-R, was directionally sub-cloned into the pEKpllacZ promoter probe vector using the restriction enzymes *Pst*I and *Sal*I, while the other PCR products were blunt-end cloned into the *Sal*I site of pEKpllacZ. The sub-cloning procedures, i.e. restriction enzyme digestions, agarose gel electrophoresis, ligation and *E. coli* JM109 transformation procedures were performed as described in Appendix B.9, B.4, B.14 and B.8, respectively. The resultant transformants were screened by restriction enzyme mapping and finally by DNA sequencing to verify the orientation of the PCR product cloned into pEKpllacZ (Appendix B.9 and B.5). In addition, the plasmids pDA012_A9 and pDA012_A10 (Figure 8) were digested with *Hin*dIII as outlined in Appendix B.9 and the resultant fragments were gel purified (Appendix B.11). The purified DNA fragments designated A9-*Hin*dIII and A10-*Hin*dIII, respectively, were blunt end cloned into the *Sal*I site of pEKpllacZ (Figure 11). β-galactosidase assays were performed in

triplicate using 100 µl aliquots of exponentially growing *E. coli* JM109 transformed with the afore-mentioned constructs as described by Miller (1992).

3.3.4.8 Multiple sequence alignment

The agarases and β-glucanases (family 16 glycoside hydrolases) shown in Figure 15 were aligned with the 290 amino acid sequence of AagA using the optimal alignment option in DNAMAN version 4.13 (Lynnon BioSoft).

3.3.5. RNA analytical methods

3.3.5.1 Recovery of RNA from *P. gracilis* B9

Total cellular RNA was prepared from wild-type *P. gracilis* B9 using the method of Ausubel *et al.* (1989). Ten milliliters of overnight cells were collected by centrifugation (10K rpm for 10 min), and resuspended in 10 ml protoplast buffer (Appendix A.2.20) to which 80 µl of lysozyme (80 mg/ml) was then added (Appendix A.2.20). The resulting protoplasts were collected by centrifugation (7K rpm for 5 min) and resuspended in 500 µl lysis buffer (Appendix A.2.20) and 15 µl of DEPC. The tubes were incubated at 37°C for 5 min before salt-saturated NaCl (Appendix A.2.20) was added to precipitate most of the SDS contained within the lysis buffer. The white precipitate was removed by centrifugation at 14K rpm for 10 min. Supernatants were collected and the nucleic acid precipitated with absolute ethanol and centrifugation. Pellets were washed in 70% ethanol to remove any residual salt and resuspended in a final volume of 20 µl of DEPC treated water.

RNA samples were treated with DNAse I (Roche) in order to remove any DNA contamination. Ten units of DNAse I was added to RNA in a final volume of 50 µl. The tubes were left at 37°C for 1 h. The RNA was recovered by adding an equal volume of phenol / chloroform / isoamylalcohol (Appendix A.2.20), centrifuging at 14K rpm for 10 min and transferring the resulting aqueous phase to a clean tube. In order to precipitate the RNA, 5 µl 3M sodium acetate (Appendix A.2.20) and 150 µl absolute ethanol was added to the tube containing the RNA. The mixture was centrifuged at 14K rpm for 10 min. Centrifugation resulted in the precipitation of the RNA which was subsequently suspended in 20 µl of DEPC treated water. RNA was quantified by reading the absorbance at 260 nm using a Spectrophotometer (Beckman).

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3.3.5.2 Primer extension analysis of *aag*A

RNA was isolated as described in section 3.3.5.1. from a 24 h old *P. gracilis* B9 culture. The oligonucleotide primer (100 ng) 5' TGCTTCTTTAACTAGTGCGAGTG 3' was used to reverse transcribe RNA. The primer was end-labelled with [γ-^{32}P]-dATP using T_4 polynucleotide kinase, precipitated with 4 M ammonium acetate and absolute ethanol, and finally resuspended in TEN 600 solution (Appendix B.18).

Radiolabelled oligonucleotide (2.3 x 10^4 cpm) was added to 40 µg of total cellular RNA. The hybridization was performed at 37°C for 120 min. Reverse transciption of RNA was carried out with M-MuLV reverse transcriptase (Roche). The reaction was performed at 37°C for 1 h. The reaction mix was treated with 105 µl of RNase (10 mg/ml) to reduce the amount of total RNA in the sample and also to prevent aberrant electrophoresis of the primer extension product. Reverse transcribed product was extracted by adding 15 µl 3 M sodium acetate (Appendix A.2.20) and 150 µl phenol:chloroform:isoamyl alcohol (Appendix A.2.20). The tube was spun at 10K rpm for 10 min before the aqueous phase was removed and the DNA precipitated by the addition of 300 µl of ice-cold absolute ethanol. The tube was then spun at 10K rpm for 30 min and the DNA pellets were washed with 70% ethanol. The pellet was resuspended in 3 µl stop buffer. The DNA was denatured at 95°C for 5 min before separation on a 6% acrylamide / 7 M urea gel (Appendix B.16). The primer extension products were compared to a sequencing ladder, generated with the Sequenase sequencing kit (Amersham-Pharmacia) and the primer used in the primer extension procedure (Appendix B.16).

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3.3.5.3 Northern hybridization analysis of *aag*A

To determine the size and presence or absence of the mRNA transcript of *aag*A in relation to the growth phase of *P. gracilis* B9, total RNA was isolated from 5 and 24 h old cultures of *P. gracilis* B9 as described in section 3.3.5.1. The 550 bp *Eco*RI (6400)-*Eco*RI (6950) *aag*A internal fragment was isolated from pDA1 (section 3.3.4.2) and used as a probe against *P. gracilis* B9 total RNA. Ten micrograms of total RNA per sample was analyzed in the Northern hybridization procedure that was followed as described in Appendix B.19.

3.4 Results

3.4.1. Restriction enzyme mapping of the recombinant plasmids pDA1 and pDA3

Five *E. coli* HB101 transformants capable of hydrolyzing agar were isolated after screening the previously established *P. gracilis* B9 genomic DNA library for agarase encoding genes (Schroeder, 1994). A preliminary restriction digest performed on the five-agarolytic recombinants with three restriction endonucleases, *Eco*RI, *Pst*I, and *Sac*I, revealed that the plasmids fell into two groups. A representative of each of the groups was mapped. The first group of three plasmids mapped the same as the previously identified recombinant plasmid, designated pDA1 (Figure 1), while the other two clones harbored a new recombinant plasmid, designated pDA3 (Figure 2). Comparison of the restriction endonuclease maps of pDA1 and pDA3 revealed that the two plasmids had a similar restriction pattern located to a 2.2 kb region; i.e. the region between *Nco*I (5000)- *Eco*RI (7200) on pDA1 and *Eco*RI (7100)- *Nco*I (9300) on pDA3 were similar (Figure 1 and 2).

3.4.2. Southern hybridization studies

The 550 bp *Eco*RI-*Eco*RI fragment of the 'common region' of the two recombinant plasmids, pDA1 and pDA3, was isolated from pDA1 and used as a probe against wild-type *P. gracilis* B9 chromosomal DNA in order to verify that the inserted DNA fragments carried by these recombinant plasmids were of *P. gracilis* B9 origin. Indeed, the *Eco*RI (6400)-*Eco*RI (6950) fragment hybridized to two *Sac*I fragments of 32 and 22 kb in size (lane 1), a 8.5 kb *Pst*I fragment (lane 2), a 8.25 kb *Hin*dIII-*Pst*I fragment (lane 3) and a 9 kb *Hin*dIII fragment (lane 4) (Figure 3).

To reaffirm the restriction mapping data in section 3.4.1, i.e. that the 'common region' was indeed the same in both plasmids, the *Eco*RI (6400)-*Eco*RI (6950) probe isolated from pDA1 was hybridized to pDA3. The two 550 and 250 bp *Eco*RI fragments and the three 350, 250 and 200 bp *Eco*RI-*Sac*I fragments were faint and hence are not visible on the 1% agarose gel photograph (Figure 4A, lanes 3 and 4). As expected from the endonuclease restriction map of pDA3 (Figure 2), the 8.8 kb *Pst*I (lane 2), the 550 bp *Eco*RI (lane 3), the two *Eco*RI-*Sac*I

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fragments of 350 and 200 bp in size (the fragments migrate very close to each other and hence, appear as one long smear; lane 4) and the 12.45 kb *Sac*I (lane 5) were homologous to the probe (Figure 4B). The two 1.823 kb and 1.827 *Pst*I fragments, which migrated as a doublet through the 1% agarose gel (lane 2), and the three 7.1 kb, 4.55 kb and 250 kb *Eco*RI fragments from both the *Eco*RI and *Eco*RI-*Sac*I digests (lanes 3 and 4) were not homologous to the probe (Figure 4B). *Sac*I linearized the pDA3 and hence the 12.45 kb fragment was homologous to the probe (lane 5) (Figure 4B).

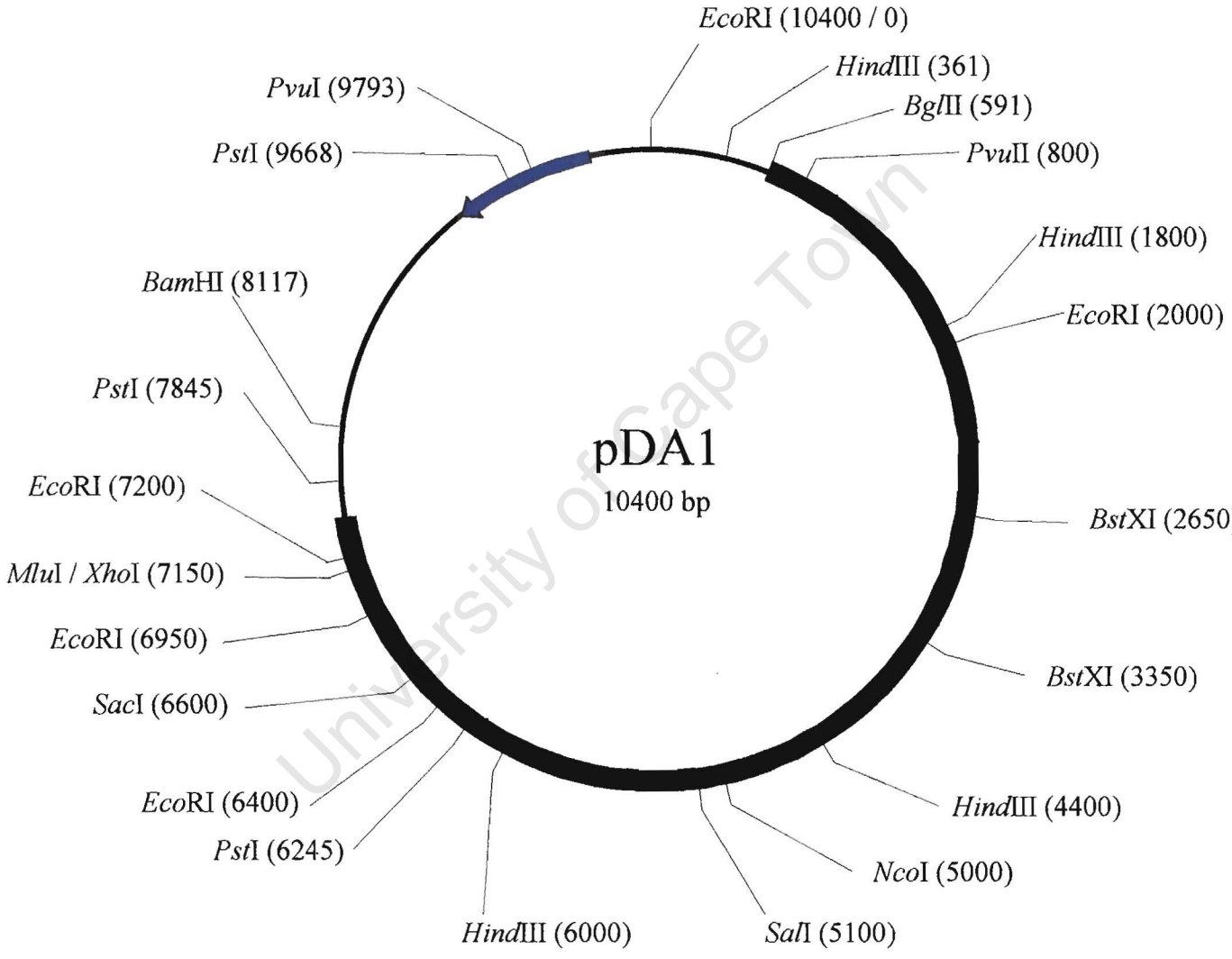

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Figure 1. Restriction enzyme map of the recombinant plasmid pDA1. The thick line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The size of the recombinant plasmid is shown in base pairs (bp). The numbers inside the brackets indicate the positions in bp of the various restriction enzyme sites. The position of the β-lactamase gene (➔) is shown.

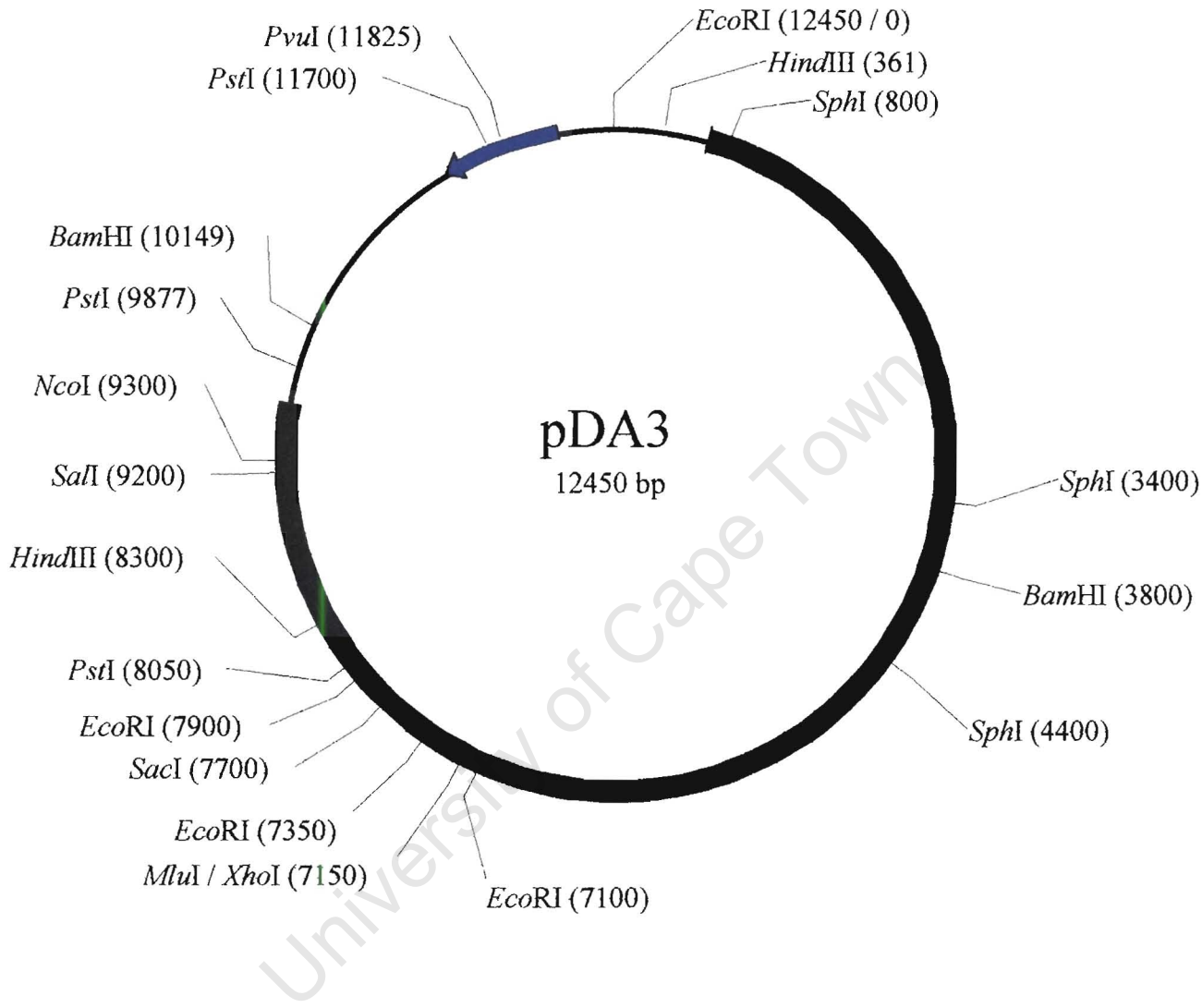

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Figure 2. Restriction enzyme map of the recombinant plasmid pDA3. The thick line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The size of the recombinant plasmid is shown in base pairs (bp). The numbers inside the brackets indicate the positions in bp of the various restriction enzyme sites. The position of the β-lactamase gene (➔) is shown.

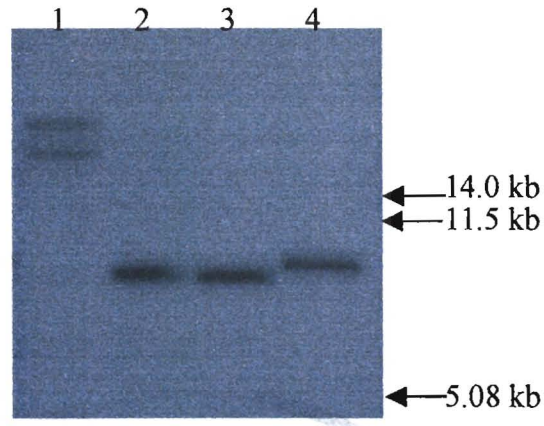

Figure 3. Southern hybridization of the 550 bp *Eco*RI (6950)-*Eco*RI (6400) restriction fragment of pDA1 against *P.gracilis* B9 genomic DNA. Genomic DNA was digested with *Sac*I (lane 1), *Pst*I (lane 2), *Hin*dIII-*Pst*I (lane 3) and *Hin*dIII (lane 4). The arrows indicate the positions and sizes in kb of the lambda markers.

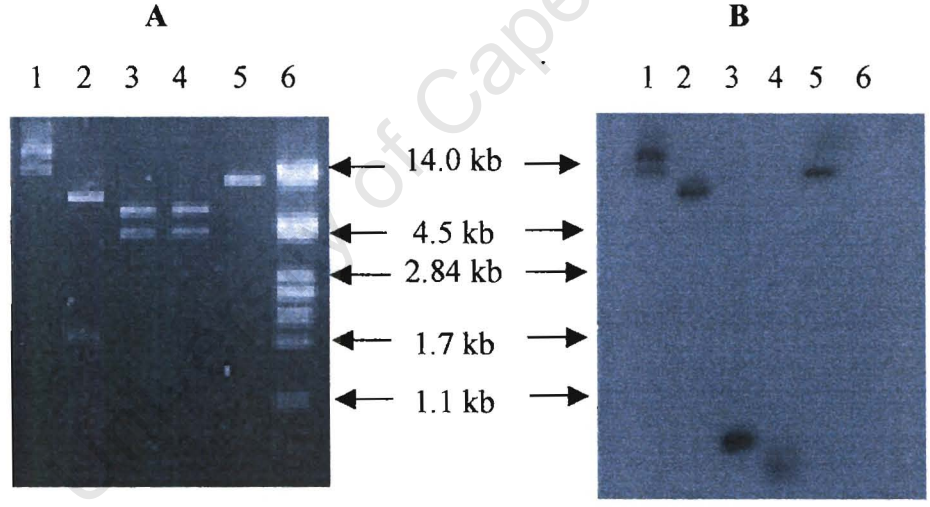

Figure 4. A) pDA3 digested with restriction enzymes *Eco*RI, *Pst*I and *Sac*I and the resulting fragments separated on a 1% TAE agarose gel.

B) Southern hybridization of the 550 bp *Eco*RI (6400)-*Eco*RI (6950) restriction fragment of pDA1 against pDA3. Lane 1: undigested pDA3, Lane 2: pDA3 digested with *Pst*I, Lane 3: pDA3 digested with *Eco*RI, Lane 4: pDA3 digested with *Eco*RI-*Sac*I, Lane 5: pDA3 digested with *Sac*I, and Lane 6: lambda DNA digested with *Pst*I. The arrows indicate the sizes in kb of the lambda markers.

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3.4.3. Deletion analysis of pDA1 and pDA3

Since both the recombinant plasmids harbored large inserts of *P. gracilis* B9 genomic DNA (pDA1 has an insert of ~7.0 kb and pDA3 an insert of ~9.0 kb) it was necessary to identify which region of the *P. gracilis* B9 DNA fragment was responsible for the agarolytic activity observed in the *E. coli* HB101 clones. This was achieved by deleting various fragments from the two recombinant plasmids and visually scoring for agarolytic activity as described in section 3.3.3. The deletion strategies that were followed are depicted in Figures 5 and 6.

Deletion of the 550 bp *Eco*RI (6400)-*Eco*RI (6950) fragment in pDA1 produced the plasmid pDA10. The *E. coli* JM109 transformed with pDA10 lost its ability to hydrolyze agar. The *E. coli* JM109 transformed with pDA12, which was generated by deletion of the 4.4 kb *Eco*RI (2000)-*Eco*RI (6400) fragment in pDA1, also lost its agarolytic activity. Deleting the 2.0 kb *Eco*RI (10400 / 0)-*Eco*RI (2000) and the 4.04 kb *Hin*dIII (361)-*Hin*dIII (4400) fragments in pDA1 to yield pDA11 and pDA15, respectively, had no effect on the agarolytic activity when transformed into *E. coli* JM109. However, the *E. coli* JM109 transformed with pDA16, generated by the deletion of the 5.63 kb *Hin*dIII (361)-*Hin*dIII (6000) fragment in pDA1, resulted in an increase in agarolytic activity in relation to pDA1 (Figure 5). Deletion of the 2.05 kb *Xho*I (7150)-*Sal*I (9200) fragment from pDA3 resulted in a loss of agarolytic activity, i.e. when pDA31 was transformed into *E. coli* JM109 no pitting of the agar was observed (Figure 6). Therefore the *Nco*I-*Eco*RI 2.2 kb region shared by the two plasmids (section 3.4.1) includes the agarase gene(s).

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3.4.4 DNA sequencing of the agarase gene cloned from *P. gracilis*

In order to characterize the agarolytic gene(s) encoded by pDA1 and pDA3, a 2.75 kb *Hin*dIII-*Xho*I restriction enzyme fragment from pDA11 was sub-cloned into the multiple cloning site (MCS) of Bluescript KS and the subsequent plasmid was designated pDA012 (Figure 7). pDA012 was then sequentially deleted from both ends of the insert and a few of the resultant deletion plasmids were sequenced (Figure 8). All the sequences generated were assembled with DNASIS software version 2.1 (Hitachi Software Engineering), while the completed assembled sequence was analyzed using DNAMAN version 4.13 (Lynnon BioSoft).

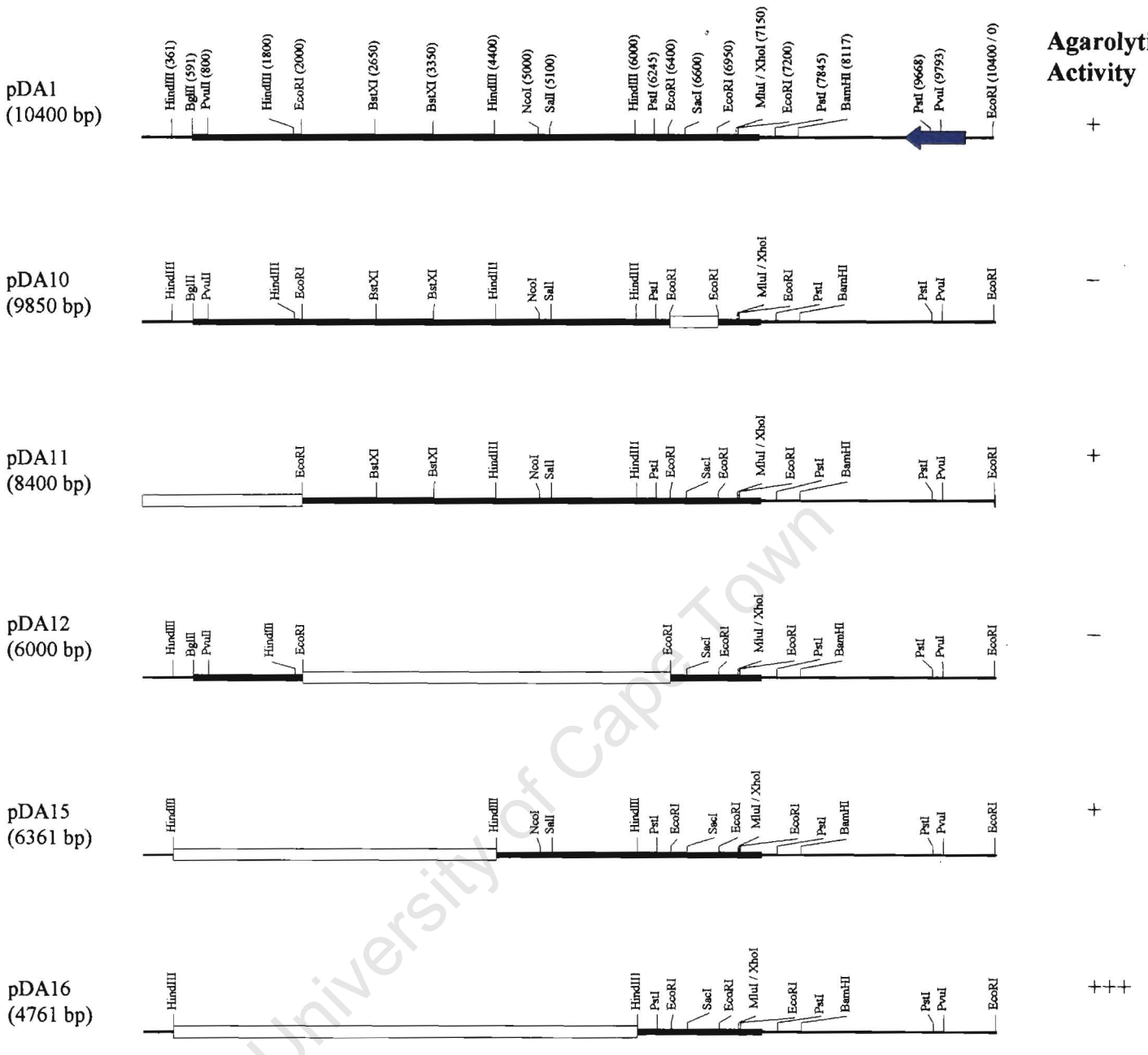

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Figure 5 Deletion mutagenesis of pDA1. Various regions of *P. gracilis* B9 DNA were deleted in pDA1. (□): represents the fragment deleted to create plasmids pDA10, pDA11, pDA12, pDA15, and pDA16. The thick solid line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The position of the β-lactamase gene (➔) is shown. The plus or minus signs represent the presence or absence of agarolytic activity, respectively. The size of the recombinant plasmid is shown in base pairs (bp). The positions (in bp) of the various restriction enzyme sites are indicated in the pDA1 linear restriction map.

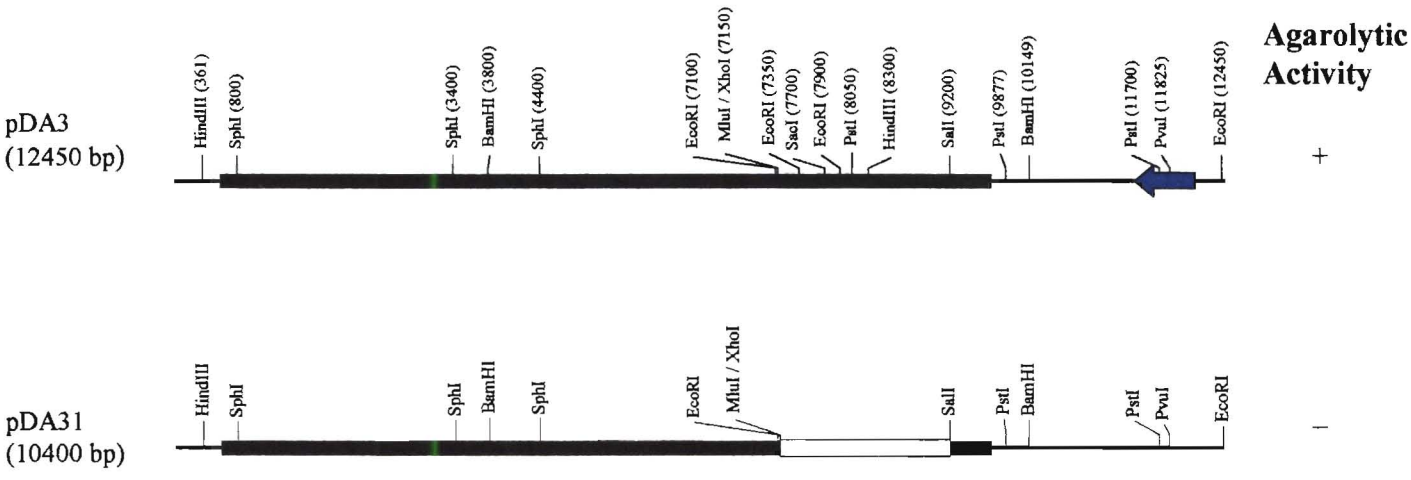

Figure 6 Deletion mutagenesis of pDA3. (□): represents the fragment deleted to produce pDA31. The thick solid line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. The position of the β-lactamase gene (➔) is shown. The plus sign indicates agarolytic activity, whereas the minus sign represents the absence of agarolytic activity. The sizes of the recombinant plasmids are shown in base pairs (bp). The positions (in bp) of the various restriction enzyme sites are indicated in the pDA3 linear restriction map.

Upon translation of the nucleotide sequence in all six reading frames, a single ORF was found in the region of the cloned *P. gracilis* B9 DNA associated with agarolytic activity in *E. coli* JM109. The ORF began at the ATG codon at position 1792 and continued to position 2664. It codes for a protein of 290 amino acids and thus a hypothetical size of 31.87 kDa (Figure 9). The protein possesses features commonly associated with signal peptides of secreted proteins, as seen from the hydrophobicity plot (Figure 10). The putative peptidase cleavage site is between residues 21 and 22, thus yielding a mature protein of 269 amino acids and thus a molecular mass of 30.23 kDa (Figure 9). A putative Shine Dalgarno sequence, GGAGA, at position 1781-1785, was located upstream from the initiation codon ATG. This sequence resembles the putative ribosome-binding site normally regarded as the Shine Dalgarno sequence (Shine and Dalgarno, 1975). The 14 bp inverted repeat at positions 2748-2761 and 2767-2780 has a calculated ΔG of -16.90K cal/mol (determined using DNAMAN for Windows version 4.13, Lynnon Biosoft). This region of DNA has the capability for haipin formation, suggesting that this stem-loop could function as a Rho-independent transcriptional terminator (Figure 9).

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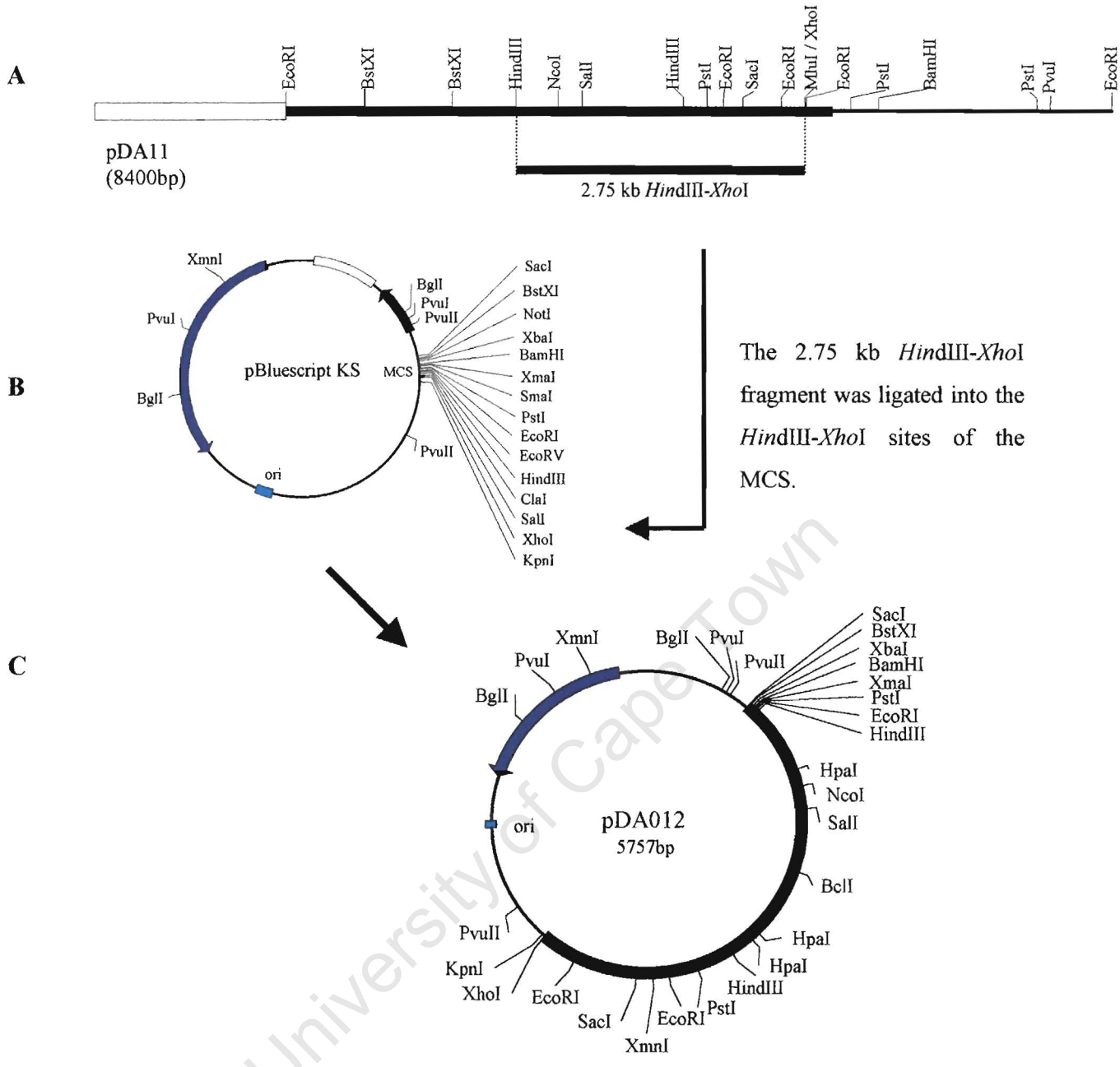

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Figure 7. Construction of pDA012. The sizes of the recombinant plasmids are shown in base pairs (bp). A) 2.75 kb *Hin*dIII-*Xho*I fragment from pDA11. (□): represents the fragment deleted from pDA1 to produce pDA11. The thick solid line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pEcoR251 DNA. B) The 2.75 kb *Hin*dIII-*Xho*I fragment was ligated into the *Hin*dIII-*Xho*I sites of the cloning vector pBluescript KS. The positions of the β-lactamase (➔) and the β-galactosidase (➔) genes are shown, as well as the two origins of replication, i.e. M13 ori (□) and ori (■). C) The resulting plasmid was designated pDA012. The thick black solid line represents cloned 2.75 kb *Hin*dIII-*Xho*I *P. gracilis* B9 fragment whereas the thin line represents pBluescript KS DNA. Enzyme restriction site positions are based on sequence data.

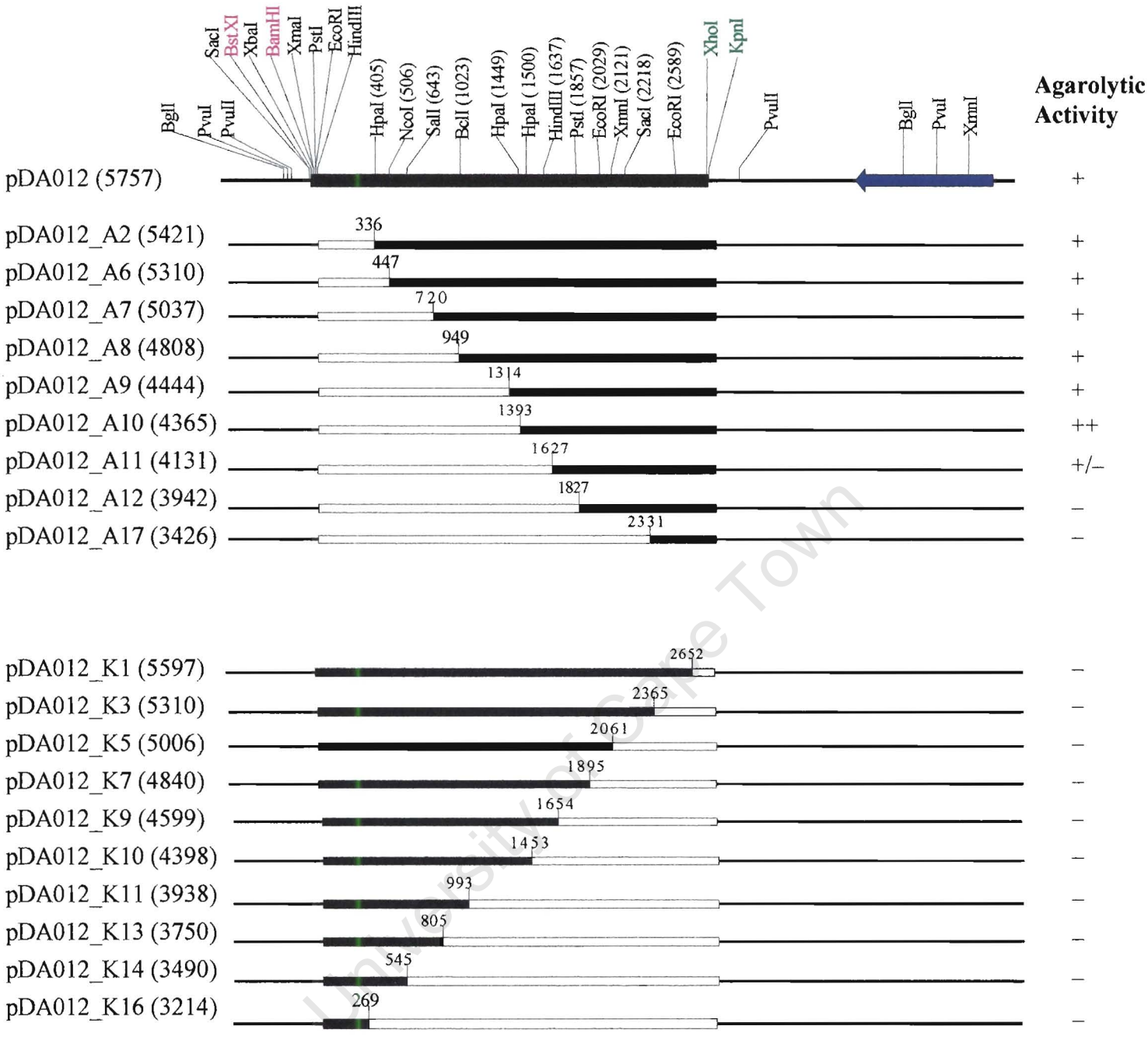

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Figure 8 pDA012 was deleted sequentially from the *Bam*HI restriction site and the resultant plasmids were designated pDA012_A(n). pDA012 was also deleted sequentially from the *Xho*I restriction site and the resultant plasmids were designated pDA012_K(n). The positions (in bp) of the various restriction enzyme sites (based on sequence data) and the sizes of the recombinant plasmids indicated in brackets. The positions of the Heinikoff shorts are shown above each construct. (□): represents the deleted fragments. The thick solid line represents cloned *P. gracilis* B9 DNA whereas the thin line represents pBluescript KS. The plus or minus signs represent the presence or absence of agarolytic activity, respectively.

```
                                                                    A9
1261    ATTGTAAAAATAAATTATTTTTCAATTTATACATCAAGTTATAAAAACAATCCAACCTTG
1321    CTTCATTAAAAATATCTCTCCAGTTCCAATATTAGGTATTTATTAGCAGTAGTAATATCT
                    A10
1381    CTATTTTGATAAAAGTTAAACTGATTGATACAGAGAAAAAACATCTCGCCCAAGCAAAAA
1441    CACAATGTTAACAGGTGCGATTAAATTATTAACAAAATAAATATTTAATTATCTATTGTT
1501    AACAATGTGTAATTGTGCAAATATTAAACAGTTGCTTTGGTGTTACTTTAAAATCAGTAC
1561    AACAAAGTTACATTACACACTATTTCACTTTTATTAAAACGTATCCATGTCGGTGTGCTT
          A11          HindIII
1621    ATGCTGGTATTTTCATAAGCTTGAGTTTGAATATGGATACAAATAATAGAAGGTACACAC
1681    AAAAGAGATTGTTTCATCTAGGGCCTGTTTATCTTTCGATGATTAAATTCACTAAAGTCA
                                                          SD
1741    CTCGCACTAGTTAAAGAAGCATATCTACATTAATTTGCATGGAGATTTTATATGAATATA
   1                                                          M  N  I
                                    A12
1801    TTAAAACTACTATCCTGTTCTACTTGCGCAATACTCTGCACAGCAACACATGCTGCAGAT
   4     L  K  L  L  S  C  S  T  C  A  I  L  C  T  A  T  H  A *A  D
1861    TGGGACGCATATAGTATTCCGGCTTCTGCTGGATCAGGTAAAACATGGCAATTACAAACT
  24     W  D  A  Y  S  I  P  A  S  A  G  S  G  K  T  W  Q  L  Q  T
1921    GTTTCCGACCAATTTAACTACCAAGCCGGTACTTCAAATAAACCGGCAGCATTTACCAAT
  44     V  S  D  Q  F  N  Y  Q  A  G  T  S  N  K  P  A  A  F  T  N
1981    CGTTGGAATGCTTCGTATATTAATGCTTGGCTTGGGCCTGGTGATACTGAATTCAGTTCA
  64     R  W  N  A  S  Y  I  N  A  W  L  G  P  G  D  T  E  F  S  S
2041    GGTCATTCCTACACTACTGGTGGTGCGTTAGGCCTTCAGGCAACTGAAAAAGCAGGAACA
  84     G  H  S  Y  T  T  G  G  A  L  G  L  Q  A  T  E  K  A  G  T
2101    AATAAAGTGCTTTCGGGAATTGTTTCTTCAAAAGCAACTTTTACATACCCACTTTATCTT
 104     N  K  V  L  S  G  I  V  S  S  K  A  T  F  T  Y  P  L  Y  L
2161    GAGGCAATGGTAAAACCGAGTAATAACACTATGGCTAATGCCGTATGGATGCTGAGCTCT
 124     E  A  M  V  K  P  S  N  N  T  M  A  N  A  V  W  M  L  S  S
2221    GATTCAACTCAAGAAATTGATGCAATGGAGTCCTACGGCAGTGATCGTGTAGGGCAAGAG
 144     D  S  T  Q  E  I  D  A  M  E  S  Y  G  S  D  R  V  G  Q  E
2281    TGGTTTGACCAACGTATGCACGTTAGTCACCATGTTTTTATACGTGAGCCATTTCAAGAT
 164     W  F  D  Q  R  M  H  V  S  H  H  V  F  I  R  E  P  F  Q  D
2341    TACCAACCAAAAGACGCAGGCGCATGGGTATACAATAGCGGTGAAACATACCGAAATAAA
 184     Y  Q  P  K  D  A  G  A  W  V  Y  N  S  G  E  T  Y  R  N  K
2401    TTTCGTCGCTACGGTGTACATTGGAAGGACGCATGGAACCTAGATTACTATATTGATGGT
 204     F  R  R  Y  G  V  H  W  K  D  A  W  N  L  D  Y  Y  I  D  G
2461    GTATTAGTTCGCAGCGTTTCGGGTCCGAATATAATTGATCCTGAAGGCTATACAGGCGGC
 224     V  L  V  R  S  V  S  G  P  N  I  I  D  P  E  G  Y  T  G  G
2521    ACAGGGCTAAATAAACCAATGCACATCATTTTAGATATGGAACATCAACCTTGGCGTGAT
 244     T  G  L  N  K  P  M  H  I  I  L  D  M  E  H  Q  P  W  R  D
2581    GTAAAACCGAATTCAACCGAGCTAGCTGATTCAAACAAAAGTATATTTTGGATTGATTGG
 264     V  K  P  N  S  T  E  L  A  D  S  N  K  S  I  F  W  I  D  W
2641    GTACGTGTTTACAAAGCAAACTAAGTCATTCTAAAATATTTGTAATACTAGGTTTTATTA
 284     V  R  V  Y  K  A  N  *
2701    CTTCTCGTTATACGACACGGAGCAATAAAATTTAAGCTCCCCAAAACTACTTAATGCGGC
2761    TATTACAGCCGCATTAAGTATAATTAACCTGAACTCTGGATAGTAAATCTATCTCGAGC
```

Figure 9 Nucleotide sequence of the coding region of *aag*A situated on the 2.75 kb *Hin*dIII-*Xho*I fragment of pDA012. The Shine-Dalgarno sequence is underlined and labelled as SD. The initiation codon, ATG, and the 5' end of the respective Heinikoff shortened sequences are in bold. The *Hin*dIII restriction site (red) and the position of the putative signal sequence peptidase cleavage site (*) are shown. The putative termination signal is highlighted in blue. *aag*A has been assigned the accession number U61972.

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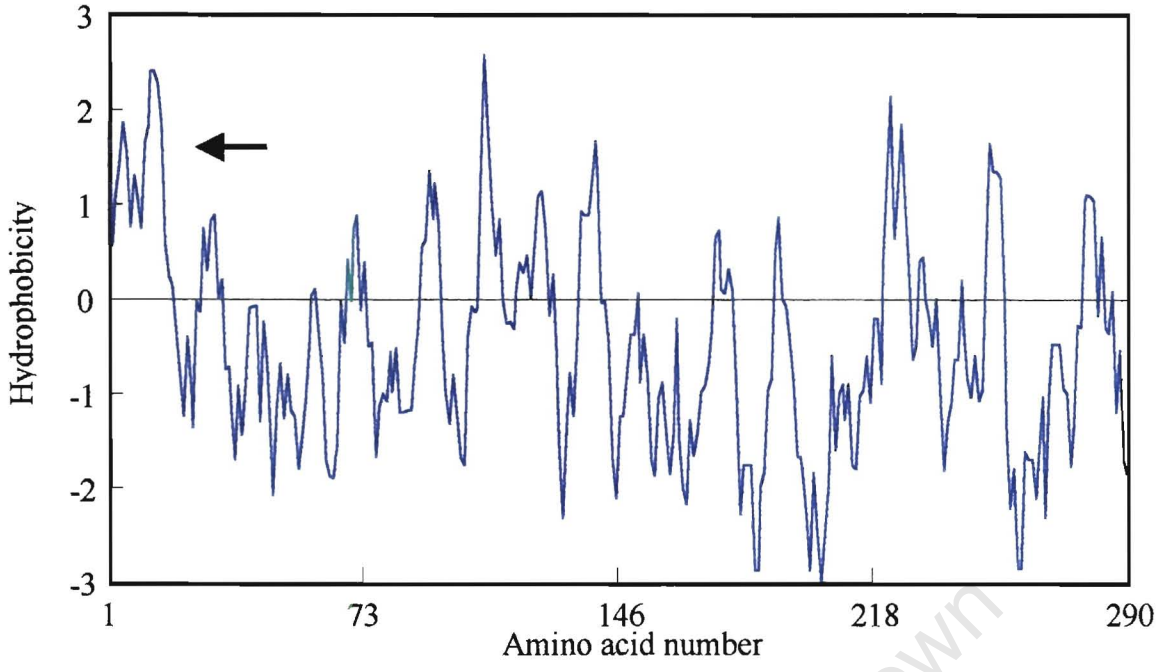

Figure 10 Hydrophobicity plot of the deduced 290 amino acid sequence of the β-agarase of *P. gracilis* B9. Arrow: proposed signal peptide.

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3.4.5 Identification of the putative promoter of *aag*A

Various constructs representing DNA fragments of various lengths situated upstream of the start codon (ATG) of *aag*A (A9-R, A10-R, A11-R, A11.5-R and A11.7-R) were amplified by PCR (Figure 11). These PCR products were sub-cloned into the pEKpllacZ promoter probe vector. The PCR product, A9-R, was directionally sub-cloned into the pEKpllacZ promoter probe vector using the restriction enzymes *Pst*I and *Sal*I. The resultant plasmid was designated pEKA9. The other PCR products were blunt-end cloned into the *Sal*I site of pEKpllacZ. The resultant plasmids were designated pEKA10, pEKA11, pEKA11.5, and pEKA11.7. The recombinant pEKpllacZ plasmids that harbored PCR constructs that were inserted in the opposite orientation were designated pEK10A, pEK11A, pEK11.5A, and pEK11.7A. In addition, the two *Hin*dIII fragments (A9-*Hin*dIII and A10-*Hin*dIII) from the plasmids pDA012_A9 and pDA012_A10, respectively, were blunt end cloned into the *Sal*I site of pEKpllacZ. The plasmids were designated pEKHA9 and pEKHA10, respectively. All the constructs were verified by restriction enzyme mapping and by sequencing of the inserted DNA. β-galactosidase assays were performed

on *E. coli* cultures harboring the above constructs and the levels of enzyme activity compared (Figure 12).

A 10.5 fold increase in β-galactosidase activity, 348 to 3674 Miller units, occurred when the sequence upstream of the 5' end of A10 was deleted, i.e. difference in activity between *E. coli* JM109 transformed with pEKA9 and *E. coli* JM109 transformed with pEKA10, respectively (Figure 12, columns 1 and 2). Similarly, *E. coli* JM109 transformed with pEKHA9 had lower activity (951 Miller units) than *E. coli* JM109 transformed with pEKHA10 (2254 Miller units) (columns 6 and 7). An increase in enzyme activity was also observed in the deletion mutants, i.e. *E. coli* JM109 transformed with pDA012_A9 had less agarolytic activity when compared to *E. coli* JM109 transformed with pDA012_A10 (Figure 8).

A 2.5 fold reduction in β-galactosidase activity, 3674 to 1435 Miller units, occurred when the sequence upstream of the 5' end of A11 is deleted, i.e. difference in activity between *E. coli* JM109 transformed with pEKA10 and *E. coli* JM109 transformed with pEKA11, respectively (Figure 12, columns 2 and 3). A reduction of enzyme activity was also observed in the deletion mutants, i.e. *E. coli* JM109 transformed with pDA012_ A11 had less activity when compared to *E. coli* JM109 transformed with pDA012_A10 (Figure 8).

The *E. coli* transformants harboring pEKA11, pEKA11.5 and pEKA11.7 all appear to be promoterless despite the higher β-galactosidase activity that was measured in relation to the pEKpllacZ control, since the PCR products that had been cloned in the opposite orientation also resulted in similar β-galactosidase activities (Figure 12, columns 3-5, 8-11 and 12). A reduction in agarolytic activity was also apparent when *E. coli* JM109 harboring pDA012_A11 was compared to *E. coli* JM109 harboring the shorter deleted constructs (pDA012_A2 to pDA012_A10) (Figure 8).

In section 3.4.3, *E. coli* JM109 transformed with pDA16, generated by the deletion of the 5.63 kb *Hin*dIII (361)-*Hin*dIII (6000) fragment in pDA1, resulted in an increase in agarolytic activity in relation to pDA1. Thus, this result indicates the presence of a putative promoter downstream of the *Hin*dIII restriction site. However, the increase in agarolytic activity exhibited by *E. coli* JM109 harboring pDA16 was a consequence of the strong rightward lambda promoter on pEcoR251 (Figure 13). When pDA16 is transformed into *E. coli* K514λ (Figure 13, colony

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number 2), i.e. expressing the lambda repressor, the zone of agar hydrolysis disappears. Thus, the promoter of *aag*A lies upstream of the *Hin*dIII (1637) restriction site, which is 155 nucleotides upstream of the start codon of *aag*A.

In order to approximate the position of the promoter of *aag*A, a 23-mer oligonucleotide complementary to the mRNA transcribed from *aag*A was used in primer extension analysis. The primer extension placed the start site of the *aag*A gene transcript at 184 nucleotides upstream of the putative translational start site (Figure 14). Although a conserved promoter region could not be identified, the sequences TATTAA at nucleotide positions –16 to –11 and TTACAT at –40 to –35 could possibly serve as the -10 and –35 regions of a putative promoter, respectively (Harley and Reynolds, 1987).

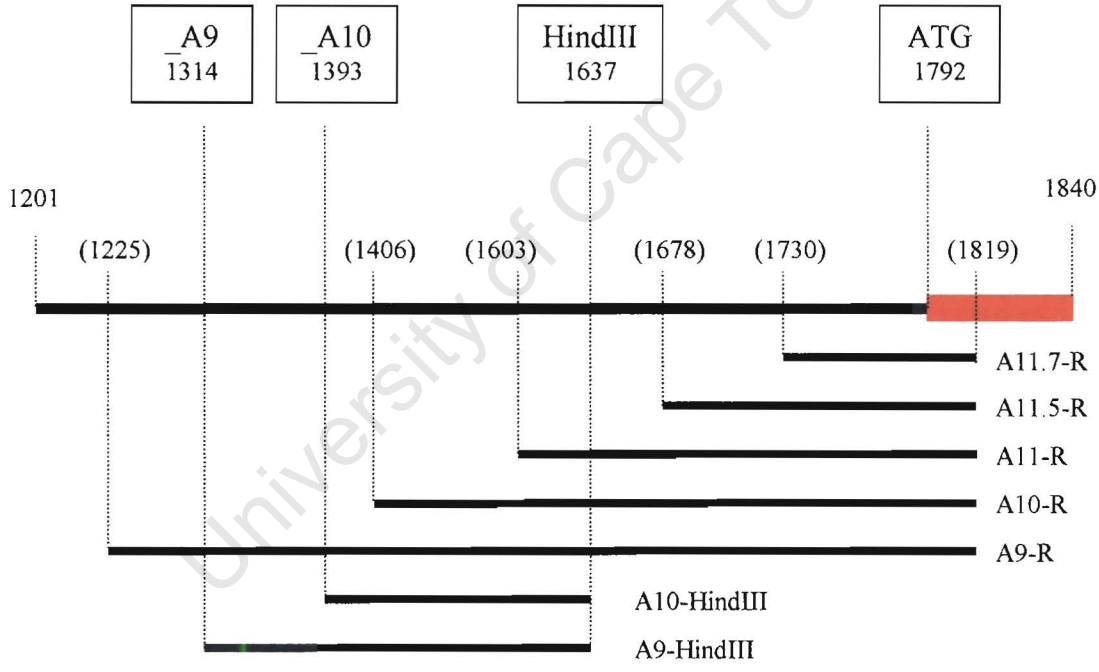

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Figure 11 PCR strategy for promoter analysis. The numbers indicated in brackets are the positions (in bp) of the various PCR products in relation to the 2.75 kb *Hin*dIII-*Xho*I fragment of pDA012. The boxes indicate the 5' position of two of the deletion constructs, pDA012_A9 and pDA012_A10, and the positions of the *Hin*dIII restriction site and the start codon of *aag*A (positions are in bp). The 5' end of *aag*A (■) is shown.

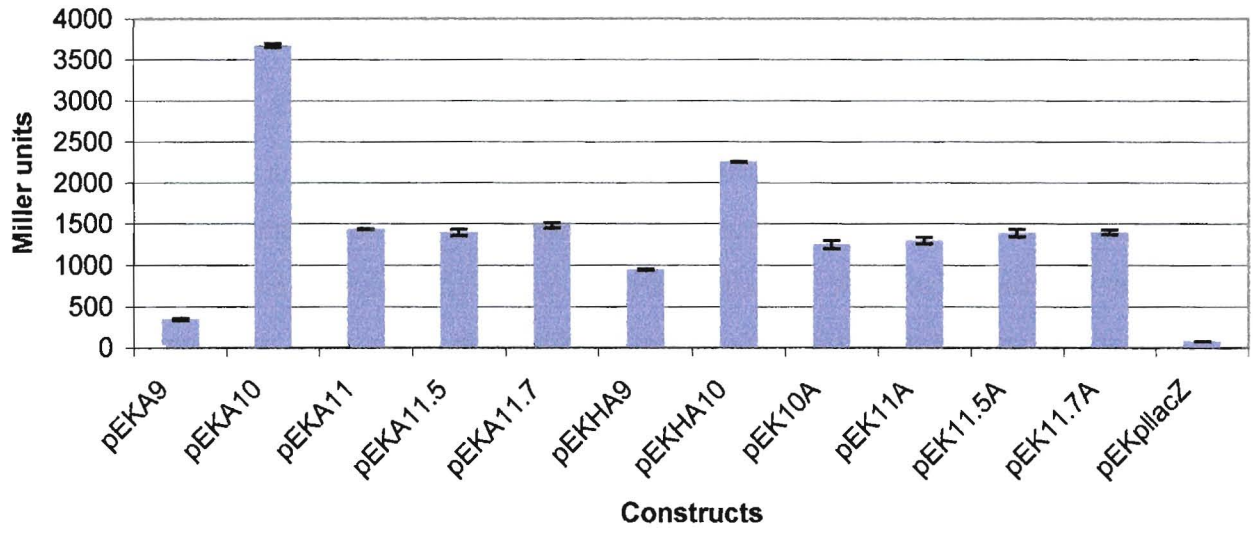

Figure 12. Promoter analysis of the *aag*A gene. Each data point represents the mean of 3 values, while the error bars represent the standard deviation of the mean.

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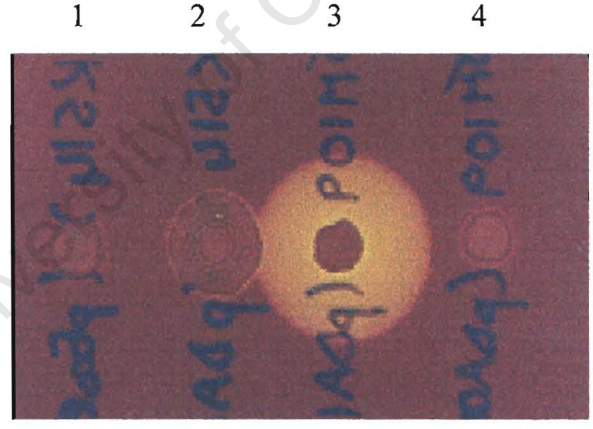
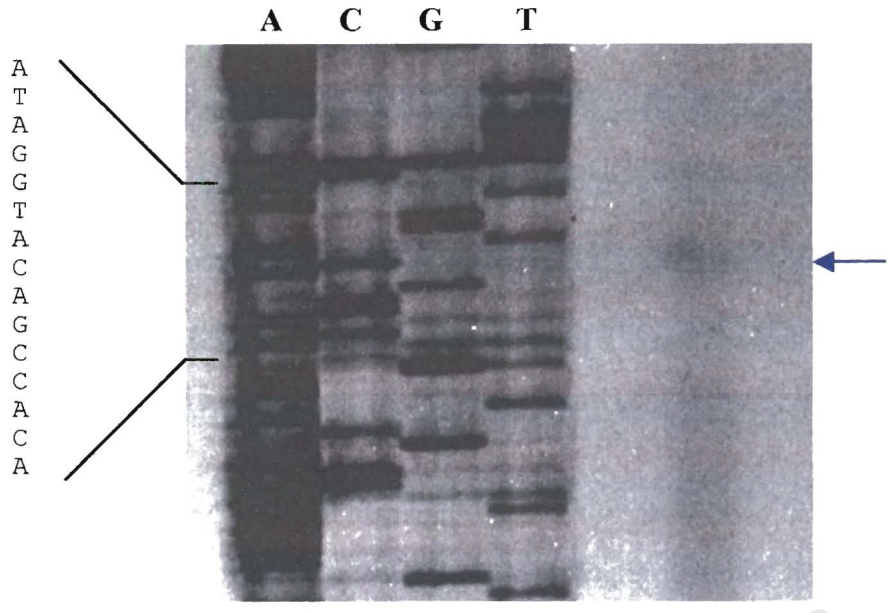


Figure 13 Agarolytic activity of 5-day-old transformants stained with Gran's Iodine. *E. coli* strains transformed with the following plasmids: Colony number 1, *E. coli* K514λ (pEcoR251); colony number 2, *E. coli* K514λ (pDA16), colony number 3, *E. coli* JM109 (pDA16); and colony number 4, *E. coli* JM109 (pDA012_A12).

A



B

```
1261    ATTGTAAAAATAAATTATTTTTCAATTTATACATCAAGTTATAAAAACAATCCAACCTTG
1321    CTTCATTAAAAATATCTCTCCAGTTCCAATATTAGGTATTTATTAGCAGTAGTAATATCT
                                                                A9
                                                                A10
1381    CTATTTTGATAAAAGTTAAACTGATTGATACAGAGAAAAAACATCTCGCCCAAGCAAAAA
1441    CACAATGTTAACAGGTGCGATTAAATTATTAACAAAATAAATATTTAATTATCTATTGTT
1501    AACAATGTGTAATTGTGCAAATATTAAACAGTTGCTTTGGTGTTACTTTAAAATCAGTAC
                -35                             -10             +1
1561    AACAAAGTTACATTACACACTATTTCACTTTTATTAAAACGTATCCATGTCGGTGTGCTT
                A11     HindIII
1621    ATGCTGGTATTTTCATAAGCTTGAGTTTGAATATGGATACAAATAATAGAAGGTACACAC
1681    AAAAGAGATTGTTTCATCTAGGGCCTGTTTATCTTTCGATGATTAAATTCACTAAAGTCA
                                                        SD
1741    CTCGCACTAGTTAAAGAAGCATATCTACATTAATTTGCATGGAGATTTTATATGAATATA
   1                                                        M  N  I
                                        A12
1801    TTAAAACTACTATCCTGTTCTACTTGCGCAATACTCTGCACAGCAACACATGCTGCAGAT
   4     L  K  L  L  S  C  S  T  C  A  I  L  C  T  A  T  H  A  A  D
```

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Figure 14 A) Mapping of the transcriptional start site of *aag*A by primer extension analysis. The sequence ladder on the left is the reverse complement of the *aag*A nucleotide sequence. The DNA sequence in the region of the transcriptional start site is indicated. The complementary base of the transcriptional start site is indicated in blue in the sequence and by the arrow.

B) The putative –10 and –35 regions (blue), the putative Shine-Dalgarno sequence (underlined and labelled as SD) are shown. The predicted transcriptional start site of *aag*A is indicated (+1) at position 1608. The initiation codon, ATG, and the 5' end of respective Heinikoff shortened sequences are indicated in bold. A 23-mer oligonucleotide complementary to the mRNA transcribed from *aag*A is shown in bold italics. The *Hin*dIII restriction site is shown in red.

3.4.6 Homology searches

In order to determine the putative identity of the protein encoded by the ORF included in the 2.75 *Hin*dIII-*Xho*I fragment of pDA012, we searched several databases for homologous sequences. The ORF was found to have 76% and 85% identity to the β-agarase (*dag*A, M73783) from *Pseudoalteromonas atlantica* ATCC 19262^T (IAM 12927^T) at the DNA and amino acid levels, respectively. It also shared a lesser amino acid identity with the two β-agarases (*aga*A, AF098954, and *aga*B, AF098955) from *Cytophaga drobachiensis* and the β-agarase (*dag*A, P07883) from *Streptomyces coelicolor* A3(2); i.e. 51, 44 and 34%, respectively (Table 2). The other sequenced β-agarases were found to not have significant homology to the *P. gracilis* B9 enzyme since they it did not appear on the blast search. However, a few endo-1,3(4)-β-glucanases were found to have homology to AagA (Table 2). The multiple sequence alignment showed significant sequence similarities occurring at specific regions of the peptide chain, with Glu^{148} (AagA numbering) aligning with the Glu that is strictly conserved in family 16 hydrolases (Barbeyron *et al.*, 1994; and Barbeyron *et al.*, 1998) (Figure 15).

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Table 2 The similarities as obtained from a BLAST search of the GENBANK database, with the 290 amino acid sequence of AagA

Accession number	Protein/Organism	Sequence identity (%)
M73783	β -agarase / *P. atlantica* ATCC 19262^T	85
AF098954	β -agarase A / *C. drobachiensis*	51
AF098955	β -agarase B / *C. drobachiensis*	44
P07883	β -agarase / *S. coelicolor* A3(2)	34
AB038772	Endo-β-galactosidase C / *C. perfringens*	25
P23903	Endo-1,3-β-glucanase A1 / *B. circulans*	22
JN0772	Endo-1,3-β-glucanase / *B. circulans*	22
X89732	Endo-1,3(4)-β-glucanase / *C. thermocellum*	21

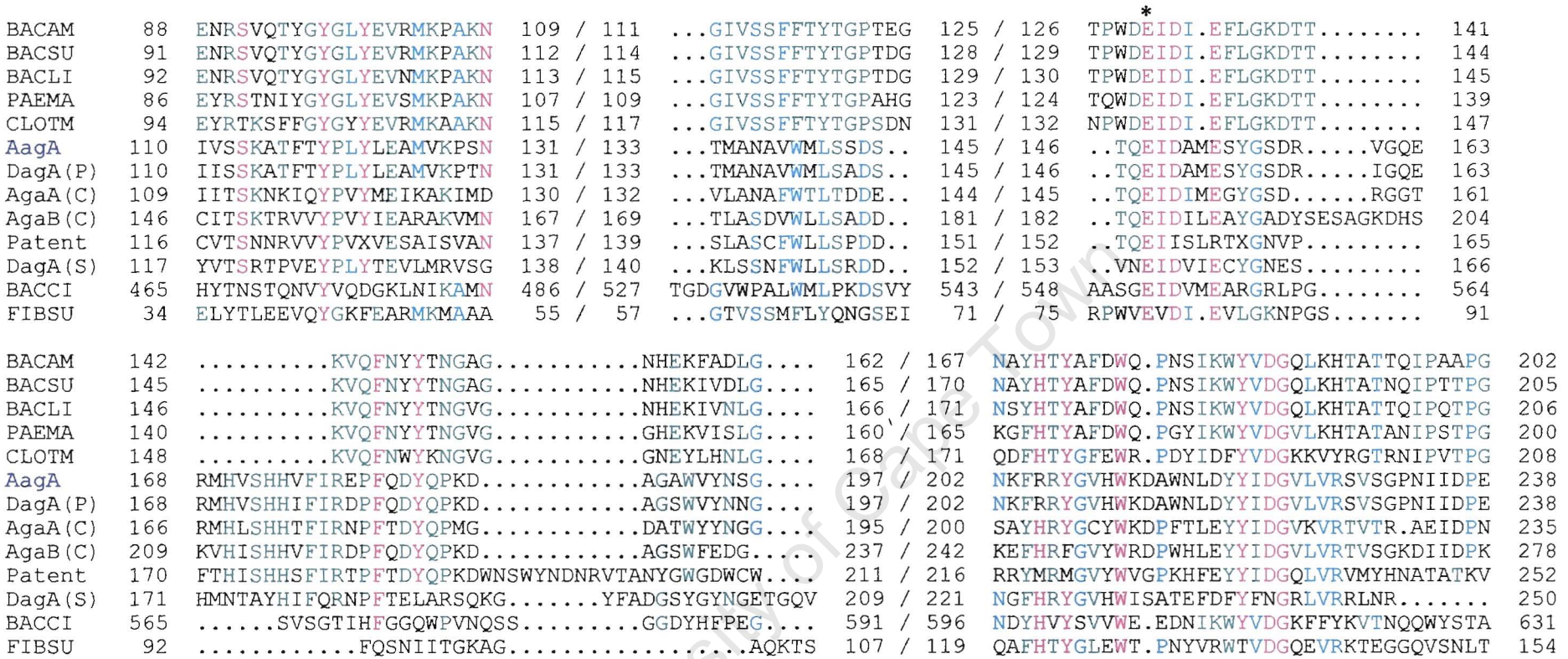

Figure 15 Multiple sequence alignment of β-agarases and representatives of the family 16 hydrolases. The amino acids highlighted in pink, blue and green represent sequence identities of >75%, >50%, and >33%, respectively. BACAM: 1,3(4)-β-glucanase from *B. amyloliquefaciens* (P07980); BACSU: 1,3(4)-β-glucanase from *B. subtilis* (P04957); BACLI: 1,3(4)-β-glucanase from *B. licheniformis* (P27051); PAEMA: 1,3(4)-β-glucanase from *B. macerans* (P23904); CLOTM: 1,3(4)-β-glucanase B from *C. thermocellum* (P29716): AgaA: β-agarase from *P. gracilis* B9 (U61972); DagA(P): β-agarase from *P. atlantica* ATCC 19262^{T} (M73783); AgaA(C): β-agarase A from *C. drobachiensis* (AF098954); AgaB(C): β-agarase B from *C. drobachiensis* (AF098955); Pat: β-agarase from *Flavobacterium* sp. NR19 (AR034164); DagA(S): β-agarase from *S. coelicolor* A3(2) (P07883); BACCI: 1,3-β-glucanase A1 from *B. circulans* (P23903); and FIBSU: 1,3-β-glucanase from *F. succinogenes* (P17989). The asterisk indicates the position of the strictly conserved Glu residue of family 16 glycoside hydrolases (Barbeyron *et al.*, 1994; Barbeyron *et al.*, 1998).

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3.4.7 Northern hybridization studies

To determine the size and presence or absence of the mRNA transcript of *aag*A in relation to the growth phase of *P. gracilis* B9, total cellular RNA was prepared from wild-type *P. gracilis* B9 grown in BM (Chapter 2, Figure 8A), at two points of its growth phase. The first extraction was at time 5 h, where no agarolytic activity was detected. The second sample was taken at 24 h, where agarolytic activity was detected. A single transcript of 1.2 kb in size was detected in the 24 h sample by Northern hybridization analysis (Figure 16B, lane 3).

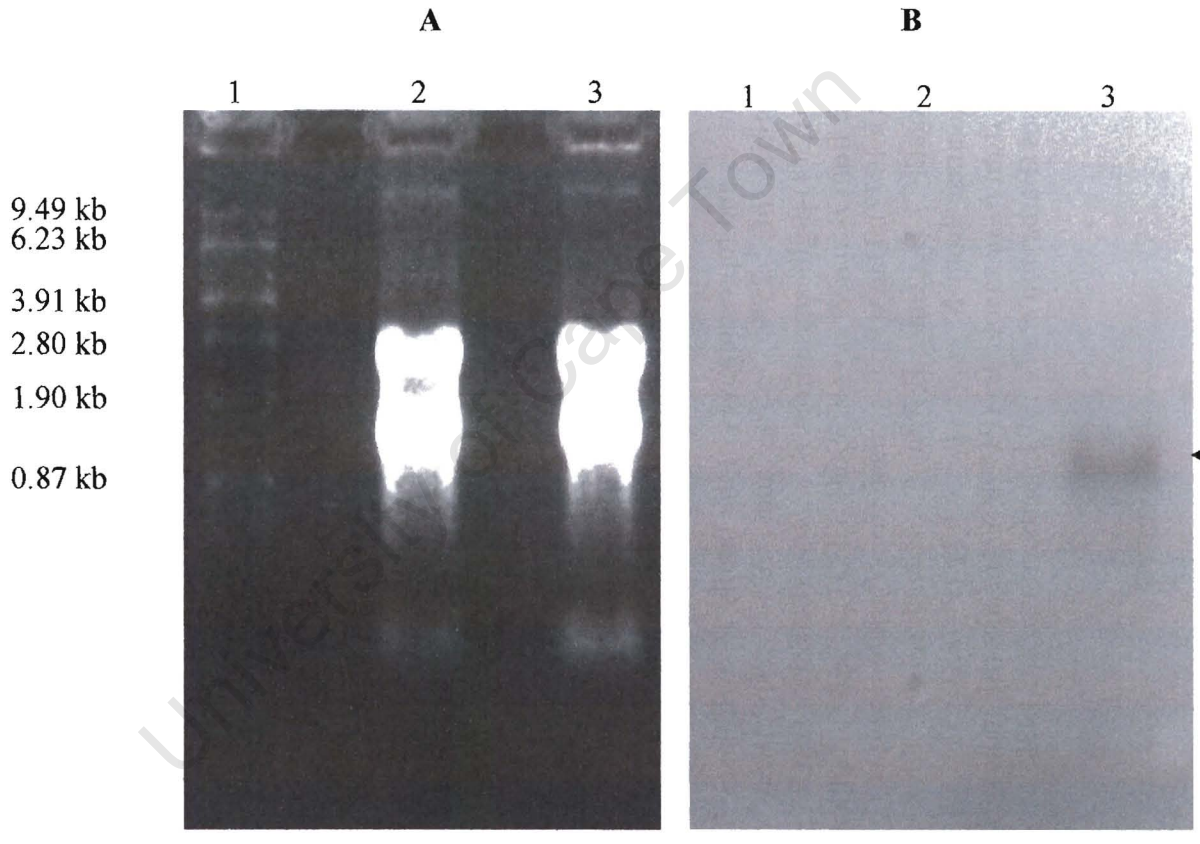

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Figure 16 A) Denaturing agarose gel and B) corresponding autoradiograph indicating the synthesis of *aag*A mRNA during stationary phase of growth. Lane 1, RNA molecular mass standards; lane 2, 10 µg of total RNA isolated from *P. gracilis* B9 after 5 h of growth; lane 3, 10 µg of total RNA isolated from *P. gracilis* B9 after 24 h of growth. An internal *Eco*RI-*Eco*RI fragment from the *aag*A gene was labeled with [α-^{32}P]dCTP and used as a probe. Arrow: single transcript of 1.2 kb in size. The sizes of the RNA standards, in kilobase pairs (kb), are shown.

3.4 Discussion

The *Pseudoalteromonas gracilis* B9 genomic library was screened for agarolytic activity by visual inspection for pitting of the agar. Five agar-digesting *E. coli* clones were obtained. Three of the clones encoding agarase activity contained the plasmid designated as pDA1, while the other two clones contained another plasmid, designated pDA3. Restriction endonuclease maps of pDA1 and pDA3 were constructed. The restriction maps revealed that the two plasmids had a region of DNA in common. Southern hybridization studies performed on pDA1 and pDA3 not only confirmed that the cloned DNA was of *P. gracilis* B9 origin, but it also confirmed the homology shared between the two plasmids. Subsequent deletion analysis performed on pDA1 and pDA3 revealed the 'common region' to be the location of the agarolytic activity on the plasmids. Thus, the two plasmids contain stretches of *P. gracilis* B9 genomic DNA which encodes the agarase gene(s) and the flanking genes either immediately upstream or downsteam of the agarase gene(s).

The agarase-encoding region was sub-cloned from pDA1 into the pBluescript KS cloning vector. The subsequent plasmid, pDA012, was then sequentially deleted from both ends of the inserted DNA and the resultant deletion plasmids sequenced. The nucleotide sequences of the various constructs were assembled and the completed sequence was analyzed. A BLAST search of the GENBANK database showed that the ORF situated in the *P. gracilis* B9 DNA of pDA012 had sequence identity to a number of β-agarases. The β-agarase *dag*A (M73783), from *Pseudoalteromonas atlantica* ATCC 19262^T, had the greatest similarity to the *P. gracilis* B9 gene. Therefore, it was concluded that the 873 bp ORF encoded a putative β-agarase and was designated *aag*A.

The putative promoter region of *aag*A was characterized. The pEKpllacZ promoter fusion construct, pEKA11, containing a DNA fragment 189 nucleotides upstream of the start codon of *aag*A, effectively lost the ability to express β-galactosidase activity. Thus suggesting that the *aag*A promoter had been disruption or deleted. In addition, the low levels of β-agarase expression in *E. coli* JM109 transformed with pDA012_A11 (165 nucleotides upstream of the start codon of *aag*A) was considered to be a result of transcriptional read-through on the high-copy number plasmid, pBluescript KS, since a loss of agarolytic activity occurred when pDA16

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(155 nucleotides upstream of the start codon of *aag*A) was transformed into *E. coli* K514λ. Hence, the promoter region lies upstream of the *Hin*dIII (1637) restriction site, which is 155 nucleotides upstream of the start codon of *aag*A.

In order to approximate the position of the promoter of *aag*A, primer extension analysis was performed on the total cellular RNA isolated from the 24 h old culture of *P. gracilis* B9. Northern hybridization studies confirmed that *aag*A is expressed during the late stationary phase of growth (section 2.4.3). The 1.2 kb mRNA transcript was detected at 24 h and not at 5 h following inoculation of the culture medium. The primer extension analysis placed the start site of the *aag*A gene transcript 184 nucleotides upstream of the putative translational start site. This is consistent with the location of the *aag*A promoter as indicated by the promoter probe studies. Although a conserved promoter region could not be identified, the sequences TATTAA at nucleotide positions –16 to –11 and TTACAT at –40 to –35 could possibly serve as the -10 and –35 regions of a putative *aag*A promoter, respectively (Harley and Reynolds, 1987). In addition, the mRNA transcript size is consistent with the predicted size of 1.16 kb calculated from the sequence data, i.e. the distance between the rho-independent terminator hairpin and the location of the transcriptional start site.

In general, the area upstream of the putative promoter is A+T rich (A+T=73%). The α subunit of *E. coli* RNA polymerase participates in promoter recognition through specific interactions with UP element DNA (A+T rich tracts), a region upstream of the recognition hexamers for the σ subunit (the –10 and –35 hexamers) (Ross *et al.*, 1998). UP elements have been described to have large positive (30- to 70-fold) effects on promoter activity. The majority of these UP elements occur between –40 and –60, with the best functioning close to the –35 hexamer rather than further upstream (Ross *et al.*, 1998; and Aiyar *et al.*, 1998). Site directed mutagenesis and gel-shift retardation assays could be employed in the future to ascertain whether the A+T rich region upstream of the putative promoter indeed contains an UP element.

In addition, the region situated between the 5' ends of pDA012_A9 and pDA012_A10 (also A+T rich: 74%) appears to have a negative effect on transcription. An increase in enzyme activity was observed in the _A10 deletion mutant, i.e. *E. coli* JM109 transformed with pDA012_A9 had less agarolytic activity when compared to an *E. coli* JM109 transformed with pDA012_A10. Similarly, a 10.5 fold increase in β-galactosidase activity, 348 to 3674 Miller units, occurred

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when the sequence upstream of the 5' end of A10 was deleted, i.e. difference in activity between *E. coli* JM109 transformed with pEKA9 and *E. coli* JM109 transformed with pEKA10, respectively. Once again, site directed mutagenesis and gel-shift retardation assays could be employed in the future to ascertain the role this A+T rich region has with respect to the regulation of *aag*A. Future work could also determine whether this promoter region plays a role in catabolite repression, which affects expression of the *P. gracilis* B9 β-agarase gene (section 2.4.3). However, glucose repression of the β-agarase from *Streptomyces coelicolor* A3(2), DagA, acted at a post-transcriptional level, probably at the secretion step; i.e. glucose did not inhibit *dag*A transcription but the newly synthesized protein was degraded intracellularly (Parro and Mellado, 1994). Nonetheless, the regulatory processes acting in the promoter region of *aag*A will differ to what has been described for *dag*A, since five different promoters were shown to control *dag*A's transcription (Buttner *et al.*, 1987; and Parro and Mellado, 1993). In addition, these five different promoters are recognized by at least three different holoenzymes of the RNA polymerase (Buttner *et al.*, 1988; and Brown *et al.*, 1992). Thus, it remains to be seen whether the A+T-rich area plays a role in catabolite repression of *aag*A expression in *P. gracilis* B9.

The primary translational product of the *aag*A gene is predicted to be a protein of 290 amino acids. The NH_2 terminus of the protein may function as a signal sequence, allowing for export of the extracellular β-agarase. The *dag*A and *agr*A genes of *Streptomyces coelicolor* A3(2) and *Pseudoalteromonas atlantica* T6c encode for preprocessed β-agarases with signal peptides of 30 and 20 amino acid residues, respectively (Buttner *et al.*, 1987; and Belas, 1989). The putative peptidase cleavage site of pre-AagA could be located between residues 21 and 22, thus yielding a mature protein of 269 amino acids with a molecular mass of 30.23 kDa.

The two β-agarases (*aga*A, AF098954, and *aga*B, AF098955) from *Cytophaga drobachiensis*, the β-agarase (*dag*A, M73783) from *Pseudoalteromonas atlantica* ATCC 19262^T and the β-agarase (*dag*A, P07883) from *Streptomyces coelicolor* A3(2) are all members of the family 16 glycoside hydrolases (Barbeyron *et al.*, 1994; Barbeyron *et al.*, 1998; Coutinho and Henrissat, 1999). Site directed mutagenesis within the 1,3(4)-β-glucanase gene of *Bacillus licheniformis* (BACLI) showed that Glu^{134} plays an important role in the catalytic domain of this family 16 hydrolase (Planas *et al.*, 1992). Barbeyron *et al.* (1994) performed multiple sequence alignments with family 16 hydrolases and found a strict conservation of Glu in relation to Glu^{134} of BACLI amongst the hydrolases. In addition, a conserved motif of two Glu residues (of which the afore

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mentioned Glu is one) separated by 3 or 4 amino acids, depending on whether they are bacterial lichenases and plant xyloglucan endotransglycosylases or galactanases and laminarinases respectively, are characteristic of the catalytic site of family 16 glycoside hydrolases (Barbeyron et al., 1998). The sequence similarities observed between the β-agarases and the family 16 glycoside hydrolases suggests that AagA from *P. gracilis* B9 and the unnamed β-agarase from *Flavobacterium* sp. NR19 are new members of the family 16 glycoside hydrolases. The sequence alignment revealed that both agarases contained the strictly conserved Glu^{148} (AagA numbering) or Glu^{134} (BACLI numbering).

Sequential deletions generated from the C-terminal end of the β-agarase, PjaA, of *Pseudomonas* sp. W7 resulted in the eventual loss of agarase activity of the resultant truncated proteins, indicating the importance of the C-terminal end in agarolytic activity (Lee *et al.*, 2000). Sequential deletions of the C-terminal of AagA resulted in immediate loss of agarolytic activity, even when just the last three amino acids of AagA were removed. Thus, the Glu^{148} and the C-terminus are important for the agarolytic activity of AagA. The majority of hydrolases have from two to six or more domains, i.e. catalytic domain, substrate-binding domain, etc. (Warren, 1996). A group of extracellular proteins, the immunoglobulin A protease family, contain a large helper domain at the C-terminal end. The helper domain functions as an anchor by anchoring the mature domain to the outer surface while assisting translocation of the protein across the outer membrane (Pugsley *et al.*, 1990). Thus, the C-terminus of AagA might have a similar function in the translocation of AagA across the outer membrane.

The screening of the *P. gracilis* B9 genomic library, as described in this chapter, resulted in the successful cloning of an extracellular β-agarase from *P. gracilis* B9. However, it is known from literature that at least another agarase, or more likely two agarases, an α- and β-agarase, are required for the complete hydrolysis of agar (section 1.3.1). These agarases are usually cell bound. In addition, more than one extracellular agarase can be produced by a specific bacterium (Hofsten and Malmqvist, 1975; Sugano *et al.*, 1994; Nomura *et al.*, 1998; and Araki *et al.*, 1999). Nonetheless, we were only successful in identifying one extracellular agarase from *P. gracilis* B9. Similarly, the screening method employed here also failed to identify *E. coli* transformants that contained intracellular agarases. Kong *et al.* (1997) described the cloning and expression of two intracellular agarases from *Pseudomonas* sp. W7. The *E. coli* transformants were screened by first sonicating the cells. The sonicated cells were plated on an agar plate and screened with

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an iodine solution. Zones of clearing were noticed around the two clones. Therefore, future work might entail the possible identification of the intracellular agarases of *P. gracilis* B9, by screening the genomic library as described by Kong *et al.* (1997).

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CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF THE β(1-4) AGARASE PROTEIN FROM BOTH *PSUEDOALTEROMONAS GRACILIS* B9 AND THE *ESCHERICHIA COLI* JM109 (pDA16) TRANSFORMANT

CONTENTS

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4.1 Summary

A novel β(1→4) agarase, which hydrolyzes the β(1→4) linkages of agarose to yield predominately neoagarotetraose, was isolated from both the wild-type bacterium, *Pseudoalteromonas gracilis* B9, and an *E. coli* transformant, *E. coli* JM109 harboring the recombinant plasmid pDA16. *In vitro* transcription/translation of recombinant plasmids encoding an agarolytic gene from *P. gracilis* B9 in *E. coli* JM109, designated *aag*A, resulted in a single band (M_r, 31,500) following SDS-PAGE. The extracellular β-agarases were purified 35- and 22-fold from the wild-type *P. gracilis* B9 and the *E. coli* JM109 (pDA16) transformant by using gel filtration chromatography and a combination of gel filtration and ion-exchange chromotography, respectively. Both purified proteins exhibited a single band (M_r, 30,000) following SDS-PAGE. The optimum temperature and pH for both enzymes was 50°C and pH 7, respectively. However, their stability profiles at 37°C were different. Thin-layer chromatography (TLC) of the digestion products produced by both enzymes showed that both enzymes have the same substrate specificity; i.e. they both hydrolyze the β(1→4) linkages of agarose to yield predominately neoagarotetraose, with saccharides the size of neoagarotetraose and smaller not subject to hydrolysis. Based on the similarities shared between the two enzymes, i.e. substrate specificity, mode of action, enzyme size and enzyme characteristics, the β(1→4) agarase (AagA) purified from the extracellular media of the *E. coli* JM109 (pDA16) transformant (isolated from a *P. gracilis* B9 genomic library) was considered the extracellular β(1-4) agarase purified from *P. gracilis* B9. In addition, TLC of the agarolytic activity of the extracellular fraction of *P. gracilis* B9 grown in various media is indicative of the action of the purified β(1→4) agarase.

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4.2 Introduction

The mechanism whereby the marine bacterium *Pseudoalteromonas atlantica* ATCC 19262^T (IAM 12927^T) hydrolyzes agar involves three enzymes, an extracellular enzyme and two intracellular enzymes (Day and Yaphe, 1975;and Groleau and Yaphe, 1977; and Morrice *et al.*, 1983a and b). The first enzyme in this pathway is the extracellular enzyme, β-Agarase I, which initially cleaves the agarose moiety of agar in an endo-acting mechanism yielding predominantly the tetramer, neoagarotetraose, as the major end product. β-Agarase I was shown to be specific for regions of the polymer that contain a minimum of one unsubstituted neoagarobiose unit, where it hydrolyzes the β(1→4) linkages at the reducing end of this moiety. It was also shown to be able to slowly degrade the neoagarohexaose but not the neoagarotetraose. β-Agarase I is the predominant extracellular species that acts on solutions of agar and higher neoagaro-oligosaccharides, as well as upon agar in the gel state.

The β-Agarase I was purified by Morrice *et al.* (1983b) from the bacterial growth medium. SDS-PAGE gel electrophoresis indicated a single protein with a molecular weight of 32 000. Both the crude extracellular extract (Yaphe, 1975) and the purified β-Agarase I (Morrice *et al.*, 1983b) showed activity over a pH range of 5 to 8 with an optimum activity for the crude extract at pH 6 and the purified protein at pH 7. The enzyme was shown to be stable at temperatures at and below 30°C over a 2 h period. The enzyme is unstable at temperatures of 40°C and above, with increasing instability the higher the temperature (Morrice *et al.*, 1983b). In 1996, the nucleotide sequence of the gene encoding the β-Agarase I was submitted to the GENBANK database under the accession M73783. The gene was designated *dag*A.

A number of other extracellular agarases have been characterized (section 1.3.1). Some of these extracellular agarases had similar characteristics to that of the β-agarase I from *P. atlantica* ATCC 19262^T, while others were very different. Some bacteria export more than one agarase into the surrounding media, each of which have different substrate specificities yielding different end-products upon agar hydrolysis (Hofsten and Malmqvist, 1975; Sugano *et al.*, 1994; Nomura *et al.*, 1998; and Araki *et al.*, 1999). Screening of the genomic library of *P. gracilis* B9 produced a single agarase gene, which had significant homology to an extracellular β-agarase gene from *P. atlantica* ATCC 19262^T (Chapter 3). Thus, the aim of this chapter was to firstly confirm that the β-agarase cloned from *P. gracilis* B9 into *E. coli* JM109 was responsible for the extracellular

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agarolytic phenotype observed in Chapter 2. In order to do so, the extracellular agarase(s) produced by *P. gracilis* B9 was purified and subsequently characterized. Secondly, the size of the protein product (AagA) of the cloned β-agarase gene, *aag*A, from *P. gracilis* B9 (Chapter 3) was determined. Thirdly, AagA was in turn purified from the *E. coli* JM109 (pDA16) transformant, and subsequently characterized. Consequently, AagA was compared to the extracellular β-agarase(s) purified from *P. gracilis* B9.

The protocols employed for purification of the *P. gracilis* B9 agarase(s) included common protein purification methods, i.e. gel filtration and ion-exchange chromatography. Thin-layer chromatography was one of the tools used to characterize the specificity of the enzymes. Yaphe and co-workers successfully used this technique to evaluate the individual end-products of agar hydrolysis as a function of agarase activity (Duckworth and Yaphe, 1970). Other characteristics of the *P. gracilis* B9 agarase(s), such as optimum temperature, pH and stability, were also determined. Finally, the characteristics of the extracellular β-agarase(s) of *P. gracilis* B9 were compared to the other agarases described in the literature.

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4.3 Material and Methods

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

4.3.1 Size determination of AagA using *in vitro* transcription/translation

The plasmid pBluescript KS and selected sequential deletion mutants (pDA012_A9, pDA012_A10, pDA012_A12, pDA012_K3, and pDA012_K5) were transcribed and translated *in vitro*, using the Promega *E. coli* S30 extract system for circular DNA in the presence of ^{35}S methionine. Reaction mixes were incubated for 1 h at 30°C before the ^{35}S labeled proteins were precipitated with acetone and the pellets resuspended in 30 µl SDS-PAGE sample buffer. Samples were separated on a 12% SDS-PAGE gel and stained for 15 min with Coomassie blue R250 (Appendix A.2.25) and destained in 7% glacial acetic acid (Appendix A.2.25) prior to drying.

4.3.2 Purification of the extracellular agarase from *P. gracilis* B9

A preliminary investigation of the characteristics of the extracellular agarase from *P. gracilis* B9 was performed as follows. An O/N culture of strain B9 was inoculated into 100 ml of BM and incubated for 30 h at 22°C on an orbital shaker at 100 rpm. The cells were harvested (10K rpm for 15 min at 4°C) and the supernatant was collected. The supernatant was divided into 10 ml aliquots, which were dialyzed against buffers with differing pH's (Appendix A.2.23). The agarolytic activity of these samples was determined as described in section 4.3.5. The aliquot with the enzyme at the optimum pH was tested at different ionic strengths of phosphate buffer by dialyzing 1 ml aliquots againsts a range of 0-100 mM phosphate buffer (pH 7) (Appendix A.2.23) at 4°C and subsequently determining their agarolytic activities. The incubation temperature of the agarase assay was varied between 4-50°C in order to determine the temperature at which the agarase is optimally active. Finally, the stability of the enzyme(s) at 4 and 22°C was tested over 3 days by determining the agarolytic activity every day.

Six, 1 l *P. gracilis* B9 cultures were grown in BM for 30 h at 22°C. The cells were harvested by centrifugation (10K rpm for 15 min at 4°C) and a total of 5,6 l of supernatant was collected. The following procedures were carried out at 4°C. The supernatant was adjusted to a final

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ammonium sulphate saturation of 85% (559 g/l) by adding ammonium sulphate (Merck) in increments of 250 g (Englard and Seifter, 1990). The precipitate was collected by centrifugation (10K rpm for 30 min), resuspended in 45 ml of 20 mM Tris-Cl buffer (pH 7) (Appendix A.2.23) and dialyzed multiple times against 5 l of the same buffer.

The following procedures were performed at 20°C. A column (3 cm x 1 m) of Sephadex G100 (Pharmacia) that had been equilibrated with 20 mM Tris-Cl (pH 7) was prepared. The dialyzed ammonium sulphate concentrate was applied to the column and 5 ml fractions were collected with a Gilson FC 204 Fraction Collector (Gilson Medical Electronics, Inc.). The agarolytic activity was determined every 5th fraction, while the absorbance at 280 nm was read every 2nd fraction with a Beckman spectrophotometer. The active fractions were pooled (250 ml, total volume) and the resultant volume was reduced with an Amicon Centricon PM10 filter system to a final volume of 11 ml.

A column (3 cm x 28 cm) of Sephadex G75 (Pharmacia) that had been equilibrated with 20 mM Tris-Cl (pH 7) was prepared. The active concentrate (11 ml) was applied to the column and 1.8 ml fractions were collected with a Gilson FC 204 Fraction Collector (Gilson Medical Electronics, Inc.). The agarolytic activity was determined every 5th fraction, while the absorbance at 280 nm was read every 2nd fraction with a Beckman spectrophotometer. The active fractions were pooled (18 ml, total volume) and concentrated to 5 ml with an Amicon Centricon PM10 filter system. Finally, the 5 ml active concentrate was dialyzed against 10 mM phosphate buffer, pH 7 (Appendix A.2.23).

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4.3.3 Purification of the AagA from *E. coli* JM109 (pDA16)

Five, 1 l *E. coli* (pDA16) cultures were grown in LB (ampicillin) for 24 h at 22°C on an orbital shaker at 100 rpm. The cultures were centrifuged (10K rpm for 15 min at 4°C) and 4,7 l supernatant was collected. The following procedures were done at 4°C. The supernatant was adjusted to a final ammonium sulphate saturation of 85% (559 g/l) ammonium sulphate in increments of 250 g (Englard and Seifter, 1990). The precipitate was collected by centrifugation (10K rpm for 30 min), resuspended in 40 ml of 20 mM Tris-Cl buffer (pH 7) and dialyzed multiple times against 5 l of the same buffer.

The following procedures were carried out at 20°C. A column (3 cm x 28 cm) of DEAE-Sephadex A-50 (Pharmacia) was activated with 5 M NaCl and then equilibrated with 20 mM Tris-Cl (pH 7) (Rossomando, 1990). The dialyzed ammonium sulphate concentrate was applied to the column and the column was washed with 250 ml of 20 mM Tris-Cl (pH 7). The proteins were eluted from the column in a stepwise fashion with an increasing NaCl molarity, i.e. 250 ml volumes of 0.1 M NaCl, 0.25 M NaCl, 0.5 M NaCl and 1 M NaCl (Appendix A.2.23) were passed through the columns, respectively. Five ml fractions were collected with a Gilson FC 204 Fraction Collector (Gilson Medical Electronics, Inc.). The agarolytic activity was determined every 5th fraction, while the absorbance at 280 nm was read every 2nd fraction with a Beckman spectrophotometer. The resultant total volume (200 ml) was reduced with an Amicon Centricon PM10 filter system to a final volume of 45 ml.

A column (6 cm x 1 m) of Sephadex G75 that had been equilibrated with 20 mM Tris-Cl (pH 7) was prepared. The active concentrate (45 ml) was applied to the column and 5 ml fractions were collected with a Gilson FC 204 Fraction Collector (Gilson Medical Electronics, Inc.). The agarolytic activity was determined every 5th fraction, while the absorbance at 280 nm was read every 2nd fraction with a Beckman spectrophotometer. The active fractions were pooled (250 ml, total volume) and concentrated to 4 ml with an Amicon Centricon PM10 filter system. Finally, the 4 ml active concentrate was dialyzed against 10 mM phosphate buffer, pH 7.

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4.3.4 Bradford assay for protein quantitation

Protein concentrations were determined by the Bradford method (Appendix B.20).

4.3.5 Ferricyanide assay for quantitation of agarase activity

Agarase activity was determined by the Ferricyanide reducing sugar assay (Appendix B.6). The incubation period used to assay the activity of the chromatography fractions was reduced to 30 min. Therefore, the agarolytic activity (U) of the chromatography fractions was expressed as µg galactose produced per ml per 30 min at 37°C.

Agarase activity (U) for the determination of the optimum conditions for enzyme activity was generally expressed as µg galactose produced per ml per min at 37°C, except where the

temperature of the assay was altered to determine the optimum reaction temperature of the agarase.

4.3.6 SDS-polyacrylamide gel electrophoresis

SDS-PAGE analysis was performed with a 12% gel in accordance with the Laemmli method (Appendix B.21.1). The proteins were detected by silver staining using a modified procedure devised by Sammons *et al.* (1981) (Appendix B.22).

4.3.7 Zymogram detection of the extracellular agarase

SDS-PAGE was performed (Appendix B.21.1) with the following modification: a final concentration of 0.1% agarose (Appendix A.2.23) was incorporated into the separating gel matrix. After electrophoresis, the gel was soaked in 10 mM phosphate buffer (pH 7) at 22°C. The buffer was replaced hourly for 3 h before incubation at 37°C for 12 h. Finally, zones of hydrolysis were visualized by staining the gel with Gran's Iodine (Appendix A.2.15).

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4.3.8 Optimum pH of the extracellular agarase

Sixty ng aliquots of purified enzyme were made up to 300 µl with 0.1 M phosphate buffers with pH values of 5 to 8 (Appendix A.2.23). The enzyme activity was determined as described in section 4.3.5, except that the PIPES buffer was omitted and the incubation time was reduced to 30 min. Agarolytic activity (U) was expressed as µg galactose produced per ml per min at 37°C.

4.3.9 Optimum reaction temperature of the extracellular agarase

Sixty ng aliquots of purified enzyme were suspended in 300 µl PIPES buffer. The incubation temperature and incubation time of the enzyme assay described in section 4.3.5 was altered. A temperature range of 4 to 75°C was tested over a 30 min period. Agarolytic activity (U) was expressed as µg galactose produced per ml per min at the tested temperatures.

4.3.10 Thermostability of the extracellular agarase at various temperatures

Aliquots of purified enzyme were placed at temperatures of 4, 22, 37 and 50°C. At time intervals of 0, 2, 6, 8, 10, 20, 30, 45, and 60 min, 60 ng of the enzyme was removed. The activities of the enzyme aliquots was determined as described in section 4.3.5, except that the volume of PIPES buffer was adjusted to obtain a final volume of 300 µl (consistent for all the tests) and the incubation time was reduced to 30 min. Agarolytic activity (U) was expressed as µg galactose produced per ml per min at 37°C.

4.3.11 Thin-Layer Chromatography analysis of the agarase hydrolysates

Purified enzyme (600 ng) was added to 100 µl freshly prepared 1% agarose substrate (Appendix A.2.12) and 200 µl 20 mM PIPES solution (Appendix A.2.12). Similarly, 600 ng of purified enzyme was added to 50 µl of each of three oligosaccharides (2.5 µg / µl; final concentration) and 140 µl 20 mM PIPES solution. The 200 µl (final volume) reaction mixes were incubated at 37°C for 1 h. TLC was performed on Silica gel 60 aluminium foil (Merck) and developed with the solvent *n*-butanol : acetic acid : water (Appendix A.2.27). The resulting saccharides were visualised after spraying with naphthoresorcinol reagent (Appendix A.2.27). Each of the three oligosaccharides (1.25 µg), i.e. neoagaroheaxose, neoagarotetraose and neoagarobiose, was used as molecular size markers (Sigma).

In an attempt to verify whether the 1.5 fold increase in agarolytic activity detected during late stationary phase of growth in media supplemented with a reducing sugar (section 2.4.3) was a result of an increase in production of a specific agarase or a number of agarases, the cells of the 40 h cultures of strain B9 grown in either BM or BM supplemented with D-glucose were harvested and their respective supernatants collected as described in section 4.3.3. The harvested cells were resuspended in 10 ml of 10 mM phosphate buffer (pH 7). Cell suspensions were sonicated for three cycles for 30-seconds with a 30 second interval between each cycle to prevent overheating of cell extracts. The resulting cell extracts were centrifuged (10K rpm for 10 min) and the supernatants collected. The soluble cellular extracts were dialyzed multiple times against 1 l of 10 mM phosphate buffer (pH 7). One hundred µl of culture supernatants and cellular extracts were added to 100 µl of 1% agarose solution and incubated at 37°C for 1 h. The

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reaction mixes were dot-blotted onto a silica gel in aliquots of 1 µl, until 3 µl was loaded per sample. The silica gel was treated as described above.

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4.4 Results

4.4.1 Characteristics of the extracellular agarase(s) from *P. gracilis* B9

In order to successfully purify active extracellular agarase(s) from *P. gracilis* B9, it was necessary to perform a set of preliminary experiments. Preliminary characterization of the extracellular agarolytic activity associated with the *P. gracilis* B9 growth medium showed that the buffers required for purification of active enzyme(s) should have a neutral pH (Figure 1A) and a low ionic strength (Figure 1C). Assay temperatures of either 37 or 50°C could be used (Figure 1B) and much of the purification procedure could be performed at room temperature (22°C, Figure 1D).

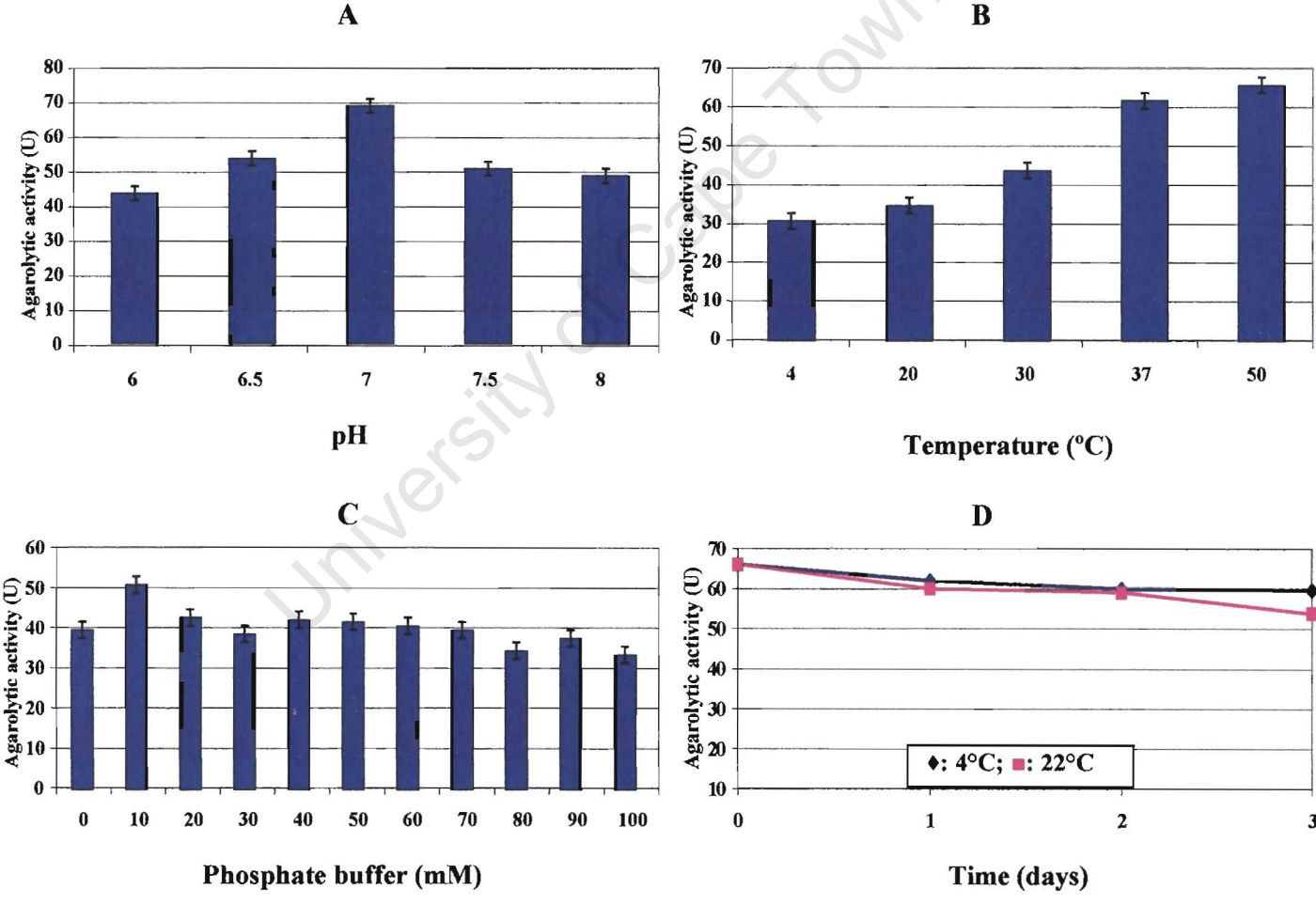

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Figure 1 The effect of abiotic factors on the activity of the *P. gracilis* B9 extracellular agarase(s). Agarolytic activity (U) is expressed as µg galactose/ml/h at 37°C, except for (B), where the reaction temperatures were changed accordingly. The error bars represent the standard deviation of the mean.

4.4.2 Purification and characterization of an extracellular agarase from *P. gracilis* B9

In order to purify sufficient amounts of enzyme, 6 l of *P. gracilis* culture medium supernatant was saturated with ammonium sulphate and the precipitate harvested. The precipitate was resuspended in and dialyzed against 20 mM Tris-Cl (pH 7). The suspension was subjected to gel filtration chromatography on a Sephadex G100 column and the active fractions (75 to 120) were collected (Figure 2). The active concentrate was subjected to another gel filtration column, Sephadex G75 (Figure 3). The active fractions (40 to 50) were pooled and concentrated by Amicon filtration. An aliquot of the ammonium sulphate concentrate and the final concentrate were analyzed by SDS-PAGE (Figure 4A). Multiple bands of different sizes in lane 2 were present in the crude ammonium sulphate precipitate. The final active concentrate (lane 3) exhibited a single protein band of 30 kDa in size. The zymogram confirmed that the 30 kDa protein was an agarolytic enzyme (Figure 4B). The overall yield of enzyme obtained from the extracellular fraction (supernatant) was 15% for a purification of 35-fold (Table 1).

The optimum pH and temperature required for maximal activity of the extracellular agarase purified from *P. gracilis* B9 was pH 7 and 50°C, respectively (Figure 5 and 6). The effect of temperature on the stability of the purified agarase showed that the enzyme is stable at 4 and 22°C over a 60 min period, while storage at temperatures of 37 and 50°C resulted in a gradual to rapid decline in activity, respectively (Figure 7). TLC revealed that the agarase secreted by *P. gracilis* B9 hydrolyzes the β(1→4) linkages of agarose to predominantly yield neoagarotetraose as the major end product (Figure 8, lane 2). In addition, the enzyme hydrolyzed neoagarohexaose to produce the neoagarotetraose and neoagarobiose (Figure 8, lane 3). Whereas, the enzyme did not hydrolyze either neoagarotetraose or neoagarobiose (Figure 8, lanes 4 and 5).

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4.4.3 Characterization of the substrate specificity of the extracellular agarase(s) of *P. gracilis* B9

TLC was used to firstly verify whether a similar mode of action to the purified extracellular β(1→4) agarase could be observed in the extracellular fraction of *P. gracilis* B9. Secondly, we wanted to determine whether the 1.5 fold increase in agarolytic activity observed during the late stationary phase of growth in media supplemented with a reducing sugar (section 2.4.3) was due to an increase in production of a specific agarase such as the purified β(1→4) agarase or a

number of agarases. Hence, the cells from 40 h cultures of strain B9 grown in either BM or BM supplemented with D-glucose were harvested and their respective supernatants collected. TLC of the extracellular fractions of *P. gracilis* B9 grown under the two conditions both have similar agarose hydrolysis end-products, with neoagarotetraose the predominant specie (Figure 8, lanes 6 and 8). This was a similar agarose hydrolysis profile to that observed for the purified β(1→4) agarase from *P. gracilis* B9. Thus, the results suggested that the extracellular β(1→4) agarase is produced by *P. gracilis* B9 under both growth conditions and that the presence of reducing sugar in the growth media induces the bacterium to synthesize the extracellular β(1→4) agarase.

TLC of the cellular extracts of *P. gracilis* B9 grown under the afore-mentioned conditions both resulted in similar agarose hydrolysis end-products; i.e. predominately neoagarobiose (Figure 8, lanes 7 and 9). This is indicative of the presence of at least one other β(1→4) agarase, which is located intracellularly in *P. gracilis* B9.

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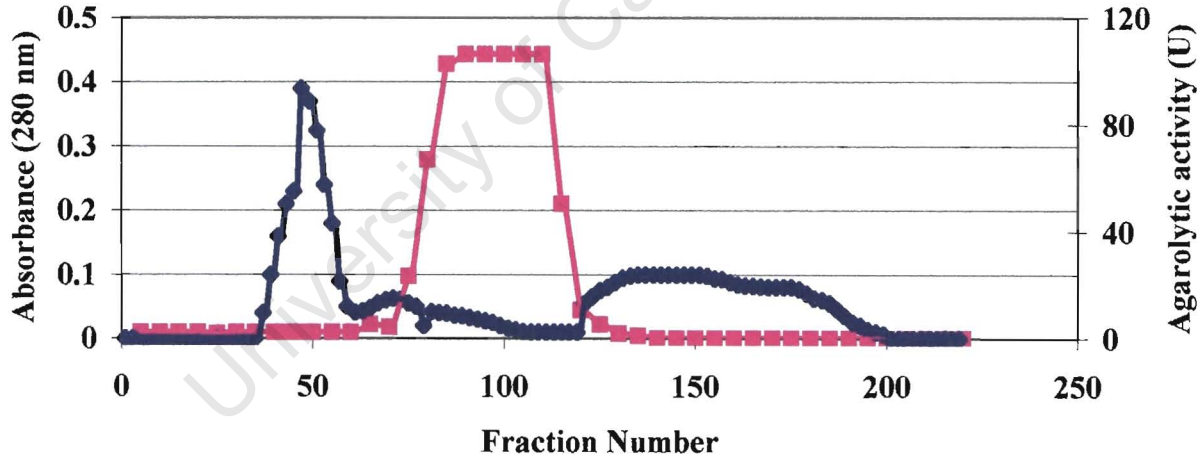

Figure 2 Gel filtration chromatography of ammonium sulphate concentrate of *P. gracilis* B9 culture supernatant on Sephadex G100 (3 cm x 1m). Protein (♦) was monitored by absorbance at 280 nm and agarolytic activity (■) was assayed as described in section 4.3.5. Agarolytic activity (U) is expressed as μg galactose/ml/30 min at 37°C.

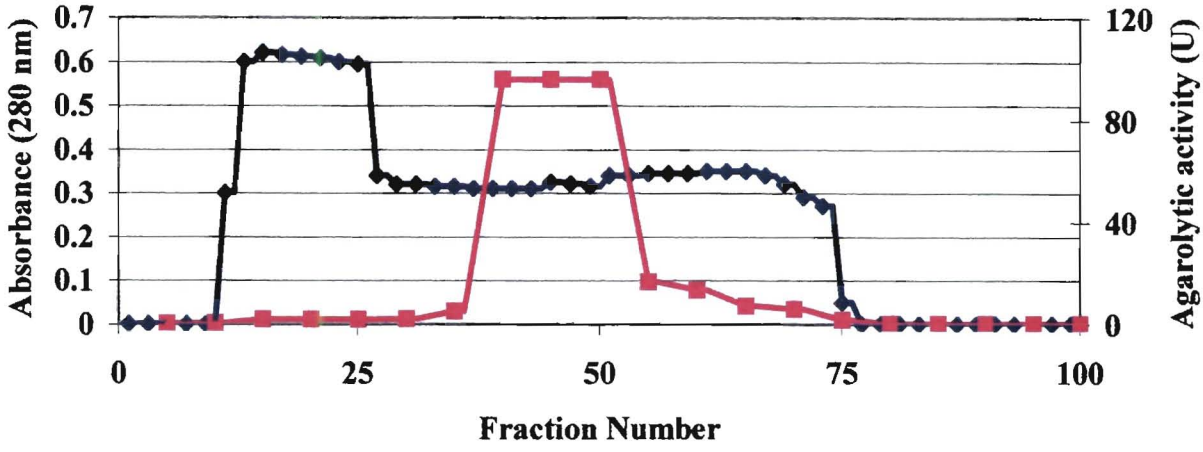

Figure 3 Gel filtration chromatography of the active concentrate (post Sephadex G100 chromotography) of *P. gracilis* B9 on Sephadex G75 (3 cm x 28 cm). Protein (♦) was monitored by absorbance at 280 nm and agarolytic activity (■) was assayed as described in section 4.3.5. Agarolytic activity (U) is expressed as µg galactose/ml/30 min at 37°C.

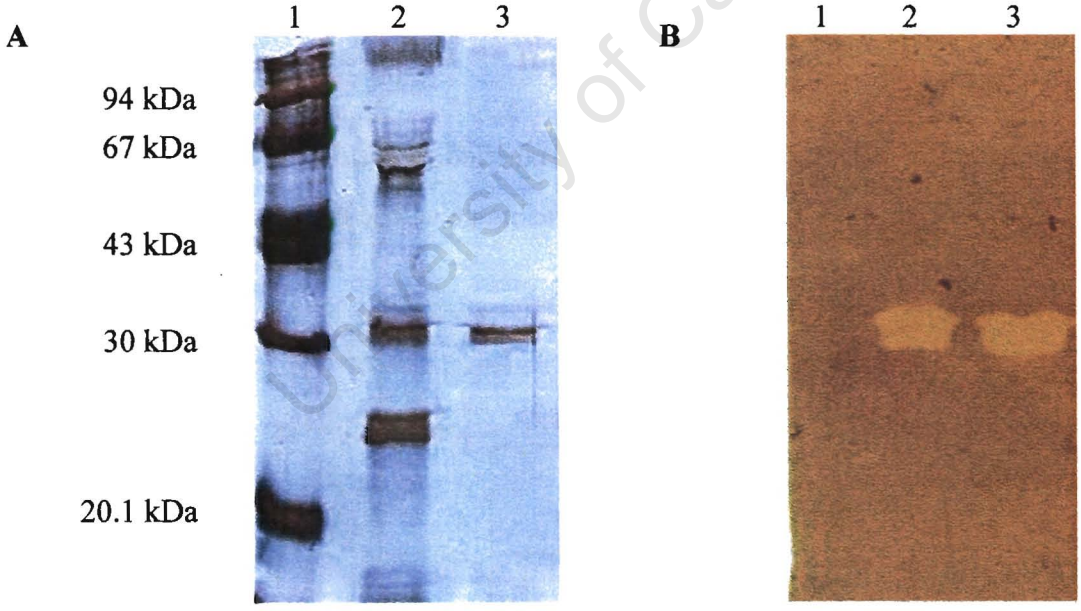

Figure 4 A) SDS-PAGE of samples obtained at different stages during purification of the extracellular agarase of *P. gracilis* B9. B) Zymogram of samples depicted in (A). Lane 1: molecular mass markers, lane 2: ammonium sulphate concentrate, and lane 3: final concentrate. The proteins were visualized by silver staining, while the enzymatically active bands were detected by staining with Gran's Iodine. The sizes of the molecular mass markers are shown in kDa.

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Table 1 Purification of extracellular agarase from *P. gracilis* B9.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture Supernatant	90.252	1570320	17399	100	1
Ammonium sulphate preparation	39.581	1324688	33468	84.36	1.92
Sephadex G100	0.798	241781	303157	15.39	17.42
Sephadex G75	0.380	230628	606517	14.68	34.85

(U): µg galactose/ml/min at 37°C

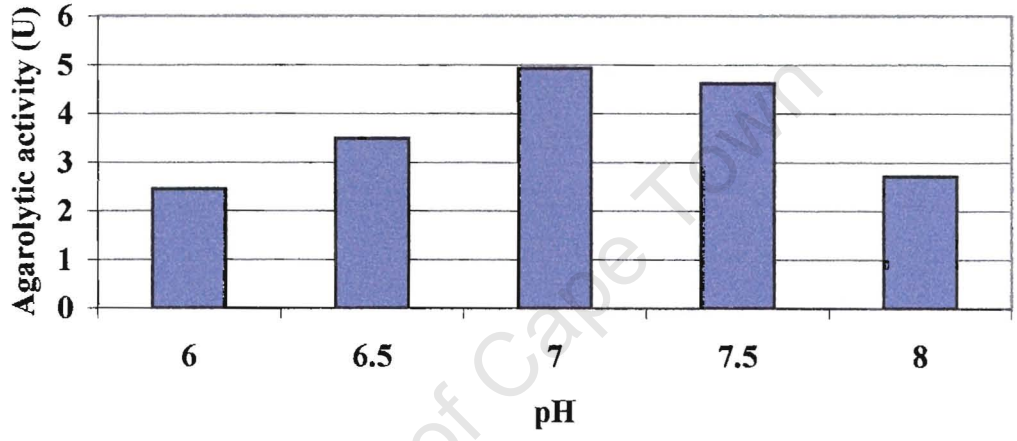

Figure 5 Effect of pH on the activity of the purified *P. gracilis* B9 extracellular agarase. Agarolytic activity (U) is expressed as µg galactose/ml/min at 37°C.

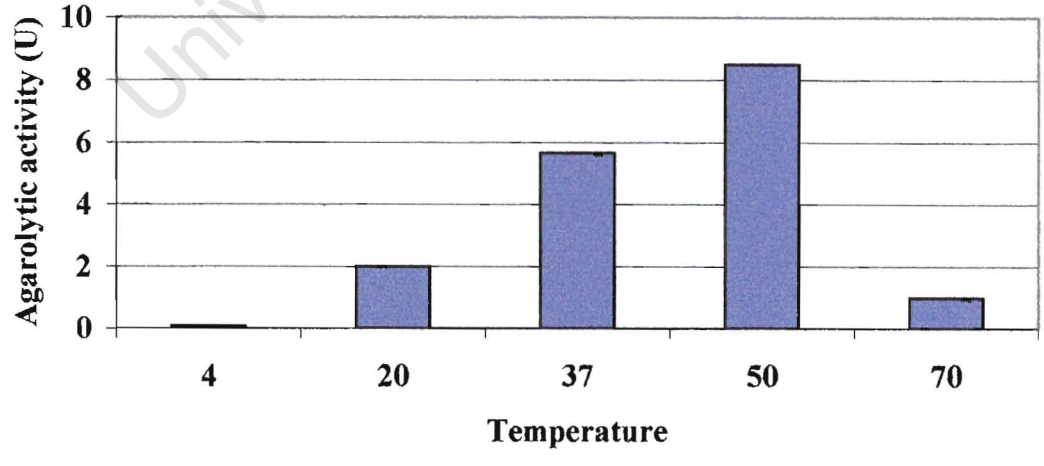

Figure 6 The effect of temperature on the activity of the purified *P. gracilis* B9 extracellular agarase. Agarolytic activity (U) is expressed as µg galactose/ml/min at the reaction temperature.

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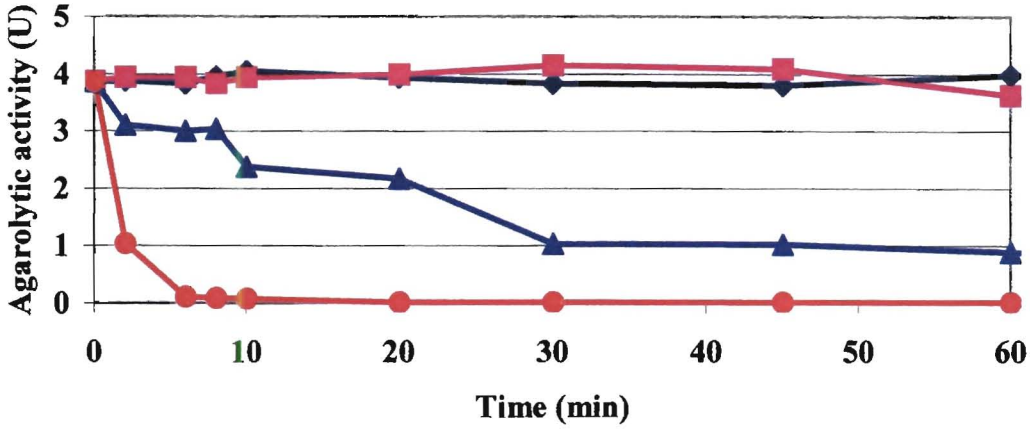

Figure 7 Stability of the purified *P. gracilis* B9 extracellular agarase over a 60 min period at the following temperatures: ♦: 4°C; ■: 22°C, ▲: 37°C, ●: 50°C. Agarolytic activity (U) is expressed as µg galactose/ml/min at 37°C.

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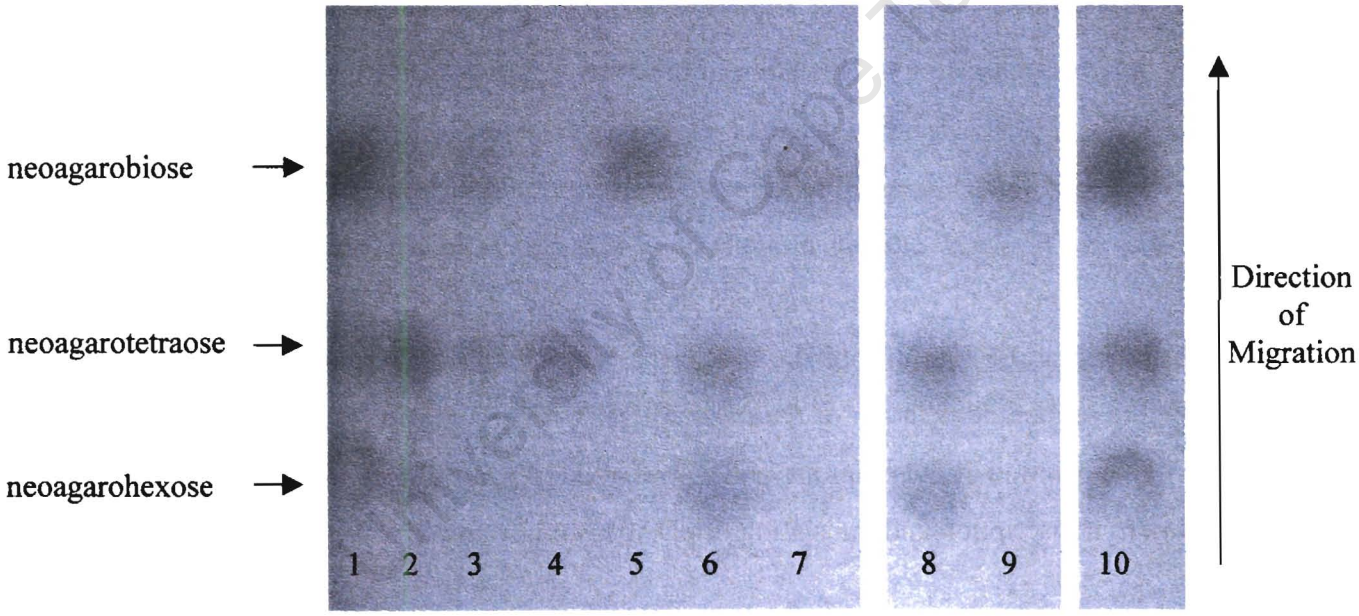

Figure 8 TLC analysis of the oligosaccharides formed following degradation of various saccharide substrates by the purified *P. gracilis* B9 agarase: Lanes 1 and 10, molecular size markers; lane 2, purified agarase incubated with agarose; lane 3, purified agarase incubated with neoagarohexose; lane 4, purified agarase incubated with neoagarotetraose; lane 5, purified agarase incubated with neoagarobiose; lane 6, supernatant of strain B9 grown in BM supplemented with D-glucose incubated with agarose; lane 7, cellular extract of strain B9 grown in BM supplemented with D-glucose incubated with agarose; lane 8, supernatant of strain B9 grown in BM incubated with agarose, lane 9, cellular extract of strain B9 grown in BM incubated with agarose.

4.4.4 Determination of the size of AagA

A number of recombinant plasmids generated as a consequence of the sequential deletion of the inserted DNA of pDA012 (section 3.4.4), which contains the ORF that encodes AagA, were used to determine the size of AagA by *in vitro* transcription/translation. *In vitro* transcription/translation of the plasmids pDA012_A9 and pDA012_A10 resulted in a 32 kDa and a 31.5 kDa protein (Figure 9; lanes 3 and 4). However, *in vitro* transcription/translation of the cloning vector pBluescript KS produced two main proteins of 32 kDa and 21 kDa in size (Figure 9, lane 7), with the larger protein being β-lactamase and the smaller protein, the α-subunit of β-galactosidase (Short *et al.*, 1988). Therefore, the presence of the 32 kDa protein (β-lactamase of the cloning vector), the absence of the 21 kDa protein and the presence of a 31.5 kDa protein (disruption of the β-galactosidase gene due to insertion of *P. gracilis* B9 DNA into the MCS of the cloning vector) following the *in vitro* transcription/translation of pDA012_A9 and pDA012_A10, suggests that the 31.5 kDa protein is the size of the cloned agarase, AagA. Similarly, *in vitro* transcription/translation of the other sequential deletion constructs of pDA012 (pDA012_A12, pDA012_K3, and pDA012_K5) produced the 32 kDa protein and not the 21 kDa protein (lanes 1, 2 and 6). In addition, *in vitro* transcription/translation of plasmids pDA012_K3 and pDA012_K5 produced smaller proteins of 19 kDa and 15 kDa in size, respectively (lanes 1 and 2). This is consistent with the sequence data of *aag*A, since the sequential deletions would result in truncation of AagA from the C-terminal end, resulting in truncated forms of AagA. Similarly, the sequential deletion of the inserted DNA of pDA012 that generated pDA012_A12 resulted in the deletion of the promoter and 5' end of *aag*A, thus explaining the absence of AagA in the *in vitro* transcription and translation of pDA012_A12 (lane 6).

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4.4.5 Purification and characterization of AagA from *E. coli* JM109 (pDA16)

In order to purify sufficient amounts of AagA, total protein from 5 l of *E. coli* JM109 (pDA16) culture supernatant was concentrated with ammonium sulphate and subjected to anion-exchange chromatography. The agarolytic activity eluted from the column with 0.1 M NaCl, the first NaCl treatment (Figure 10). The resultant active fraction (250 ml, final volume) was concentrated with an Amicon Centricon PM10 filter system to a final volume of 45 ml. The active concentrate was subjected to gel filtration chromotography, using Sephadex G75. The active fractions eluted between tubes

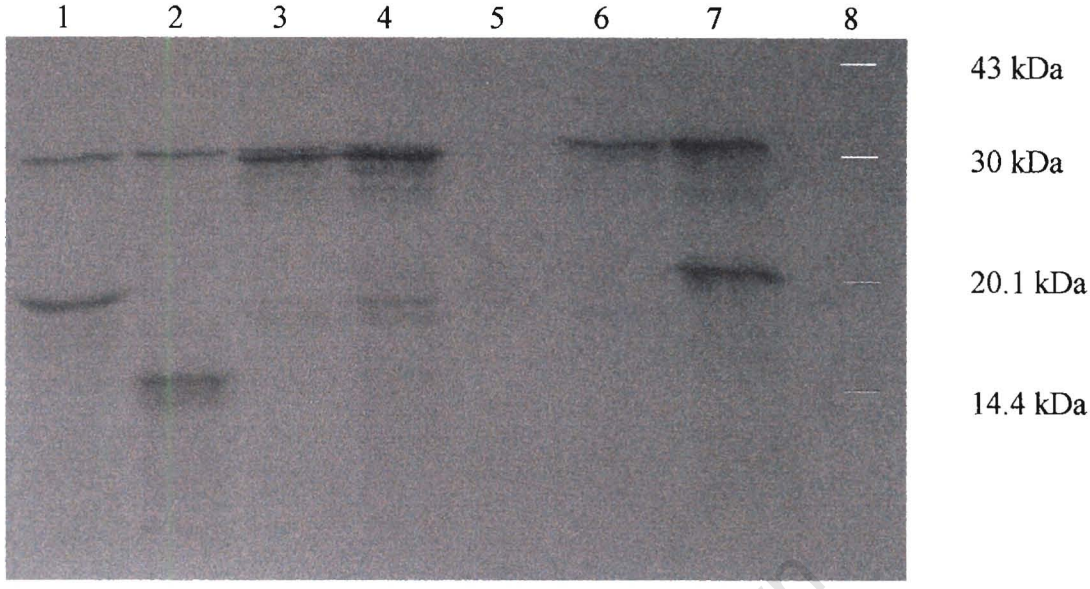

Figure 9 Autoradiograph of *in vitro* transcription/translation. Lane 1: pDA012_K3; Lane 2: pDA012_K5; Lane 3: pDA012_A9; Lane 4: pDA012_A10; Lane 5: No DNA control; Lane 6: pDA012_A12; Lane 7: pBluescript KS; and Lane 8: molecular mass standards in kDa.

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75 and 115 (Figure 11). After concentrating the active pools by Amicon filtration, an aliquot of the ammonium sulphate fraction and the final concentrate was subjected to SDS-PAGE. The final concentrate exhibited a single band at 30 kDa (Figure 12A, lane 1). The zymogram confirmed that the purified protein was an agarolytic enzyme (Figure 12B). The overall yield from the extracellular fraction (supernatant) was 21.4% for a purification of 21.65-fold (Table 2).

The optimum pH and temperature for maximal activity of the purified AagA was pH 7 and 50°C, respectively (Figure 13 and 14). AagA is stable over a 60 min period at temperatures of 4, 22 and 37°C while a rapid decline in activity was observed at 50°C (Figure 15). TLC revealed that AagA hydrolyzes β(1→4) linkages in agarose to predominantly yield neoagarotetraose as the major end product (Figure 16, lanes 1). In addition, AagA hydrolyzed the neoagarohexaose to produce neoagarotetraose and neoagarobiose (Figure 16, lane 2). AagA did not hydrolyze either neoagarotetraose or neoagarobiose (Figure 16, lanes 3 and 4).

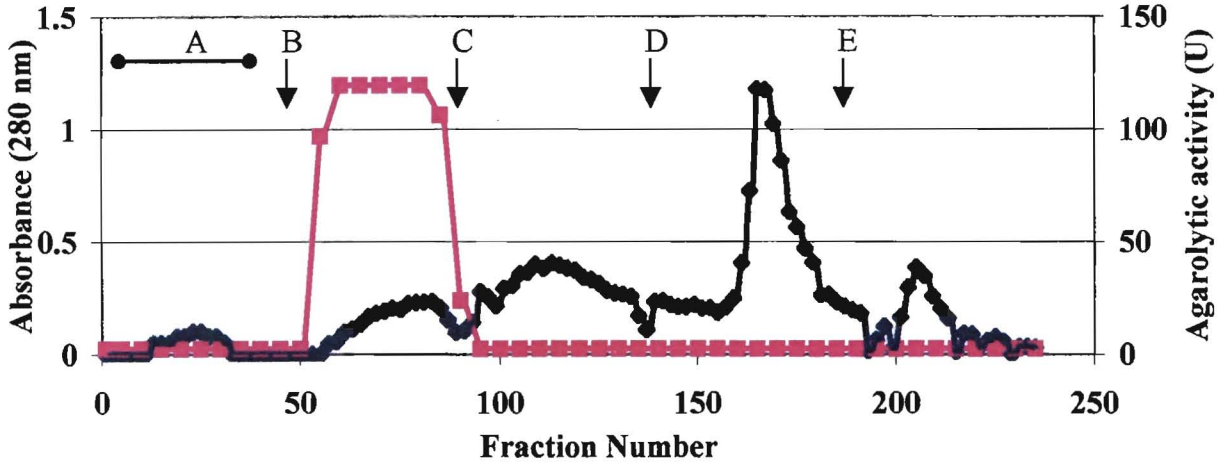

Figure 10 Ion-exchange chromatography of ammonium sulphate concentrate of *E. coli* JM109 (pDA16) culture supernatant on DEAE-Sephadex A-50 (3 cm x 28 cm). A: load (active concentrate) and wash (20 mM Tris-Cl, pH 7); B: addition of 0.1M NaCl; C: addition of 0.25M NaCl; D: addition of 0.5M NaCl; E: addition of 1M NaCl. Protein (♦) was monitored by absorbance at 280 nm and agarolytic activity (■) was assayed as described in section 4.3.5. Agarolytic activity (U) is expressed as µg galactose/ml/30 min at 37°C.

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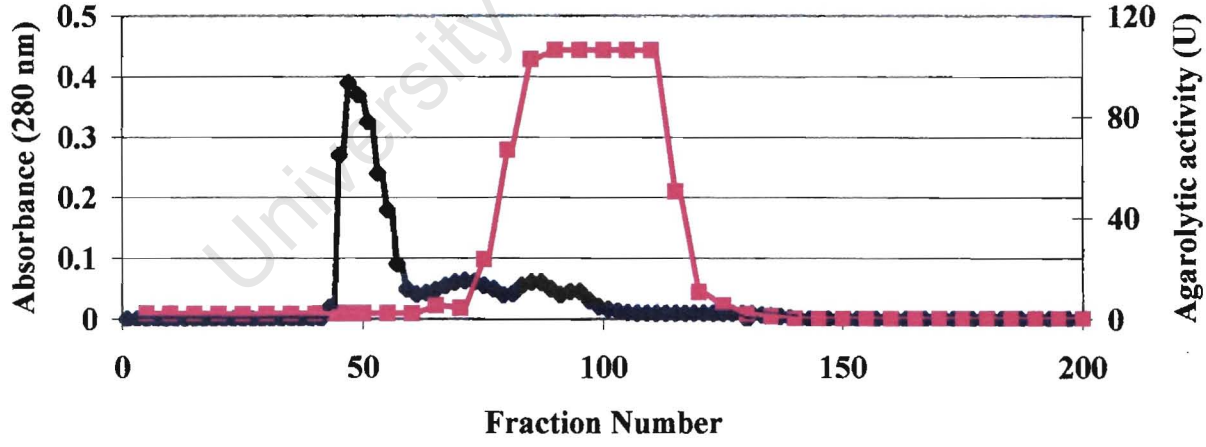

Figure 11 Gel filtration chromatography of the active concentrate (post DEAE-Sephadex A50 chromotography) of *E. coli* JM109 (pDA16) on Sephadex G75 (3 cm x 1m). Protein (♦) was monitored by absorbance at 280 nm and agarolytic activity (■) was assayed as described in section 4.3.5. Agarolytic activity (U) is expressed as µg galactose/ml/30 min at 37°C.

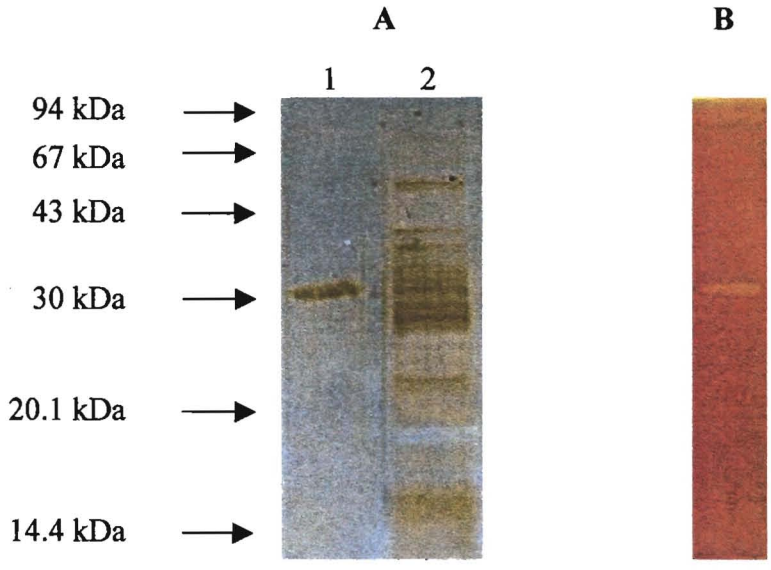

Figure 12 A) SDS-PAGE of samples obtained at different stages during purification of AagA from the *E. coli* JM109 transformant. B) Zymogram of the final concentrate depicted in (A) (lane 1). Lane 1: final concentrate, and lane 2: ammonium sulphate concentrate. The proteins were visualized by silver staining, while the enzymatically active band were detected by staining with Gran's Iodine. The sizes and positions of the molecular mass markers are shown in kDa and by the arrows, respectively.

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Table 2 Purification of AagA from *E. coli* (pDA16).

Purification step	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Recovery (%)**	**Purification (fold)**
Culture Supernatant	135.86	4331310	31881	100	1
Ammonium sulphate preparation	86.63	3479770	40168	80.34	1.26
Sephadex A50	2.148	1321480	615214	30.51	19.3
Sephadex G75	1.343	926990	690238	21.4	21.65

(U): µg galactose/ml/min at 37°C

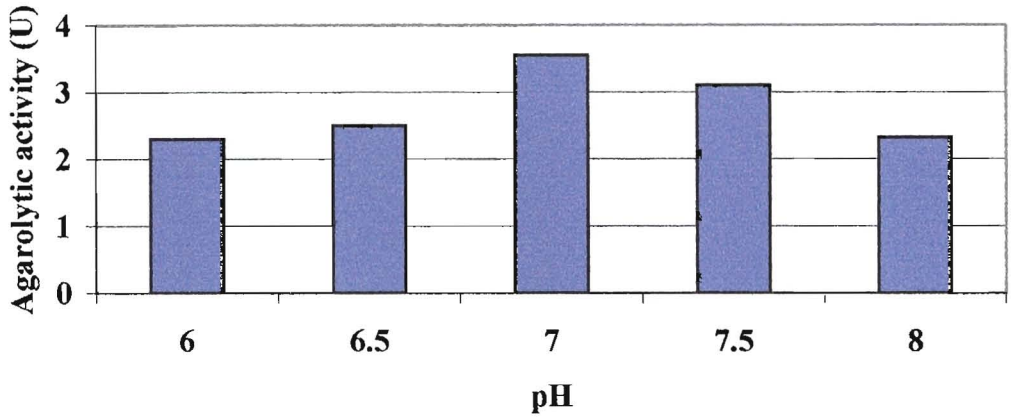

Figure 13 Effect of pH on the activity of purified AagA. Agarolytic activity (U) is expressed as µg galactose/ml/min at 37°C.

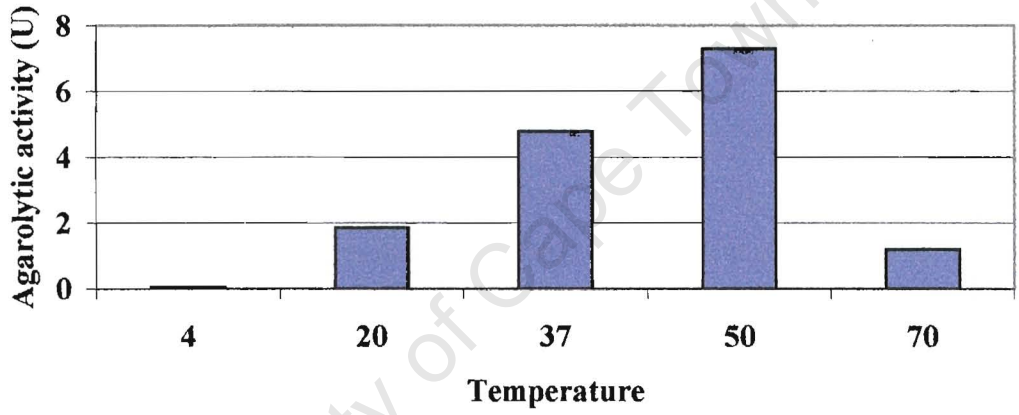

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Figure 14 The effect of temperatures on the activity of purified AagA. Agarolytic activity (U) is expressed as µg galactose/ml/min at the reaction temperature.

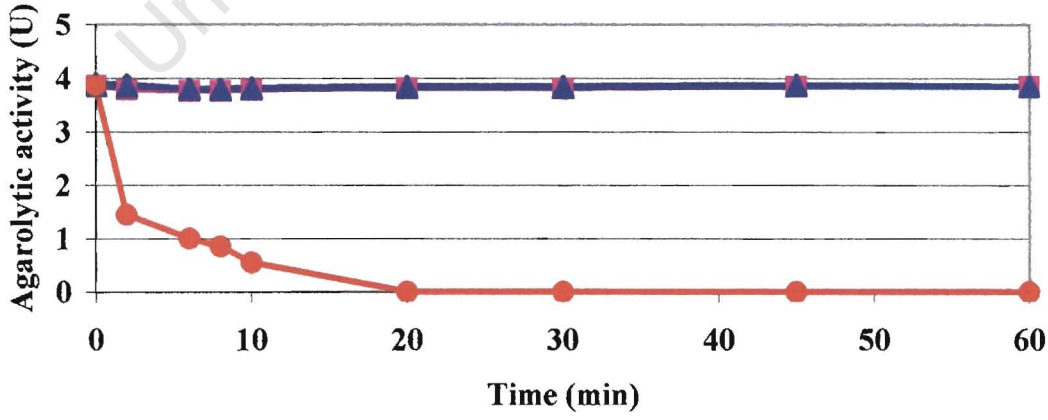

Figure 15 Stability of the purified AagA over a 60 min period at the following temperatures: ♦: 4°C; ■: 22°C, ▲: 37°C, ●: 50°C. Agarolytic activity (U) is expressed as µg galactose/ml/min at 37°C.

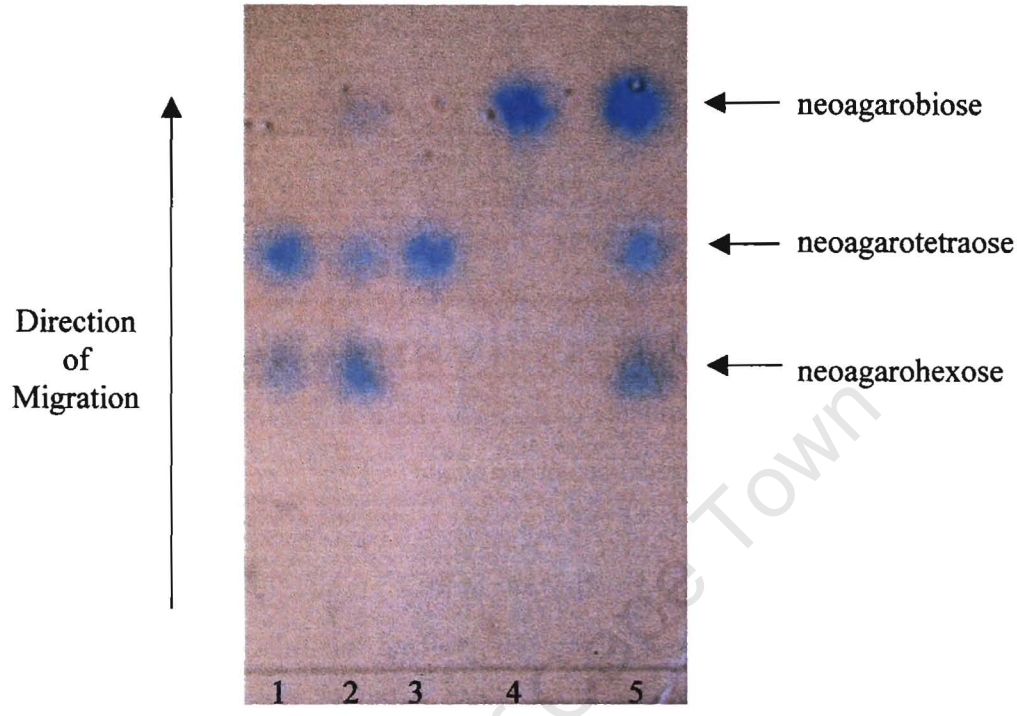

Figure 16 TLC analysis of the oligosaccharides formed following AagA degradation of various saccharide substrates: Lane 1, purified agarase incubated with agarose; lane 2, purified agarase incubated with neoagarohexose; lane 3, purified agarase incubated with neoagarotetraose; lane 4, purified agarase incubated with neoagarobiose; and lane 5, molecular size markers.

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4.5 Discussion

In vitro transcription/translation of the cloning vector pBluescript KS produced two main products. The 32 kDa product is the β-lactamase and the second smaller product, is the 21 kDa α-subunit of the β-galactosidase. Since the original plasmid, pDA012, was generated by insertion of a 2,8 kb DNA fragment carrying the agarolytic region from pDA1 into the multiple cloning site of pBluescript KS, synthesis of the α-subunit of the β-galactosidase was disrupted. Hence, the sequential deletion mutants of pDA012 (pDA012_A9, pDA012_A10, pDA012_A12, pDA012_K3, and pDA012_K5) were negative for the 21 kDa protein. *In vitro* transcription/translation of the two plasmids, pDA012_K3 and pDA012_K5, produced the 32 kDa β-lactamase protein and a smaller protein of 19 kDa and 15 kDa in size, respectively. This was consistent with the DNA sequence data, since these constructs had been shortened from the 3' end of *aag*A, thus producing truncated proteins.

In vitro transcription/translation of the plasmids, pDA012_A9 and pDA012_A10, again produced the 32 kDa β-lactamase protein, as well as the full length 31.5 kDa preprocessed protein, pre-AagA. This observed size is consistent with the 290 amino acid protein encoded by *aag*A, which would have a theoretical size of 31.87 kDa (section 3.4.4). The absence of the 31.5 kDa protein following *in vitro* transcription/translation of pDA012_A12 is consistent with the sequencing data which showed that the *aag*A gene of pDA012_A12 has its promoter and 5' end deleted.

In vitro transcription/translation of *aag*A (870 bp) suggests that the protein has an apparent molecular mass of 31.5 kDa. The *dag*A and *agr*A genes from *S. coelicolor* A3(2) and *P. atlantica* T6c, respectively, both encode for a pre-processed β-agarase which includes signal peptides (Buttner *et al.*, 1987; and Belas, 1989). A putative signal sequence peptidase cleavage site was identified from the deduced amino acid sequence of *aag*A (Chapter 3). Consequently, cleavage of the putative signal sequence would produce a mature AagA protein of 30.2 kDa.

A 30 kDa extracellular β-agarase was purified from the extracellular fractions of both *P. gracilis* B9 and the transformant *E. coli* JM109 (pDA16). Thus, the sizes of the purified β-agarases are identical and they are consistent with the theoretical size predicted for the mature β-agarase (30.2 kDa). In addition, zymogram analysis confirmed that the proteins purified from both *E.*

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coli JM109 (pDA16) and *P. gracilis* B9 were agarolytic. TLC data confirmed that the extracellular agarase purified from *P. gracilis* B9 was indeed the β(1→4) agarase that was cloned into *E. coli*; i.e. both agarases hydrolyzed the β(1→4) linkages of agarose to predominately produce neoagarotetraose. However, the β(1→4) agarase only hydrolyses saccharides larger than neoagarotetraose. This is consistent with the extracellular β-agarase type I enzyme from *P. atlantica* ATCC 19262^T (Day and Yaphe, 1975; and Morrice *et al.*, 1983a).

The levels of agarolytic activity increased 1.5 fold during the late stationary phase of *P. gracilis* B9 growth in media supplemented with a reducing sugar as opposed to non-supplemented media (section 2.4.3). This increased activity appears to be the result of induction of AagA, i.e. the extracellular β(1→4) agarase purified from *P. gracilis* B9 is more than likely the extracellular β(1→4) agarase isolated from the *P. gracilis* B9 genomic DNA library in *E. coli* and purified from the *E. coli* JM109 (pDA16) transformant, since the TLC data confirmed that the mode of agar hydrolysis by *P. gracilis* B9 was identical in both media. In addition, only one extracellular agarase was purified from the extracellular fraction of *P. gracilis* B9 and only one extracellular agarase was found after screening a genomic DNA library of *P. gracilis* B9 for agarase genes (Chapter 3). Therefore, the 1.5 fold increase in agarolytic activity was a result of induction of AagA synthesis and not due to the expression of another extracellular agarase.

The end products of agarose hydrolysis by the cellular extract of *P. gracilis* B9 is indicative of the presence of at least a second agarase that hydrolyzes agarose to predominately neoagarobiose. This enzyme is probably a neoagarotetraose hydrolase, similar to the β-agarase II of *P. atlantica* ATCC 19262^T, which also has agarase activity (Day and Yaphe, 1975; and Morrice *et al.*, 1983a).

AagA has a pH and temperature optimum of 7 and 50°C, respectively. However, the enzyme is not stable at the optimum reaction temperature of 50°C. Therefore, even though the rate of hydrolysis is more efficient at 50°C, it is only sustained over a short period of time. Although the activity was less efficient at lower temperatures, the enzyme could hydrolyze the substrate for a longer period of time. The significance of this high temperature optimum of the agarase for the bacterium is not obvious, especially since the average water temperature at Saldanha Bay is 15°C (Bolton, 1986). However, due to environmental conditions experienced during the summer months in the upper surface layer of the water column at Saldanha Bay, where the water

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temperature could rise to and persist above 20°C (Anderson *et al.*, 1996a), it would be a distinct advantage for the bacterium if an important enzyme like the extracellular agarase could function and be stable at these higher temperatures. Hence, it could be hypothesized that abiotic conditions prevalent at Saldanha Bay may have selected for organisms that can cope or flourish in this environment. Thus, the 50°C reaction temperature optimum of AagA could be an indirect consequence of the overall ability of the bacterium to adapt and hence produce an enzyme that can function at temperatures above 20°C. In addition, due to the efficiency of the enzyme at 50°C, we believe that AagA has potential as a tool in molecular biological applications.

The difference in the stability of AagA purified from *P. gracilis* B9 and from *E. coli* JM109 (pDA16), suggests that processing of the pre-protein is different in *E. coli* compared to the wild-type *P. gracilis* B9. The addition or subtraction of a few amino acids as a result of incorrect or no cleavage of the signal peptide, might affect the tertiary structure of the folded protein, which will in turn either result in a stronger or weaker conformation of the extracellular protein.

An alternate explanation for the difference in the stability of AagA purified from *P. gracilis* B9 and from *E. coli* JM109 (pDA16), is that the two enzymes might be different and that *P. gracilis* B9 produces two extracellular β(1→4) agarase(s). The β(1→4) agarases, II and IV, of *Pseudomonas* sp. O-148 were distinguished from each other by the use of a more sulphated form of agarose when determining their substrate specificities and the resulting oligosaccharides were analyzed using ^{13}C-NMR spectroscopy (Nomura *et al.*, 1998). In addition, multiple extracellular agarases have been identified in other bacteria (Hofsten and Malmqvist, 1975; Sugano *et al.*, 1994; and Araki *et al.*, 1999). Despite the overwhelming evidence that the two purified enzymes from both *P. gracilis* B9 and the *E. coli* JM109 (pDA16) transformant are the same, i.e. the identical size and the similar characteristics of the enzymes, in conjunction with the purification of a single extracellular agarase from the growth media of *P. gracilis* B9, amino acid sequencing of the two enzymes will conclusively verify the identities of the enzymes. Thus, future work should entail sequencing of the two purified proteins. However, for purposes of comparison, I will discuss them as being the same enzyme.

Table 3 compares the characteristics of the extracellular agarases that have been reported in the literature (section 1.3.1). The characteristics of the extracellular β(1→4) agarase purified from *P. gracilis* B9 are very similar to the extracellular β(1→4) agarase of *P. atlantica* ATCC 19262^{T}.

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Table 3 Comparison of the characteristics of AagA to that of the extracellular agarases described in Chapter 1.

Bacterium	Gene	Size (kDa)	pH (opt)	Temp. (opt)	Temp. (stability)	Mode of action	Predominant end products of agarose hydrolysis	End products of neoagarooctaose hydrolysis	Hydrolysis of Porphyran	Group
Pseudoalteromonas gracilis B9	*aag*A	30	7	50°C	<30°C	β(1-4) linkages	neoagarotetraose			
Pseudoalteromonas atlantica ATCC 19262^T	*dag*A	32	7		<30°C	β(1-4) linkages	neoagarotetraose	neoagarotetraose	+	
Cytophaga sp.NCMB 1327			7.2	40°C		β(1-4) linkages	neoagarotetraose	neoagarotetraose	+	
Pseudomonas sp. W7	*pja*A	59	7.8	20-40°C		β(1-4) linkages	neoagarotetraose			
Alteromonas sp. C-1		52	6.5			β(1-4) linkages	neoagarotetraose			
Pseudomonas-like sp.	IIb	63	6.7	43°C		β(1-4) linkages	neoagarotetraose	neoagarotetraose		I
Pseudomonas sp. O-148	II					β(1-4) linkages	neoagaro-oligosaccharides ≥ neoagarotetraose			
Pseudomonas sp. O-148	IV					β(1-4) linkages#				
Pseudoalteromonas antarctica N-1		33	7		<30°C	β(1-4) linkages	neoagarohexaose and neoagarotetraose			
Vibrio sp. P0-303	a	87.5	6.5-7.5	38-55°C		β(1-4) linkages	neoagarohexaose and neoagarotetraose			
Vibrio sp. P0-303	c	57								na
Vibrio sp. P0-303	b	115				β(1-4) linkages	neoagarobiose			
Vibrio sp. AP-2		20	5.5		<45°C	β(1-4) linkages	neoagarobiose			
Vibrio sp. JT0107	*aga*A	107	8	30°C	<40°C	β(1-4) linkages	neoagarotetraose and neoagarobiose			
Vibrio sp. JT0107	0072	72	8	30°C		β(1-4) linkages	neoagarotetraose and neoagarobiose			
Agarbacterium pastinator 2AC			7			β(1-4) linkages	neoagarotetraose and neoagarobiose			II
Cytophaga flevensis		26.5	6.3	30°C		β(1-4) linkages	neoagarotetraose and neoagarobiose	neoagahexaose and neoagarobiose	–	
Pseudomonas-like sp.	I	210*	6.7	38°C		β(1-4) linkages	neoagarohexaose and neoagarobiose			
Alteromonas agarlyticus GJ1B	*aga*A	360*	7.2		<45°C	α(1-3) linkages	agarotetraose			III
Streptomyces coelicolor A3(2)	*dag*A	28 –32.2	7.5			β(1-4) linkages				na
Pseudoalteromonas atlantica T6c	*agr*A	57.5				β(1-4) linkages				na
Microscilla (4 strains)		66-68	6.5-6.8	27- 33°C						na

blank: data not available; *: dimer; #: depending on the degree of sulphation; na: not assigned

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Four other extracellular β(1→4) agarases from *Cytophaga* sp. NCMB 1327, *Pseudomonas*-like sp. (IIb), *Pseudomonas* sp. W7 and *Alteromonas* sp. C-1 have similar characteristics, especially with respect to substrate specificity, since they all hydrolyze agarose to predominantly yield neoagarotetraose as the major end-product (Table 3). In addition, even though the four extracellular β(1→4) agarases of *P. antarctica* N-1, *Vibrio* sp. P0-303 (a) and *Pseudomonas* sp. O-148 (II and IV) hydrolyze agarose to predominantly yield different major end-products, they share a common characteristic with the afore-mentioned group in that they are limited by the disaccharide and thus, neoagarotetraose is the smallest end-product generated by these strains (Table 3).

The extracellular β(1→4) agarases of *Vibrio* sp. P0-303 (b), *Vibrio* sp. JT0107 (0072), *Vibrio* sp. AP-2, *Agarbacterium pastinator* 2AC, *Cytophaga flevensis* and *Pseudomonas*-like (I) are not limited by the disaccharide and thus, neoagarobiose is the common end-product generated by these strains following agarose hydrolysis. The other extracellular β(1→4) agarases of *Vibrio* sp. P0-303 (c), *Streptomyces coelicolor* A3(2), *P. atlantica* T6c, and *Microscilla* are not well characterized with respect to their agarose hydrolysates and thus, they were not assigned to a grouping. The extracellular agarase of *Alteromonas agarlyticus* GJ1B differed completely from the other extracellular agarases in that it hydrolyzed agarose at the α(1→3) linkages to yield agarosaccharides as its end-products, with agarotetraose as the predominant specie.

The extracellular agarases could therefore be divided into three groups. The first group was defined by the inability to hydrolyze neoagarotetraose and neoagarobiose, the second, by the inability to hydrolyze neoagarobiose, and the third, by the ability to hydrolyze the α(1→3) linkages in agarose. The Group I extracellular agarases are also more stable at lower temperatures (<30°C) and have a higher temperature optima (μ40°C), while the Group II extracellular agarases are stable at higher temperatures (<40°C) and generally have lower temperature optima (~30°C). A few members of Group I cleave the central β(1→4) linkage of neoagarooctaose to produce neoagaroteraose and can also hydrolyze a highly substituted form of agarose, porphyran. A member of Group II preferentially hydrolyzes the exterior β(1→4) linkage of neoagarooctaose to produce neoagarohexaose and neoagarobiose. In addition, the agarase could not hydrolyze porphyran. Despite these observations, more thorough analyses are required of the extracellular agarases belonging to these groups to determine whether these differences are genuine or not.

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The amino acid sequence homology of these extracellular agarases is consistent with this form of grouping (section 3.4.6). The extracellular agarases which fell into the family 16 glycoside hydrolase occur in the first grouping, Group I; i.e. the extracellular agarases from *P. gracilis* B9, *P. atlantica* ATCC 19262^T and *Pseudomonas* sp. W7. Therefore, the amino acid sequence data predicts that the extracellular agarase of *S. coelicolor* A3(2) will also fall into Group I. The extracellular agarases that weren't members of the family 16 glycoside hydrolase fell into the second grouping, Group II, i.e. the extracellular agarase of *Vibrio* sp. JT0107 (AgaA). The extracellular agarase of *P. atlantica* T6c is also predicted to fall into Group II. We believe that this method of grouping is more consistent than those proposed by Vera *et al.* (1998), who sorted the agarases according to size. As more extracellular agarases are identified and characterized, the significance of this form of grouping will be verified.

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CHAPTER 5

IN SITU DETECTION OF THE β(1-4) AGARASE OF *PSEUDOALTEROMONAS GRACILIS* B9 IN INFECTED *GRACILARIA GRACILIS*

CONTENTS

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5.1 Summary

Polyclonal antibodies against the β-agarase purified from *E. coli* JM109 transformed with pDA16 were generated in a rabbit. Western hybridization confirmed that the cloned agarase was the extracellular β-agarase of *P. gracilis* B9. The anti-AagA antibodies cross-hybridized only to the 30 kDa protein band present in the extracellular extract of *P. gracilis* B9, confirming that under defined growth conditions, AagA is the only β-agarase secreted into the growth medium. The observed relationship between disease symptoms exhibited by infected *G. gracilis* and the agarolytic phenotype of *P. gracilis* B9 was confirmed. Comparison of bleached thalli from infected alga to unbleached thalli of healthy *G. gracilis*, showed a weakening of the cell structure in the former plants. Immuno-gold labeled antibodies localized the agarase *in situ* to the cell walls of bleached *G. gracilis*. A more direct correlation was noticed between the severity of the symptoms of bleaching and the degree of cell wall degradation. Thus, the weakening observed in the cell structure was attributed to degradation of the mucilaginous component of the cell wall of bleached thalli.

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5.2 Introduction

A cross-section through a mature vegetative thallus reveals two to three layers of small cortical cells followed by an inner medullary region, where the large medullary cells are highly vacuolated (Fredericq and Hommersand, 1989). The cell wall of *G. gracilis* is composed of a number of constituents (Craigie, 1990), of which the relatively well-characterized polysaccharide constituent consists of at least two components, a fibrillar and a mucilaginous component. The latter component is regarded as a non- or para-crystalline matrix in which the former is embedded (Mackie and Preston, 1974). The fibrillar portion is composed mainly of a long glucose chain that results in a cellulosic network with xylose and mannose occurring as lateral chains along the cellulosic backbone. The mucilaginous component consists of agar (Bellanger *et al.*, 1990).

The cellulosic fibrillar network, being rigid in nature, plays an important role in the structure and hence the strength of the thallus. The mucilaginous agar component, due to its flexibility, has a subtler structural role. Christiaen *et al.* (1987) showed that *G. gracilis* cultivated under growth promoting conditions consists mainly of charged polymers, while agarose becomes the major compound when it is cultivated under poor growth conditions. Within a seasonal cycle of growth, a continuous turnover of agar polymers in the cell wall of *G. gracilis* can occur. The changes in agar composition play an important structural function. In the case of high concentrations of agarose precursors, the network structure of the polysaccharide gel in the matrix is low, allowing for cell wall expansion, i.e. short thinner fragments at higher temperatures as a consequence of high growth rates during summer. In contrast, when the agar has a high content of agarose, the three-dimensional structure of the molecule forms a rigid cell wall, i.e. thick thallus observed at low temperatures due to slow growth rates during winter. The strong gel obtained under winter conditions may contribute to the physical resistance of plants growing under adverse environmental conditions (Christiaen *et al.*, 1987).

As outlined in Chapter 1, the South African *Gracilaria* industry which solely depends on the natural resources of *G. gracilis* at Saldanha Bay has experienced a number of setbacks over the last decade or so. Even the pilot studies undertaken by the SRU, whose mandate is to investigate the viability of cultivating *Gracilaria* in Saldanha Bay, experienced a number of setbacks. Subsequently, investors are very cautious in establishing commercial scale farms at Saldanha Bay as encouraged by the SRU. Thus, the future of mariculture of *G. gracilis* at Saldanha Bay

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depends on understanding and consequent prevention of the collapses experienced by *Gracilaria* at Saldanha Bay, as such collapses would be financially devastating.

A number of seaweed diseases attributed to bacteria are discussed in Chapter 1. Other than the disease affecting *G. gracilis* at Saldanha Bay, four other incidences were attributed to agarolytic bacteria (Toncheva-Panova and Ivanova, 1997; Largo *et al.*, 1995; Lavilla-Pitogo, 1992; and Friedlander and Gunkel, 1992). However, the role of agarases in the virulence mechanism of these bacterial pathogens has yet to be determined.

Immunocytochemistry has been used to detect tissue constituents or antigens *in situ* with the use of labeled antibodies. Colloidal gold probes, usually gold spheres of between 2 and 40 nm in diameter, are generally used as markers for detecting antigens *in situ* in conjunction with a wide range of microscopical and non-microscopical preparative techniques. These gold probes are used for the localization of specific proteins. Labelling produced by this technique is usually distinct and highly sensitive. The history of colloidal gold marker systems dates back to the early 1970's when Faulk and Tayor used immunocytochemical probes to identify surface antigens on Salmonellae. Soon thereafter, the use of an indirect labeling technique in which secondary antibodies were adsorbed on to the colloidal gold and used to detect primary antibodies already bound to the antigen was developed. Advancements in this field by Romano, Roth, Geoghegan and Ackerman towards the late 1970's and early 1980's enabled successful complexing of colloidal gold with a wide variety of proteins (Beesley, 1989).

In this chapter, I'll describe the use of the *in vitro* assay developed by Jaffray and Coyne (1996), in conjuction with colloidal gold immunocytochemistry, as tools to evaluate the role the *P. gracilis* B9 β-agarase plays in the infection process.

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5.3 Materials and Methods

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

5.3.1 Antibody production against purified AagA

Polyclonal antibodies against purified AagA protein were obtained by immunizing a rabbit with 150 ug of AagA, purified from the *E. coli* JM109 (pDA16) transformant, together with Freund's incomplete adjuvant (Ausubel *et al.*, 1989). Four injections containing 150 ug AagA each were given intravenously every 7 days over a period of four weeks. Beginning on the day of the second injection, continuing on a weekly basis over a ten-week period, the rabbit was bled to obtain serum containing polyclonal antibodies against AagA.

5.3.2 Western hybridization analysis to determine antibody specificity

The first three bleeds were tested for the presence of anti-AagA antibodies. Serial dilutions of purified AagA were "dot-blotted" in triplicate onto nitrocellulose membranes and the membranes were subsequently air-dried. Western blot analysis was performed as follows: To prevent non-specific binding of antisera, the membranes were blocked by immersion in 100 ml blocking solution (Appendix A.2.28) for 2 h at room temperature. The three bleeds were diluted 1:100 in blocking solution and each membrane was incubated separately in each bleed for 1 h at room temperature. The membranes were washed 3x in 100 ml 1x PBS (Appendix A.2.2) for 15 min per cycle. Diluted alkaline phosphatase-goat anti rabbit IgG conjugate supplied by Sigma (diluted 1:5000 in blocking solution), was added in one container to the membranes and incubated for 1 hr at room temperature. The membranes were washed 3x in 100 ml PBS as described previously. Freshly prepared developing substrate solution (Appendix A.2.28) was added to the membranes. Colour development was allowed to proceed for a few minutes. Rinsing the membranes in water stopped the reaction. All the membranes were stopped simultaneously.

O/N cultures of *P. gracilis* B9, *E. coli* JM109 (pDA16) and *E. coli* K514λ (pDA16) were inoculated into 100 ml flasks containing BM for strain B9 and LB (ampicillin) for the two *E. coli* transformants. The flasks were incubated for 24 h at 22°C. The cells were harvested (10K rpm for 15 min at 4°C) and the supernatants collected. The supernatants were adjusted to a final

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ammonium sulphate saturation of 85% with 50 g of ammonium sulphate at 4°C. The precipitates were collected by centrifugation (10K rpm for 30 min) and resuspended in 1 ml of 10 mM phosphate buffer (pH 7) respectively. The suspensions were dialyzed multiple times against 100 ml of the resuspension buffer.

In addition, the harvested cells of the afore-mentioned cultures were resuspended in 10 ml of 10 mM phosphate buffer (pH 7). Cell suspensions were treated to three cycles of sonication for 30-seconds with a 30 second interval in between each cycle to prevent overheating of cell extracts. The resulting cell extracts were centrifuged (10 K rpm for 10 min) and the supernatants collected. The soluble cellular extracts were dialyzed multiple times against 1 l of 10 mM phosphate buffer (pH 7) at 4°C.

The extracellular and cellular extracts, in conjunction with the purified agarases, were separated on 12% SDS-PAGE in accordance with the Laemmli method (Appendix B.21.1). The resulting proteins were transferred to a nitrocellulose membrane using the Hoeffer electroblotting apparatus as outlined in Appendix B.21.2. Western blot analysis was performed as described above, except that the fifth bleed was used. The bleed employed in the Western hybridization experiment was first absorbed against *E. coli* JM109 whole cell extracts and the resultant protein conjugants were collected by centrifugation (10K rpm for 10 min at 22°C). The supernatant was diluted 1:100 in blocking solution and applied to the respective membranes, which were subsequently incubated between 3-12 h at room temperature.

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5.3.3 Assay for identification of bacterial pathogens of *G. gracilis*

Axenic *G. gracilis* was generated and used in a pathogenicity assay as described by Jaffray and Coyne (1996) (Appendix B.1). The axenic *Gracilaria* was injected (five thalli per treatment) with either a 100 µl aliquot of SSW, a 100 µl aliquot culture of strain B9 or a 100 µl aliquot of a non-agarolytic bacterial epiphyte of *G. gracilis*, isolate SS5g (Appendix B.1). Axenic *Gracilaria* was also injected with 3 µg of AagA (10 µl) purified from the *E. coli* JM109 (pDA16) transformant. All the samples were incubated at 22°C. The samples were monitored daily and symptoms recorded. After 5 days, the injected ends (5 mm) of two thalli per treatment were prepared for light and transmission electron microscopy as described below.

5.3.4 Ultrastructure evaluation and colloidal gold immunolabelling

5.3.4.1 Sample fixation and embedding schedule

The procedure described by Dykstra (1993) was adapted for the preparation of pathogenicity assay samples. The 6 mm thalli were washed in a base buffer (bb) (Appendix A.2.11) to remove any excess material. The samples were immersed in a fixative of 2.5% glutaraldehyde in bb (Appendix A.2.11), 5-10 times the sample volume, O/N at 4°C. The tissues were rinsed twice (5 min each) in bb. The samples were post-fixed in 1% osmium tetroxide (Appendix A.2.11) for 1 h at 22°C. The tissues were rinsed in water twice (5 min each). Dehydration of the samples was carried out by passing them through the following alcohol dilution series:

30% ethanol, 5 min
50% ethanol, 5 min
70% ethanol, 5 min
80% ethanol, 5 min
90% ethanol, 5 min
95% ethanol, 5 min
100% ethanol, 10 min (twice)
100% acetone, 10 min (twice)

The samples were then infiltrated with Spurr resin (Spurr, 1969) as follows:

Spurr resin and 100% acetone (1:1), O/N
Spurr resin and 100% acetone (3:2), 7 h
100% Spurr resin, O/N
New 100% Spurr resin the next morning.

The thalli were placed individually in moulds and covered with Spurr resin. The samples were polymerized in a 60°C oven for two days after which the polymerized wedges were stored at 22°C.

5.3.4.2 Post-embedding schedule for transmission electron microscopy

Ultra-thin cross sections (Figure 1) of the thallus were obtained with the Leica ultracuts ultramicrotone (Leica, Cambridge Ltd) and mounted on carbon-coated nickel grids. An adapted procedure was followed as described by Slot and Geuze in Beesley (1989). The grids were first floated, section downwards, on phosphate buffered saline (PBS) containing 1% BSA (PBS BSA) (Appendix A.2.11) for 5 min. The grids were transferred to PBS containing glycine (Appendix A.2.11) for 3 min and washed twice (1 min each) with PBS BSA. Duplicate grids were floated

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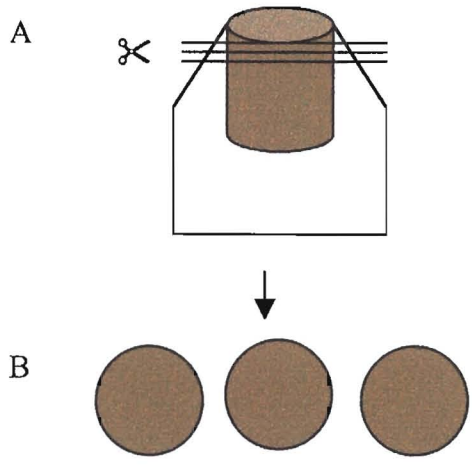

Figure 1 A) A graphic representation of the position of the thallus (brown) in the polymerized Spurr resin wedge. Scissor and horizontal lines indicate the direction of sectioning.
B) The resultant cross-sections of the thallus were analyzed.

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on anti-AagA containing serum obtained from either the first or the fifth bleed for 12 h and washed five times (1 min each) with PBS BSA containing 0.1% Tween (Appendix A.2.11). The grids were washed thrice (1 min each) with PBS BSA. The grids were then floated on a 1:50 dilution of 15 nm gold anti-rabbit probe in PBS BSA for 2 h. The grids were rinsed five times (1 min each) with PBS BSA containing 0.1% Tween, followed by three washes (1 min each) with PBS BSA. The conjugant label complexes were fixed with 1% glutaraldehyde in PBS (Appendix A.2.11) at 22°C for 3 min. The grids were rinsed five times (1 min each) in ultrapure water.

The grids were stained with 2% uranyl acetate for 10 min and washed five times (1 min each) with ultrapure water. The sections were then stained with a second stain, Reynolds lead citrate (Appendix A.2.11), for 5 min and the grids washed in a stream of ultrapure water for 2 min. The samples were visualized on a JEM-200CX transmission electron microscope (JEOL).

5.4 Results

5.4.1 Specificity of the anti-AagA antibodies

To determine which bleed had the greatest titre of anti-AagA antibiodies, a preliminary screening of the first three bleeds was done. Serial dilutions of AagA purified from the *E. coli* JM109 (pDA16) transformant were "dot-blotted" in triplicate onto nitrocellulose membranes and each membrane was probed with one of the bleeds. Antiserum obtained from the first bleed contained no anti-AagA antibodies since no positive signal was produced, even at the lowest antigen dilution (82.5 ng AagA) (Figure 2A, position 1). A slight positive reaction was detected with antisera from the second bleed (Figure 2B, position 1), while antisera from the third bleed contained increased titres of anti-AagA antibodies since it produced a relatively strong signal against the highest antigen dilution (10.3 ng AagA) (Figure 2C, position 4).

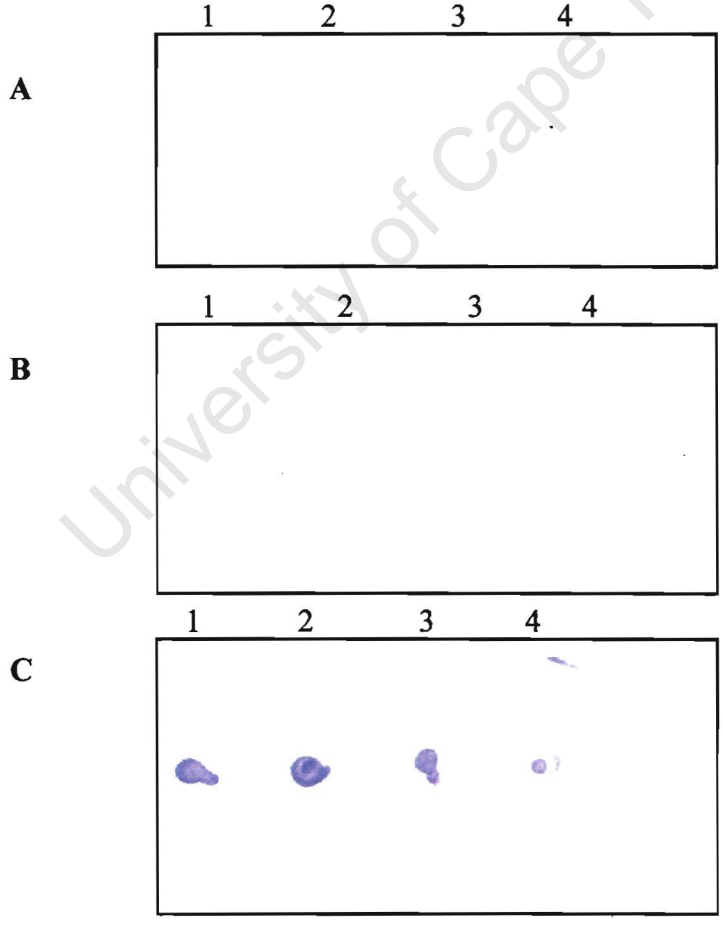

Figure 2 A preliminary 'dot-blot' Western hybridization to determine which bleed contains anti-AagA antibodies. A) Bleed 1 (week 2); B) Bleed 2 (week 3); and C) Bleed 3 (week 4). Position 1: 82.5 ng of AagA; position 2: 41.25 ng of AagA; position 3: 20.6 ng of AagA; and position 4: 10.3 ng of AagA.

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In order to determine the specificity of the polyclonal antibodies against AagA purified from the *E. coli* JM109 (pDA16) transformant, extracellular fractions of *P. gracilis* B9, *E .coli* JM109 (pDA16) and *E .coli* K514λ (pDA16) were separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. This nitrocellulose membrane was subjected to Western hybridization, which showed that the anti-AagA antibodies obtained from the 5th bleed (week 6) cross-reacted strongly to both AagA purified from the *E. coli* JM109 (pDA16) transformant and the extracellular β-agarase purified from *P. gracilis* B9, respectively (Figure 3, lanes 3 and 7). The anti-AagA antibodies detected the presence of the 30 kDa AagA in the extracellular growth medium, and not in the cellular fraction, of *E. coli* JM109 (pDA16) from where it was originally purified (lanes 4 and 8). The antibodies also detected the extracellular β-agarase present in the extracellular growth medium, and not in the cellular fraction, of the wild-type B9 strain (lanes 6 and 10). Despite pre-absorbing the 5th bleed with total *E. coli* JM109 cellular extracts, a larger protein band of 35 kDa was detected in both the *E. coli* cellular fractions (lanes 8 and 9) and the extracellular medium of *E. coli* JM109 (pDA16) (lane 4). No other proteins, were detected in the *P. gracilis* B9 extracts besides the extracellular β-agarase (Figure 3).

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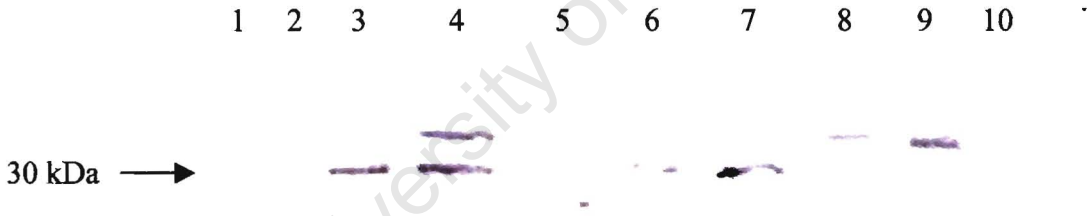

Figure 3 Western blot analysis of the 12% SDS-PAGE containing the following: lane 1: protein standards; lane 2: empty; lane 3: purified AagA from *E. coli* JM109 (pDA16); lane 4: extracellular extract (ammonium sulphate concentrated) of *E. coli* JM109 (pDA16); lane 5: extracellular extract (ammonium sulphate concentrated) of *E. coli* K514λ (pDA16); lane 6: extracellular extract (ammonium sulphate concentrated) of *P. gracilis* B9; lane 7: purified extracellular β-agarase from *P. gracilis* B9; lane 8: cellular extract of *E. coli* JM109 (pDA16); lane 9: cellular extract of *E. coli* K514λ (pDA16); and lane 10: cellular extract of *P. gracilis* B9. The position of the 30 kDa size standard is shown (arrow).

5.4.2 *In situ* localization of the *P. gracilis* B9 agarase in infected *G. gracilis*

The pathogenicity assay devised by Jaffray and Coyne (1996) was used to evaluate the effects of the agarase of *P. gracilis* B9 in the infection process. The injected (with sterile seawater) control axenic thalli, which were incubated in BM at 22°C, remained dark and healthy throughout the incubation period (Figure 4, sample 1). No bacteria were isolated from the control thallus after the 5-day incubation period of the assay. Similarly, healthy and dark thalli were observed following injection with isolate SS5g (Figure 4, sample 4). After the 5-day incubation period with bacterium SS5g, only the isolate SS5g was recovered from the injected thalli. In comparison, a 1 mm area of thallus bleaching was observed after 4 days when thalli were injected with the bacterium B9 (Figure 4, sample 3). In addition, a larger area of bleaching occurred in thalli injected with purified AagA (Figure 4, sample 2). Thallus bleaching in these thalli occurred 2 days post-injection. Only bacterium B9 was re-isolated from thalli injected with bacterium B9, while no bacteria were isolated from AagA injected thalli after the 5-day incubation period.

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Figure 4 Results of pathogenicity assay after 5 days incubation at 22°C. Sample 1, axenic thallus injected with SSW; sample 2, axenic thallus injected with AagA; sample 3, axenic thallus injected with bacterium B9; and sample 4, axenic thallus injected with bacterium SS5g. Bar indicates the length of the lesion.

The injected thallus areas, corresponding to the bleached areas in the bacterium B9 and AagA injected thalli, were washed in a base buffer to remove any excess material. The thalli were oriented as shown in Figure 1, with the infected area directed towards the narrow end of the wedge. The cross-sections were analyzed under a transmission electron microscope. Comparisons of the SSW, bacterium B9 and bacterium SS5g cross-sections revealed no apparent differences in the cell structure. However, a comparison between any of these three cross-sections and that of the cross-sections of AagA injected thalli, revealed a clear disruption in the algal cell structure, i.e. the cell walls of the AagA injected thalli generally appeared more swollen when compared to the bacterium SS5g injected thalli, for example (Figure 5 and 6).

Immuno-gold labeled antibodies revealed that the β-agarase was associated with the cell walls of bleached *G. gracilis*. The immuno-gold was localized to the intercellular matrix of the cell wall of the AagA and bacterium B9 infected thalli (Figure 7B and 7C). Immuno-gold was not detected in the cell walls of the thalli that had been injected with either SSW or bacterium SS5g (Figure 7A).

The fibrillar component of the intercellular matrix between cortical and medullary cells was disrupted in the severely bleached thallus, i.e. in the cross-sections of thalli that had been injected with AagA, the fibrillar appearance of the cell walls had been lost (Figures 7C). Even though the immuno-gold was localized to the intercellular matrix of the cell wall of the thalli injected with bacterium B9, the fibrillar appearance of the intercellular matrix remained evident (Figure 7B). Similarly, the fibrillar nature of the intercellular matrix in the thalli injected with either SSW or bacterium SS5g were also still evident (Figure 7A).

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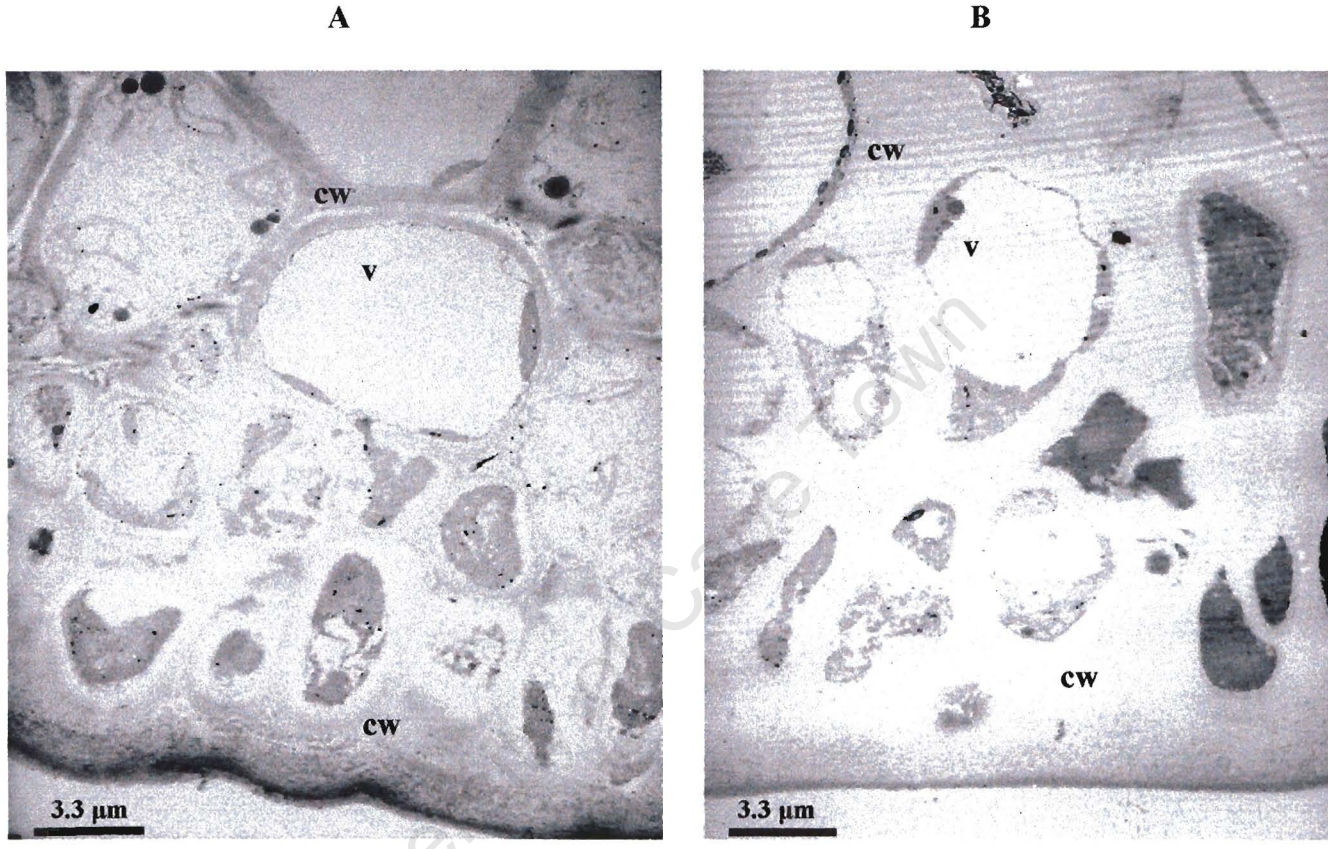

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Figure 5 Transmission electron micrographs of: A) a cross-section through a mature thallus injected with the bacterium SS5g. B) a cross-section through a mature thallus injected with AagA. (v): vacuoles and (cw): cell-wall.

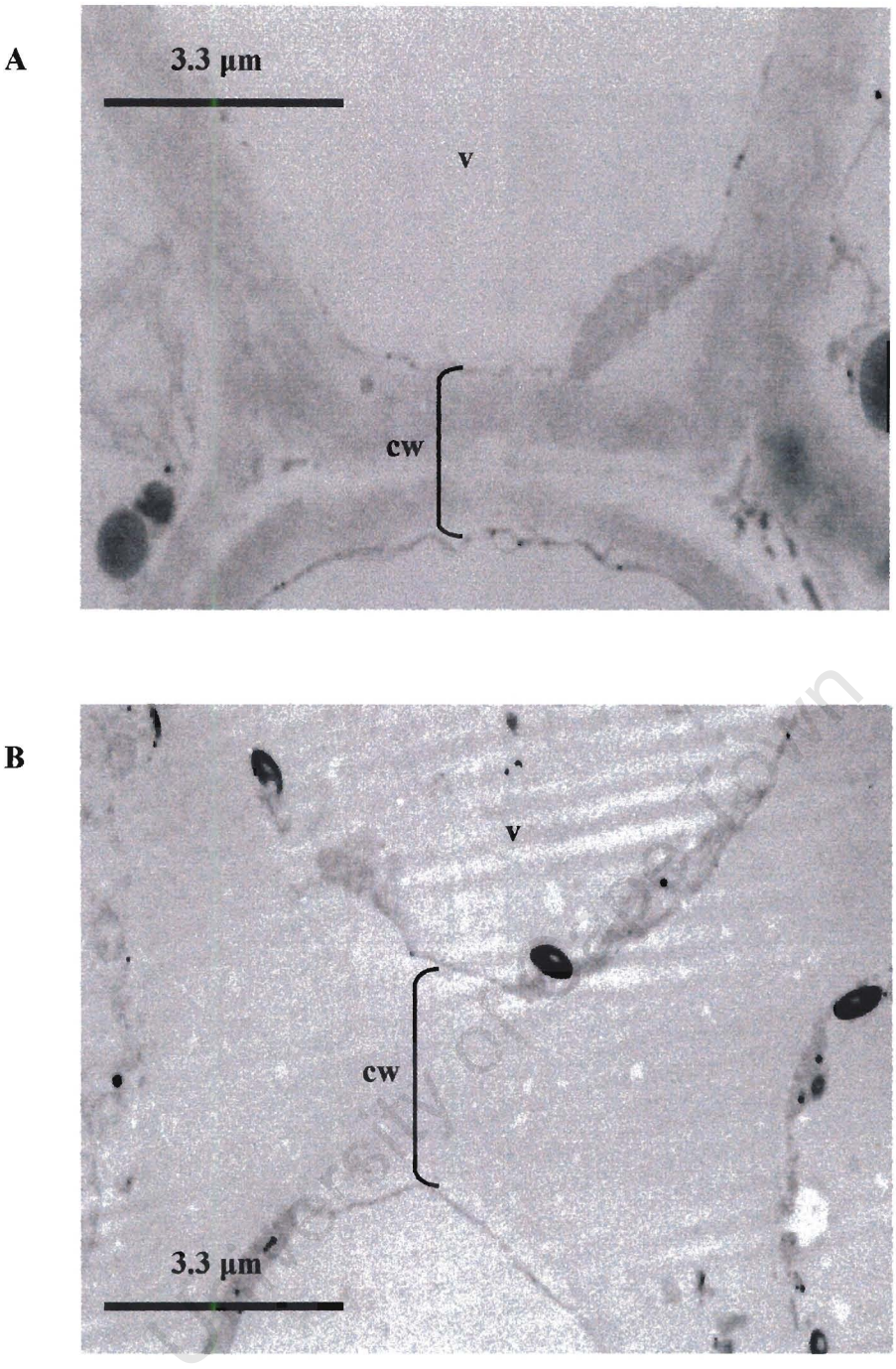

Figure 6 Transmission electron micrographs of: A) a cross-section through a mature thallus injected with the bacterium SS5g; and B) a cross-section through a mature thallus injected with AagA. (v): vacuoles, (cw): cell-wall.

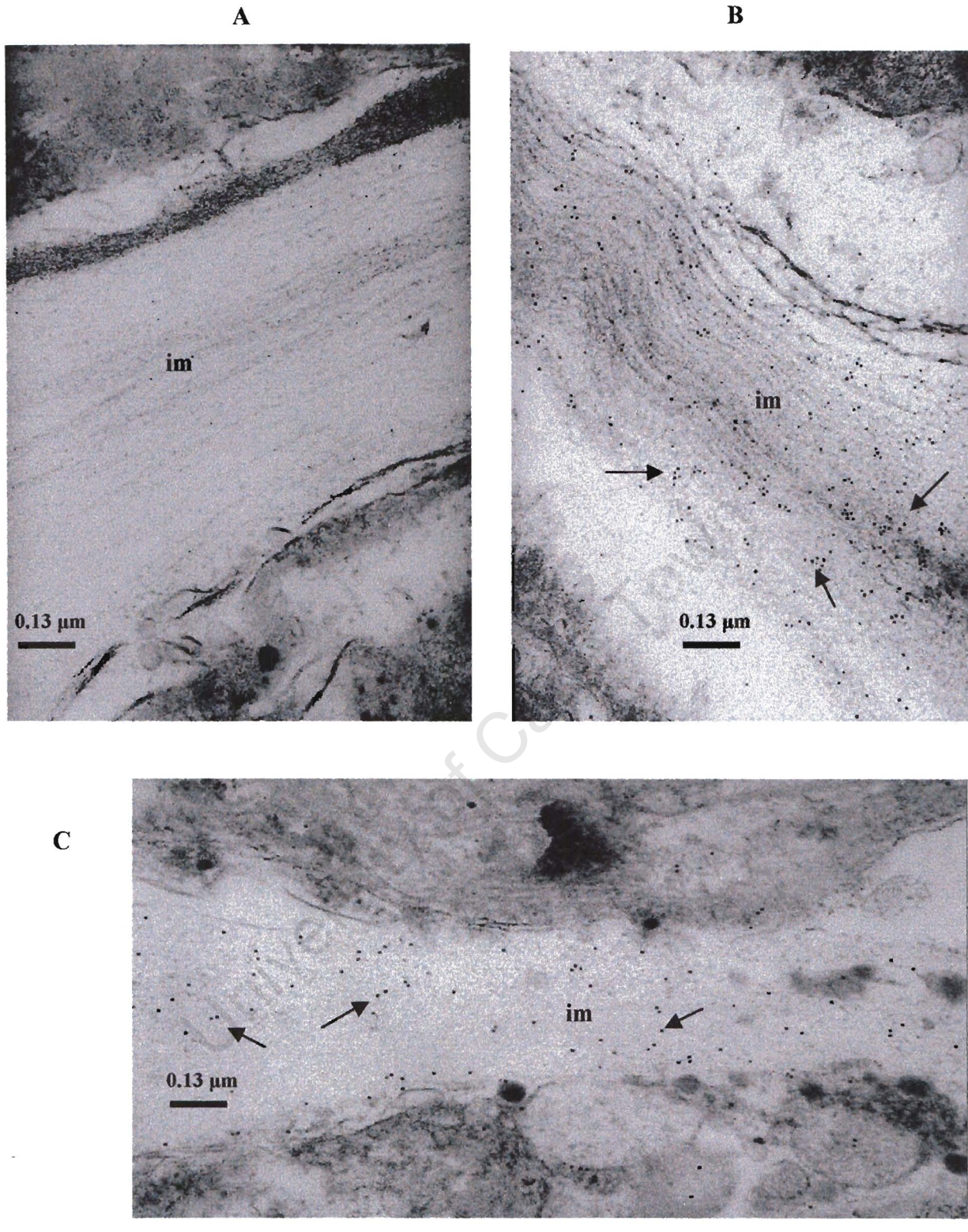

Figure 7 Transmission electron micrographs of: A) a cross-section through a mature thallus injected with SSW; B) a cross-section through a mature thallus injected with the bacterium B9; and C) a cross-section through a mature thallus injected with AagA. (im): intercellular matrix. Arrows: 15 nm gold particles.

5.5 Discussion

The polyclonal antibodies raised against the β-agarase, AagA, purified from the *E. coli* JM109 (pDA16) transformant specifically cross-reacted with AagA and the extracellular β-agarase secreted and purified from *P. gracilis* B9. This result is consistent with the earlier data presented in Chapter 4, i.e. the identical enzyme sizes, the identical substrate specificity data etc., that the extracellular β-agarase from *P. gracilis* B9 is more than likely the β-agarase (AagA) that was cloned into *E. coli*. Once again, amino acid sequencing of both the purified agarases should conclusively verify this observation. In addition, the 30 kDa band was the only protein detected in the extracellular extract of *P. gracilis* B9. Therefore, AagA is the only β-agarase secreted into the medium under the growth conditions tested. However, we cannot rule out the possibility that under different growth conditions, other extracellular agarases might be produced by *P. gracilis* B9. For example, when the culture conditions of *Vibrio* sp. JT0107 were changed, another the agarase, designated agarase 0072, was produced by the bacterium (Sugano *et al.*, 1995). The absence of AagA in the extracellular medium of the *E. coli* K514λ (pDA16) transformant confirmed earlier data (section 3.4.5) that expression of *aag*A is under the control of the lambda promoter on pDA16; thus explaining the repression of gene expression when pDA16 was transformed into an *E. coli* strain expressing the lambda repressor.

The observed relationship between disease symptoms exhibited by infected *G. gracilis* and the agarolytic phenotype of *P. gracilis* B9, was confirmed. Comparison of the severely bleached AagA injected thalli to the slightly bleached bacterium B9 injected thalli, the non-bleached SSW injected thalli and the non-bleached bacterium SS5g injected thalli, showed disruption of the cell structure in the severely bleached AagA injected thalli. The cell wall appeared to have weakened, i.e. it could not maintain its ultrastructure upon treatment with gluteraldehyde in the sample preparation schedule, thus appearing swollen in comparison to the other afore-mentioned samples. Immuno-gold labeled antibodies localized the β-agarase, *in situ*, to the intercellular matrix of the cell walls of thalli injected with either AagA or the bacterium B9. The more severe the symptoms of bleaching, the greater the disruption of the fibrillar component of the cell walls. The cellulosic fibrillar cell wall component functions in concert with the mucilaginous agar component of the cell wall to provide the thallus with strength (Christiaen *et al.*, 1987). One could hypothesize that the degradation of the mucilaginous component by the β-agarase weakened the overall structure and consequently, resulted in the eventual collapse of the fibrillar

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component in the AagA injected sample. The swollen appearance observed in the cell wall of AagA injected thalli could be attributed to overall loss in cell wall strength of the bleached thalli.

Weinberger *et al.* (1999) showed that *Gracilaria conferta* responded with an oxidative burst, a rapid increase in respiration and halogenation when it detected the breakdown products of agar, neoagarohexaose and neoagarotetraose. Neoagarohexaose elicited a release of hydrogen peroxide resulting in an immediate increase in algal brominating activity. Bleached thallus tips appeared a few hours after the addition of neoagarohexaose. These observations are consistent with our results. The end-products released as a result of the agarase activity on the mucilaginous component of the cell wall could elicit a similar response. Thus, the extensive thallus bleaching as a result of injected pure enzyme (AagA) into the thallus of *G. gracilis*, and the thallus bleaching that occurred as a consequence of the secretion of *P. gracilis* B9 extracellular agarase into the thallus of *G. gracilis*, could be due to the macroalga responding to the end-products (neoagarohexaose and neoagarotetraose) produced by the extracellular β-agarase of *P. gracilis* B9 as a consequence of agar degradation.

The site of injection of the thalli employed in the pathogenicity assay was the central medullary cells. In the case of the AagA and bacterium B9 injected thalli, the β-agarase was detected in both the medullary and cortical cell walls. This indicates that the enzyme spread through the cell wall from the site of injection. In addition, no bacteria were located away from the site of injection. Thus, it could be hypothesized that the β-agarase is secreted into the thalli by the bacterial pathogen, where the enzyme is localized to the mucilaginous component (agar) within the cell wall, where hydrolysis of the agar then takes place. The breakdown products of the agar hydrolysis by the extracellular β-agarase, namely neoagarohexaose and neoagarotetraose, are then released from the polymer into the surrounding media, possibly by diffusion stimulated by wave action. The bacterial pathogen hydrolyses the oligosaccharides further by means of its cell bound enzyme(s).

The extracellular β-agarase was localized to the *G. gracilis* cell wall following injection with bacterium B9. However, the ultimate effect of the enzyme on the thallus had not yet taken place; i.e. complete disruption of the cellulosic fibrillar appearance as a consequence of the degradation of the mucilaginous agar component of the cell wall and the severe thallus bleaching. However, at an incubation temperature of 30°C, the characteristic bleached phenotype was observed (section 2.4.1). Therefore, *P. gracilis* B9 has the potential of being a pathogen of *G. gracilis*.

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Although the water temperature at Saldanha Bay does not reach temperatures of 30°C, other environmental factors (Chapter 2) may collectively contribute to the disease of *G. gracilis* observed at Saldanha Bay. We've demonstrated the importance of the agarolytic phenotype of *P. gracilis* B9 as a key virulence factor in the disease affecting *G. gracils* at Saldanha Bay. However, other virulence factors (cellular processes or features) more than likely work in concert with the agarase in eliciting disease. This is supported by the large body of evidence that exists concerning host-pathogen interactions (Taylor, 1998; Casadevall and Pirofski, 2000). Whether it is pathogens infecting plants or animals, the individual components of host-pathogen interactions are shared, either conceptually or mechanistically. Criteria such as the ability to colonize, damage, infect and cause disease all contribute to the successful pathogenesis of a host.

It is difficult to pinpoint the exact factors that cause disease of *G. gracilis* at Saldanha Bay since the initial effects of disease cannot be detected with the naked eye. Only once the disease has progressed to the stage of thallus bleaching and breakage, are the symptoms visible. However, by this time the agar component of the cell wall is degraded and the crop rendered useless. *In situ* monitoring of the extracellular β-agarases at various times during the growth season of *G. gracilis*, either in natural or cultivated populations at Saldanha Bay, would allow for exact determination of environmental cues that result in the collapse of the *G. gracilis* populations. Advances in immunocytochemistry techniques have allowed for the development of non-microscopical techniques such as pregnancy diagnostic kits (Organon Teknika, Belgium) (Beesley, 1989). Similarly, an *in situ* agarase detection kit could be developed to assist potential farmers in early detection of the onset of disease and thus allow for early harvesting, which would in turn circumvent the complete collapse of the entire *Gracilaria* crop and thus avoid financial ruin.

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CHAPTER 6

GENERAL DISCUSSION

The future of mariculture of *G. gracilis* at Saldanha Bay depends on a better understanding, and possible prevention, of the collapses experienced by the *Gracilaria* at Saldanha Bay. The aim of this study was to isolate the gene(s) that encode the agarolytic activity associated with an epiphytic bacterial pathogen of *G. gracilis* from Saldanha Bay. Upon characterization, the enzyme(s) would be used as a tool to elucidate the virulence mechanism, and thus, strategy employed by the bacterium in eliciting disease in *G. gracilis*.

Overall, we were fairly successful in the initial aims of this study. An agarolytic bacterium (isolate B9), which was isolated from *G. gracilis* from Saldanha Bay, was used in an assay developed by Jaffray and Coyne (1996). Since thallus bleaching is one of the main symptoms experienced during the collapses of *G. gracilis* at Saldanha Bay, it could be concluded that the bacterium B9 is indeed a putative pathogen of *G. gracilis*. In addition, the 16S rDNA sequence, the physical and the phenotypic characteristics of the bacterium B9 suggested that it is a member of the genus *Pseudoalteromonas*. We propose that strain B9 is significantly different in comparison to the phenotypic characteristics of the other members of the *Pseudoalteromonas* genus. Hence strain B9 was designated the species name *gracilis*, after the seaweed from which it was isolated, *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine et Farnham. However, this is a tentative assignment since the DNA-DNA hybridization data is still outstanding.

In an endeavor to clone the gene(s) responsible for the agarolytic activity of *P. gracilis* B9, a genomic library of *P. gracilis* B9 was constructed in *E. coli* HB101. This library led to the successful cloning, sequencing and characterization (at the molecular level) of an extracellular β-agarase, AagA, from *P. gracilis* B9. The comparison of the β-agarase from *P. gracilis* B9 to a number of β-agarases and β-glucanases of the family 16 glycoside hydrolases from a number of bacteria, led to the assignment of most of the β-agarases cloned to date to the family 16 glycoside hydrolases. From the substantial amount of data collected in this study, we concluded that AagA is the extracellular β-agarase purified from the extracellular fraction of *P. gracilis* B9. However, the difference in the enzyme stability data between the two purified enzymes appears to dispute this conclusion. As discussed earlier in this thesis, protein sequencing of the two β-

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agarases should conclusively determine whether our conclusion is valid or not. Another approach could entail mutating *aag*A in *P. gracilis* B9 by site-directed mutagenesis. We did in fact attempt to do just this but were unsuccessful in a number of approaches (data not shown). We attempted mutating *aag*A in *P. gracilis* B9 using homologous recombination (Herrero *et al.*, 1990) through the transfer of a recombinant transposable vector from the donor *E. coli* SM10 (λpir) to the recipient *P. gracilis* B9 (Miller and Mekalanos, 1988). We also attempted the transfer of the recombinant transposable vector via electroporation and modified $CaCl_2$ methods (Miller and Nickoloff, 1994; and Belas *et al.*, 1988). However, none of these methods proved successful. Thus, future work should entail sequencing of the respective purified proteins, which will unequivocally confirm the identity of the two purified proteins.

To further characterize the extracellular β-agarase of *P. gracilis* B9, *in vitro* experiments were performed on *P. gracilis* B9 cultured in a variety of media so as to ascertain the expression profile(s) of the agarolytic activity by *P. gracilis* B9. A number of interesting expression profiles were discovered. For example, extracellular β-agarase expression in *P. gracilis* B9 is regulated via end-product and catabolite repression. *P. gracilis* B9 extracellular β-agarase production is also affected by substrate composition.

To address the final aim of the study, polyclonal antibodies against the purified β-agarase, AagA, was used as a tool to elucidate the virulence mechanism employed by the bacterial pathogen in eliciting disease in *G. gracilis*. The observed relationship between the disease symptoms exhibited by infected *G. graciils* and the agarolytic phenotype of *P. gracilis* B9 was confirmed. Immuno-gold labeled antibodies localized the β-agarase, *in situ*, to the intercellular matrix of the cell walls of the thalli injected with AagA and the bacterium B9. The more severe the symptoms of bleaching, the greater the disruption of the fibrillar component of the cell walls was observed.

From all the data collected from the *in vitro* growth studies of *P. gracilis* B9 and the *in situ* localization of AagA to the cell walls of *G. gracilis*, the following hypotheses could tentatively be made. It could be hypothesized that since agarase production occurs primarily during stationary phase of growth, the initial low levels of agarase production serves as "scavengers" to detect for the presence or absence of the polymers. Hence, it is conceivable that *P. gracilis* B9 would produce the agarase at low levels, detect the polymer (directly or indirectly) and ultimately regulate the agarase expression accordingly, since enzyme synthesis and export is energetically expensive. Once the polymer is detected, large amounts of enzyme are

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subsequently produced. However, the bacterium will not expend energy unnecessarily. As mentioned earlier, the bacterium will utilize the freely available substrates first.

The onset of disease in *G. gracilis* in Saldanha Bay occurs during the summer where high water temperatures and oligotrophic waters predominate. The network structure of the polysaccharide gel in the matrix is low in the cell wall of the thalli (Christiaen *et al.*, 1987). The afore-mentioned network structure is low because of the substituted form of agar. The higher agarolytic activity that is observed when *P. gracilis* B9 is grown in the presence of the more substituted form may be due to adaptation of *P. gracilis* B9 to these conditions. In addition, this study has clearly shown the important effect carbohydrates have on agarase production. Therefore, it was hypothesized that during the conditions that occur in Saldanha Bay during summer, the bacterium could be subjected to low levels of utilizable, energy inexpensive carbohydrates with agar as its only readily available source of carbon. Since the bacterium is in oligotrophic conditions, it will mostly be in the stationary phase of growth where agarase production is initiated. In addition, we observed that the agarase spreads through the *G. gracilis* cell wall from the site of injection and no bacteria were located away from the site of injection. Thus, it could be hypothesized that the β-agarase is secreted into wounds present on the thalli by the bacterial pathogens that have colonized the algal surface in the vicinity of these wounds. These wounds could be due to the action of grazers (macro or micro) or the continuous movement and abrasion that can occur as a result of wave action. Hence, these wounds would serve as entry points (analogous to injecting the bacterium or agarase into the thalli in the pathogenicity assay) for the eventual spread of the agarase. The β-agarase is localized to the mucilaginous component (agar) within the cell wall, where hydrolysis of the agar then takes place. The breakdown products of agar hydrolysis by the extracellular β-agarase, namely neoagarohexaose and neoagarotetraose, are then released from the polymer into the surrounding media, possibly by diffusion stimulated by wave action. The bacterial pathogen hydrolyses the oligosaccharides further with its cell bound enzyme(s).

Once the water quality improves and agar is no longer the sole source of carbon available to the bacterium, agarase production is either reduced to low levels or completely switched off. Over the following winter and summer seasons, the few left over strands of thalli regenerate vegatatively, thus replenishing the macroalgal populations until conditions are suitable for the infectious process to repeat itself again.

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The above hypotheses still need to be verified and the *in vitro* techniques described in this study could be used as tools for a thorough and detailed examination of the causes of the disease experienced by *G. gracilis* at Saldanha Bay. *In situ* monitoring of the extracellular β-agarases at various times during the growth season of *G. gracilis*, either in natural or cultivated populations at Saldanha Bay, would allow for the exact determination of environmental cues that result in the eventual collapse of the *G. gracilis* populations. Similarly, the role of other pathogenic agents in relation to the agarolytic virulence mechanism adopted by bacterial pathogens of *G. gracilis* could be investigated.

Finally, an *in situ* agarase detection kit could be developed to assist potential farmers in early detection of the onset of disease and thus allow for early harvesting, which would in turn circumvent the complete collapse of the entire *Gracilaria* crop and thus avoid financial ruin.

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APPENDIX A

MEDIA AND SOLUTIONS

CONTENTS

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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.

Ultrapure water used was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system.

A.1 Media

A.1.1 Basal Media (BM)

NaCl (Saarchem)	30.0 g
$MgCl_2.6H_2O$ (Saarchem)	2.3 g
KCl (Saarchem)	0.3 g
Casamino acids (Difco)	5 g
Yeast extract (Biolab)	1 g
Agar (Biolab)	0.5 g
water to	1 l

In BM supplement with a reducing sugar, 2 g of D-glucose (Saarchem) or D-galactose (Saarchem) were added. In BM lacking agar, the agar component was omitted. In BM were the agar substituted for agarose (Hispanagar D1 LE), the 0.5 g of agar was substituted with 0.5 g agarose. The pH of the all the media was adjusted to 7.0 with 1 M NaOH.

A.1.2 Basal Agar (BA)

NaCl	30.0 g
$MgCl_2.6H_2O$	2.3 g
KCl	0.3 g
D-glucose	2 g
Casamino acids	5 g
Yeast extract	1 g
Agar	20 g
water to	1 l

The pH of the all the media was adjusted to 7.0 with 1 M NaOH prior to adding the agar and autoclaving.

A.1.3 Media for Carbohydrate utilization test

Peptone (Difco)	2.0 g
NaCl	30 g
K_2HPO_4 (Saarchem)	0.3 g
1% Bromophenol blue (w/v) (Merck)	3 ml
water to	1 l

The pH is adjusted to 7.1 before adding the bromophenol blue. The carbohydrates 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.

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A.1.4 Media for Nitrate reduction and denitrification test

D-glucose	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.

A.1.5 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.

A.1.6 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

A.1.7 Media for Carrageenase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Casamino acids	0.5 g
Yeast extract	0.1 g
D-glucose	0.2 g
Carrageenan (Sigma)	2.0 g
water to	100 ml

A.1.8 Media for Gelatinase activity test

NaCl	3.00 g
$MgCl_2.6H_2O$	0.23 g
KCl	0.03 g
Gelatin (Merck)	12 g
water to	100 ml

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A.1.9 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

A.1.10 Luria-Bertani broth (LB)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
water to	1 l

A.1.11 Luria-Bertani agar (LA)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
water to	1 l

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A.2 Solutions

A.2.1 Antibiotic solutions in growth media

- Ampicillin (Sigma) (100 mg/ml)
  Dissolve 2 g in 20 ml water. Filter sterilize and store aliquots at 4°C.
  Dilute 1:1000 into media for final concentration of 100 µg/ml.

- Amphotericin (Sigma) (10mg/ml)
  Dissolve 0.1 g in 10 ml water. Filter sterilize and store aliquots at 4°C.
  Dilute 1:1000 into media for final concentration of 10 µg/ml.

- Kanamycin (Sigma) (30 mg/ml)
  Dissolve 0.6 g in 20 ml water. Filter sterilize and store aliquots at –20°C.
  Dilute 1:1000 into media for final concentration of 30 µg/ml.

A.2.2.General stock solutions

- 0.5 M EDTA

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

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

- 1 M Tris base

Tris (Roche)	12.1 g
water to	100 ml

- 1 M Tris-HCl

Tris	12.1 g
water to	100 ml

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

- TE buffer (Tris-EDTA)

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

- EtBr 10 mg/ml (Ethidium Bromide)

Etbr (Sigma)	0.1 g
water to	10 ml

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

- 1 M $MgCl_2$

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

- 70% EtOH

absolute EtOH (Merck)	70 ml
water to	100 ml

Do not autoclave. Store at –20°C

- 10% Ammonium persulfate

Ammonium persulfate (Pharmacia)	1 g
water to	10 ml

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

- 10x Phosphate buffered saline (PBS)

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

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

- 1x PBS (dilute 10x PBS 1:10 with water)

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A.2.3 Solutions for *In vitro* assay to detect bacterial pathogens of *G. gracilis*

- 1% Povidone-iodine (PVP-I) solution

Povidone iodine (Merck)	1 g
Sterile water to	100 ml

Do not autoclave and store in dark.

- Antibiotic 'cocktail' for generating axenic *G. gracilis*
  - Penicillin G (Sigma) (100 mg/ml)
    - Dissolve 1 g in 10 ml water. Filter sterilize and store aliquots at 4°C.
    - Dilute 1:200 into SSW for final concentration of 500 µg/ml.
  - Streptomycin (Roche) (100 mg/ml)
    - Dissolve 2 g in 20 ml water. Filter sterilize and store aliquotes at 4°C.
    - Dilute 1:200 into SSW for final concentration of 500 µg/ml.
  - Kanamycin (Sigma) (100 mg/ml)
    - Dissolve 1 g in 10 ml water. Filter sterilize and store aliquots at –20°C.
    - Dilute 1:200 into SSW for final concentration of 500 µg/ml.
  - Nalidixic acid (Sigma) (0.1 mg/ml)
    - Dissolve 1 mg in 10 ml water. Filter sterilize and store aliquots at 4°C.
    - Dilute 1:200 into SSW for final concentration of 0.5 µg/ml.
  - Cefotaxine (Claforan®, Roussel) (250 mg/ml)
    - Dissolve 5 g in 20 ml water. Filter sterilize and store aliquots at –20°C.
    - Dilute 1:1000 into SSW for final concentration of 2.5 mg/ml.

The above antibiotics were combined in the their stipulated dilutions into seawater that had been autoclaved (SSW).

A.2.4 Solutions for Gram-staining procedure

- Crystal violet

Crystal violet (Merck)	2 g
absolute EtOH	10 ml
Ammonium oxalate (BDH)	4 g
water to	500 ml

- Gram iodine

Iodine (Saarchem)	1g
Potassium iodide (Saarchem)	2 g
water	245 ml
5% Sodium bicarbonate (Saarchem)	60 ml

- Acetone solution

absolute EtOH	250 ml
Acetone (Saarchem)	250 ml

- Counter stain

Safranin (Merck)	0.5 g
absolute EtOH	100 ml

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A.2.5 Solutions for Catalase and oxidase tests

- Kovac's reagent

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

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

- 3% Hydrogen peroxide solution

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

Freshly prepared. Do not autoclave.

A.2.6 Solution for Cellulase activity test

- Congo Red

Congo Red (BDH)	0.1 g
sterile water	100 ml

A.2.7 Solutions for Starch hydrolysis assay

- 0.5% starch

Starch (BDH)	0.5 g
sterile water	100 ml

Freshly prepared. Do not autoclave.

- DNS Reagent

3,5 Dinitrosalicylic acid (Sigma)	2.65 g
NaOH	4.95 g
Rochelle salts (Na K Tartrate) (Merck)	76.5 g
Phenol (Merck)	1.9 g
Na-metabisulphite (Sigma)	2.075 g
sterile water to	354 ml

Dissolve 3,5 Dinitrosalicylic acid, NaOH and Rochelle salts completely in sterile water before adding the other constituents (dissolving them in turn). The phenol in melted at 50°C. A 3 ml sample must be titrated to the end point with 5-6 ml 0.1 M HCl using phenothalein as an end-point indicator. If less HCl is required, then solid NaOH must be added to the DNS solution at the rate of 2 mg / ml. The DNS must be stored in the dark under N_2.

A.2.8 Solutions for Chromosomal DNA extractions

- 10% SDS (Sodium dodecyl sulphate)

SDS (BDH)	10 g
water to	100 ml

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

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- Proteinase K (20 mg/ml)

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

Do not autoclave. Store at –20°C.

- 5 M NaCl

NaCl	29.22 g
water to	100 ml

- CTAB/NaCl

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

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

- Chloroform (Merck) / isoamyl alcohol (Merck)

Mix at ratio 24:1

- RNase (10 mg/ml)

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

Heat for 15 min at 100°C and allow to cool slowly to room temperature. Do not autoclave. Aliquot into eppendorf tubes and store at –20°C.

A.2.9 Solution for Agarose gel electrophoresis

- 50x TAE (Tris-acetate buffer)

Tris	242 g
Glacial acetic acid (Saarchem)	57.1 ml
0.5 M EDTA	100 ml
water to	1 l

A.2.10 Solutions for Sequencing

- 10x Tris-Borate-EDTA buffer (TBE)

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

- 2 M NaOH

NaOH	80 g
water to	1 l

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- 3 M Na-acetate

Na-acetate (Merck)	204.0 g
water to	500 ml

Dissolve sodium acetate in 400 ml and adjust pH with glacial acetic acid to 5.2. Make up volume to 500 ml.

- Silane

Silane	50 µl
Glacial acetic acid	30 µl
water	300 µl
absolute EtOH	9.62 ml

A.2.11 Solutions for Electron microscopy

- 2% uranyl acetate (pH 5) solution

uranyl acetate (Sigma)	5 g
100 % Methanol (BDH)	25 ml

Filter sterilize before use. Store at 4°C.

- base buffer (bb)

10x PBS (pH 7.4)	10 ml
NaCl	2.34 g
ultrapure water to	100 ml

- 2.5% glutaraldehyde

25% gluteraldehyde (Sigma)	1 ml
10x bb	1 ml
sterile ultrapure water	10 ml

Do not autoclave and freshly prepared.

- 1% osmium tetroxide

osmium tetroxide (Sigma)	0.1 g
ultrapure water to	10 ml

Do not autoclave.

- PBS / 1% BSA (PBS BSA)

10x PBS (pH 7.4)	1 ml
BSA (Roche)	0.1 g
sterile water	10 ml

Filter sterilized and freshly prepared.

- PBS containing glycine (neutralizing buffer)

10x PBS (pH 7.4)	1 ml
glycine (Saarchem)	15 mg
sterile water	10 ml

Filter sterilized and freshly prepared.

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- PBS BSA containing 0.1% Tween 20 (v/v)

10x PBS (pH 7.4)	1 ml
BSA	0.1 g
Tween 20 (Saarchem)	10 µl
sterile water	10 ml

Filter sterilized PBS / BSA (8 ml), add Tween and adjust volume to 10 ml. Freshly prepared.

- 1% gluteraldehyde

25% gluteraldehyde	100 µl
10x PBS (pH 7.4)	250 µl
sterile ultrapure water	2.5 ml

Do not autoclave and freshly prepared.

- Reynolds lead citrate

Lead citrate (Sigma)	1.33 g
Na citrate (Saarchem)	1.76 g
ultrapure water	30 ml
1 M NaOH	8 ml
ultrapure water to	50 ml

Dissolve lead citrateand sodium citrate in 30 ml water and shake for 1 min. Allow solution to stand for 30 min before adding NaOH. Make up to 50 ml.

A.2.12 Solutions for Ferricyanide assay for reducing-sugars

- Mineral 1 solution

K_2HPO_4 (Saarchem)	6.0 g
water to	1 l

- Mineral 2 solution

NaCl	12.0 g
$(NH_4)_2SO_4$ (Roche)	12.0 g
KH_2PO_4	6.0 g
$CaCl.2H_2O$ (Saarchem)	2.5 g
$MgSO_4.7\ H_2O$ (Merck)	2.5 g
water to	1 l

- 20 mM PIPES solution

PIPES (Merck)	0.6 g
Mineral 1 solution	5.0 ml
Mineral 2 solution	5.0 ml
ultrapure water to	100 ml

Adjust pH to 6.8 with 10 M NaOH to dissolve the PIPES. Adjust volume to 100 ml.

- 1% Agarose substrate

20 mM PIPES solution	90 ml
agarose	1 g
water to	100 ml

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- Stop reagent

Na_2HPO_4. $7H_2O$ (Saarchem)	1 g
NaOH (Saarchem)	1 g
water to	100 ml

Replace monthly and need not be autoclaved.

- Colour reagent

$K_3[Fe(CN)_6]$ (Merck)	116 mg
NaOH	220 mg
Water to	100 ml

Store in dark and replace monthly. Need not be autoclaved.

A.2.13 Solutions for Large scale preparation of plasmid DNA

- Solution 1

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

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

- Solution 2

NaOH	0.8 g
SDS	1 g
sterile water to	100 ml

Make fresh weekly.

- Solution 3

KOA (Saarchem)	147 g
water to	500 ml

Dissolve potassium acetate in 250 ml water, pH to 4.8 using glacial acetic acid and adjust volume to 500 ml.

- Salt saturated isopropanol

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

Shake up and stand overnight. Do not autoclave.

A.2.14 Solutions for Transformation of *E. coli*

- 0.1 M $MgCl_2$

1 M $MgCl_2$	10 ml
water to	100 ml

- 0.1 M $CaCl_2$

$CaCl_2.2H_2O$ (Saarchem)	1.47 g
water to	100 ml

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A.2.15 Solution for Detecting agarase activity

- Gran's Iodine

Iodine	5 g
Potassium iodide	10 g
water to	500 ml

Dissolve the iodine and potassium iodide in 100 ml water. Adjust the volume to 500 ml with water. Do not autoclave and store in dark.

A.2.16 Solution for Restriction enzyme digestions

- Gel tracking dye

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

A.2.17 Solution for Ammonium acetate precipitation

- 7.5 M Ammonium acetate

Ammonium acetate (Saarchem)	262.8 g
water to	500 ml

Dissolve ammonium acetate in 400 ml water, pH to 7.5 using glacial acetic acid and adjust volume to 500 ml.

A.2.18 Solutions for Southern hybridization analysis

- 0.25 M HCl

HCl (Saarchem)	21.35 ml
water to	1 l

Do not autoclave.

- 0.4 M NaOH

NaOH	16 g
water to	1 l

- 0.4 M NaOH / 1 M NaCl

NaOH	16 g
NaCl	58.44 g
water to	1 l

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

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

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

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- STE (Sodium chloride-Tris EDTA)

0.1 M NaCl	2.92 g
TE buffer	500 ml

- Sephadex G-50

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

- Tracking dye

Dextran Blue 2000 (Pharmacia)	0.3 g
NaCl	0.029 g
Orange G (BDH)	0.1 g
water to	10 ml

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

Na_2 HPO_4. $7H_2O$ (Saarchem)	134 g
85% H_3PO_4 (Saarchem)	4 ml
water to	1 l

- 25% SDS (Sodium dodecyl sulphate)

SDS	250 g
water to	1 l

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

- Church pre-hybridization buffer (pre CHB)

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

- Church hybridization buffer (CHB)

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

- Wash buffer A (WBA)

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

- Wash buffer B (WBB)

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

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A.2.19 Solutions for Heinikoff shortening

- Exonuclease III buffer

1 M Tris-HCl (pH 7.6)	660 µl
1 M $MgCl_2$	6.6 µl
water to	10 ml

- 10x S1 nuclease buffer

KOA (Saarchem)	0.98 g
NaCl	11.7 g
Glycerol (Saarchem)	50 ml
$ZnSO_4$ (Saarchem)	0.3 g
water to	100 ml

Adjust the pH to 4.6 with glacial acetic acid.

- S1 nuclease mix (enough for 15 tubes)

10x S1 nuclease buffer	24.6 µl
sterile H_2O	155.4 µl
S1 nuclease (add prior to use)	36 U

- S1 nuclease stop

1 M Tris base	3 ml
0.5 M EDTA	1 ml
water to	10 ml

- Klenow mix

1M Tris-HCl (pH 7.6)	0.2 ml
1M $MgCl_2$	70 µl
water to	10 ml

A.2.20 Solutions for RNA extractions

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

- Sterile DEPC treated water

DEPC (Sigma)	1 ml
water to	1 l

- 0.5 M EDTA

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

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

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- 1M Tris-HCl

Tris	12.1 g
DEPC	0.1 ml
water to	100 ml

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

- TE buffer (Tris-EDTA)

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

- 1 M Sucrose

Sucrose	34.23 g
DEPC treated water to	100 ml

- Ethidium Bromide (EtBr) (10 mg / ml)

Etbr	0.1 g
DEPC treated water to	10 ml

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

- 70% EtOH

absolute EtOH	70 ml
DEPC treated water to	100 ml

Do not autoclave. Store at –20°C

- Protoplast buffer

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

Do not autoclave. Store at 4°C.

- Lysozyme (80 mg / ml)

Lysozyme (Roche)	0.8 g
DEPC treated water to	10 ml

Do not autoclave. Aliquot into eppendorf tubes. Store at –20°C

- Lysis buffer

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

Store at room temperature

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- Saturated NaCl

NaCl	40 g
DEPC	0.1 ml
water to	100 ml

Stir until solution reaches saturation

- Phenol / chloroform / isoamylalcohol (25:24:1)

Mix at ratio 25:24:1
Do not autoclave. Store at 4°C

- 3 M Na-acetate

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

Dissolve sodium acetate in 400 ml and adjust pH with glacial acetic acid to 5.2. Add DEPC and make up volume to 500 ml.

A.2.21 Solutions for Labeling oligonucleotide for primer extension

- 1 M Na-acetate

Na-acetate	68.02 g
DEPC	0.5 ml
water to	500 ml

Dissolve sodium acetate in 400 ml and adjust pH with glacial acetic acid to 5.2. Add DEPC and make up volume to 500 ml.

- 1 M DTT (Dithiothreitol)

DTT (Sigma)	0.3 g
Na-acetate (pH 5.2)	0.2 ml
DEPC treated water to	20 ml

Filter sterilize and store aliquots at –20°C

- 1 M Spermidine

Spermidine (Sigma)	2.9 g
DEPC treated water	20 ml

Filter sterilize and store aliquots at –20°C

- 1 M $MgCl_2$

$MgCl_2.6H_2O$	20.3 g
DEPC	0.1 ml
water to	100 ml

- 10x T_4 polynucleotide kinase buffer

1 M Tris, pH 7.6	1 ml
1 M $MgCl_2$	200 µl
1 M DTT	200 µl
1 M spermidine	200 µl
0.5 M EDTA	4 µl
DEPC treated water	396 µl

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- 4 M Ammonium acetate

Ammonium acetate	30.8 g
DEPC	0.1 ml
water to	100 ml

- TEN 600

NaCl	0.35g
DEPC	10 µl
TE buffer to	10 ml

A.2.22 Solutions for Northern hybridization analysis

- 5x MOPS

MOPS (Sigma)	20.6 g
50 mM Na-acetate	800 ml
0.5 M EDTA (pH 8)	10 ml
DEPC treated water to	1 l

Combine MOPS and Na-acetate, adjust pH to 7 with 2M NaOH and add EDTA. Make the volume up to 1 l with DEPC treated water and filter sterilize through 0.22 µm filter. Store in dark.

- 7.5 mM NaOH

NaOH	0.31 g
DEPC	1 ml
water to	1 l

- 20x SSC (Sodium chloride trisodium citrate)

NaCl	17.5 g
Tri-Na citrate	8.82 g
DEPC	0.1 ml
water to	100 ml

Dissolve NaCl and Tri-Na citrate in 80 ml water. Adjust pH to 7.4 with NaOH. Add DEPC and make up to 100 ml with water.

- 2x SSC / 0.1% SDS

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

Do not autoclave.

A.2.23 Solutions for Determining agarase activity

- 0.1 M Phosphate buffer at various pH's

(A) 0.2 M monobasic sodium phosphate solution

NaH_2PO_4 (Saarchem)	27.8 g
water to	1 l

(B) 0.2 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 200 ml.

x	y	pH
87.7	12.3	6.0
68.5	31.5	6.5
39.0	61.0	7.0
16.0	84.0	7.5
5.3	94.7	8.0

- Phosphate buffer (pH 7) at various ionic strengths (dilute 0.1 M with water)

- 20 mM Tris-Cl (pH 7)

1 M Tris-HCl (pH 7)	20 ml
water to	1 l

- 1 M NaCl

NaCl	58.4 g
20 mM Tris-Cl (pH 7) to	1 l

- 0.5 M / 0.25 M / 0.1 M NaCl (dilute 1 M NaCl accordingly)

- 1% Agarose solution

Agarose	1 g
4x Separating gel buffer	100 ml

A.2.24 Solutions for Determining protein concentration using Bradford assays

- Bovine serum albumin (BSA) (1 mg / ml)

BSA	0.01 g
Sterile water	10 ml

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

- 0.15 M NaCl

NaCl	0.88 g
water to	100 ml

- Coomassie brilliant blue solution

Coomassie brilliant blue G250 (Saarchem)	100 mg
absolute EtOH	50 ml
Phosphoric acid (Saarchem)	100 ml
water to	1 l

In a 1 l volumetric flask dissolve Coomassie 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

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A.2.25 Solutions for SDS-PAGE gels

- 4x Separating gel buffer

Tris base	18.17 g
10% SDS	4 ml
water to	100 ml

Adjust pH to 8.8 with HCl and add water to a final volume of 100 ml.

- 4x Stacking gel buffer

Tris base	6.06 g
10% SDS	4 ml
water to	100 ml

Adjust pH to 6.8 with HCl and add water to a final volume of 100 ml.

- 10x SDS-PAGE Running buffer

Tris base	30 g
Glycine	144 g
10% SDS	100 ml

- 2x SDS-PAGE sample buffer

Glycerol	2 ml
10% SDS	2 ml
Bromophenol blue	0.25 mg
4x Stacking gel buffer	2.5 ml
β-mercaptoethanol (Merck)	0.5 ml
water to	10 ml

- Coomassie blue dye staining solution

Coomassie ^{R} brilliant blue R250 (Sigma)	2.5 g
Isopropanol (Merck)	250 ml
Glacial acetic acid	100 ml
water to	1 l

Do not autoclave.

- Destaining solution

Glacial acetic acid	70 ml
water to	1 l

Do not autoclave.

A.2.26 Solutions for Silver staining of polyacrylamide gels

- Gel-soaking solution

100% ethanol	80 ml
glacial acetic acid	10 ml
ultrapure water to	200 ml

Do not autoclave.

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- Silver nitrate solution

Silver nitrate (BDH)	1.6 g
ultrapure water to	8 ml

Always make fresh and do not autoclave.

- 0.36% NaOH solution

NaOH	0.9 g
ultrapure water to	250 ml

- Ammoniacle solution

0.36% NaOH	42 ml
37% NH_3 solution (Sigma)	2.8 ml
Silver nitrate solution	8 ml
ultrapure water to	200 ml

42 ml 0,36% NaOH was added to a clean 250 ml glass measuring cylinder and 2.8 ml NH_3 solution (37%) added drop wise to this while stirring. Silver nitrate solution (8 ml) was added slowly to this solution and the volume made up to 200 ml with ultrapure water. The solution was always used within an hour of making it.

- Silver nitrate developing solution

Citric acid (Merck)	25 mg
37% Formaldehyde	95 µl
ultrapure water to	500 ml

Do not autoclave.

A.2.27 Solutions for TLC

- Developing solvent

n-butanol (Merck)	500 ml
glacial acetic acid	250 ml
water	250 ml

- Naphthoresorcinol reagent

Solution A

Naphthoresorcinol (Sigma)	0.04 g
EtOH to	20 ml

Solution B

EtOH	37.5 ml
sulphuric acid (Merck)	10 ml

Combine solution A and B in 1:2 ratio (20 ml : 40 ml), allow to cool and use immediately.

A.2.28 Solutions for Western blot analysis

- Blocking solution

Non-fat dried milk	1 g
10 x PBS	10 ml
sterile water to	100 ml

Do not autoclave

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- Developing substrate solution

Nitroblue tetrazolium (Sigma)	75 mg
5-Bromo-4-chloro-3-indolyl phosphate (Sigma)	50 mg
1 M Tris-HCl (pH 9.2)	1 ml
1 M $MgCl_2$	50 µl
1 M NaCl	1 ml
ultrapure water to	100 ml

Freshly prepared. Do not autoclave.

A.2.29 Solution for Electroblotting proteins onto nitrocellulose

- Blotting buffer

Tris	6 g
Glycine	200 ml
water to	2 l

Do not autoclave.

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APPENDIX B

STANDARD METHODS

CONTENTS

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B.1 *In vitro* assay to detect bacterial pathogens of *G. gracilis*

(Jaffray and Coyne, 1996)

B.1.1 Generation of axenic *G. gracilis*

All *G. gracilis* samples used in the assays were collected from Saldanha Bay. Eleven, 5 cm length quantities of healthy *G. gracilis* (dark red and free of epiphytic algae) were immersed in 500 ml autoclaved water for 4 h. The macroalgae were then immersed in 100 ml 1% Povidone-iodine solution (Appendix A.2.3) for 60 s and finally incubated for 24 h at 12°C in 100 ml SSW containing a 'cocktail' of antibiotics (Appendix A.2.3). The treated macroalgae were rinsed thoroughly in SSW and one 5 cm thallus per treatment was tested for axenicity by rubbing the thallus across the surface of BA plate (Appendix A.1.2). These plates were incubated for up to 14 days at 22°C, during which time they were monitored for growth of bacterial colonies.

B.1.2 Pathogenicity Assay

The candidate bacterial isolates were cultured in 10 ml BM (Appendix A.1.1) at 22°C for 16 h on an orbital shaker (Gallenkamp) at 100 rpm. One hundred microlitres of each culture was subsequently transferred to 100 ml BM in an Erlenmeyer flask and incubated under the same conditions until the culture reached stationary phase (10 h). The axenic thalli were injected at one end with 100 µl of bacterial suspension using a sterile needle, to a depth of approximately 5 mm. Five thalli were injected per experiment. Experimental controls were 5 thalli injected with SSW. The injected thalli were placed in an Erlenmeyer flask containing 50 ml BM without agar and incubated at 22°C and 30°C. The thalli were scored for the appearance of symptoms up to 5 days. At the same time, in order to satisfy Koch's postulates, one of the thalli was removed and a piece of the thalli was incubated on the surface of a BA plate. The plate cultures were scored for the growth of bacterial colonies identical in appearance to the isolate originally injected into the thallus.

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B.2 Large scale preparation of bacterial genomic DNA

(Ausubel *et al.*, 1989 unit 2.4)

Grow 100 ml culture of bacterial strain O/N. Pellet the cells for 10 min at 4 000 rpm and discard the supernatant. Resuspend the cells in 9.5 ml TE buffer (Appendix A.2.2). Add 0.5 ml 10% SDS and 50 µl of 20 mg / ml proteinase K (Appendix A.2.8), mix and incubate 1 h at 37°C. Add 1.8 ml of 5 M NaCl (Appendix A.2.8) and mix thoroughly. Add 1.5 ml CTAB / NaCl (Appendix A.2.8) solution and mix thoroughly. Incubate for 20 min at 65°C. Extract with an equal volume of chloroform / isoamyl alcohol (Appendix A.2.8). Centrifuge at 6 000 rpm for 10 min, room temperature. Transfer aqueous phase to a clean tube. Precipitate DNA by adding 0.6 volumes isopropanol (Merck). Centrifuge at 10 000 rpm for 10 min. Wash pellet with 1 ml 70% EtOH (Appendix A.2.2). Resuspend DNA in 1 ml TE buffer with 10 µl RNAse (Appendix A.2.8). Measure the DNA concentration on a spectrophotometer (Appendix B.12.1)

B.3 PCR of 16S rDNA fragment from *P. gracilis* B9

B.3.1 Primers used for strain B9 16S rDNA amplification

F1 5′CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCI TGG CTC AG 3′

F3 5′CGC CAG GGT TTT CCC AGT CAC GAC GCC AGC AGC CGC GGT AAT AC 3′

F5 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′

R3 5′CAG GAA ACA GCT ATG ACC ACG AGC TGA CGA CAI CCA TG 3′

R5 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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B.3.2 PCR Protocol

Prepare a master mix as follows:

Reagents (Roche)	Volume (µl)
B9 genomic DNA (50 ng / µl)	30
Mg (25 mmol)	24
PCR buffer (10x)	60
Taq polymerase (5 U / µl)	6
dNTP's (25 mM)	6
sterile water	414
TOTAL	540

Aliquots (90 µl) were sub divided into 5 PCR eppendorf tubes. The following primers of 5 µl each 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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B.4 Agarose gel electrophoresis

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

Melt agarose in 1x TAE (Appendix A.2.9) by heating in microwave and swirling to ensure even mixing. Agarose concentrations can vary from 1% for separating plasmid DNA fragments to 0.8% for separating larger chromosomally restriction enzyme digested DNA fragments. Add Ethidium bromide solution (Appendix A.2.2) 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 1 to 10 V / cm until the dye in the loading buffer reach the end of the gel.

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B.5 Automated Sequencing

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

B.5.1 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′

Forward universal pBluescript KS/SK primer: 5'AAT ACG ACT CAC TAT AGG GCG AAT 3'

Reverse universal pBluescript KS/SK primer: 5'GAG CGG ATA ACA ATT TCA CAC AGG 3'

B.5.2 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 200 µl 0.5 µl PCR tubes. In separate microfuge tubes, 1 µg 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 lid (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

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).

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B.5.3 Preparing the sequencing gel

Urea (Pharmacia)	19 g
1.5x TBE buffer (Appendix A.2.10)	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 A.2.2)	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 polymerize for 2 h.

B.5.4 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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B.6 Ferricyanide assay for reducing-sugars

(Park and Johnson, 1949)

In duplicate, 100 µl culture supernatant was added to 100 µl freshly-prepared 1% agarose substrate (Appendix A.2.12) and 200 µl 20 mM PIPES (piperazine-*N*-*N*'-bis(2-ethanesulfonic acid)) solution (Appendix A.2.12) and incubated at 37°C for 1 h. The reaction was terminated by the addition of 200 µl of Stop reagent (Appendix A.2.12), after which 300 µl Colour reagent (Appendix A.2.12) was added. The tubes were incubated in a boiling water bath for 2.5 min. The tubes were subsequently cooled at 22°C and the absorbance determined at 420 nm. Agarolytic enzyme activity (agarolytic units: U) was expressed as µg galactose produced per ml per h, where the concentration of galactose was determined from a standard curve of absorbance at 420 nm versus galactose concentration (µg galactose/ml).

B.7 Large scale preparation of plasmid DNA

Two methods were routinely used for large scale preparation of plasmid DNA.

B.7.1 CsCl-EtBr gradient centrifugation

(Taken from Ish-Horowicz and Burke, 1981)

Inoculate 5 ml Luria broth (LB) containing appropriate antibiotic selection (Appendix A.2.1) with a single colony of *E. coli* containing the desired plasmid. Grow at 37°C with vigorous shaking O/N. Inoculate 200 ml LB containing selection agent with 1 ml of O/N culture. Grow at for 8-12 h at 37°C or until the culture is saturated. Collect cells by centrifuging 10 min at 6 000 rpm, 4°C. Resuspend the cells in 4 ml solution 1 (Appendix A.2.13) and leave at room temperature for 10 min. Add 8 ml solution 2 (Appendix A.2.13) and mix well by shaking. Leave on ice for 10 min. Add 6 ml solution 3 (Appendix A.2.13), shake well and leave on ice for 10 min. Centrifuge at 10 000 rpm for 10 min and transfer the supernatant to a clean SS34 centrifuge tube. Add 1 volume of isopropanol and incubate at room temperature for 10 min. Pellet the DNA by centrifugation at 15 000 rpm for 15 min. Wash the pellet with 10 ml 70% EtOH. Resuspend DNA in 4.2 ml TE buffer.

Add 4.62 g CsCl and mix to dissolve. Centrifuge for 10 min at 10 000 rpm. Transfer the supernatant to a clean SS34 tube. Add 200 µl of 10 mg / ml EtBr and adjust the refractive index to 1.396. Fill two Vti65 tubes and centrifuge at 55 000 rpm O/N at 20°C. Visualize DNA bands under UV (310 nm) and remove the plasmid band using a 2 ml syringe to a clean eppendorf tube. Add an equal volume of salt saturated isopropanol (Appendix A.2.13). Discard the top phase. Add two volumes of sterile water and one volume isopropanol. Incubate on ice for 10 min. Precipitate DNA by centrifuging at 14 000 rpm for 15 min. Wash pellet with 500 µl 70% EtOH. Resuspend pellet in 100 µl TE buffer and store at 4°C. Measure the DNA concentration on a spectrophotometer (Appendix B.12.1).

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B.7.2 Nucleobond AX 100

(Nucleobond AX handbook "Properties and applications" (Macherey-Nagel, Düren)

All buffers supplied in kit. Inoculate 100 ml LB containing selection agent with 1 ml of O/N culture. Resuspend the bacterial cell pellet in buffer S1 (4 ml). Add buffer S2 (4 ml). Mix gently and incubate it at room temperature for 5 min. Add buffer S3 (4 ml). Mix gently and incubate on ice for 5 min. Centrifuge at high speed (15 000 rpm for 40 min at 4°C). Remove the supernatant carefully from the white precipitate. Equilibrate the cartridge with buffer N2 (2 ml). Load the

supernatant on a Nuleobond AX 100 cartridge. Wash the cartridge with buffer N3 (2 x 4 ml). Elute the plasmid DNA with buffer N5 (2 ml). Precipitate the plasmid DNA with 0.7 volumes of isopropanol and centrifuge at high speed (13 000 rpm for 15 min at at 4°C). Wash the pellet with 70% EtOH, dry briefly and redissolve in TE buffer. Measure the DNA concentration on a spectrophotometer (Appendix B.12.1).

B.8 Transformation of *E. coli*

B.8.1 Preparation of competent *E. coli* cells

(Taken from Dagert and Ehrlich, 1979)

Inoculate a single colony of freshly streaked *E. coli* into a 5 ml LB and shake at 37°C for 2.5 h. Inoculate this starter culture into 100 ml pre-warmed LB and grow at 37°C until the OD_{600} reaches 0.35 (approximately 3.5-4.0 x 10^7 cells / ml). Transfer the culture to a GSA tube and centrifuge for 5 min at 5 000 rpm, 4°C. Decant the supernatant and resuspend the cells in 100 ml ice cold 0.1 M $MgCl_2$ (Appendix A.2.14). Leave on ice for 1 min. Collect the cells as before and resuspend in 50 ml 0.1 M $CaCl_2$ (Appendix A.2.14). Incubate on ice for 2 h. Collect the cells as before and resuspend them in 10 ml 0.1 M $CaCl_2$. Aliquot 100 µl into 1.5 ml eppendorf tubes and store at –70°C.

B.8.2 Transformation of competent cells

(Taken from Dagert and Ehrlich, 1979)

Add 1 to 50 ng of plasmid DNA to 100 µl of competent cells. Leave on ice for 10 min. Heat-shock cells at 42°C for 2 min or 37°C for 5 min. Add 0.9 ml LB and allow expression at 37°C for 30 to 60 min. Plate 100 µl of cells on LA containing antibiotic selection. Incubate on LA plates at 37°C O/N.

B.9 Restriction endonuclease digestions

(Ausubel *et al.*, 1989 unit 3.1)

The restriction enzyme buffers are supplied with their respective enzymes were obtained from Roche and Amersham. Pipette 0.1 to 4 µg of either plasmid or chromosomal DNA into a clean

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eppendorf tube. Add 2 µl restriction enzyme buffer. Adjust the volume to 18 µl with sterile water. Add restriction enzyme nuclease (1 to 5 U / µg DNA) to a final volume of 20 µl. Incubate the reaction mixture for 1 to 2 h at 37°C. Stop the reaction by adding 5 µl gel tracking dye (Appendix A.2.16).

In order to perform multiple restriction enzyme digestions, first cleave with one of the restriction enzymes, precipitate the products using ammonium acetate precipitation (Appendix B.10) and finally cleave with the second restriction enzyme. In order to perform partial restriction enzyme digestions, use 20 µg of DNA and only 10 U of restriction enzyme. Incubate for 1 h at 37°C before stopping the reaction.

B.10 Ammonium acetate precipitation of DNA

(Coyne *et al.*, 1996)

Precipitation of DNA is carried out by adding half the volume of 7.5 M ammonium acetate, pH 7.5 (Appendix A.2.17) to the DNA suspension. Incubate at room temperature for 15 min. Centrifuge at 14 000 rpm for 15 min. Transfer the supernatant to a clean eppendorf tube. Add 2.5x volumes 100% EtOH and incubate at –20°C for 30 min. Centrifuge at 14 000 rpm for 30 min at room temperature. Wash the DNA pellet with 70% EtOH. Resuspend DNA in 10 µl of TE. Determine the DNA concentration via the Ethidium bromide fluorescent quantitation method (Appendix B.12.2).

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B.11 Geneclean: Purifying DNA from TAE agarose gels

(Guide to Protocols Procedures, BIO 101)

All reagents supplied in kit. Excise band from agarose gel. Add 3 volumes of NaI and incubate at 55°C to melt gel. Add GLASSMILK suspension and leave to stand for 2 min at 22°C. Pellet GLASSMILK / DNA complex (5 s). Wash pellet with NEW wash (3 times, 5 s spins). Elute DNA with eluting buffer. Measure the DNA concentration on an agarose gel (Appendix B.12.2).

B.12 Quantitation of DNA and RNA samples

(Coyne *et al.*, 1996)

B.12.1 Spectrophotometric quantitation of DNA and RNA

Perform a DNA or RNA scan of the DNA/RNA solution between 310-220 nm to determine the UV light absorbance of the sample. The absorbance peak 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 and 40 µg / ml for single stranded DNA or RNA.

B.12.2 Ethidium bromide fluorescent quantitation of DNA

Prepare three λ DNA standards with known concentrations: 5 ng /10 µl, 10 ng / 10 µl and 20 ng / 10 µl. Load 10 µl from each standard with 2.5 µl gel tracking dye into the wells of a 1% TAE agarose gel. Prepare several dilutions of DNA sample of unknown concentration in 10 µl. Add 2.5 µl gel tracking dye and load next to the standards on the agarose gel. Electrophorese the samples at 100 V for 5 min. Visualize 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 you load 10 µl of a 1/10 dilution of the DNA sample, which corresponds to an intensity equivalent to that of the 10 ng standard, the DNA sample will have a concentration of 10 ng / µl.

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B.13 Southern hybridization procedure

(Reed and Mann, 1985)

B.13.1 Southern transfer of DNA from agararose gel onto nitrocellulose membrane

Soak the agarose gel in 2x volumes 0.25 M HCl (Appendix A.2.18) for 5 min at room temperature. Rinse the gel in 2x volumes of water. Saturate 10 sheets (25 x 20 cm) Whatman 3MM paper with 0.4 M NaOH (Appendix A.2.18). Place sheets on top of an inverted gel-casting tray, which has been placed in a tray covered with Saran wrap. Add 0.4 M NaOH / 1 M NaCl (Appendix A.2.18) to the tray so that the ends of the Whatman paper are submerged. Invert the gel and place on top of the saturated Whatman paper. Ensure that no air bubbles remain trapped. Cut Hybond N+ nylon membrane (15 x 20 cm) (Amersham). Wet membrane in water and place on gel, ensuring that all air bubbles are removed. Cover the edges with Saran wrap. Place 3x

sheets (20 x 15 cm) Whatman 3MM paper over the membrane, followed by a 10 cm stack of dry absorbant paper towel. Place a glass plate on top of the towels, followed by a 0.2 to 0.4 kg weight. Blot O/N. Mark wells of the gel on the membrane with a pencil and rinse the membrane 2x SSC (Appendix A.2.18) for 5 min at room temperature. Air-dry the membrane on dry Whatman paper and store between 2 sheets of Whatman 3MM sheets at 4°C.

B.13.2 Labelling DNA by random prime labelling

(Protocol used with Roche labelling kit)

Reagents supplied in the kit. Denature 25 ng of DNA fragments by heating for 10 min at 95°C and subsequent cooling on ice. Add 3 µl of dATP, dGTP, dTTP mixture and 2 µl of reaction mixture. Add 5 µl 3000 Ci/mmol [α-^{32}P] dCTP and make up the reaction volume to 19 µl with sterile water. Add 1 µl of Klenow enzyme and incubate the reaction mixture at 37°C for 30 min. Stop the reaction by heating to 65°C for 10 min. Separate the labelled DNA from the unincorporated nucleotides using a spin column (Appendix B.13.3).

B.13.3 Separation of radioisotope labelled DNA from unincorporated nucleotides using the spin column procedure

(Ausubel *et al.*, 1989 unit 3.4)

Plug the bottom of a 1 ml disposable syringe with a small amount of sterile glass wool. Prepare a Sephadex G-50 (Appendix A.2.18) column with bed volume of 0.9 ml in the syringe. Wash the column with 0.1 ml STE (Appendix A.2.18). To the labelled DNA sample add 10 µl of tracking dye (Appendix A.2.18) and 40 µl STE buffer. Place an eppendorf at the bottom of a bench top centrifuge and place the syringe containing the Sephadex column inside the eppendorf, so that the syringe will empty inside the tube. Load the DNA onto the column and centrifuge for 4 min at 14 000 rpm. The Blue dextran in the tracking dye will move with the labelled probe and will empty into the eppendorf tube, whereas the Orange G will remain with the unincorporated nucleotides on the column. Determine the specific activity of the labelled DNA by counting 1 µl of probe in 2 ml scintillation fluid. Specific activity is expressed as counts per minute (cpm) / µg DNA.

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B.13.4 Prehybridization, hybridization and washing of Southern blots

(Church and Gilbert, 1984)

Seal the Hybond N+ membrane containing transferred DNA in a plastic bag along with 0.2 ml of Church pre-hybridization buffer (pre CHB) (Appendix A.2.18) per cm^2 of membrane. Incubate the sealed bag at 65°C for 1 h with agitation. Denature the labelled probe by heating at 100°C for 10 min and place on ice. Remove pre CHB from the plastic bag and add fresh Church hybridization buffer (CHB) (Appendix A.2.18) (50 µl / cm^2 membrane) to the bag along with 1 x 10^6 cpm / ml of labelled probe. Remove any bubbles and heat-seal the bag. Hybridize O/N at 65°C with agitation.

Wash membranes with Wash buffer A (WBA) and Wash buffer B (WBB) (Appendix A.2.18) at 65°C for 10 min and monitor the radioactivity between each wash on the membrane using a Geiger counter. Once the radiation reach 200-500 cpm the membrane is sealed in a new plastic bag and placed in an X-ray cassette containing enhancer screens. Expose the membrane to X-ray film and develop in an automatic X-ray film processor.

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B.14 Ligations

(Coyne *et al.*, 1996)

B.14.1 Intramolecular ligations

To re-circularize plasmid DNA, use approximately 1 pmol of DNA. Add 2 µl 10x ligation buffer (Roche). Adjust volume to 18 µl with sterile water. Add 2 U of T_4 ligase (Roche) to a final volume of 20 µl and incubate reaction mix at 15°C, O/N.

B.14.2 Intermolecular ligations

In order to polymerize two distinct DNA fragments the total DNA concentration (vector plus insert) should not exceed 10 pmol. Use ratios of vector to insert in the order of 1:1 to 1:4 pmol. To an eppendorf add the vector and insert DNA. Add 2 µl of 10x ligation buffer (Roche). Add 2 U of T_4 ligase (Roche) to a final volume of 20 µl. When ligating DNA fragments with cohesive ends incubate reaction mixes at 15°C. When joining blunt-ended DNA, use 10x more enzyme and incubate the reaction mixes at room temperature.

B.14.3 Repairing 3' or 5' overhanging ends to generate blunt ends

(Ausubel *et al.*, 1989 unit 3.5)

In a 20 µl reaction, digest 0.1 to 4 µg of DNA with restriction endonuclease (Appendix B.9). Add 1 µl of 0.5 mM dNTPs. Add 1 µl of Klenow and incubate at 30°C. Stop the reaction by heating to 75°C for 10 min of by adding 1 µl of 0.5 M EDTA (Appendix A.2.2).

B.15 Heinekoff shortenings

The plasmid DNA was precipitated with ammonium acetate (Appendix B.10) and resuspended in exonuclease III buffer (Appendix A.2.19). To this, 150 U of exonuclease III was then added after which 4.5 µl aliquots of DNA were removed at 20 second intervals, transferred to tubes containing SI nuclease mix (Appendix A.2.19) and incubated for 30 min at 22°C. The reactions were stopped through the addition of 1.75 µl of S1 nuclease stop (Appendix A.2.19) and placed at –70°C for 10 min. In order to confirm the extent of the shortening reactions, 2 µl from every 2nd time point was removed and run on a 1% agarose gel as described in Appendix B.4. In order to allow blunt-end ligations to proceed, overhangs generated by restriction enzymes were filled in with dNTPs using Klenow. This was achieved by the addition of 1.7 µl of klenow mix (Appendix A.2.19) to each tube as well as 1 µl of Klenow (1 U / µl). The reactions were left to proceed for 3 min at room temperature before 1 µl of dNTP's (0.5 mM) was added and the tubes incubated for 5 min at room temperature.

In order to re-circularize the shortened DNA fragments, ligations were performed at 15°C O/N as described in Appendix B.14. Half of each ligation mix was transformed into competent *E. coli* HB101 (Appendix B.8) and the resulting transformants were selected on LA containing ampicillin. Plasmid DNA isolated from transformants was cleaved with the restriction enzyme *Pvu*II in order to identify pBluescript KS containing the desired shortened inserts.

B.16 Manual Sequencing

(Coyne *et al.*, 1996)

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B.16.1 PCR primers for sequencing

Forward universal pBluescript KS/SK primer: 5'AAT ACG ACT CAC TAT AGG GCG AAT 3'
Reverse universal pBluescript KS/SK primer: 5'GAG CGG ATA ACA ATT TCA CAC AGG 3'
Oligonucleotide primer (Primer extension): 5' TGC TTC TTT AAC TAG TGC GAG TG 3'

B.16.2 Sequenase sequencing protocol

(Sequenase sequencing kit (Amersham-Pharmacia)-Sanger *et al.*, 1977)

Ten micrograms of plasmid DNA was alkaline denatured in 2 M NaOH (Appendix A.2.10) for 30 min at 37°C. The denatured templates were precipitated with 0.3 M sodium acetate (final concentration) (Appendix A.2.10), and absolute ethanol at -70°C, washed in 70% ethanol to remove excess salt and air-dried. To the dried DNA pellets, annealing buffer, sterile water and either a forward or reverse primer was added in a total volume of 10 µl. The forward and the reverse universal pBluescript KS primers were used for all sequencing reactions. Annealing of the primer to single stranded DNA templates was performed at 37°C for 30 min and subsequently, the tubes were slowly cooled to room temperature.

The following components (supplied in the kit) were added to each of the primed DNA templates to allow for the labeling reaction: DMSO, lab mix, enzyme dilution buffer, T_7 DNA polymerase and labelled [α-^{35}S]-dATP. Labeling was performed at room temperature for 10 min. Reactions were then terminated as follows: Four termination tubes (A, C, G, T) were prepared on ice. These contained extension mixes as well as the relevant ddNTP for termination. Aliquots of 4.5 µl were transferred from the labeling reaction tubes to each of the four termination tubes. The tubes were incubated for 5 min at 37°C before stop buffer was added to each of the tubes.

B.16.3 Preparing the sequencing gel

Mark the outer surface of two sequencing glass plates using tape, and wash the inner surfaces of both plates with detergent. Add 1 ml silane (Appendix A.2.10) to the front plate and spread evenly over the entire inner surface. Allow to dry for 5 min and wash gently with EtOH. Arrange spacers on bottom plate and position the silanized top plate over the bottom plate. Clamp the two

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plates together and place on gel pouring apparatus. Prepare the following gel mix for 6% PAGE / 7 M Urea sequencing gel:

- 40% Acrylogel (BDH) 7.4 ml
- Urea (Merck) 25.7 g
- 10x TBE (Appendix A.2.10) 5 ml
- water to 50 ml

Filter the gel mix through a 0.8 µm Millipore filter. Add 200 µl 10% ammonium persulphate and 45 µl TEMED. Pour mix between glass plates. Insert flat edge of spacer into the top of the gel at the top of the plate and leave the gel to polymerize.

B.16.4 Running of sequencing gels

Warm 2 liters of TBE in microwave on high for 6 min. Remove all clamps from glass plates as well as the bottom spacer. Fill the bottom space of the gel with TBE. Clamp the plates onto a sequencing gel tank and fill the bottom and top tanks with prewarmed TBE buffer. Place shark tooth comb (points facing down) between the tanks until the tips of the teeth pierce the gel. Load 4 µl of sequencing samples and run for 30 min to 6 h at 42 mA. On completion of the run, remove the buffer from the bottom tank. Remove plates from gel tank and use a spatula to separate the two plates to expose the gel. Place precut Whatman 3MM filter paper on gel. Check that gel is adhering to filter paper before removing paper and gel from the sequencing plate. Dry the gel at 75°C for 1 h on dryer. Expose to X-ray film to view sequences.

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B.17 PCR of the promoter region of *aag*A

B.17.1 Primers used for promoter region amplifications

A9: 5′CTG CAG GAT CCT AGC ATA GCT C 3′

A10: 5′CTG CAG GAT CCA GAG AAA AAA CAT CT 3′

A11: 5′CTG CAG GAT CCA TGT CGG TGT GC 3′

A11.5: 5′CTG CAG GAT CCC ACA AAA GAG A 3′

A11.7: 5′CTG CAG GAT CCA CTA AAG TCA CTC GCA C 3′

R: 5′TTG CAC GCG TCG ACC AGG ATA GTA G 3′

Arrows indicating the position of the *Pst*I and *Sal*I restriction sites for the five forward and one reverse primer, respectively.

B.17.2 PCR Protocol

Prepare a master mix as follows:

Reagents (Roche)	Volume (µl)
pDA1 DNA (1 µg /µl)	1
Mg (25 mmol)	12
PCR buffer (10x)	30
Taq polymerase (5 U / µl)	3
dNTP's (25 mM)	3
sterile water	215
TOTAL	264

Aliquots (44 µl) were sub divided into 5 PCR eppendorf tubes. The following primers of 3 µl each were added to the 5 PCR tubes:

Tube number	Primer (10 µM)
1	A9 and R
2	A10 and R
3	A11 and R
4	A11.5 and R
5	A11.7 and R

PCR cycle profile was setup as follows:

Temperature (°C)	Time (s)	Cycles
96	240	1
96	30	
50	45	
72	60	30
72	300	1

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The PCR was performed in a thermal cycler with a heated lid (Hybaid). The PCR reactions were purified with High Pure PCR Product Purification Kit Protocol (Roche).

B.18 Labelling of oligonucleotide primer for primer extension

(Ausubel *et al.*, 1989 unit 4.8)

To an eppendorf tube, add the following reagents:

- 1 µl of DEPC treated water
- µl of T_4 10x polynucleotide kinase buffer (Appendix A.2.21)
- 1 µl of 0.1 M DTT (Appendix A.2.21)
- 1 µl of 1 mM spermidine (Appendix A.2.21)
- 100 ng / µl oligonucleotide primer
- 100 µCi of [γ-^{32}P] ATP
- 20-30 U / µl T_4 polynucleotide kinase
- Final volume 15 µl.

In order to avoid possible precipitation of the oligonucleotide primer, the spermidine and oligonucleotide should not be pre-mixed. Incubate the reaction mix at 37°C for 1 h. Stop the reaction by adding 2 µl 0.5 M EDTA and TE buffer to a final volume of 25 µl. Incubate for 5 min at 65°C to inactivate the kinase. Precipitated the labelled oligonucleotide by adding 25 µl 4 M ammonium acetate (Appendix A.2.21) and 250 µl ice cold 100% ethanol. Leave O/N at -20°C. Centrifuge at 14 000 rpm for 15 min and resuspended the pellet in 25 µl of DEPC treated water (Appendix A.2.21). Repeat the precipitation and finally, resuspend the pellet in 100 µl of TEN 600 buffer (Appendix A.2.21). Determine the specific activity of the labelled oligonucleotide by counting 1 µl in 2 ml scintillation fluid in a Beckman scintillation counter.

B.19 Northern hybridization procedure

All the solutions, apparatus and glassware were treated as recommended by Sambrook *et al.* 1989 for RNA applications (Appendix A.2.20).

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B.19.1 Preparation of RNA for electrophoresis

(Sambrook *et al.* 1989, unit 7.43)

To an eppendorf, add the following:

- Total RNA (10 µg) x µl
- 5x MOPS buffer (Appendix A.2.22) 2 µl
- formaldehyde (Merck) 3.5 µl
- formamide (Merck) 10 µl

Heat the samples for 15 min at 65°C. After denaturation, add 2 µl tracking dye and 0.5 µl Ethidium bromide solution (10 mg / ml).

B.19.2 Electrophoresis of RNA through gels containing formaldehyde

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

Melt 0.8 g agarose in 35 ml DEPC treated water by heating in microwave and swirling to ensure even mixing. Agarose concentration was reduced to 1.5% by the addition of formaldehyde (10 ml) and 5x MOPS buffer (11 ml) to have a final volume of 50 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 MOPS bufferto cover the gel. Load RNA samples into the wells of the gel. Attach leads so that DNA migrates into the gel toward the anode. Run the gel at 3-4 V / cm until the bromophenol blue in the loading buffer reach the end of the gel.

B.19.3 Northern transfer of RNA from an agarose gel onto nitrocellulose membrane

(Reed and Mann, 1985; and Sambrook *et al.* 1989, unit 7.46)

Soak the gel in 2x volumes DEPC treated water at room temperature. Rinse the gel several times in 2x volumes of DEPC treated water. Soak the gel in 2x volumes 7.5 mM NaOH for 20 min (Appendix A.2.22). Soak the gel in 2x volumes 20x SSC (Appendix A.2.22) for 45 min. Saturate 10 sheets (10 x 15 cm) Whatman 3MM paper with 1x SSC. mPlace sheets on top of an inverted

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gel-casting tray, which has been placed in a tray covered with Saran wrap. Add 1x SSC to the tray so that the ends of the Whatman paper are submerged. Invert the gel and place on top of the saturated Whatman paper. Ensure that no air bubbles remain trapped. Cut Hybond N+ nylon membrane (9 x 12 cm) (Amersham). Wet membrane in water and place on gel, ensuring that all air bubbles are removed. Cover the edges with Saran wrap. Place 3x sheets (9 x 12 cm) Whatman 3MM paper over the membrane, followed by a 10 cm stack of dry absorbant paper towel. Place a glass plate on top of the towels, followed by a 0.2 to 0.4 kg weight. Blot O/N.

Mark wells of the gel on the membrane with a pencil and rinse the membrane 2x SSC / 0.1% SDS (Appendix A.2.22) for 5 min at room temperature. Air-dry the membrane on dry Whatman paper and irradiate the membrane @254 nm for 5 min to fix the RNA onto the membrane. Store the membrane between 2 sheets of Whatman 3MM sheets. The DNA probes for hybridization were prepared as described in Appendix B.13.3

B.19.4 Prehybridization, hybridization and washing of Northern blots

As described in Appendix B.13.4, except the hybridization and washing temperature of 42°C was used.

B.20 Bradford protein assay for protein quantitation

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

Aliquot (in duplicate) the following amounts of BSA (Appendix A.2.24) and 0.15 M NaCl (Appendix A.2.24) 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 A.2.24) to the standard and sample tubes.

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Vortex for 5 seconds. Allow the tubes to stand at room temperature for 5 min. Determine the OD_{595} of all the samples and plot a standard curve of OD_{595} versus protein concentration, using the standards. Use the curve to determine the protein concentration of the sample.

B.21 Western hybridization procedure

B.21.1 Preparing and running denaturing SDS-PAGE gels

(Ausubel *et al.*, 1989 unit 10.2)

Combine the following reagents for a 12% separating gel mix in a glass beaker. The reagents are as follows:

- 40% acrylogel 4 ml
- 4x separating gel buffer (Appendix A.2.25) 2.5 ml
- sterile water to 9.95 ml
- 10% ammonium persulfate (Appendix A.2.2) 50 µl
- TEMED 15 µl

Pour the separating gel mix into the assembled gel plates, leaving sufficient space at the top for the stacking gel. Gently overlay the gel mix with 0.1% SDS. After polymerization, remove the overlay and rinse the surface of the separating gel to remove unpolymerized acrylamide.
Prepare the 5% stacking gel mix as follows:

- 40% acrylogel 625 µl
- 4x stacking gel buffer (Appendix A.2.25) 1.25 ml
- sterile water to 4.97 ml
- 10% ammonium persulfate 25 µl
- TEMED 15 µl

Pour the stacking gel mix and insert the comb immediately. After the stacking gel has polymerized, remove the comb and rinse the wells to remove any unpolymerized acrylamide. Place the assembled gel into the electrophoresis apparatus and fill the tank with SDS-PAGE running buffer (Appendix A.2.25). Prepare protein samples by adding 5 µl of SDS-PAGE sample buffer (Appendix A.2.25) to 10 µl of protein sample. Denature protein samples by boiling for 3 min at 96°C. Load samples into the bottom of the wells. Run the gel at constant current of 15 mA

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in the stacking gel and 30 mA in the separating gel. After electrophoresis, visualize the protein bands in the gel by staining with Coomassie blue dye (Appendix A.2.25) for 15 min at 37°C. Destain the gel in destaining solution (Appendix A.2.25). Dry the gel for 45 min at 70°C using a gel dryer. Alternatively, silver stain the gel (Appendix B.22) and photograph.

B.21.2 Electroblotting of proteins onto a nitrocellulose membrane

(Towbin *et al.*, 1979)

Remove the SDS-PAGE gel from glass plate and soak the gel in blotting buffer (Appendix A.2.29) for 1 h. Pre-wet the nitrocellulase membrane in blotting buffer and place gel on membrane. Ensure that no air bubbles remain trapped. Cut four sheets of Whatman 3MM filter paper (10 x 5 cm) and soak in blotting buffer. Sandwhich the membrane and gel between the filter paper. Clamp the entire sandwhich between two perforated sheets of perspex. Load assembly into the transblot chamber filled with blotting buffer. Ensure that the nitrocellulose is towards the anode (+). Transfer at 15 V for 4 h.

B.22 Silver staining protocol

(Sammons *et al.*, 1981)

The following steps were all performed with gentle agitation of the gel on a rotary shaker. The SDS-PAGE gel was soaked O/N in gel-soaking solution (Appendix A.2.26). It was subsequently fixed in 8.33% gluteraldehyde (Merck) for 30 min, after which it was rinsed for 3 h in ultrapure (Milli-Q) water, with fresh water added to the gel every 20 min. The silver stain (Appendix A.2.26) was prepared during the last hour of rinsing and the gel stained for 30 min. It was subsequently rinsed in ultrapure water for 1 h at the most, replacing the rinse-water with fresh water every 20 min. To check whether the gel had been sufficiently washed, 5 ml of a saturated NaCl solution (Appendix A.2.26) was added to 5 ml of the rinse water and observed for the formation of a white precipitate. When no more white precipitate was visible (usually after 2 to 3 washes), sufficient freshly made developer (Appendix A.2.26) was added to the gel to cover it. When protein bands became visible (5-10 min), the developer was poured off, and ultrapure water added to stop the reaction.

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LITERATURE CITED

Abbott, I. A. (1996) Ethnobotany of seaweeds: clues to uses of seaweeds. *Hydrobiologia* **326/327**: 15-20.

Aiyar, S. E., Gourse, R. L. and Ross, W. (1998) Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase α subunit. *Proc. Natl. Acad. Sci., USA* **95**: 14652-14657.

Akagawa-Matsushita, M., Itoh, T., Katayama, Y., Kuraishi, H. and Yamasato, K. (1992a) Isoprenoid quinone composition of some marine *Alteromonas*, *Marinomonas*, *Deleya*, *Pseudomonas* and *Shewanella* species. *J. Gen. Microbiol.* **138**: 2275-2281.

Akagawa-Matsushita, M., Matsuo, M., Koga. Y. and Yamasato, K. (1992b) *Alteromonas altantica* sp. nov. and *Alteromonas carrageenovora* sp. nov., bacteria that decompose algal polysaccharides. *Int. J. Syst. Bacteriol.* **42**: 621-627.

Akagawa-Matsushita, M., Koga, Y. and Yamasato, K. (1993) DNA relatedness among nonpigmented species of *Alteromonas* and synonymy of *Alteromonas haloplanktis* (Zobell and Upman 1944) Reichelt and Baumann 1973 and *Alteromonas tetraodonis* Simidu et al. 1990. *Int. J. Syst. Bacteriol.***43**: 500-503.

Altschul, S., Gish, W., Miller, W., Meyers, E. and Lipman, D. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Anderson, R. J., Levitt, G. J. and Share, A. (1996a) Experimental investigations for the mariculture of *Gracilaria* in Saldanha Bay, South Africa. *J. Appl. Phycol.* **8**: 421-430.

Anderson, R. J., Levitt, G. J., Dawes, C. P. and Simons, R. H. (1992) Experimental growth of *Gracilaria* in Saldanha Bay, South Africa. In *Proceedings of the first international workshop on sustainable seaweed resource development in Sub-Saharan Africa* (Mshigeni, K. E., Bolton, J., Critshley, A. and Kiangi, G., eds.), pp. 19-36, Windhoek, Namibia.

Anderson, R. J., Monteiro, P. M. S. and Levitt, G. J. (1996b) The effect of localised eutrophication on competition between *Ulva lactuca* (Ulvaceae, Chlorophyta) and a commercial resource of *Gracilaria verrucosa* (Gracilariaceae, Rhodophyta). *Hydrobiologia* **326/327**: 291-296

Anderson, R. J., Simons, R. H. and Jarman, N. G. (1989) Commercial seaweeds in Southern Africa: a review of utilization and research. *S. Afr. J. Mar. Sci.* **8**: 277-289.

Anderson, R. J., Smit, A. J. and Levitt, G. J. (1999) Upwelling and fish-factory waste as nitrogen sources for suspended cultivation of *Gracilaria gracilis* in Saldanha Bay, South Africa. *Hydrobiologia* **398/399**: 455-462.

Andrews, J. H. (1976) The pathology of marine algae. *Biol. Rev.* **51**: 211-253

University of Cape Town

Aoki, T., Araki, T. and Kitamikado, M. (1990) Purification and characterization of a novel β-agarase from *Vibrio* sp. AP-2. *Eur. J. Biochem.* **187**: 461-465.

Araki, T., Hayakawa, M., Lu, Z., Karita, S. and Morishita, T. (1998) Purification and characterization of agarases from a marine bacterium, *Vibrio* sp. PO-303. *J. Mar. Biotechnol.* **6**: 260-265.

Armisen, R. (1995) World-wide use and importance of *Gracilaria*. *J. Appl. Phycol.* **7**: 231-243.

Atlas, R. M. and Bartha, R. (1987) Microorganisms in their natural habitats: air, water and siol microbiology. In *Microbial Ecology: fundamentals and applications* (2nd edition), pp. 263-304. The Benjamin/Cummings Publishing Company, Inc, California.

Austin, B. (1988) Identification. In *Methods in Aquatic Bacteriology* (Austin, B., ed.) pp. 95-112, John Wiley and Sons Ltd., Great Britain.

Ausubel, S. F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) Current protocols in Molecular Biology. Green Publishing associates and Wiley-Interscience, Harvard Medical School, USA.

Bailey, W. R. and Scott, E. G. (1966) Staining formulae and procedures. In *Diagnostics Microbiology*. The C. V. Mosby Company, Saint Louis.

Barbeyron, T., Gerard, A., Potin, P., Henrissat, B. and Kloareg, B. (1998) The Kappa-Carrageenase of the Marine Bacterium *Cytophaga drobachiensis*. Structural and Phylogenetic Relationships Within Family-16 Glycoside Hydrolases. *Mol. Biol. Evol.* **15(5)**: 528-537.

Barbeyron, T., Henrissat, B. and Kloareg, B. (1994) The gene encoding the kappa-carrageenase of *Alteromonas carrageenovora* is related to β-1,3-1,4-glucanases. *Gene* **139**: 105-109.

Baumann, L., Baumann, P., Mandel, M. and Allen, R. (1972) Taxonomy of aerobic marine Eubacteria. *J. Bacteriol.* **110**: 402-429.

Baumann, P. Baumann, L., Bowditch, R. D. and Beaman, B. (1984) Taxonomy of *Alteromonas*: *A. nigrifaciens* sp. nov., nom. rev.; *A. macleodii*; and *A. haloplanktis*. *Int. J. Syst. Bacteriol.* **34**: 145-149.

Beesley, J. E. (1989) Colloidal Gold: A new perpective for cytochemical marking. In *Royal Microscopical Society Microscopy Handbooks 17*, Oxford University Press.

Belas, R. (1989) Sequence analysis of the *agr*A gene encoding β-agarase from *Pseudomonas atlantica*. *J Bacteriol.* **171**: 602-605.

Belas, R., Bartlett, D. and Silverman, M. (1988) Cloning and gene replacement mutagenesis of a *Pseudomonas atlantica* agarase gene. *Appl. Environ. Microbiol.* **54**: 30-37.

University of Cape Town

Bellanger, F., Verdus, M. C., Henocq, V. and Christiaen, D. (1990) Determination of the composition of the fibrillar part of *Gracilaria verrucosa* (Gracilariales, Rhodophyta) cell wall in order to prepare protoplasts. *Hydrobiologia* **204/205**: 527-531.

Bhattacharya, D., Elwood, H. J., Goff, L. J. and Sogin, M. L. (1990) Phylogeny of *Gracilaria lemaneiformis* (Rhodophyta) based on sequence analysis of ITS small subunit ribosomal RNA coding region. *J. Phycol.* **26**: 181-186.

Bibb, M. J., Jones, G. H., Joseph, R., Buttner, M. J. and Ward, J. M. (1987) The agarase gene (*dag*A) of *Streptomyces coelicolor* A3(2): affinity purification and characterization of the cloned gene product. *J. Gen. Microbiol.* **133**: 2089-2096.

Bird, C. J. (1995) A review of recent taxonomic concepts and developments in the Gracilariaceae (Rhodophyta). *J. Appl. Phycol.* **7**: 255-267.

Bird, C. J. and Kain, J. M. (1995) Recommended names of included species of Gracilariaceae. *J. Appl. Phycol.* **7**: 335-338.

Bird, C. J. and Rice, E. L. (1990) Recent approaches to the taxonomy of the Gracilariaceae (Gracilariales, Rhodophyta) and the *Gracilaria verrucosa* problem. *Hydrobiologia* **204/205**: 111-118.

Bird, C. J., Ragan, M. A., Critchley, A. T., Rice. E. L. and Gutell, R. R. (1994) Molecular relationships among the Gracilariaceae (Rhodophyta): further observations on some undetermined species. *Eur. J. Phycol.* **29**: 195-202.

Bird, C. J., Rice, E. L., Murphy, C. A., Liu, Q. Y. and Ragan, M. A. (1990) Nucleotide sequences of 18S ribosomal RNA genes from the red algae *Gracilaria tikvahiae* McLachlan, *Gracilaria verrucosa* (Hudson) Papenfuss and *Gracilariopsis* sp. *Nucleic Acids Res.* **18**: 4023-4033.

Bixler, H. J. (1996) Recent developments in manufacturing and marketing carrageenan. *Hydrobiologia* **326/327**: 35-57.

Bolton, J. J. (1986) Marine Phylogeography of the Benguela upwelling region on the west coast of Southern Africa: A temperature dependent approach. *Bot. Mar.* **29**: 251-256.

Booth, W. E. and Hoope, H-G. (1985) Epiphytic bacterial activity on different macroalgal species determined by tritiated thymidine incorporation. *Bot. Mar.* **27**: 47-56.

Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. and McMeekin, T. A. (1997) Diversity and association of phychrophilic bacteria in Antarctic sea ice. *Appl. Environ. Microbiol.* **63**: 3068-3078.

Bowman, 1998 *Pseudoalteromonas prydzensis* sp. nov., a psychrotrophic, halotolerant bacterium from Antarctic sea ice. *Int. J. Syst. Bacteriol.* **48**: 1037-1041.

University of Cape Town

Bozal, N., Tudela, E., Rosselló-Mora, R., Lalucat, J. and Guinea. J. (1997) *Pseudoalteromonas antarctica* sp. nov., isolated from an Antarctic coastal environment. *Int. J. Syst. Bacteriol.* **47**: 345-351.

Branch, G. M., Griffiths, C. L., Branch, M. L. and Beckley, L. E. (1999) Two Oceans. A guide to the marine life of Sothern Africa, pp. 330-331. David Philip Publishers (Pty) Ltd.

Brown, K. L., Wood, S. and Buttner, M. J. (1992) Isolation and characterization of the major vegetative RNA polymerase of *Streptomyces coelicolor* A3(2); renaturation of a sigma subunit using GroEL. *Mol. Microbiol.* **6**: 1133-1139.

Brylinsky, M. (1977) Release of dissolved organic matter by some marine macrophytes. *Mar. Biol.* **39**: 213-220.

Buck, J. D., Meyers, S. P. and Leifson, E. (1963) *Pseudomonas (Flavobacterium) piscicida* Bein comb. nov. *J. Bacteriol.* **86**: 1125-1126.

Buttner, M. J., Fearnley, I. M. and Bibb, M. J. (1987) The agarase (*dag*A) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. *Mol. Gen. Genet.* **209**: 101-109.

Buttner, M. J., Smith, A. M. and Bibb, M. J. (1988) At least three different RNA polymerase holoenzymes direct transcription of the agarase gene (*dag*A) of *Streptomyces coelicolor* A3(2). *Cell* **52**: 599-607.

Casadevall, A. and Pirofski, L-A. (2000) Host-Pathogen Interactions: Basic Concepts of Microbial Commensalism, Colonization, Infection, and Disease. *Infection and Immunity* **68**: 6511-6518

Chan, K. Y., Baumann, L., Garza, M. M. and Baumann, P. (1978) Two new species of *Alteromonas*: *Alteromonas espejiana* and *Alteromonas undina*. *Int. J. Syst. Bacteriol.* **28**: 217-222.

Chapman, V. J. and Chapman, D. J. (1980) Agar-agar. In *Seaweeds and their Uses* (Chapman, V.J. and Chapman, D. J., eds.), pp148-193, Chapman and Hall, New York.

Christiaen, D., Stadler, T., Ondarza, M. and Verdus, M. C. (1987) Structures and functions of the polysaccharides from the cell wall of *Gracilaria verrucosa* (Rhodophyceae, Gigartinales). *Hydrobiologia* **151/152**: 139-146.

Christie, N. D. (1981) Primary production in Langebaan Lagoon. In *Estuarine ecology with particular reference to southern Africa* (Day, J. H.ed.), pp. 101-115. Cape Town, Balkema.

Church, G. and Gilbert, W. (1984) Genomic sequencing. *Proceedings of the National Academy of Sciences, U.S.A.* **81**: 1991-1995.

Cole, K. M. (1990) Chromosomes. In *Biology of Red Algae* (Cole, K.M. and Sheath, R.G., eds.), pp. 73-101, Cambridge University Press, Cambridge.

University of Cape Town

Corre, S., Prieur, D., Chamroux, S., Floch, J-Y. and Hourmant, A. (1989) Étude préliminaire des communauté bactériennes epiphytes de Laminara digitata. *Can. J. Microbiol.* **35**: 740-743.

Correa, J. A., Flores, V. and Sánchez, P. (1993) Deformative disease in *Iridaea laminariodes* (Rhodophyta): gall development associated with an endophytic *Cyanobacterium*. *J. Phycol.* **29**: 853-860.

Correa, J.A. (1996) Disease in seaweeds: an introduction. *Hydrobiologia* **326/327**: 87-88.

Coutinho, P. M. and Henrissat, B. (1999) Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html

Coyne, V., James, M., Reid, S. and Rybicki, E. (1996) *Molecular biology techniques manual* (4th ed.), University of Cape Town, Cape Town.

Craigie, J. S. and Correa, J. A. (1996) Etiology of infectious diseases in cultivated *Chondrus crispus* (Gigartinales, Rhodophyta). *Hydrobiologia* **326/327**: 97-104.

Craigie, J. S. (1990) Cell walls. In *Biology of Red Algae* (Cole, K.M. and Sheath, R.G., eds.), pp. 221-258, Cambridge University Press, Cambridge.

Cundell, A.M., Sleeter, T. D. and Mitchell, R. (1977) Microbial populations associated with the surface of the brown alga *Ascophyllum nodosum*. *Microb. Ecol.* **4**: 81-91.

Dagert, M. and Ehrlich, S. D. (1979) Prolonged incubation in calcium chloride improves the competence of *Escherichia coli*. *Gene* **6:** 23-28.

Day, D. F. and Yaphe, W. (1975) Enzymatic hydrolysis of agar: purification and characterization of neoagarobiose hydrolase and *p*-nitrophenyl α-galactoside hydrolase. *Can. J. Microbiol.* **21**: 1512-1518.

DeFrank, J. J., Beaudry, W. T., Cheng, T-C., Harvey, S. P., Stroup, A. N. and Szafraniec, L.L. (1993) Screening of halophilic bacteria and *Alteromonas* species for organophosphorus hydrolysing enzyme activity. *Chem.-Biol. Interactions* **87**: 141-148.

De Vos, P., Van Landschoot, A., Segers, P., Tytgat, R., Gillis, M., Bauwens, M., Rossau, R., Goor, M., Pot, B., Kersters, K., Lizzaraga, P. and De ley, J. (1989) Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridisations. *Int. J. Syst.Bacteriol.* **39**: 35-49.

Duckworth, M. and Turvey, J. R. (1969a) An extracellular agarase from a *Cytophaga* species. *Biochem. J.* **113**: 139-142.

Duckworth, M. and Turvey, J. R. (1969b) The specificity of an agarase from a *Cytophaga* species. *Biochem. J.* **113**: 693-696.

Duckworth, M. and Turvey, J. R. (1969c) The action of a bacterial agarase on agarose, porphyran and alkali-treated porphyran. *Biochem. J.* **113**: 687-692.

University of Cape Town

Duckworth, M. and Yaphe, W. (1970) Thin-layer chromatographic analysis of enzymic hydrolysis of agar. *J. Chromatogr.* **49**: 482-487.

Duckworth, M. and Yaphe, W. (1971) The structure of agar. *Carbohyd. Res.* **16**: 189-197.

Dykstra, M. J. (1993) A manual of applied techniques for biological electron microscopy. Plenum Press, New York and London.

Eikmanns, B. J., Klienertz, E., Liebl, W. and Sahm, H. (1991) A family of *Corynebacterium glutamicum* / *Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**: 93-98.

Enger, Ø, Nygaard, H., Solberg, M., Schei, G., Nielsen, J. and Dundas, I. (1987) Characterization of *Alteromonas denitrificans* sp. nov. *Int. J. Syst. Bacteriol.* **37**: 416-421.

Englard, S. and Seifter, S. (1990) Precipitation Techniques. In *Methods in Enzymology, vol. 182, Guide to protein purification* (Deutscher, M. P. ed.), pp. 285-300. Academic Press, Inc.

Engledow, H. R. and Bolton, J. J. (1992) Environmental tolerances in culture and agar content of *Gracilaria verrucosa* (Hudson) Papenfuss (Rhodophyta, Gigartinales) from Saldanha Bay. *S. Afr. J. Bot.* **58**: 263-267.

Felsenstein, J. (1995) PHYLIP (Phylogeny Inference Package) Version 3.57c. University of Washington, Seattle.

Fox, G. E., Wisotszkey, J. D. and Jurtshuk, P. Jr (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**: 166-170.

Fredericq, S. and Hommersand, M. H. (1989) Proposal of the Gracilariales, ord. nov. (Rhodophyta) based on an analysis of the reproductive development of *Gracilaria verrucosa*. *J. Phycol.* **25**: 213-227.

Freshwater, D. W., Fredericq, S., Butler, B. S., Hommersand, M. H. and Chase, M. W. (1994) A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proc. Natl. Acad. Sci. USA* **91**: 7281-7285.

Friedlander, M. and Gunkel, W. (1992) Factors leading to thallus disintegration and the control of these factors in *Gracilaria* sp. In *Proceedings of the 4th German-Israeli Status Seminar* (Moav, B., Hilge, B. and Rosenthal, H., eds.), pp. 221-243, EAS special publication No.17, Oostende.

Fukasawa, S., Dunlap, P. V., Baba, M. and Osumi, M. (1987) Identification of an agar-digesting, luminous bacterium. *Agric. Biol. Chem.* **51**: 265-268.

Garbary, D. J. and Gabrielson, P. W. (1990) Taxonomy and evolution. In *Biology of Red Algae* (Cole, K.M. and Sheath, R.G., eds.), pp. 477-498, Cambridge University Press, Cambridge.

University of Cape Town

Gauthier, 1976 *Alteromonas rubra* sp. nov., a new marine antibiotic-producing bacterium. *Int. J. Syst. Bacteriol.* **26**: 459-466.

Gauthier, 1977 *Alteromonas citrea*, a new Gram negative, yellow-pigmented species from seawater. *Int. J. Syst. Bacteriol.* **27**: 349-354.

Gauthier, M. J. and Breittmayer, V. A. (1979) A new antibiotic-producing bacterium from seawater: *Alteromonas aurantia* sp. nov. *Int. J. Syst. Bacteriol.* **29**: 366-372.

Gauthier, 1982 Validation of the name *Alteromonas luteoviolacea*. *Int. J. Syst. Bacteriol.* **32**: 82-86.

Gauthier, G., Gauthier, M. and Christen, R. (1995) Phylogenetic analysis of the genera *Alteromonas*, *Shewanella*, and *Moritella* using genes encoding the small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int. J. Syst. Bacteriol.* **45**: 755-761.

Ghadi, S. C., Muraleedharan, U. D. and Jawaid, S. (1997) Screening for agarolytic bacteria and development of a novel method for *in situ* detection of agarase. *J. Mar. Biotechnol.* **5**: 194-200.

Goff, L. J., Moon, D. A. and Coleman, A. W. (1994) Molecular delineation of species and species relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). *J. Phycol.* **30**: 521-537.

González, M., Montoya, R., Candia, A., Gómez, P. and Cisternas, M. (1996) Organellar DNA restriction fragment length polymorphism (RFLP) and nuclear random amplified polymorphic DNA (RAPD) analyses of morphotypes of *Gracilaria* (Gracilariales, Rhodophyta) and Chile. *Hydrobiologia* **326/327**:229-234.

Groleau, D. and Yaphe, W. (1977) Enzymatic hydrolysis of agar: purification and characterization of β-neoagarotetraose hydrolase from *Pseudomonas atlantica*. Can. J. Microbiol. **23**: 672-679.

Ha, J-C., Kim, G-T., Kim, S-K., Oh, T-K., Yu, J-H. and Kong. I-S. (1997) β-agarase from *Pseudomonas* sp. W7: purification of the recombinant enzyme from *E. coli* and the effects of salt on its activity. *Biotechnol. Appl. Biochem.* **26**: 1-6.

Harley, C. B. and Reynolds, R. P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Res.* **15**: 2343-2361.

Herrero, M., De Lorenzo, V. and Timmis, K. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *J. Bacteriol.* **172**: 6557-6567.

Hofsten, B. v. and Malmqvist, M. (1975) Degradation of agar by a Gram-negative bacterium. *J. Gen. Microbiol.* **87**: 150-158.

University of Cape Town

Holmström, C., James, S., Neilan, B. A., White, D. C. and Kjelleberg, S. (1998) *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int. J. Syst. Bacteriol.* **48**: 1205-1212.

Hugh, R. and Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. *J. Bacteriol.* **66**: 24

Ish-Horowicz, D. and Burke, J. (1981) Rapid and efficient cosmid cloning. *Nucleic Acids Res.***9**: 2989-2998.

Isaac, W. E. (1942) Seaweeds of possible economic importance in the Union of South Africa. *J. S. Afr. Bot.* **8**: 225-236.

Isaac, W. E. (1956) The ecology of *Gracilaria confervoides* (L.) Grev. In South Africa with special reference to its ecology in the Saldanha-Langebaan Lagoon. In *The Second International Seaweed Symposium, Trondheim*, (Braarud, T. and Sørensen, N. A., eds.), pp. 173-185, Pergamon, London.

Isaac, W. E. and Molteno, C. J. (1952) Seaweed resources of South Africa. *S. Afr. J. Bot.* **18**: 85-92.

Ivanova, E. P., Kiprianova, E. A., Mikhailov, V. V., Levanova, G. F., Garagulya, A. D., Gorshkova, N. M., Vysotskii, M. V., Nicolau, D. V., Yumoto, N., Taguchi, T. and Yoshikawa, S. (1998) Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description. *Int. J. Syst. Bacteriol.* **48**: 247-256.

Ivanova, E. P., Chun, J., Romanenko, L. A., Matte, M. E., Mikhailov, V. V., Frolova, G. M., Huq, A. and Colwell, R.R. (2000) Reclassification of *Alteromonas distincta* Romanenko *et al.* 1995 as *Pseudalteromonas distincta* comb. nov. *Int. J. Syst. Evol. Microbiol.* **50**: 141-144.

Izumi, K. (1972) Chemical heterogeneity of agar from *Gracilaria verrucosa*. *J. Biochem.* **72**: 135-140.

Jaffray, A. E. and Coyne, V. E. (1996) Development of an *in situ* assay to detect bacterial pathogens of the red alga *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham. *J. Appl. Phycol.* **8**: 409-414.

Jaffray, A. E., Anderson, R. J., and Coyne, V. E. (1997) Investigation of bacterial epiphytes of the agar-producing red seaweed *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham from Saldanha Bay, South Africa and Lüderitz, Namibia. *Bot. Mar.***40**: 569-576.

Jaffray, A. E. (1999) The investigation of bacterial pathogens of the red macroalga *Gracilaria gracilis* and its response to bacterial infection. *Thesis for the degree of Doctor of Philosophy in the Microbiology Department*. University of Cape Town, Cape Town.

Jensen, P. R., Kauffman, C. A. and Fenical, W. (1996) High recovery of culturable bacteria from the surfaces of marine algae. *Mar. Biol.* **126**: 1-7

University of Cape Town

Kain, J. M. and Destombe, C. (1995) A review of the life history, reproduction and phenology of *G. gracilis*. *J. Appl. Phycol.* **7**: 269-281.

Kendall, K. and Cullum, J. (1984) Cloning and expression of an extracellular-agarase from *Streptomyces coelicolor* A3(2) in *Streptomyces lividans* 66. *Gene* **29**: 315-321.

Kirimura, K., Masuda, N., Iwasaki, Y., Nakagawa, H., Kobayashi, R., and Usami, S. (1999) Purification and characterization of a novel β-agarase from an alkalophilic bacterium, *Alteromonas* sp. E-1. *J. Biosci. Bioeng.* **87**: 436-441.

Kivelä, H. M., Männistö, R. H., Kalkkinen, N. and Bamford, D. H. (1999) Purification and protein composition of PM2, the first lipid-containing bacterial virus to be isolated. *Virology* **262**: 364-374.

Knutsen, S. H., Myslabodski, D. E., Larsen, B. and Usov, A. I. (1994) A modified system of nomenclature for red algal galactans. *Bot. Mar.* **37**: 163-169.

Kong, J-Y., Hwang, S-H., Kim, B-J., Bae, S-K. and Kim, J-D. (1997) Cloning and expression of an agarase gene from a marine bacterium *Pseudomonas* sp. W7. *Biotechnol. Lett.* **19**: 23-26.

Kong, M. K. and Chan, K-Y. (1979) A study on the bacterial flora isolated from marine algae. *Bot. Mar.* **22**: 83-97.

Largo, D. B., Fukami, K. and Hishijima, T. (1995) Occasional pathogenic bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus alvarezii* and *Euchuema denticulatum* (Solieraceae, Gigartinales, Rhodophyta). *J. Appl. Phycol.* **7**: 545-554.

Lavilla-Pitogo, C. R. (1992) Agar-digesting bacteria associated with 'rotten thallus syndrome' of *Gracilaria* sp. *Aquaculture* **102**: 1-7.

Laycock, R. A. (1974) The detrital food chain based on seaweeds. 1. Bacteria associated with the surface of Laminaria Fronds. *Mar. Biol.* **25**: 223-231.

Lee, S-B., Park, J-H., Yoon, S-C., Kim, J-M. and Kong, I-S. (2000) Sequence analysis of a β-agarase gene (*pja*A) from *Pseudomonas* sp. isolated from marine environment. *J. Biosci. Bioeng.* **89**: 485-488.

Lemos, M. L., Toranzo, A. E. and Barja, J. L. (1985) Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. *Microb. Ecol.* **11**: 149-163.

Leon, O., Quintana, L., Peruzzo, G., and Slebe, J. C. (1992) Purification and properties of an extracellular agarase from *Alteromonas* sp. strain C-1. *Appl. Environ. Microbiol.* **58**: 4060-4063.

Ludwig, W. and Schleifer, K-H. (1999) Phylogeny of *Bacteria* beyond the 16SrRNA standard. *ASM News.* **65**: 752-757.

Mackie, W. and Preston, R. D. (1974) Cell wall and intercellular region polysaccharides. In *Algal physiology and biochemistry* (Stewart, W.D.P. ed.), pp. 40-85. Botanical Monographs Vol 10, Blackwell Scientific Publ..

University of Cape Town

Malmqvist, M. (1978) Purification and characterization of two different agarose-degrading enzymes. *Biochim. Biophys. Acta.* **537**: 31-43.

Mariani, P., Tolomio, C., Baldan, B. and Braghetta, P. (1990) Cell wall ultrastructure and cation localization in some benthic marine algae. *Phycologia* **29**: 253-262.

Mazure, H. G. F. and Field, J. G. (1980) Density and ecological importance of bacteria on kelp fronds in an upwelling region. *J. Exp. Mar. Biol. Ecol.* **43**: 173-182.

McBride, D. L., Kugrens, P. and West, J. A. (1974) Light and electron microscopy observations on red algal galls. *Protoplasma* **79**: 249-264.

McHugh, D. J. (1984) Marine phycoculture and its impact on the seaweed colloid industry. *Hydrobiologia* **116/117**: 351-354.

McHugh, D. J. (1991) Worldwide distribution of commercial resources of seaweeds including *Gelidium*. *Hydrobiologia* **221**: 19-29.

Meulen, H. J. van der and Harder, W. (1975) Production and characterization of the agarase of *Cytophaga flevensis*. *Antonie van Leeuwenhoek.* **41**: 431-447.

Meulen, H. J. van der and Harder, W. (1976) Characterization of the neoagarotetra-ase and neoagarobiase of *Cytophaga flevensis*. *Antonie van Leeuwenhoek.* **42**: 81-94.

Miller, G. L. (1959) Use of Dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chemistry* **31**: 426-428.

Miller, J. H. (1992) Procedures for working with lac. In *A short course in bacterial genetics: A laboratory manual and handbook for E. coli and related bacteria*, pp. 71-80. Cold Spring Harbor Laboratory Press.

Miller, V. and Mekalanos, J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *V. cholerae* requires ToxR. *J. Bacteriol.* **170**: 2575-2583.

Miller, E. M. and Nickoloff, J. A. (1994) *Escherichia coli* Electrotransformation. In *Methods in Molecular Biology: Electroporation protocols for microorganisms* (Nickoloff, J. A. ed.) pp 105-113 Vol 47, Humana Press Inc., Totowa, NJ.

Morrice, L. M., McLean, M. W., Long, W. F. and Williamson, F. B. (1983a) β-agarases I and II from *Pseudomonas atlantica*. Substrate specificities. *Eur. J. Biochem.* **137**: 149-154.

Morrice, L, M., McLean, M. W., Williamson, F. B. and Long, W. F. (1983b) β-agarases I and II from *Pseudomonas atlantica*. Purifications and some properties. *Eur. J. Biochem.* **135**: 553-558

Morrice, L. M., McLean, M. W., Long, W. F. and Williamson, F. B. (1983c) An investigation using β-agarases I from *Pseudomonas atlantica* and ^{13}C-NMR Spectroscopy. *Eur. J. Biochem.* **133**: 673-684.

University of Cape Town

Mow-Robinson, J. M. and Rheinheimer, G. (1985) Comparison of bacterial populations from the Kiel Fjord in relation to the absence of benthic vegetation. *Bot. Mar.* **28**: 29-39.

Moyer, C. L., Dobbs, F. C. and Karl, D. M. (1995) Phylogenetic diversity of the bacterial community from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.* **61**: 1555-1562.

Murano, E. (1995) Chemical structure and quality of agars from *Gracilaria*. *J. Appl. Phycol.* **7**: 245-254.

Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, p., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E. and Zavarzin, G. A. (1990) Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *Int. J. Syst. Bacteriol.* **40**: 213-215.

Naganuma, T., Coury, D. A., Polne-Fuller, M., Gibor, A. and Horikoshi, K. (1993) Characterization of agarolytic *Microscilla* isolates and their extracellular agarases. *System. Appl. Microbiol.* **16**: 183-190.

Nikolaeva, E. V., Usov, A. I. and Tambiev, A. H. (1999) Study on the marine agarolytic bacterium-infusorium association. *Marine Biol.* **135**: 407-410.

Nomura, K., Naitoh, Y., Muramatsu, S., Yoshizawa, Y., Tsunehiro, J., Fukui, F. and Itoh, M. (1998) New sulphated oligosaccharides produced by *Pseudomonas* β-agarase from *Gracilaria verrucosa* polysaccharide. *Biosci. Biotechnol. Biochem.* **62**: 1190-1195.

Oliveira, E. C. de and Plastino, E. M. (1994) Gracilariaceae. In *Biology of Economic Algae* (Akatsuka, I., ed.), pp. 185-226, SPB Academic Publishing, The Hague, Netherlands.

Olsen, G. J., Woese, C. R. and Overbeek, R. (1994) Minireview. The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol.***176**: 1-6.

Page, R. D. M. (1998) TREEVIEW software version 2.1. (http://taxonomy.zoology.gla.ac.za)

Park, J. T. and Johnson, M. J. (1949) A submicrodetermination of glucose. *J. Biol. Chem.* **181:**149-151.

Parro, V. and Mellado, R. P. (1993) Heterologous recognition in vivo of promoter sequences from the *Streptomyces coelicolor dag*A gene. *FEMS Microbiol. Lett.* **106**: 347-356.

Parro, V. and Mellado, R. P. (1994) Effects of glucose on agarase overproduction by *Streptomyces*. *Gene* **145**: 49-55.

Parro, V., Vives, C, Godia, F. and Mellado, R. P. (1997) Overproduction and purification of an agarase of bacterial origin. *J. Biotechnol.* **58**: 59-66.

Pizarro, A. (1986) Recent advances in the management and field cultivation of *Gracilaria* sp. In Chile. *Monografias Biologicas* **4**: 63-96.

University of Cape Town

Planas, A., Juncosa, M., Lloberas, J. and Querol, E. (1992) Essential catalytic role of Glu134 in endo-β-1,3-1,4-D-glucan 4-glucanohydrolase from *B. licheniformis* as determined by site-directed mutagenesis. *FEBS Lett.* **308**: 141-145.

Potin, P., Richard, C., Rochas, C. and Kloareg, B. (1993) Purification and characterization of the α-agarase from *Alteromonas agarlyticus* (Cataldi) comb. nov., strain GJ1B. *Eur. J. Biochem.* **214**: 599-607.

Pugsley, A. P. D., Enfert, C., Reyss, I. And Kornacker, M. G. (1990) Genetics of extracellular proptein secretion by gram-negative bacteria. *Annu. Rev. Genet.* **24**: 67-90.

Ragan, M. A., Bird, C. J., Rice, E. L., Gutell, R. R., Murphy, C. A. and Singh, R. K. (1994) A molecular phylogeny of the marine red algae (Rhodophyta) based on the nuclear small-subunit rRNA gene. *Proc. Natl. Acad. Sci. USA* **91**: 7276-7280.

Reed, K. and Mann, D. (1985) Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13:** 7207-7221.

Rees, D. A. (1972) Shapely Polysaccharides. The eighth Colworth medal lecture. *Biochem. J.* **126**: 257-273.

Rees, G. N., Rainey, F. A. and Harfoot, C. G. (1994) Characterization of a novel obligate anaerobe that ferments agar. *Arch. Microbiol.* **162**: 395-400.

Reichelt, J. L. and Baumann, P. (1973) Change of the name *Alteromonas marinopraescens* (Zobell and Upham) Baumann et al. to *Alteromonas haloplanktis* (Zobell and Upham) comb. nov. and assignment of strain ATCC 23821 (*Pseudomonas enalia*) and strain c-A1 of De Vos and Oginsky to this species. *Int. J. Syst. Bacteriol.* **23**: 438-441.

Renn, D. W. (1990) Seaweeds and biotechnology – inseparable companions. *Hydrobiologia* **204/205**: 7-13.

Rheinheimer, G. (1984) Bacterial ecology of the North and Baltic Seas. *Bot. Mar.* **27**: 277-299.

Rieper-Kirchner, M. (1989) Microbial degradation of North Sea macroalgae: field and laboratory studies. *Bot. Mar.* **32**: 241-252.

Rosell, K. G. and Srivastava, L. M. (1987) Fatty acids as antimicrobial substances in brown algae. *Hydrobiologia* **151/152**: 471-475.

Ross, W., Aiyar, S. E., Salomon, J. and Gourse, R. L. (1998) *Escherichia coli* promoters with UP elements of different strengths: modular structure of bacterial promoters. *J. Bacteriol.* **180**: 5375-5383.

Rossomando, E. F. (1990) Ion-exchange chromotography. In *Methods in Enzymology, vol. 182, Guide to protein purification* (Deutscher, M. P. ed.), pp. 309-317. Academic Press, Inc.

Rotmann, K. W. G. (1990) Saldanha Bay, South Africa: recovery of *Gracilaria verrucosa* (Gracilariales, Rhodophyta). *Hydrobiologia* **204/205**: 325-330.

University of Cape Town

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. N. Y.

Sammons, D. W., Adams, L. D. and Nishizawa, E. E. (1981) Ultrasensitive silvarbased color staining of polypeptides in polyacrylamide gels. *Electrophoresis.* **2**: 135.

Sampietro, A. R. and Vattuone de Sampietro, M. A. (1971) Characterization of the agarolytic system of *Agarobacterium pastinator. Biochim. Biophys. Acta.* **244**: 65-76.

Sanger, F., Nicklen, S. and Carlson, A. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci., USA* **74**: 5463-5467.

Santelices, B. and Doty, M. S. (1989) A review of *Gracilaria* farming. *Aquaculture* **78**: 95-133.
Sastry, V. M. V. S. and Rao, G.R.K. (1994) Antibacterial substances from marine algae: successive extraction using benzene, chloroform and methanol. *Bot. Mar.* **37**: 357-360.

Sawabe, T., Makino, H., Tatsumi, M., Nakano, K., Tajima, K., Iqbal, M. M., Yumoto, I., Ezura, Y. Christen, R. (1998) *Pseudoalteromonas bacteriolytica* sp. nov., a marine bacterium that is the causative agent of red spot disease of *Laminaria japonica. Int. J. Syst. Bacteriol.* **48**: 769-774.

Sawabe, T., Tanaka, R., Iqbal, M. M., Tajima, K., Ezura, Y., Ivanova, E. P. and Christen, R. (2000) Assignment of *Alteromonas elyakovii* KMM 162^T and five strains isolated from spot-wounded fronds of *Laminaria japonica* to *Pseudoalteromonas elyakovii* comb. nov. and the extended description of the species. *Int. J. Syst. Evol. Microbiol.* **50**: 265-271.

Scholfield, C. I., Gacesa, P., Price, J. H., Russell, S. J. and Bhoday, R. (1991) Restriction fragment length polymorphisms of enzymically-amplified small-subunit rRNA-coding regions from *Gracilaria* and *Gracilariopsis* (Rhodophyta) – a rapid method for assessing 'species' limits. *J. Appl. Phycol.* **3**: 329-334.

Schroeder, D. C. (1994) Cloning and characterization of the agarase genes of an epiphytic bacterial strain which is a pathogen of the red alga, *Gracilaria verrucosa. Thesis for the degree of Bachelor of Science (Honours) in the Microbiology Department.* University of Cape Town, Cape Town.

Shiba, T. and Taga, N. (1980) Heterotrophic bacteria attached to seaweeds. *J. Exp. Mar. Biol. Ecol.* **47**: 251-258.

Shine, J., and Dalgarno, L. (1975) Determination of cistron specificity in bacterial ribosomes. *Nature* (London) **254:**34-38.

Shieh, W. Y. and Jean, W. D. (1998) *Alterococcus agarolyticus*, gen. nov., sp.nov., a halophylic thermophilic bacterium capable of agar degradation. *Can. J. Microbiol.* **44**: 637-645.

Short, J., Fernandez, J., Sorge, J. and Huse, W. (1988) λZAP: A bacteriophage λ expression vector with *in vivo* excision properties. *Nucleic Acids Res.* **16**: 7583-7600.

University of Cape Town

Sieburth, J.M. (1964) Antibacterial substances produced by marine algae. *Dev. Ind. Microbiol.* **5**: 124-135.

Sieburth, J.M. (1969) Studies on algal substances in the sea. III. The production of extracellular organic matter by littoral marine algae. *J. Exp. Mar. Biol. Ecol.* **3**: 290-309.

Simidu, U., Kita-Tsukamoto, K., Yasumoto, T. and Yotsu, M. (1990) Taxonomy of four marine bacterial strains that produce tetrodotoxin. *Int. J. Syst. Bacteriol.* **40**: 331-336.

Simons, R. H. (1977) The algal flora of Saldanha Bay. *Trans. Roy. Soc. S. Afr.* **42**: 462-482.

Smibert, R. M. and Krieg, N. R. (1981) General characteristics. In *Manual of Methods for General Bacteriology* (Gerhardt, P. R., Murray, G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R. and Briggs Phillips, G., eds). American Society for Microbiology, New York.

Spurr, A. R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**: 31-43.

Stackebrandt, E. and Goebel, B. M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**: 846-849.

Stackebrandt, E., Liesack, W. and Witt, D. (1992) Ribosomal RNA and rDNA sequence analyses. *Gene.* **115**: 255-260.

Steentoft, M., Irvine, L. M. and Farnham, W. F. (1995) Two terete species of *Gracilaria* and *Gracilariopsis* (Gracilariales, Rhodophyta) in Britain. *Phycologia* **34**: 113-127.

Stegenda, H. (1997) Seaweeds of the south african west coast, (Hall, A.V. ed.). © Bolus Herbarium, University of Cape Town, Creda Press.

Sugano, Y., Hagae, H., Inagaki, K., Yamamoto, T., Terada, I. and Yamazaki, Y. (1995) Production and characteristics of some new β-agarases from a marine bacterium, *Vibrio* sp. strain JT0107. *J. Ferment. Bioengin.* **79**: 549-554.

Sugano, Y., Kodama, H., Terada, I., Yamazaki, Y., and Noma, M. (1994) Purification and characterisation of a novel enzyme, α-neoagarooligosaccharide hydrolase (α-NAOS hydrolase), from a marine bacterium, *Vibrio* sp. strain JT0107. *J. Bacteriol.* **176**: 6812-6818.

Sugano, Y., Matsumoto, T. and Noma, M. (1994) Sequence analysis of the *aga*B gene encoding a new β-agarase from *Vibrio* sp. strain JT0107. *Biochim. Biophys. Acta.* **1218**: 105-108.

Sugano, Y., Terada, I., Arita, M., Noma, M., and Matsumoto, T. (1993a) Purification and characterisation of a new agarase from a marine bacterium, *Vibrio* sp. strain JT0107. *Appl. Environ. Microbiol.* **59**: 1549-1554.

University of Cape Town

Sugano, Y., Matsumoto, T. Kodama, H. and Noma, M. (1993b) Cloning and sequencing of *aga*A, a unique agarase 0107 gene from a marine bacterium, *Vibrio* sp. strain JT0107. *Appl. Environ. Microbiol.* **59**: 3750-3756.

Taylor, C. B. (1998) Defense Responses in Plants and Animals- More of the Same. *The Plant Cell.* **10**: 873-876.

Teather, R. M. and Wood, P. J. (1982) Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* **43**: 777-789.

Toncheva-Panova, T. and Ivanova, J. (1997) A bacterial pathogen of *Rhodella reticulata*. *J. Appl. Microbiol.* **83**: 707-711.

Towbin, K., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci., USA* **76**: 4350-4354.

Tsujibo, H., Orikoshi, H., Shiotani, K., Hayashi, M., Umeda, J., Miyamoto, K., Imada, C., Okami, Y. and Inamori, Y. (1998) Characterization of chitinase C from a marine bacterium, *Alteromonas* sp. strain 0-7, and its corresponding gene and domain structure. *Appl. Environ. Microbiol.* **64**: 472-478.

Ulitzur, S. (1972) Rapid determination of DNA base composition by ultraviolet spectroscopy. *Biochim. Biophys. Acta* **272**: 1-11.

Uyenco, F. R., Saniel, L. S. and Jacinto, G. S. (1981) The 'ice-ice' problem in seaweed farming. In *Proceedings of the Xth International Seaweed Symposium* (Levring, T., ed.), pp. 625-630, Walter de Gruyter & Co., Berlin.

Van Landschoot, A. and De Ley, J. (1983) Intra- and intergeneric similarities of the rRNA cistrons of *Alteromonas*, *Marinomonas* (gen. nov.) and some other Gram-negative bacteria. *J. Gen. Microbiol.* **129**: 3057-3074.

Vera, J., Alverez, R., Murano, E., Slebe, J. C. and Leon, O. (1998) Identification of a marine agarolytic *Pseudoalteromonas* isolate and characterization of its extracellular agarase. *Appl. Env. Microbiol.* **64**: 4378-4383.

Warren, R. A. J. (1996) Microbial hydrolysis of polysaccharides. *Annu. Rev. Microbiol.* **50**: 183-212.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P. and Trüper, H. G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**: 463-464.

Weinberger, F., Friedlander, M. and Gunkel, W. (1994) A bacterial facultative parasite of *Gracilaria conferta*. *Dis. Aquat. Org.* **18**: 135-141.

University of Cape Town

Weinberger, F., Hoppe, H-G. and Friedlander, M (1997) Bacterial induction and inhibition of a fast necrotic response in *Gracilaria conferta* (Rhodophyta). *J. Appl. Phycol.* **9**: 277-285.

Weinberger, F., Friedlander, M. and Hoppe, H-G. (1999) Oligoagars elicit a physiological response in *Gracilaria conferta* (Rhodophyta). *J. Phycol.* **35**: 747-755.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**: 697-703.

Wichels, A., Biel, S. S., Gelderbom, H. R., Brinkhoff, T., Muyzer, G. and Schütt, C. (1998) Bacteriophage diversity in the North Sea. *Appl. Environ. Microbiol.* **64**: 4128-4133.

Woese, C. R. (1987) Bacterial evolution. *Microbiol. Rev.* **51**: 221-271.

Yaphe, W. (1957) The use of agarase from *Pseudomonas atlantica* in the identification of agar in marine algae (Rhodophyceae). *Can. J. Microbiol.* **3**: 987-993.

Yoshikawa, K., Takadera, T., Adachi, K., Nishijima, M. and Sano, H. (1997) Korormicin, a novel antibiotic specifically active against marine gram-negative bacteria, produced by a marine bacterium. *J. Antibiotics Tokyo* **50**: 949-953.

Yoshinaga, I, Kawai, T. and Ishida, Y. (1997) Analysis of algicidal ranges of the bacteria killing the marine dinoflagellate *Gymnodinium mikimotoi* isolated from Tanabe Bay, Wakayama Pref., Japan. *Fisheries Science (Tokyo)* **63**: 94-98.

Young, K. S., Bhattacharjee, S. S. and Yaphe, W. (1978) Enzymic cleavage of the α-linkages in agarose, to yield agaro-oligosaccharides. *Carbohydr.Res.* **66**: 207-212.

Zabeau, M. and Stanley, K. K. (1982) Enhanced expression of the cro-β-galactosidase fusion under the control of the PR promoter of the bacteriophage lambda. *Embo J.* **1:** 1217-1224

Zobell, C. E. and Upham, H. C. (1944) A list of marine bacteria including descriptions of sixty new species. *Bulletin of the Scripps Institute of Oceanography.* **5**: 239-292.

University of Cape Town