Identification and characterization of microorganisms associated with marine macroalgae *Splachnidium rugosum*

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A thesis submitted in fulfilment of the requirements for the degree of

MAGISTER SCIENTIAE (M.Sc.)

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DECLARATION

I, Mouna Abdalhamed Albakosh, hereby declare that “Identification and characterization of microorganisms associated with marine brown macroalgae *Splachnidium rugosum*” is my own original work, and that I have accurately reported and acknowledged all sources, and that this document has not previously, in its entirety or in part been submitted at any university in order to obtain an academic qualification.

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....

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ABSTRACT

Marine macroalgae are known to carry diverse bacterial communities which interact with their hosts in both harmful and beneficial ways. Algae hosts provide the bacteria with a rich source of carbon in the form of carbohydrate polysaccharides such as fucoidan, agar and alginate, which the bacteria enzymatically degrade. *Splachnidium rugosum* is a brown alga (Phylum: Phaeophyta) that grows exclusively in the Southern Hemisphere along the temperate shores of South Africa, New Zealand and Australia. While several studies have investigated *S. rugosum* distribution and fucoidan production, the microbiome of *S. rugosum* remains largely uncharacterized. Thus, the major objective of the present study was to isolate, identify and characterize epiphytic bacterial communities associated with *S. rugosum.* Algae were sourced from Rooi Els (Western Cape, South Africa) during winter 2012. Culture based methods relied on a range of selective marine media including marine agar, nutrient sea water agar, nutrient agar and thiosulfate-citrate-bile-salts-sucrose agar to determine the composition and uniqueness of bacterial communities associated with *S. rugosum*. Epiphytic isolates were identified to species level by 16S rRNA gene sequence analysis and encompassed 39 Gram-negative and 2 Gram-positive bacterial taxa. Isolates were classified into four phylogenetic groups, Gamma-Proteobacteria, Alpha-Proteobacteria, Firmicutes and Bacteriodetes. Bacteria belonging to the phylum Gamma-Proteobacteria were the most abundant, with *Vibrio* and *Pseudoalteromonas* being the dominant genera. Three isolates with low sequence identity (<97%) to their closest relatives could possibly represent novel species. These isolates were grouped into the genera *Shewanella*, *Sphingomonas* and *Sulfitobacter*. All bacterial isolates (41) were screened for anti-microbial activity against the following test strains: *Escherichia coli*, *Bacillus cereus*, *Staphylococcus epidermidis, Mycobacterium smegmatis Micrococcus luteus* and *Pseudomonas*

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putida. Fifteen isolates (36%) displayed antimicrobial activity against one or more of the test strains, while one isolate (*Pseudomonas* species) showed broad spectrum antimicrobial activity against all the test strains except for *E. coli.* This study provides the first account of the diversity and composition of bacterial populations on the surface of *S. rugosum,* and demonstrates the ability of these bacteria to produce antimicrobial compounds. Despite recent advances in metagenomics, this study highlights the fact that traditional culturing technologies remain a valuable tool for the discovery of novel bioactive compounds of bacterial origin.

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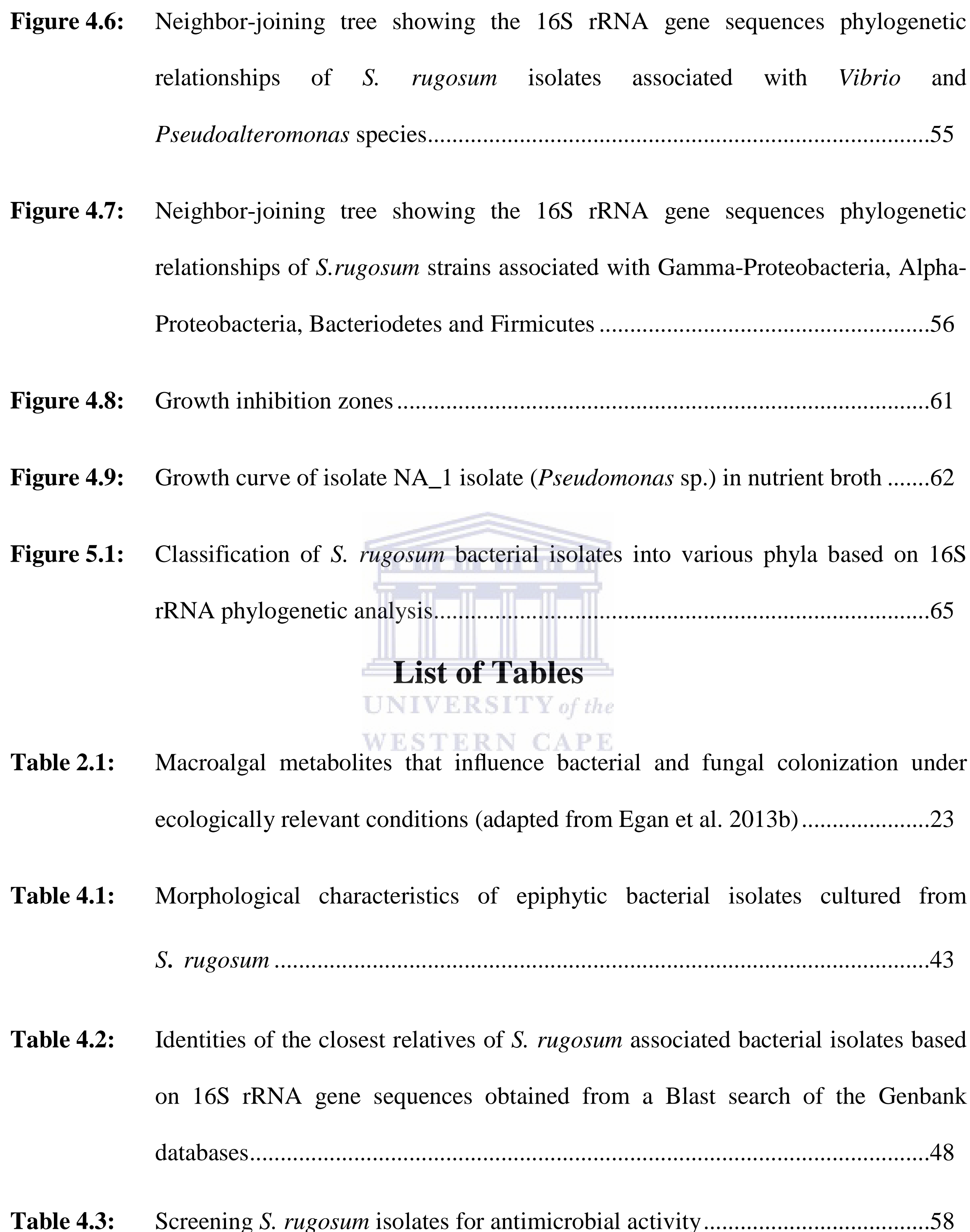

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PUBLICATION

The following article is in preparation for publication based on the research derived from this study:

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PRESENTATIONS

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Assessing the Bacterial Community Associated with the Brown Alga, *Splachnidium rugosum*. *27th Congress of the Phycological Society of Southern Africa, Qolora, South Africa 17-22 June 2012*

Identification and characterization of microorganisms associated with the brown alga *Splachnidium rugosum*. *28th Congress of the Phycological Society of Southern Africa, Cape Town, South Africa, 13-17 January 2014*

ABBREVIATIONS

Amp	ampicillin
bp	base pairs
BLAST	Basic Local Alignment Search Tool
cm^2	centimeter squre
°C	Degree Celsius
ddH_2O	Deionised distilled water
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
EDTA	Ethylenediamine tetra-acetic acid
et	*alet alia* (and other)
EtBr	Ethidium Bromide
G	gram
h/hrs	hour/hours
IPTG	Isopropyl ß-D-thiogalactopyranoside
Kb	Kilo base
LB	Luria Bertani
MA	Marine Agar
MB	Marine Broth
min	minute/s
µ	micro
ml	millilitre
mm	millimeter

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NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
ng	nanogram
nm	nanometer
NSA	Nutrient seawater agar
PCR	Polymerase chain reaction
Rpm	revolutions per minute
sec	seconds
sp.	Species
SOB	Super Optimal Broth
SOC	Super Optimal broth with catabolic repressor
SDS	Sodium Dodecyl Sulphate
TAE	Tris Acetate EDTA
TCBS	Thiosulfate Citrate Bile Sucrose Agar
TSB	Tryptic Soy Broth
Tris	Tris-hydroxymethyl-aminomethane
UV	Ultraviolet
v/v	volume per volume
V	Volts
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
x*g*	Centrifugal force

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Chapter 1

General Introduction and Project Aims

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The surfaces of marine macroalgae host huge bacterial populations consisting of unidentified bacterial species, genera and even orders (Goecke et al. 2010; Goecke et al. 2013). Little is known about the number, population composition or symbiotic interactions which exist between marine macroalgae and their surface associated microorganisms (Boyd et al. 1999b). A range of symbiotic, pathological and opportunistic interactions are reviewed in Chapter 2. The epiphytic bacterial communities associated with macroalgae are generally highly specific (Goecke et al. 2010). It has been suggested that bacterial communities protect the surface of the macroalgae against biofouling - the undesirable accumulation of micro- and microorganisms as biofilms on algal surfaces, by producing bioactive molecules (Egan et al. 2000), such as antimicrobial compounds (Penesyan et al. 2009). Marine microorganisms provide a novel source of bioactives, however, the inability to culture most marine microorganisms severely limits the bio-discovery of these compounds (Villarreal-Gomez et al. 2010). While it is possible to access genetic information using high-throughput DNA sequencing technologies, it is essential to culture the algal associated microbial communities for full taxonomic and physiological characterization.

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Marine macroalgae lack a cell-based, adaptive immune response, but have defensive capabilities such as the production of bioactive compounds to protect itself from bacterial pathogens, grazers, and biofouling agents (Goecke et al. 2010; Chapter 2). Macroalgae may also control bacterial colonization by interfering with the bacterial quorum sensing (QS) system that regulates bacterial cell-to-cell communication (Goecke et al. 2010; Chapter 2). Furthermore, algae have non-specific defence responses against bacterial pathogens similar to oxidative burst or the hyper sensitivity responses of highly developed plants (Weinberger 2007). Specific interactions

between macroalgae and their associated bacteria are reviewed in Chapter 2. Bacterial associations have been shown to affect growth and development of many algal species (Provasoli and Pintner 1972; Fries 1975; Provasoli and Pintner1980; Tatewaki et al. 1983; Marshall et al. 2006; Goecke et al. 2010), and may positively influence algal growth and provide essential nutrients and growth factors (Armstrong et al. 2001; Croft et al. 2005, 2006; Dimitrevs et al. 2006; Singh et al. 2011). Epiphytic bacterial communities can have a significant impact on the algal host's normal function and by extension, on the ecology of the habitats in which they exist.

The increasing use of macroalgae and their products enforces the need to understand the nature and severity of diseases that can be expected in macroalgae mariculture (Apt 1984). The development of an appropriate approach may provide adequate and improved protection of macroalgae in order to lower the risks of commercialization (Park et al. 2006). From this point of view, there is an urgent need to identify seaweed-associated pathogenic- and decomposing bacteria (Goecke et al. 2010). Bacterial epiphytes are furthermore an important source of secondary metabolites with multiple biological activities, including antimicrobial activities (Armstrong et al. 2001; Penesyan et al. 2009). Cultivation-based studies may provide information on the physiological characteristic of the organisms associated with algae, result in the description of new bacterial species and allow for the bioprospecting for microorganisms of biotechnological interest (Zengler et al. 2002; Joint et al. 2010).

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Splachnidium rugosum is a species of brown algae (Phylum: Phaeophyta) that grows exclusively in the Southern Hemisphere along the temperate shores of South Africa, New Zealand, Australia, and the Juan Fernandez Islands (Skottsberg 1920). This species is characterized by a sporophytic

stage of which the thallus has long, coarse mucus filled branches. *S. rugosum* produces fucoidan, a sulfated polysaccharide exhibiting diverse biological activities such as antitumor properties (Ermakova et al. 2011; Vishchuk et al. 2011). This thesis presents the first study on the microbiome of *S. rugosum* (Chapter 4). A culture dependent approach was followed as it may lead to the discovery of new species and provide an understanding into the physiological aspects of *S. rugosum* associated bacteria. The main objective of this study was the isolation and phylogenetic identification of microorganisms associated with the surface of *S. rugosum* collected from the Western Cape, South Africa using different culture media and to screen the identified microorganisms for antimicrobial activity. Project aims are summarized below:

I Isolation and morphological characterization of bacteria associated with the surface of *S. rugosum* using a range of selective marine media including marine 2216 agar (MA), nutrient seawater agar (NSA), thiosulfate-citrate-bile-salts-sucrose (TCBS) agar and nutrient agar (NA)

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II Phylogenetic analysis of the 16S rRNA gene of bacterial isolates

III Screening of bacterial isolates for antimicrobial activity against a range of bacterial pathogens

Chapter 2

Bacterial communities associated with marine macroalgae

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2.1 Marine macroalgae

Marine macroalgae or seaweeds are multi-cellular, autotrophic marine eukaryotes. They are distinguished from terrestrial plants by lacking roots, leafy shoots and vascular tissues (Graham and Wilcox 2000). Macroalgae constitute a major structural component of shallow rocky habitats in temperate waters on coastal ocean communities. They are dominant habitat formers of subtidal and intertidal temperate rocky coasts, commonly growing attached to solid substrates such as rocks (epilithic) and the surfaces of other organisms (epiphytic) (Price 1990). Macroalgae are important ecosystem engineers (Jones et al. 1994) as they support a diverse array of organisms not just as a source of food, but also at the ecosystem level as habitat formers and primary producers (Graham and Wilcox 2000). They also provide wide-ranging settling substrata for epibionts such as hydroids (Fraschetti et al. 2006), as well as a protective environment for the development of many invertebrate species, including prawns and crabs (Bulleri et al. 2002). The importance of macroalgae in coastal marine ecosystems is emphasized by the huge decline in local biodiversity due to the loss of macroalgal species in coastal environments (Schiel 2006). From a commercial perspective, macroalgal aquaculture has increased over the last few years, in particular for the Asian food market and as feed stocks for biofuel production (Borines et al. 2011).

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2.1.1 Brown algae

The brown algae (Phaeophyta) constitute the dominant macroalgae inhabiting intertidal and subtidal regions in rocky coastal ecosystems, and they display many interesting adaptations to these harsh environments (Norton et al. 1996). The majority of brown algae occur in the marine environment and only a few of the 265 genera and 1500-1200 species of brown algae exist in freshwater habitats (Clayton, 1990; Norton et al. 1996). Brown algae are especially diverse and abundant in temperate regions where species diversity is generally high (Clayton, 1990; Dawes, 1998).

Brown algae vary in morphology and can range in size from small filamentous algae such as *Ectocarpus*, to huge subtidal kelps such as *Macrocystis*, which can grow at depths greater than 60 m (Clayton, 1999; Graham et al. 2007). They are also one of only a small number of eukaryotic lineages that have evolved complex multicellularity (Cock et al. 2010). The evolution of multicellularity in this lineage is correlated with the existence of a rich array of signal transduction genes which help this organism to survive within highly variable tidal environments (Cock et al. 2010).

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2.1.1.1 *Splachnidium rugosum* and its microbiome

Splachnidium rugosum (Linnaeus) Greville is a monotypic genus in the family *Splachnidiaceae* (Greville 1830). It is a marine brown macroalgae that is most commonly found attached to rocky substrata in the mid to low intertidal zone where light is frequently the limiting resource (Clayton 1985). *S. rugosum* can be distinguished from other species of brown algae by having spotted,

elongated, cylindrical branches that resemble withered, callused fingers, filled with clear viscous mucilage (Figure 2.1). This mucus enables *S. rugosum* to withstand a high degree of desiccation stress experienced within the mid-intertidal zone.

Figure 2.1: Wild sporophytes of *Splachnidium rugosum* at Rooi Els, Western Cape, South Africa (-34° 18' 5.0004", +18° 48' 59.0004) during low tide in winter 2012. (Photograph was taken by Illana Ackerman).

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S. rugosum occurs exclusively in the southern hemisphere on temperate shores of South Africa, the Juan Fernandez Islands (Skottsberg 1920), Australia, New Zealand and on some of the northern sub-Antarctic islands (Womersley 1987) (Figure 2.2). Bacterial communities associated with *S. rugosum*, their ecological roles and interactions with algae or other organisms, and their potential as a source of bioactive compounds remain largely unknown. Moreover, *S. rugosum* is a known producer of fucoidan, a polysaccharide with a broad range of biological activities (Miller et al. 1996). The isolation of epiphytic bacteria from *S. rugosum* with potential fucoidan

hydrolyzing properties would be useful for the preparation of biologically active low molecular weight fucans in future studies.

Figure 2.2: Distribution of *S. rugosum* (highlighted in red) along the west coast of southern Africa, the southern coast of Australia, Tasmaina and New Zealand.

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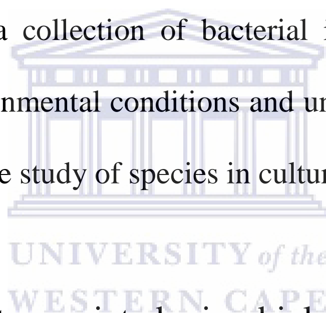
2.2. Microbial ecology: the study of microbial communities

Microbial ecology, defined as the study of the structure and physiology of microbial communities in their natural environments, has advanced considerably in recent years (Kowalchuk et al. 2007). The field of microbial ecology has previously been limited by the technical challenges involved in the observation and description of communities of microscopic cells *in situ* (Horner-Devine and Bohannan 2006). Additionally, the vast majority of microorganisms in the environment have thus far escaped cultivation (Handelsman 2004), preventing the analysis of metabolism and physiology within a laboratory setting. The

application of 16S rRNA gene amplification and sequencing to environmental samples (Schmidt et al.1991) has allowed microbiologists to unveil the enormous microbial diversity that exists within the environment (Curtis et al. 2002). Recent advances in the field of metagenomics has furthermore allowed microbial ecologists unequalled access to the metabolic and functional potential of microbial communities through the sequencing and analysis of whole community DNA (Burke et al. 2011). Metagenomics is the application of modern genomics to study microbial communities in their natural environments, bypassing the need for isolation and lab cultivation of individual species (Chen and Pachter 2005). However, much of the understanding of the functioning of bacterial communities has been derived from culturable populations. For experimental microbial ecology, a collection of bacterial isolates is essential. Expression of genes is often dependent on environmental conditions and understanding the bacterial behaviour in an ecological context requires the study of species in culture.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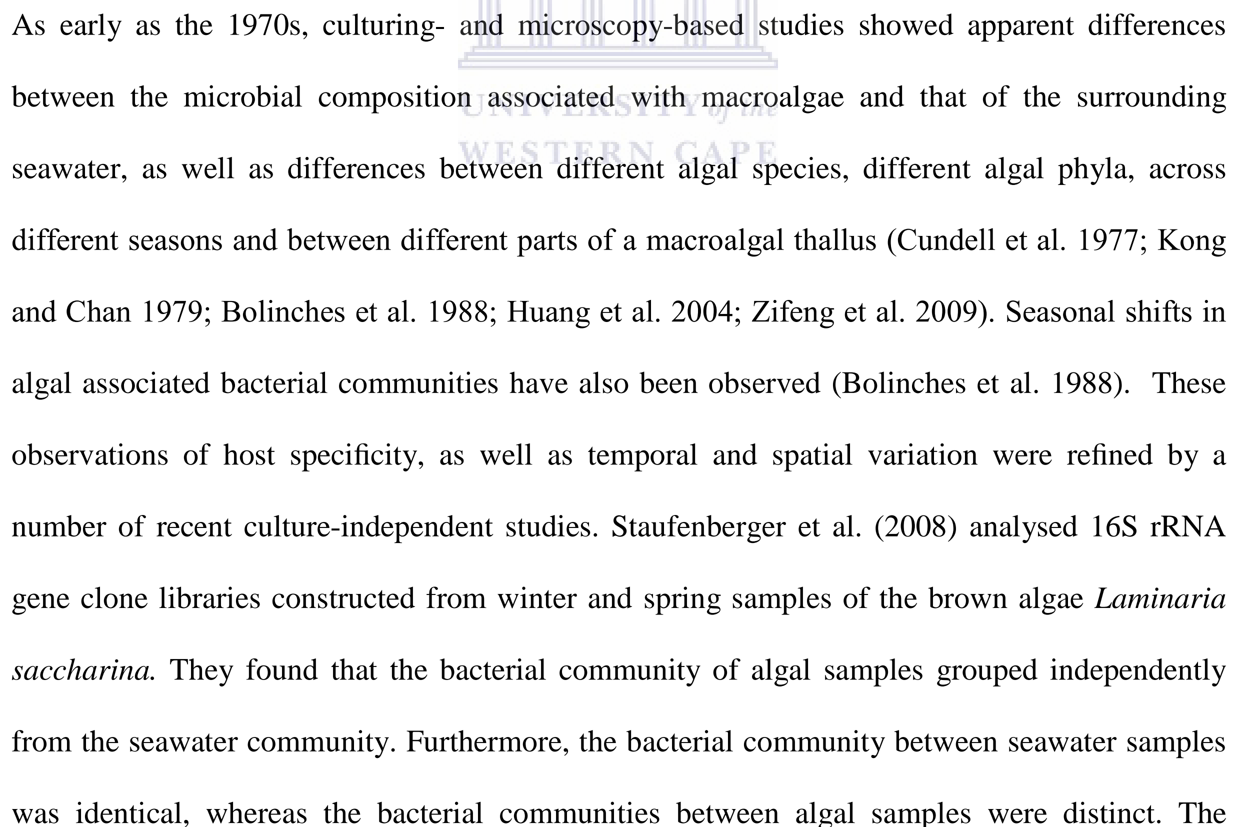
2.3 Marine macroalgae host associated microbial communities

The surfaces of marine macroalgae are colonized by diverse epiphytic microbial communities (Corre and Prieur 1990; Kong and Chan 1979; Mazure and Field 1980; Penesyan et al. 2009) dominated by bacteria from several taxonomic groups (Staufenberger et al. 2008; Tujula et al. 2010). Early studies of cultured isolates from marine macroalgae have reported the widespread presence of epiphytes belonging to the genera *Vibrio*, *Pseudomonas*, *Flavobacteria* and *Achromobacter* (Berland et al. 1969; Chan and McManus 1969; Kong and Chan 1979). While the epiphytic bacterial communities are often highly specific (Goecke et al. 2010), community structure can change rapidly due to biotic and abiotic factors. For instance, Ivanova et al. (2002) have reported a change in the microbial composition on the surface of *Fucus evanescens* from a

highly diverse bacterial community to a community dominated by only two Gamma-Proteobacterial lineages in response to nutrient enrichment. The composition of algal-microbial communities is also influenced by environmental factors such as temperature changes. For example, the surface of *Laminaria saccharina* was dominated by psychotropic bacteria during winter and by mesophilic bacteria during summer (Laycock 1974). The algal-bacterial community composition may furthermore be drastically altered when algae are exposed to biotic stress such as pathogen attack (Beleneva and Zhukova 2006). These studies suggest that the physiological and biochemical characteristics of macroalgae are a key determinant in the composition of the adhering microbial community. The composition of bacterial community structure may change based on the environmental conditions that the algae encounter.



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As early as the 1970s, culturing- and microscopy-based studies showed apparent differences between the microbial composition associated with macroalgae and that of the surrounding seawater, as well as differences between different algal species, different algal phyla, across different seasons and between different parts of a macroalgal thallus (Cundell et al. 1977; Kong and Chan 1979; Bolinches et al. 1988; Huang et al. 2004; Zifeng et al. 2009). Seasonal shifts in algal associated bacterial communities have also been observed (Bolinches et al. 1988). These observations of host specificity, as well as temporal and spatial variation were refined by a number of recent culture-independent studies. Staufenberger et al. (2008) analysed 16S rRNA gene clone libraries constructed from winter and spring samples of the brown algae *Laminaria saccharina.* They found that the bacterial community of algal samples grouped independently from the seawater community. Furthermore, the bacterial community between seawater samples was identical, whereas the bacterial communities between algal samples were distinct. The

bacterial community from different parts of the algal thallus can also be highly specific, with the younger thallus (recently growing meristem parts) having a more specific microbial population than the older phyloid parts. A more recent phylogenetic study revealed that the bacterial communities on the green algae *Ulva australis* was extremely variable between algal samples at the species level and nearly totally distinct from the planktonic seawater community (Burke et al. 2011). Similarly, Lachnit et al. (2009) found that bacterial communities from the same species of macroalgae, sourced from different habitats, were more similar than those from a different species inhabiting the same ecological niche. Their sample group consisted of six marine algal species (three brown, two red, and one green) from the Baltic and North Sea. They concluded that different species of marine macroalgae existing in the same environment carry different bacterial communities, varying in quantity and composition.

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Some microbes are found consistently as epiphytes. For example, the variability and abundance of epiphytic bacterial community associated with *U. australis* was investigated with two molecular techniques, denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (CARD-FISH). These results illustrated that members of the Alpha-Proteobacteria and the Bacteroidetes were a stable part of the associated bacterial community (Tujula et al. 2010). Numerous other studies agreed that bacterial communities on macrolagal surfaces are host-specific and proposed that marine macroalgae may control their specific epiphytic bacterial communities (Bengtsson et al. 2010; Lachnit et al. 2011; Tujula et al. 2010). Such host specific relationships are distinct from the seawater community and are likely to apply to other marine eukaryotic organisms such as marine sponges (Fieseler et al. 2004; Li et al. 2006; Taylor et al.

2007), corals (Rosenberg et al. 2007) and even terrestrial plants which are known to harbour distinct and specific microbial communities (Berg and Smalla 2009; Whipps et a. 2008).

Studies on bacterial–macroalgal associations revealed a number of new bacterial taxa (species, genera and even orders), demonstrating that marine macroalgae represent an important habitat and interesting source for the discovery of novel bacteria (Hollants et al. 2012). Goecke et al. (2010) have reported on more than 50 novel bacterial species originally isolated from macroalgae. Several of the newly described bacterial species such as *Cellulophaga fucicola* (phylum Bacteroidetes), *Leucothrix mucor* (Gamma-Proteobacteria), *Pseudoalteromonas elyakovii* (Gamma-Proteobacteria), *Tenacibaculum amylolyticum* (Bacteroidetes) and *Zobellia galactanovorans* (Bacteroidetes) have been isolated from marine macroalgae. These include *Fucus serratus* (Johansen et al. 1999), *Ulva lactuca* (Bland and Brock 1973), *Laminaria japonica* (Sawabe et al. 2000), *Avrainvillea Riukiuensis* (Suzuki et al. 2001) and *Delesseria sanguine* (Barbeyron et al. 2001), also reviewed in this paper (Hollants et al. 2012). A recent review described 101 new bacterial species from the analysis of 16S rRNA gene sequences of bacteria isolated from algal sources available on GenBank (Goecke et al. 2013). Many of these algal sources were not specified (unidentified algae), which indicates that there is definitely a great proportion of algal species that have not been studied. They found that 81% of newly described taxa came from macroalgal sources (Figure 2.3). Phylogenetic analysis showed that the bacterial species belonged to two major groups, Bacteroidetes and Proteobacteria. Fewer isolates belonging to the phyla Firmicutes, Actinobacteria, Verrucomicrobia and Planctomycetes were also present.

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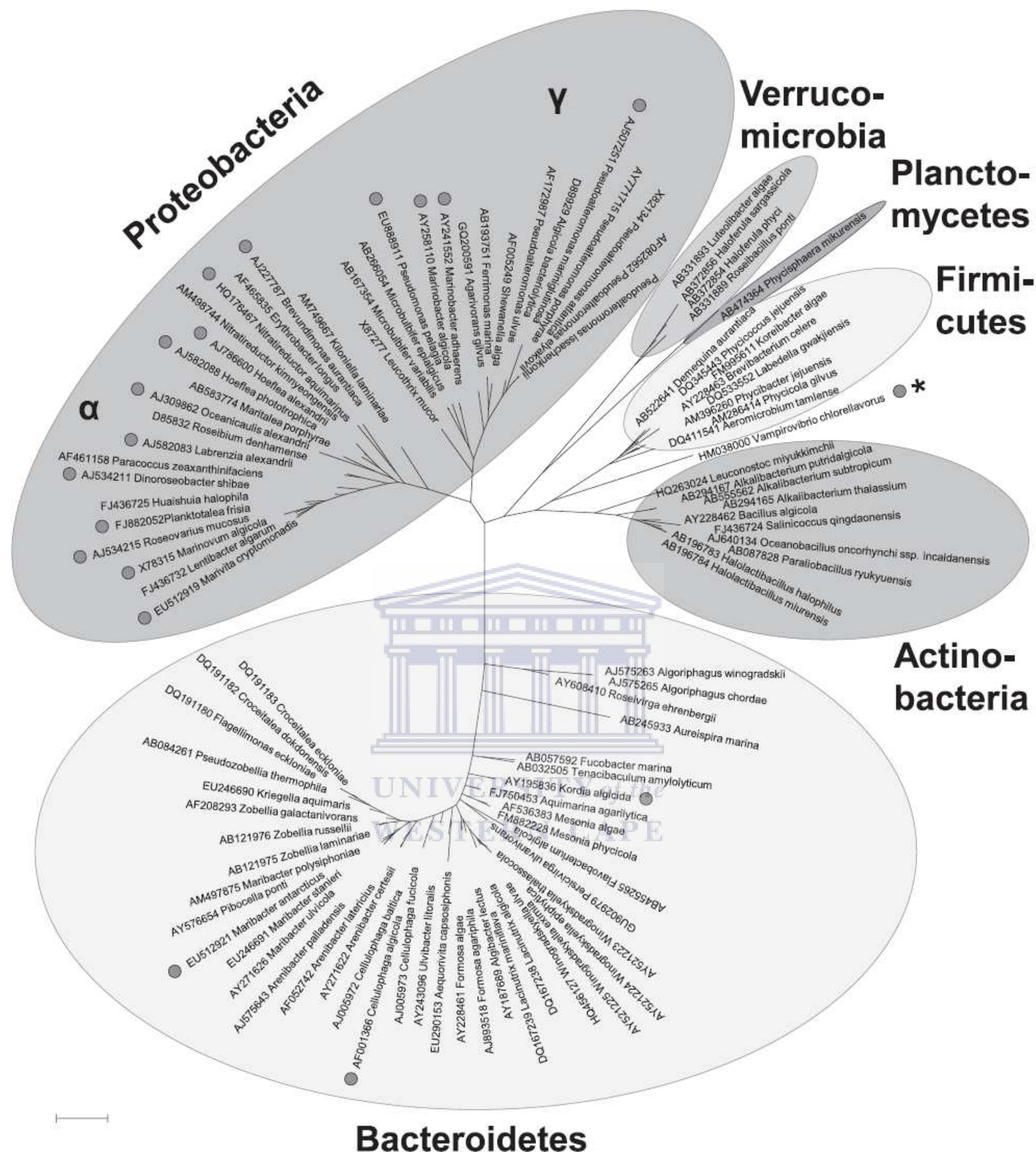
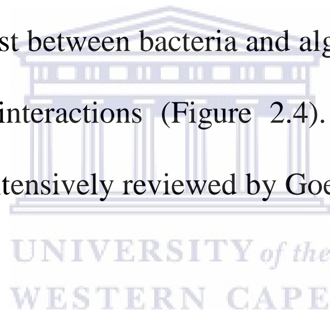


Figure 2.3: Maximum Likelihood phylogenetic tree of the 16S rRNA gene sequences from newly described bacterial species isolated from algae. Six bacterial phyla were identified: Proteobacteria with representatives of Alpha- (α) and Gamma (ϒ)-Proteobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes, Firmicutes, and Actinobacteria. One bacterial taxon (*Vampirovibrio*) described as a member of the Proteobacteria needs further taxonomical gene sequence revision (marked with an asterisk). Bacteria described from microalgal sources are marked with a grey circle. The scale bar indicates 0.1 changes per nucleotide. (Figure was taken from Goecke et al. 2013).

2.4 Macroalgal-bacterial interaction

Studies of surfaces-associated microbial communities have provided a great deal of knowledge concerning chemical interactions with their host and between members of the microbial community. Interactions between microbes and their hosts run the full spectrum from symbiotic to commensal to pathogenic (Ulvestad 2009). Beneficial symbiotic interactions between macroalgae and bacteria include the production of organic compounds by algae which are utilized by bacteria (Brock and Clyne 1984; Coveney and Wetzel 1989). Bacteria mineralize these organic substrates and provide the algae with carbon dioxide, minerals, vitamins and growth factors (Armstrong et al. 2001; Croft et al. 2005, 2006; Dimitrevs et al. 2006; Singh et al. 2011). The relationships which exist between bacteria and algae are multifaceted and can include both beneficial and detrimental interactions (Figure 2.4). The variety and nature of these chemical interactions have been extensively reviewed by Goecke et al. (2010) and Hollants et al. (2012).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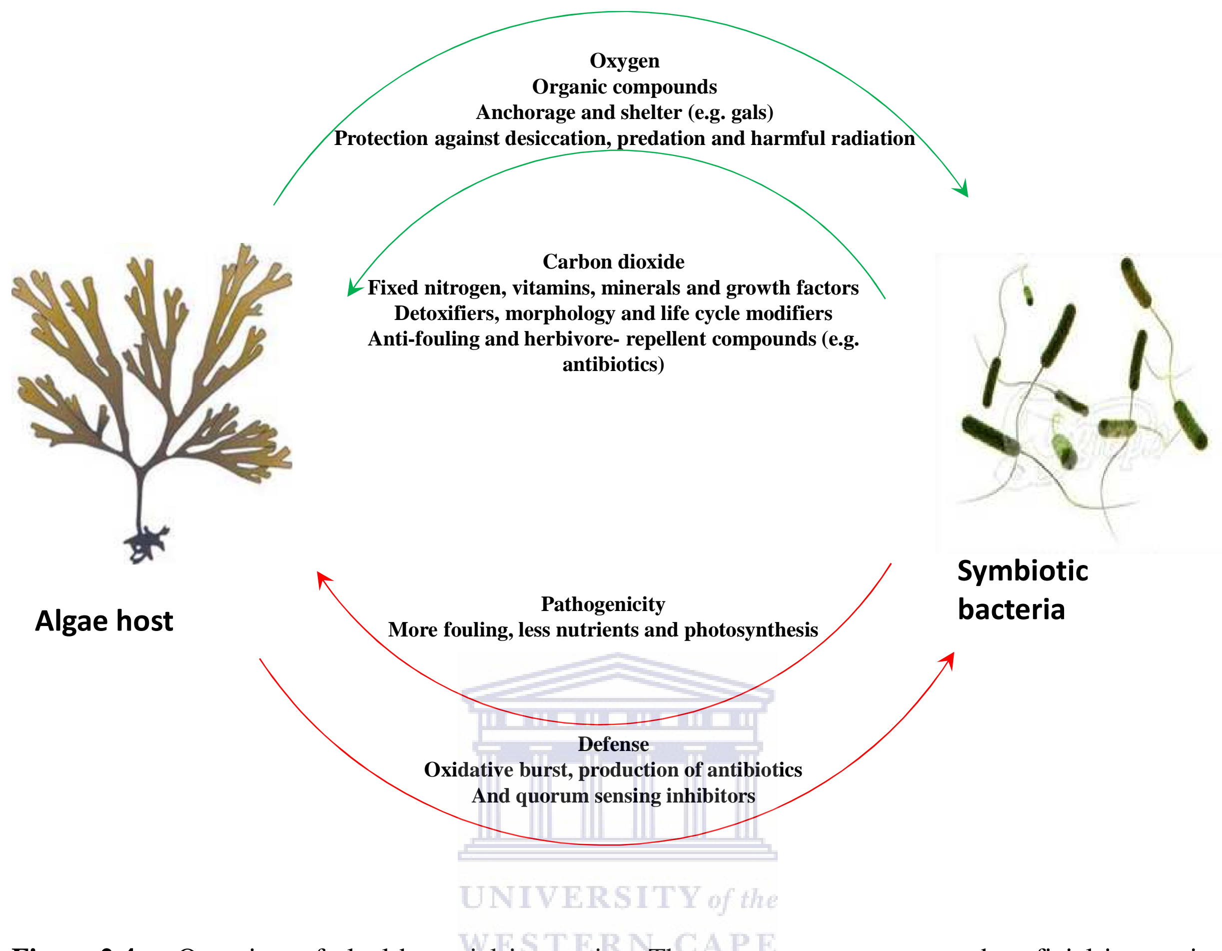

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Figure 2.4: Overview of algal-bacterial interaction. The green arrows represent beneficial interaction while the red lines represent determinant interactions between algae and bacteria. (Adapted from Hollants et al. 2012).

Several studies have shown that algal-associated bacteria are important sources of fixed nitrogen and detoxifying compounds (Chisholm et al. 1996; Riquelme et al. 1997; Goecke et al. 2010). In addition to nutritional and growth promoting effects, bacteria may also shape the morphology and life cycle of their algal host. Bacterial influences on morphogenesis have been reported in marine foliaceous green macroalgae such as *Ulvaceae* and *Monostromaceae,* and in the absence of bacteria these algae lose their typical morphology (Fries 1975; Provasoli and Pintner1980;

Tatewakiet al. 1983; Marshall et al. 2006). Similar phenomenon was also demonstrated in the red macroalgae *Dasya pedicellata* and *Polysiphonia urceolata* (Provasoli and Pintner 1972). The induction of algal morphogenesis in *Ulvaceae* and *Monostromaceae* has been shown to be controlled by a limited group of bacteria belonging the Bacteroidetes phylum, specifically *Cytophaga* and *Flavobacterium* species (Nakanishi et al. 1999; Matsuo et al. 2003). Morphogenesis effects on macroalgae have also been reported for members of the genera *Caulobacter, Vibrio, Pseudomonas, Halomonas* and *Escherichia*, as well as for Gram-positive cocci (Nakanishi et al. 1996; Marshall et al. 2006). Thallusin was the first compound identified from algae associated bacteria responsible for the induction of morphogenesis in *Monostroma* species (Matsuo et al. 2005). Thallusin demonstrates an essential symbiotic chemical interaction between macroalgae and epiphytic bacteria in the marine environment (Matsuo et al. 2005; Goecke et al. 2010). Other secondary metabolites identified include signaling and quorum sensing (QS) molecules which play a role in the completion of the algal life cycle, algal spore release and germination (Matsuo et al. 2005; Weinberger et al. 2007; Joint et al. 2007; Goecke et al. 2010). *Pseudoalteromonas porphyrae,* isolated from brown algae *Laminaria japonica* in the Sea of Japan, has been shown to improve algal growth through the production of catalase (Dimitrieva et al. 2006). It is also suggested that these epiphytic bacteria provide benefits to their host such as protection from biofouling (Egan et al. 2000; Armstrong et al. 2001; Dobretsov and Qian 2002; Rao et al. 2007). Indeed, numerous epiphytic bacteria associated with algae produce bioactive compounds which protect the algal surfaces from biofouling (Egan et al. 2000; Dobretsov and Qian 2002; Franks et al. 2006; Penesyan et al. 2009; Wiese et al. 2009). Moreover, antimicrobial compounds and QS inhibitors produced by several epiphytic bacteria act in concert with algae-derived metabolites to protect the algae surface from pathogens,

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herbivores and fouling organisms (Boyd et al. 1999b; Egan et al. 2000; Armsrong et al 2001; Rao et al. 2007; Wiese et al. 2009; Goecke et al. 2010).

Conversely, algal- bacterial interactions can also be detrimental. Some epiphytic bacteria affect the algae in a harmful manner by decomposing cell materials, like alginate, laminaran or mannitol (Dimitrieva and Dimitriev 1996; Ivanova et al. 2003; Sawabe et al. 1998b). Besides being key players in nutrient recycling processes, bacteria are also potential pathogens as they can damage algal tissue and provide entrance sites for pathogenic and harmful opportunistic bacteria (Craigie et al. 1992; Correa and McLachlan, 1994; Craigie and Correa, 1996; Wang et al. 2008). Algal cell wall degrading bacteria cause diseases and include *Alteromonas*, *Flavobacterium, Psudoalteromonas, Pseudomonas, Vibrio* and *Zobellia* species (Wang et al. 2006a; Sawabe et al. 1998a; Vairappan et al. 2001). For example, *Vibrio* species have been reported to cause disease in *Porphyra* and *Laminaria* fronds, and are considered opportunistic pathogens (Wang et al. 2008). Pathogenic bacteria can cause severe degradation of algal host cells wall and lead to seaweed mortality, causing major financial losses to seaweed mariculture (Goecke et al. 2010). From this point of view, there is an urgent need to identify seaweed-associated pathogenic and decomposing bacteria (Goecke et al. 2010). On the other hand, bacterial epiphytes represent an important source of potentially novel secondary metabolites with multiple biological activities including antimicrobial activities (Armstrong et al. 2001; Penesyan et al. 2009). Thus, if we are to understand the complex interactions between macroalgal hosts and their associated bacteria both the beneficial and harmful interactions need to be investigated.

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2.5 Antimicrobial activities of macroalgae associated bacteria

Antimicrobial activity is ubiquitous among alga-associated bacteria. Wiese et al. (2009) observed that approximately 50% of a total of 210 isolates of an epiphytic bacterial community of *Laminaria saccharina* (harvested from the Baltic Sea, Germany) exhibited antimicrobial activity against at least one test microorganism from a panel comprised of Gram-negative and Gram-positive bacteria. Burgess et al. (1999) showed that 35% of bacterial isolates from various species of macroalgae and invertebrates in Scottish waters display antimicrobial activity. While Boyd et al. (1999a) demonstrated that 21% of the surface-associated bacteria from a total of 280 strains isolated from various species of macroalgae (*Fucus serratus, Himanthalia elongata, Laminaria digitata, Palmaria palmata, Corallina officinalis, Codium fragile* subsp. *tomentosoides* and *Ulva lactuca*) in Scottish waters produced antimicrobial substances. From nine brown macroalgae, 20% of the 116 bacterial isolates produced antimicrobial substances, as did 33% of the 92 bacterial isolates from nine red macroalgae collected from Japanese waters of the Pacific Ocean (Kanagasabhapathy et al. 2006, 2008). Penesyan et al. (2009) obtained 325 bacterial isolates from the surface of *Delisea pulchra* and *Ulva australis* in Australia and 12% of the isolates showed antimicrobial activity. Theses antimicrobial activities, associated with bacteria isolated from algae, may represent a largely underexplored source of new antimicrobial compounds that would be a great interest in the search for potential new drug candidates (Penesyan et al. 2009). Antimicrobial isolates from macroalgae have been phylogenetically assigned to various genera including *Pseudomonas*, *Pseudoalteromonas*, *Stenotrophomonas*, *Vibrio*, *Aeromonas*, *Shewanella*, *Streptomyces* and *Bacillus* (Wiese et al. 2009). *Bacillus* and *Pseudoalteromonas* are widespread epiphytes on marine macroalgal surfaces (Wang et al. 2008) and numerous isolates have been implicated in the production of biologically active substances

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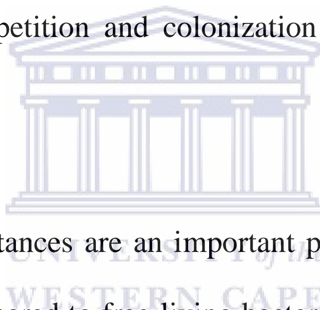
(Holmström and Kjelleberg 1999). For example, some epiphytic strains of *Pseudoalteromonas* isolated from *Ulva lactuca* were able to inhibit the growth of diverse bacteria and fungi (Egan et al. 2000). Similarly, *Pseudoalteromonas tunicate* was able to prevent biofouling by growth inhibition of other surface-associated microorganisms (Goecke et al. 2010; Rao et al. 2007) and was found to produce at least five target-specific compounds (Goecke et al. 2010). One of these compounds, violacein, is a purple pigment that inhibits protozoan grazing (Matz et al. 2008). This chemical arsenal has been shown to be important for the survival of *P. tunicate* in its highly competitive marine surface environment (Rao et al. 2007). Production of an array of active compounds against a range of target organisms is a characteristic feature of these bacteria and may mainly encourage their competition and colonization of algal surfaces (Holmström and Kjelleberg 1999).



Bacteria producing antibiotic substances are an important part of bacterial communities on the surfaces of marine organisms compared to free-living bacterial communities (Kanagasabhapathy et al. 2008; Mearns-Spragg et al. 1998; Zheng et al. 2005). However, we still have a long way to go in improving our understanding of how bacteria and their macroalgae hosts interact to provide host protection, and knowing what sort of compounds they may produce under the multifactorial natural conditions *in situ* (Bode et al. 2002). For example, Okazaki et al. (1975) have shown that a marine actinomycete (SS-228) was able to produce an antibiotic compound only when algae (*Laminaria* species) were added to the growth media. Inhibitory activities against other epiphytic bacteria are of great significance in microhabitats such as on algal surfaces, where competition for an attachment location is continuous (Lemos et al 1985; Rao et al. 2007).

2.6 Bioactive potential of brown algae and defence mechanism of macroalgae

Brown algae produce fucoidan which is a sulfated polysaccharide (Figure 2.5). Fucoidan structure are extremely diverse and this compound exhibits a wide range of biological activities including anti-tumor (Ermakova et al. 2011; Vishchuk et al. 2011), immunomodulatory (Zaporozhets et al. 2006), anti-bacterial (Shibata et al. 1999), anti-viral (Adhikari et al. 2006), anti-inflammatory (Cumashi et al. 2007), anticoagulant and antithrombotic (Mourao 2004) effects. However, despite the number of bioactivities reported, few products have been developed as commercially useful pharmaceuticals, mainly due to the high molecular weight of fucoidan and challenges associated with standardization of the polysaccharide.

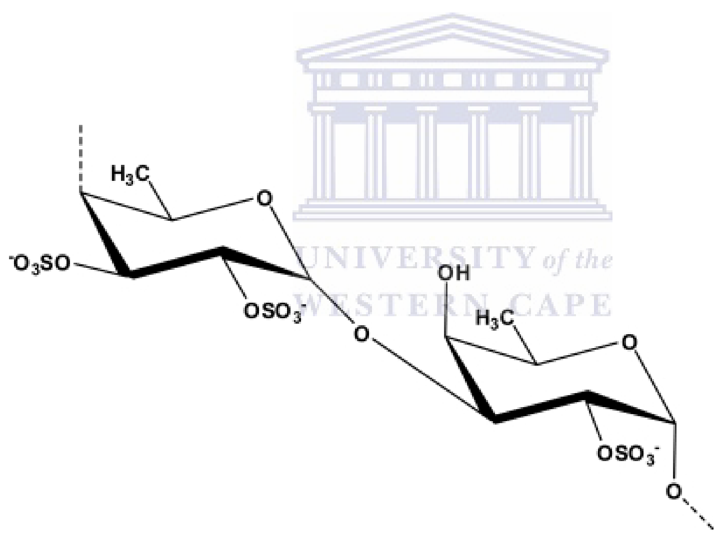


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Figure 2.5: Chemical structure of fucoidan isolated from *Fucus vesiculosus.* (Adapted from Wijesekara et al. 2010).

The search for microorganisms with potential fucoidan hydrolysing properties would be useful for the preparation of biologically active low molecular weight fucans and could play a key role in the structural analysis of fucoidans (Silchenko et al. 2013). Fucoidanases are commonly found

in bacteria associated with the surface of brown macroalgae, and a few studies have successfully purified fucoidanase (Silchenko et al. 2013). It has been shown that representatives of Proteo- and Flavobacteria were promising producers of fucoidan-degrading enzymes (Urvantseva et al. 2006). Descamps et al. (2005) isolated a marine bacterium belonging to the family *Flavobacteriaceae* from marine brown algae (*Pelvetia canaliculata*) which has the ability to degrade sulfated fucans by the secretion of fucoidan endo-hydrolases.

Macroalgae appear to rely on secondary chemical defenses against fouling and pathogenic microorganisms by the production of bioactive secondary metabolites (see review by Goecke et al. 2010). Antifouling activities have been described mainly for the algal families *Rhodophyceae*, *Phaeophyceae* and a few *Chlorophyceae,* and their potential role in mediating macroalgal-bacterial interactions have been broadly reviewed (Goecke et al. 2010; Hollants et al. 2012; Egan et al. 2013b). Macroalgae may release anti-fouling compounds into the surrounding seawater and maintain anti-grazing compounds within the thallus structure (Armstrong et al. 2001). This could be a strong selective factor for epiphytic bacterial colonizers (Egan et al. 2013b). Recent studies investigating the influence of secondary metabolites on bacterial surface colonization revealed that specific macroalgal extracts have marked effects on bacterial biofilm formation and community composition, under both laboratory and field conditions (Sneed and Pohnert 2011). A range of metabolites influencing bacterial community composition and growth on macroalgal hosts have been identified (Table 2.1). For example, the red alga *Delisea pulchra* is known for its ability to defend itself against surface colonization through the production of QS inhibitory molecules (Maximilien et al. 1998). These algae produce a set of biologically active non-toxic secondary metabolites (halogenated furanones) that inhibit acyl homoserine lactone

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(AHL)-driven QS in bacteria. Active synthesis of furanones has been shown to prevent macroalgal colonization by epiphytic bacteria (Manefield et al. 1999). Conversely, antifouling mechanisms of macroalgae do not solely rely on compounds liberated from the algae, but also on secondary metabolites produced by their associated bacteria (Lemos et al. 1985; Egan et al. 2000; Dobretsov and Qian 2002).

Table 2.1: Macroalgal metabolites that influence bacterial and fungal colonization under ecologically relevant conditions (adapted from Egan et al. 2013b).

Macroalga	Algal metabolite	References
Delisea pulchra	Halogenated furanones	Maximilien et al. 1998; Dworjanyn et al. 1999
Lobophora variegata	Cyclic lactone –lobophorolide	Kubanek et al. 2003
Asparagopsis armata	Bromoform	Paul et al. 2006
Asparagopsis armata	Dibromoacetic acid	Paul et al. 2006
Bonnemaisonia hamifera	Polyhalogenated 2-heptanone	Nylund et al. 2008
Callophycus serratus	Bromophycollides	Lane et al. 2009
Fucus vesiculosus	Fucoxanthin	Saha et al. 2011

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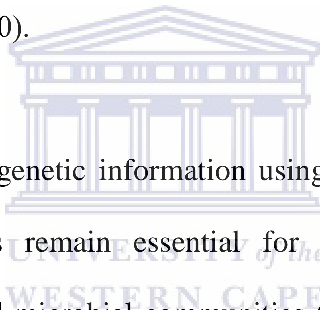
2.7 Culture based studies

Once the foundation stone of microbiology, culturing has been overshadowed by recent advances in molecular technology that enabled rapid identification and characterization of bacteria, often *in situ*. Presently, the majority of representatives of described bacterial phyla is not culturable (under standard conditions) and has been identified using culture-independent methods such as

pyrosequencing which is largely based on16S rRNA gene sequence analysis (Keller and Zengler 2004). The need for improved culturing should however not be neglected. Culture-based studies involve the isolation of microbial species from environmental samples. This remains the only way to study microbial physiology. A proper understanding of microbial physiology allows one to understand the functioning of a microorganism in its original habitat and could lead to the description of new species. Moreover, bacterial culturing is essential for the discovery of bioactives and inhibitory compounds, which may be exploited for antifouling technologies and pharmaceuticals (Armstrong et al. 2001, Penesyan et al. 2009). The advantages associated with culture-based studies from a biotechnological perspective have been reviewed extensively (Zengler et al. 2002; Joint et al 2010).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Although it is possible to access genetic information using high-throughput DNA sequencing technology, culture-based studies remain essential for full taxonomic and physiological characterization of algal associated microbial communities (Joint et al. 2010). However, within the marine environment, the ability to culture microorganisms remains a huge challenge (Joint et al. 2010). One way to increase the proportion of culturable bacteria is to incorporate the use of selective culture media (Pfeffer and Oliver 2003). A range of marine media is available which includes Marine agar, Nutrient Sea Water agar, Nutrient agar and Thiosulfate-Citrate-Bile-salts-Sucrose (TCBS) agar. TCBS agar is generally used to culture *Vibrio* species, while Marine agar is useful to culture Proteobacteria.

The Gamma-Proteobacteria are among the most identified and readily culturable microorganisms from the marine environment (Fuhrman and Hagström 2008). Li et al. (2011) compared culture-

dependent and culture-independent studies, investigating microbial composition on the marine sponge *Gelliodes carnosa.* Gamma-Proteobacteria have been identified by both approaches. Alpha-Proteobacteria and high G+C Gram positive bacteria were only identified as cultured isolates, while Beta-Proteobacteria and Cyanobacteria were only obtained from uncultured clones. Moreover, there was no overlap between the cultured isolates and the uncultured isolates at the species level. This study highlighted the significant variations in higher taxonomic phyla and even at lower phyla (e.g genera and species) when using different molecular techniques.

There has been an increasing focus on the development of new techniques to increase the number of previously uncultured microorganisms that play key roles in the environment, such as those with bioactive potential or pathogenic activity (Nakanishi et al. 1996; Dobretsov and Qian 2002; Wang et al. 2008; Wiese et al. 2009). Techniques include the use of extinction dilution in high-throughput culturing (HTC) (Kaeberlein et al. 2002), diffusion growth chambers (Kaeberlein et al. 2002) and encapsulation of cells in gel micro-droplets (Zengler et al. 2002). Other developments include the use of different gelling agents in the isolation media and the addition of ammonium to seawater-based agar (Joint et al 2010). Ammonium addition has lead to the isolation of a higher proportion of Alpha-Proteobacteria, while variation in medium composition has lead to an increased recovery of other bacterial groups such as Actinobacteria, which are of particular interest for biodiscovery (Joint et al. 2010).

It is worth noting that greatest abundance of culturable marine bacteria have been associated with planktonic organisms and seaweed (Hollant et al. 2013). On the other hand, epiphytic bacteria are more abundant on seaweed surfaces than their planktonic counterparts (Jensen et al. 1996).

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Living surfaces, such as seaweed, may be easier to colonize as they are polysaccharide rich, providing a carbon source for the associated bacteria. Agar, a product derived from seaweed, is commonly used as a gelling agent in microbiological culture media and might provide similar conditions to the surface of algae when supplemented with a low concentration of nutrients. Thus, epiphytic bacteria are thought to have a greater ability for growth on agar than planktonic species which usually inhabit the nutrient poor water column (Jensen et al. 1996).

2.8 16S rRNA gene PCR and sequencing

The 16S ribosomal RNA gene (16S rRNA) is distinct from other prokaryotic genes in that it has a number characteristic which are useful for phylogenetic studies (Dahllof 2002). Firstly, the gene is not subject to horizontal gene transfer (Moyer 2001). Secondly, it is composed of conserved, variable and hyper-variable regions enabling various phylogenetic levels to be resolved (Ludwig and Schleifer 1999). Additionally, the sequence database is the largest for any bacterial gene (Moyer 2001). Polymerase chain reaction (PCR), was discovered in 1986 by Karl Mullis and revolutionized molecular microbiology. Today, PCR is commonly used to amplify copies of 16S rRNA genes from bulk DNA extracted from environmental samples, followed by cloning and sequencing. Numerous universal PCR primers have been designed to amplify a broad spectrum of 16S rRNA genes (Muhling et al. 2008). However, no one set of primers is able to amplify all 16S rRNA genes in a sample (Isenbarger et al. 2008). A significant consideration in 16S rRNA gene sequence analysis is the choice of DNA extraction protocol. High quality DNA free of contaminants is essential, since the presence of minute concentrations of inhibitors, such as phenolics, may inhibit PCR reactions (Wilson 1997). Optimal annealing temperatures and the number of amplification cycles required needs to be optimized for each

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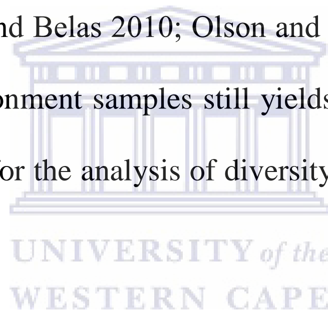
reaction (Suzuki and Giovannoni 1996). There are some limitations associated with the use of the 16S rRNA gene for phylogenetic analysis, such as the bias in primer annealing efficiency with different templates (Li et al. 2011). Many microbial species also carry multiple copies of this gene, and this microheterogeneity can complicate quantification and interpretation of molecular analysis (Dahllof et al. 2000). The conserved nature of the gene may furthermore restrict the number of closely related species that can be differentiated (Dahllof et al. 2000). Despite these limitations, 16S rRNA gene analysis from a variety of environments has lead to the discovery of many novel bacterial species (Li et al. 2006; Li et al. 2011; Wiese et al. 2009), and it is a well established approach used to characterize microbial associations with unicellular algae, corals and sponges (Geng and Belas 2010; Olson and Kellogg 2010). The analysis of 16S rRNA gene sequences from environment samples still yields valuable information, and remains the most commonly used method for the analysis of diversity in bacterial communities (Burke et al. 2011).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To the best of our knowledge, the study presented in this thesis (Chapter 4) is the first to investigate the microbiome of *S. rugosum*. A culture dependent approach was followed as it may lead to the discovery of new species and provide an understanding of physiological aspects of *S. rugosum* associated bacteria.

Chapter 3

Materials and Methods

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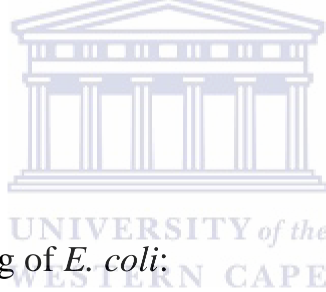
3.1 General laboratory chemicals and reagents

All culture media were supplied by Oxoid Ltd, Biolabs, Merck and Difco. Reagents were supplied by Merck Chemicals and Laboratory Supplies, Sigma-Aldrich Chemical Company or Kimix Chemical and Laboratory Supplies. DNA molecular weight markers and restriction enzyme were supplied by Fermentas and Thermo Scientific.

3.2 Media

All media were prepared prior to the isolation of bacteria associated with the surface of *S. rugosum.*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3.2.1 Media used for culturing of *E. coli*:

i. Luria-Bertani broth (LB; Sambrook and Russell, 2001): 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl

ii. Luria-Bertani agar (LA; Sambrook and Russell, 2001): solid media was prepared by the addition of 1.5% (w/v) bacteriological agar to LB

iii. Super Optimal Broth (SOB; Sambrook and Russell, 2001): 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl and 0.02% (w/v) KCl

iv. Super Optimal Broth with Catabolite repression (SOC; Sambrook and Russell, 2001) was prepared by the addition of filter sterilized 5% (w/v) 2M $MgCl_2$ and 20% (w/v) 1M D-glucose to SOB

Unless indicated otherwise, the pH was adjusted to 7.0 before autoclaving.

3.2.2 Media used for the isolation and cultivation of *S. rugosum* isolates:

i. Thiosulfate Citrate Bile Sucrose Agar (TCBS) were used for the isolation of *Vibrio* species (Pfeffer and Oliver 2003)

ii. Marine 2216 agar (MA, Difco) were used for the isolation of heterotrophic bacteria

iii. Marine 2216 broth (MB, Difco)

iv. Nutrient seawater agar (NSA): 0.3% (w/v) glycerol, 0.3% (v/v) peptone, 0.1% (w/v) yeast extract and 1.5% (w/v) bacteriological agar in filtered seawater

v. Nutrient broth (NB; Sambrook and Russell, 2001): 0.1% (w/v) meat extract, 0.2% (w/v) yeast extract, 0.5% (w/v) peptone and 0.8% (w/v) NaCl

vi. Nutrient agar (NA; Sambrook and Russell, 2001): solid media was prepared by the addition of 1.5% (w/v) bacteriological agar to NB

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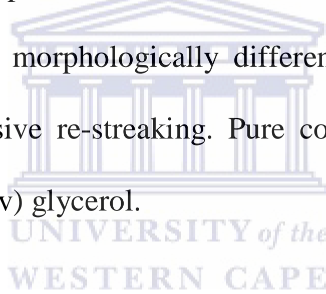
Unless indicated otherwise, the pH was adjusted to 7.0 before autoclaving. TCBS medium was not autoclaved but heated in the microwave until all components were dissolved, cooled and poured into plates.

3.3 Collection of seaweed

S. rugosum sporophytes were collected in winter on the 17th July 2012 during low tide from Rooi Els (-34° 18' 5.0004", +18° 48' 59.0004), Western Cape. The samples were transported on ice to the laboratory and processed immediately after collection.

3.4 Cultivation, isolation and storage of bacteria associated with *S. rugosum*

Seaweed was rinsed repeatedly with sterile seawater in order to remove loosely attached bacteria. Three separate sections ($1cm^2$x$1cm^2$) of the cleaned thallus were swabbed with sterile cotton applicator swabs and streaked in triplicate on different media (TCBS, MA, NA and NSA). After incubation at 25°C for 2-7 days, morphologically different bacteria were selected and pure isolates were obtained by successive re-streaking. Pure colonies were cultivated in MB and cultures stored at -80°C as 15% (v/v) glycerol.



3.5 Preparation of competent *E. coli* GeneHogs strain

Chemically competent cells were prepared according to the method of Sambrook and Russell (2001) with minor modifications. *E. coli* GeneHogs cells [genotype F- *mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ (*ara-leu*) 7697*gal*U*gal*K*rps*L (StrR) *end*A1 *nup*G*fhu*A::IS2 (Invitrogen, USA)] were cultured on LB agar and incubated overnight at 37°C. Pre-culturing was performed by inoculating a single colony into 10 ml LB broth. The culture was incubated overnight at 37°C with shaking (150 rpm) and 500 µl of the overnight culture was transferred into four 500 ml flasks containing 50 ml of LB broth each. Cultures were incubated at

37°C, shaking, until an optical density (OD at 600 nm) of 0.3-0.5 was attained. Flasks were placed on ice for 15 min and the cultures were transferred into 50 ml falcon tubes (SPL Life science). Cells were kept on ice in all subsequent steps. Cultures were centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed in 50 ml ice cold 100 mM $MgCl_2$ and incubated on ice for 1 h. Cells were collected as before and re-suspended in 3 ml ice cold 100 mM $CaCl_2$ containing 20% glycerol. Cultures were stored as 100 µl aliquots in Eppendorf tubes at -80°C.

3.6 Transformation efficiency of competent *E. coli* cells

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Plasmid (pUC 19) was extracted from *E. coli* and 250 ng of uncut plasmid was used as a positive control to determine the efficiency of *E. coli* competent cells. A tube containing 10 µL demineralized water was used as a negative control. Frozen *E. coli* GeneHogs competent cells were thawed on ice for 5 min. Plasmid DNA was added to the positive control tube and thereafter 100 µl of *E. coli* competent cells were heat shocked for 45 sec in a water bath at 42ºC. Tubes were placed immediately on ice for 4-5 min to reduce cell damage and 900 µL of SOC broth (section 3.2.1) was added to each tube. The transformation mixture was incubated for 1 hr at 37ºC with constant agitation at 150 rpm. A 1:10 serial dilution of the uncut DNA control was prepared in triplicate in SOC broth. One hundred microliters of this dilution mix and the negative control were plated onto LB agar plates supplemented with ampicillin (100 µg/ml), 1 M IPTG (isopropyl thio-ß-galactoside,), and X-Gal (20 µg/ml). White colonies were counted after overnight incubation at 37ºC.

The transformation efficiency was determined by using this formula:

TRANSFORMATION EFFICIENCY=

$$\frac{\text{number of transformants (colonies)}}{\text{microgram of plasmid DNA}} \times \frac{\text{final volume at recovery (mL)}}{\text{volume plated (mL)}} =$$

number of transformants per microgram of plasmid DNA

3.7 Genomic DNA extraction and 16S rRNA gene amplification

Colony PCR reactions were conducted for all isolates, except for isolates MA_18 and NA_1. These isolates could not be amplified using the colony PCR method, therefore genomic DNA was extracted according to Sambrook and Russell, (2001). Colony PCR was conducted according to the method used by Bauer et al. (2009). Briefly, one colony was picked and re-suspended in sterile distilled water and genomic DNA isolated by heating the cell suspension for 12 min in a boiling water bath. After cooling on ice, cell debris was removed by centrifugation (14,000 x*g* for 2 min) and 29.5 µl of the cell-free supernatant was used as the DNA template. PCR reactions were conducted in 50 µl containing 2.5 U Dream *Taq* DNA polymerase (Thermo scientific), 0.2 mM dNTPs, 1 × Dream *Taq* Buffer and 0.1 mM of each forward and reverse primer. Amplification of the near complete 16S rRNA gene was performed with the universal forward primer E9f („5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer 1489r (5'-TACCTTGTTACGACTTCA-3') using the following PCR cycling conditions: 5 min at 94°C, 30 sec at 94°C, 30 sec at 56°C, 90 sec at 72°C for 30 cycles and a final step 10 min at 72°C.

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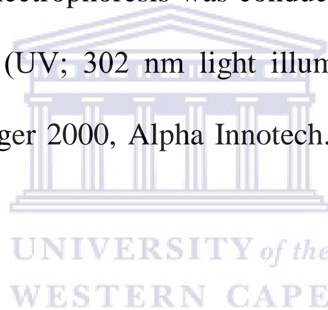
3.8 Agarose gel electrophoresis

PCR products amplified from bacteria associated with *S. rugosum* were separated on an 0.8% (w/v) agarose gel at 75 V according to the method by Sambrook and Russell (2001). 16S rRNA gene fragments with a size of 1.5 kb were expected. Briefly, agarose gels 0.8% (w/v) were prepared in Tris-Acetate EDTA (TAE) buffer (pH 8) containing 0.2% (w/v) Tris-base, 0.5 % (v/v) glacial acetic acid and 1% (v/v) 5M EDTA. Ethidium bromide (0.04 µL/ml) was added to the molten agarose to allow post-electrophoresis visualization of nucleic acids. Sample preparation involved mixing 1x loading buffer (2% (v/v) glycerol and 0.5 mg/ml bromophenol orange) with the DNA samples. Electrophoresis was conducted in 1x TAE buffer at 75 V. Gels were visualized using ultraviolet (UV; 302 nm light illumination) and photographed with a digital imaging system (Alphalmager 2000, Alpha Innotech. USA.) λ DNA digested with *Pst*I was used as a DNA size marker.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3.9 DNA extraction from agarose gels and PCR product purity

DNA extraction from agarose gels was performed by excision of bands which were approximately 1500 bp in size under long wavelength UV light (365 nm). DNA was purified from gel slices using the Nucleospin gel and PCR clean up kit (Machery-Nagel) according to the manufacturer's instructions.The purity of the PCR product was verified by gel electrophoresis (0.8%) (w/v) prior to cloning into the pGEM®-T Easy cloning vector.

3.10 Ligation of PCR product into cloning vector

Ligation was performed using the pGEM ®-T Easy Vector System. The pGEM®-T Easy vector (Figure 3.1) is a linearized vector with T overhangs at the insertion site, containing an ampicillin resistance gene and codes for ß-galacosidase (Promega, USA). The ligation reaction contained 5 µL of 2 × Rapid ligation Buffer, 1µL of T4 DNA ligase and 1 µL of PGEM®-T Easy Vector (50 ng). The PCR product was added to the mixture in 1:3 ratio of vector to insert and deionized water was added to a final volume of 10 µL. The mixture was incubated overnight at 4ºC.

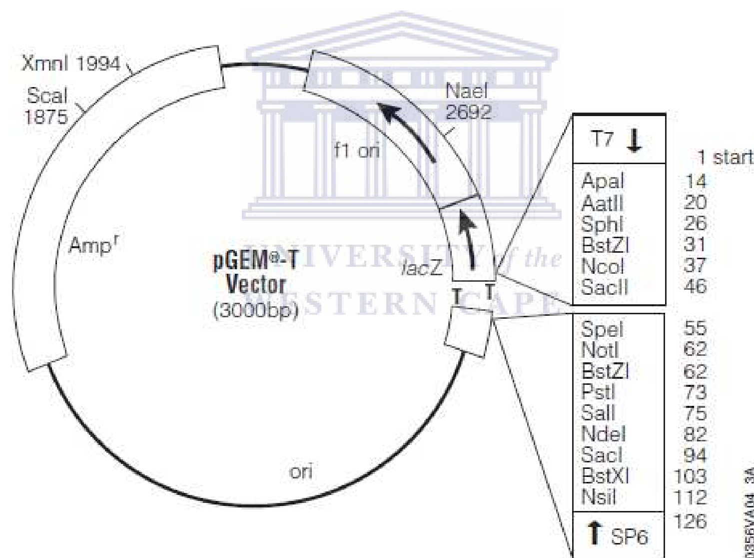

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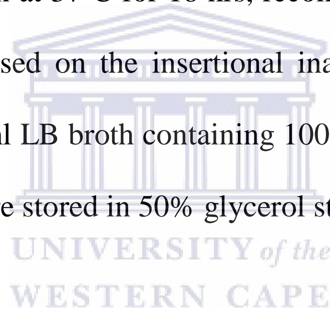
Figure 3.1: PGEM®-T Easy Vector map

3.11 Transformation of competent *E. coli* GeneHogs cells

Recombinant plasmids were transformed into chemically competent *E. coli* GeneHogs cells using heat shock transformation. An Eppendorf tube containing 100 µL of chemically competent cells was removed from -80°C and thawed on ice. Ten microlitres (10 µl) of the ligation mixture (section 3.10) was added to the thawed cells and gently mixed. The mixture was heat shocked in a water bath at 42°C for 45 sec and placed on ice for 4-5 min. After the addition of 890 µL SOC broth, transformation mixtures were incubated for 1 hr at 37°C with constant agitation at 150 rpm and plated onto LB agar plates supplemented with ampicillin (100 µg/ml), IPTG (1 M), and X-Gal (20 µg/ml). After incubation at 37ºC for 18 hrs, recombinant transformants were selected by blue/white colour selection based on the insertional inactivation of the lacZ gene. Single colonies were inoculated into 10 ml LB broth containing 100 µg/ml Amp and incubation at 37ºC at 150 rpm. Overnight cultures were stored in 50% glycerol stocks at -80°C.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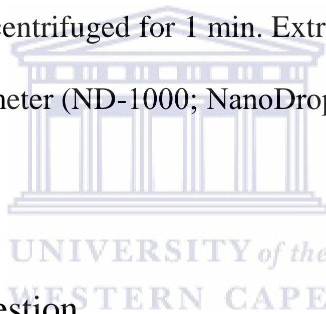
3.12 Plasmid DNA extractions

Plasmid DNA was extracted from overnight cultures using the plasmid mini-prep kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions with slight modifications. An *E. coli* GeneHogs clone harboring the recombinant plasmid pGEM ®-T Easy vector was streaked from a frozen glycerol stock onto the surface of LB agar plates containing Amp (100 µg/ml) and incubated overnight at 37°C. A single bacterial colony was inoculated into 10 ml LB broth containing Amp (100 µg/ml). Following overnight incubation for 18 hrs at 37ºC with constant shaking at 230 rpm, cells were harvested by centrifugation at 13 500 rpm for 90 sec at

4°C. This was repeated twice. The bacterial pellet was re-suspended in 0.25% ml of Buffer P1 and 0.25 ml of Buffer P2 was added to the suspension. Sealed tubes were inverted 4-6 times, 0.35 ml of Buffer N3 was added and mixed thoroughly by inverting 4-6 times. Samples were centrifuged at maximum speed (18 000 x*g*) for 10 min. The supernatant was transferred to a QIAprep spin column, washed with 700 µL of Buffer PB and centrifuged for 1 min.The flow-through from the column was discarded and 0.75 ml of Buffer PE was added to the QIAprep spin column and centrifuged for 1 min. Again the flow-through was discarded and the column was centrifuged for an additional 1 min to remove residual wash buffer. Finally, plasmid DNA was eluted with 50 µL of elution buffer in a sterile 1.5 ml microcentrifuge tube and incubated for 1 min at room temperature and was centrifuged for 1 min. Extracted plasmid DNA was quantitated using the NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, USA).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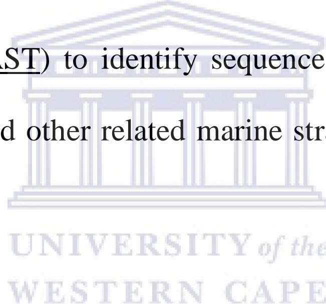
3.13 Restriction enzyme digestion

The presence of the desired 1.5 kb insert (16S rRNA gene) within the recombinant plasmid was confirmed before plasmids were subjected to sequence analysis. Restriction enzyme digestion was performed according to the manufacturer's instructions (Fermentas) with *Eco*RI (Fermentas). Reaction included *Eco*RI buffer (5 mM Tris-HCL, 1 mM MgCl2, 10 mM NaCl, 0.002% Triton x-10, 0.01 mg/ml BSA), 1U restriction enzyme *Eco*RI per 50 ng of amplicon DNA in reaction volumes of 10 µL. Reactions were incubated overnight to allow for complete digestion in a waterbath at 37°C. Restriction enzyme digestion products were separated by gel electrophoresis in 0.8% (w/v) agarose gels.

3.14 Sequencing analysis of the 16S rRNA gene

Recombinant plasmids containing 16S rRNA gene inserts were sequenced using Invitrogen primers M13 Forward (5´CCCAGTCACGACGTTGTAAAACG-3') and M13 Reverse (5´AGCGGATAACAATTTCACACAGG-3´), and analyzed on an ABI PRISM 377 automated DNA sequencer at the Central Analytical Facility at Stellenbosch University. Full-length consensus sequences were assembled using DNAMAN analysis software version 4.15 (LynnonBioSoft). The E9f forward and 1489r reverse primers were removed before alignment using DNAMAN analysis software. Sequences were compared to the 16S rRNA gene sequences in the GenBank database using the advanced Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) to identify sequences with a high degree of similarity. The 16S rRNA gene sequences and other related marine strains were aligned using DNAMAN analysis software.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3.15 Construction of the phylogenetic tree using MEGA5.2

To obtain approximate phylogenetic affiliations for the sequenced isolates, each sequence was subjected to BLAST analysis against the GenBank non-redundant nucleotide database (www.ncbi.nlm.nih.gov/BLAST/). Sequences from this study and closest relatives in GenBank were aligned using MEGA5.2 (Tamura et al. 2011) and the Fast Aligner function (Align by ClustalW) (Thompson et al. 2002). All alignments were manually edited. Neighbour joining (Saitou and Nei 1987) bootstrap-based phylogenetic analysis was conducted in MEGA 5.2 using near full-length (1500 bp) sequences only. Robustness of tree topologies was tested by parsimony-based bootstrap analysis (1000 resamplings, Felstein 1985).

3.16 Screening of *S. rugosum* isolates for antimicrobial activity

S. rugosum isolates were tested for anti-microbial activity against bacterial pathogens including *Escherichia coli* (1699), *Bacillus cereus* (clinical isolate), *Staphylococcus epidermidis* (clinical isolate), *Micrococcus luteus* (clinical isolate) and *Pseudomonas putida* (ATCC27853) using the antibiotic overlay assay technique (Bauer et al. 2005; Fyfe et al. 1984). Individual colonies of each isolate were selected and streaked onto agar plates. Cultures were incubated at 25°C for two to three days. Each test strain was inoculated into TSB and incubated at 37°C overnight except *M. smegmatis* which was incubated at 30°C for two days. One hundred microlitres (100 µl) of each test strain culture was inoculated into 100 ml of sloppy agar (3% Tryptic soy broth, 0.2% agar), and approximately 10 ml of the sloppy agar was poured onto agar plates inoculated with the *S. rugosum* bacterial isolates. The plates were incubated at 30°C for 16 hr. Antimicrobial activity was assessed by the presence of a clearance zone of growth inhibition around the colony of the marine isolate. Inhibition zones were evaluated according to the size of the zone of inhibition. One plus (+) indicated a small zone of inhibition of approximately 2 mm, two pluses (++) indicated a medium zone of inhibition of approximately 6 mm and three pluses (+++) indicated a large zone of inhibition of approximately 10 mm.

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3.17 Growth parameters of antimicrobial producing isolateNA_1

The antimicrobial producing bacterial isolate (NA_1) was grown in NB at 28°C and 147 rpm. Growth and antimicrobial activity were measured over 24 h. Briefly, media (three biological replicates) was inoculated at OD_{600} = 0.1 with a late logarithmic culture and samples were taken hourly. Cell density (OD_{600}) was measured with a spectrophotometer (THERMO BIOmatter 3,

USA) and antibacterial activity was assessed in cell free supernatant after centrifugation (14 000 x*g* for 2 min). Supernatant (10 µl aliquots) were spotted onto the surface of sloppy agar (3% Tryptic soy broth, 0.75% agar) previously seeded with 0.1% (v/v) *Mycobacterium smegmatis* (LR22) culture. The plates were incubated at 30°C for 16 h and inspected for zones of growth inhibition.

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Chapter 4

Results and Discussion

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4.1 Isolation and morphological characterization of bacteria associated with the surface of *Splachnidium rugosum*

Epiphytic microorganisms were isolated from several *Splachnidium rugosum* thalli and cultured between 1-7 days on different growth media. Each isolate was assigned a code depending on the culture media. Forty nine epiphytic bacteria were isolated from *S. rugosum* and sub-cultured to obtain pure cultures. The morphological characteristics of these isolates are presented in Table 4.1. Images showing selected isolates on plates are presented in Figure 4.1. The highest number of isolates was found on marine 2216 agar (MA). Other growth media include nutrient seawater agar (NSA), thiosulfate-citrate-bile-salts-sucrose agar (TCBS) which is highly selective for *Vibrio* species (Pfeffer and Oliver, 2003), and nutrient agar (NA). It is apparent that MA medium was more successful than others for culturing high numbers of bacterial isolates. Gram stain analysis showed that all isolates were Gram-negative, except isolates MA_9 and MA_20 which were Gram-positive. Previous studies have shown that the majority of marine bacteria associated with intertidal seaweeds *Delisea pulchra, Ulva australis* and *Laminaria digitata* are Gram-negative (Penesyan et al. 2009; Salaün et al. 2010). Conversely, a study on microbial communities associated with marine sediment has presented evidence that most bacteria from this environment are Gram-positive (Gontanget al. 2007). Bacterial community composition is clearly influenced by the host environment.

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Table 4.1: Morphological characteristics of epiphytic bacterial isolates cultured from *S. rugosum.*

Bacterial isolates	Colony colour	Colony size	Colony texture	Plate Media	Duration of growth (Days)
TCBS_1	Yellow	Small	Hard	TCBS	1-3
TCBS_2	Yellow	Large	Hard	TCBS	1-2
TCBS_3	Yellow	Large	Hard	TCBS	1-4
TCBS_4	Blue green centre	Medium	Hard	TCBS	1-3
TCBS_5	Blue green centre	Medium	Hard	TCBS	1-2
TCBS_6	Blue green centre	Small	Hard	TCBS	1-2
MA_1	Red	Small	Soft	MA	2-6
MA_2	Cream	Medium	Soft	MA	1-2
MA_3	Cream	Small	Soft	MA	1-3
MA_4	Orange	Small	Soft	MA	1-4
MA_5	Brown	Small	Soft	MA	1-2
MA_6	Cream	Small	Soft	MA	1-2
MA_7	Cream	Small	Soft	MA	1-2
MA_8	Cream	Small	Soft	MA	1-2
MA_9	White	Small	Soft	MA	1-2
MA_10	Brown	Small	Soft	MA	1-2
MA_11	Brown	Small	Soft	MA	1-2
MA_12	Cream	Small	Soft	MA	1-2
MA_13	Cream	Small	Soft	MA	1-2
MA_14	Beige	Small	Soft	MA	1-2
MA_15	Brown	Tiny	Soft	MA	1-2
MA_16	Brown	Small	Soft	MA	1-2
MA_17	Brown	Small	Soft	MA	1-2
MA_18	Brown	Small	Soft	MA	1-2
MA_19	Cream	Small	Soft	MA	1-2
MA_20	White	Small	Soft	MA	1-2
MA_21	Beige	Small	Soft	MA	1-2
MA_22	Brown	Small	Soft	MA	1-2
MA_23	Cream	Small	Soft	MA	1-2
MA_24	Cream	Tiny	Soft	MA	1-2
MA_25	Cream	Small	Soft	MA	1-2
MA_26	Cream	Small	Soft	MA	1-2
MA_27	Cream	Small	Soft	MA	1-2
MA_28	Cream	Small	Soft	MA	1-2
MA_29	Brown	Small	Soft	MA	1-2
NA_1	Lemon	Small	Soft	NA	1-2
NSA_1	Brown	Medium	Soft	NSA	1-2
NSA_2	Bright yellow	Small	Soft	NSA	4-7
NSA_3	Yellow	Small	Soft	NSA	2-3
NSA_4	Yellow	Medium	Soft	NSA	2-3
NSA_5	White	Small	Soft	NSA	2-3
NSA_6	White	Medium	Soft	NSA	2-3
NSA_7	White	Small	Soft	NSA	2-3
NSA_8	Yellow	Small	Soft	NSA	1-2

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NSA_9	Brown	Small	Soft	NSA	1-2
NSA_10	White	Medium	Soft	NSA	2-3
NSA_11	White	Small	Soft	NSA	2-3
NSA_12	White	Small	Soft	NSA	2-3
NSA_13	White	Small	Soft	NSA	1-2

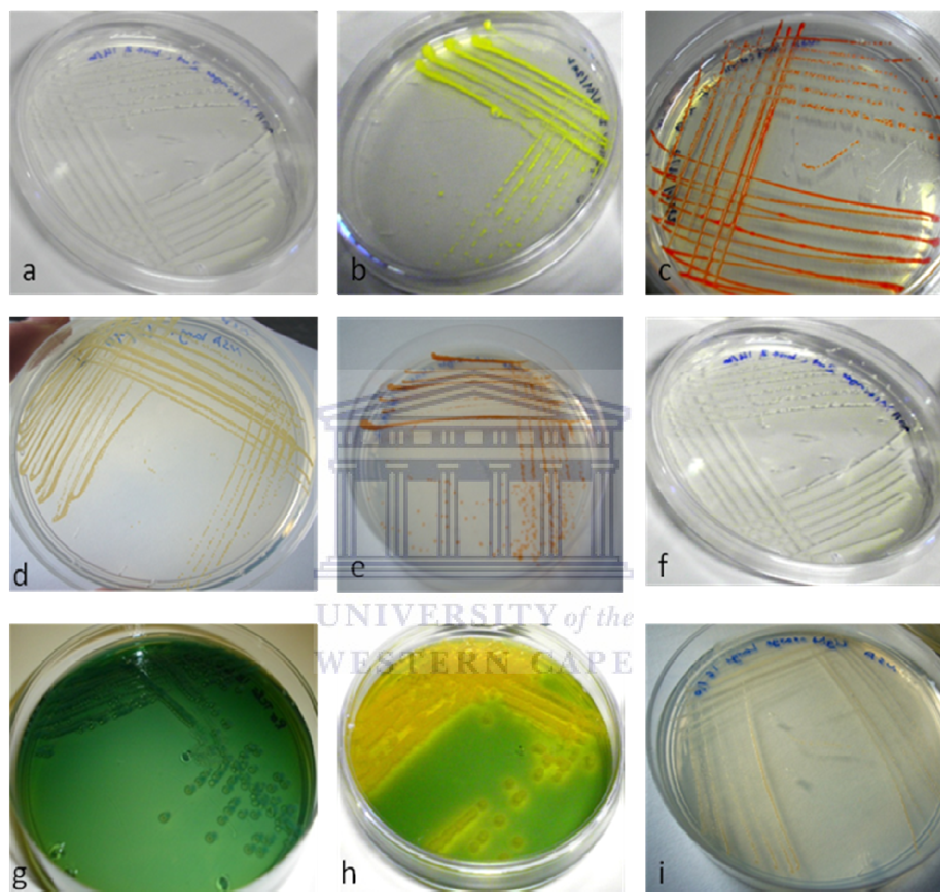

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Figure 4.1 Images showing selected isolates on plates that were cultivated. (a) Beige isolate on MA, (b) Bright yellow isolate on NSA, (c) Red isolate, (d) Brown isolate, (e) Orange isolate, (f) White isolate on NSA, (g) Isolate with green blue centre on TCBS, (h) Yellow isolate on TCBS and (i) Cream isolate on MA.

4.2 16S rRNA PCR amplification

Amplification of the near complete 16S rRNA gene was performed with primers E9f (5'-GAGTTTGATCCTGGCTCAG-3') and 1489r (5'-TACCTTGTTACGACTTCA-3') which bind to highly conserved regions of the 16S rRNA gene (Weisburg et al. 1991; Ludwig and Schleifer1999). From a total of 49 isolates, the 16S rRNA gene was successfully amplified from 39 isolates by the colony PCR technique described by Bauer et al. (2009). A representative gel image showing some of the 16S rRNA PCR products is displayed in figure 4.2.

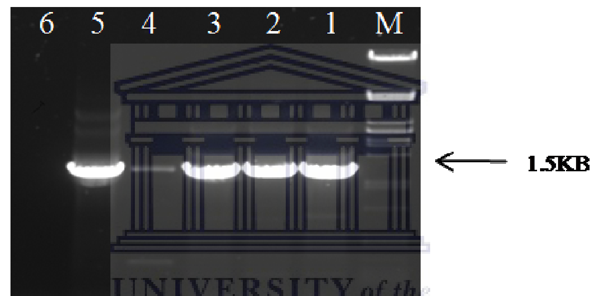

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Figure 4.2: 16S rRNA colony PCR of selected bacterial isolates from *S. rugosum*. Lane M, DNA marker (λ *Pst*I); Lanes1-4, 16S rRNA colony PCR products; Lane 5, positive control (*E. coli*); Lane 6, negative control.

16S rRNA genes of the remaining 10 isolates (TCBS_3, MA_1, MA_4, MA_14, MA_15, MA_18, MA_19, MA_21, MA_22 and NA_1) could not be amplified by colony PCR, despite the procedure being repeated thrice. These isolates were highly pigmented, especially the red (MA_1) and the orange (MA_4) isolates (Table 4.1). Pigments have been shown to inhibit the activity of Taq polymeras during PCR reactions (Monroe et al. 2013). Successful amplification were subsequently achieved with two of these isolates (MA_18 and NA_1) using genomic DNA extracted by a method described by Sambrook and Russell (2001), as the template (Figure 4.3).

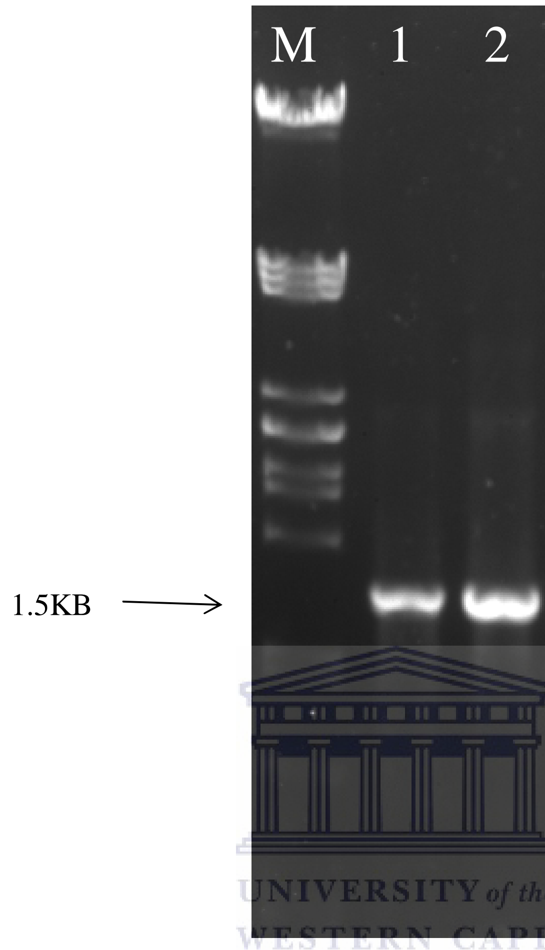

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Figure 4.3: 16S rRNA gene PCR product using extracted genomic DNA as template.Lane M, DNA molecular weight marker (λ *Pst*I); Lanes 1 and 2, 16S rRNA gene products from isolates MA_18 and NA_1, respectively.

The 1500 bp PCR products were purified from agarose gels using a Nucleospin Gel and PCR clean-up kit (Machery-Nagel). DNA purity was confirmed by agarose gel electrophoresis prior to cloning into pGEM ® -T Easy vector (Figure 4.4).

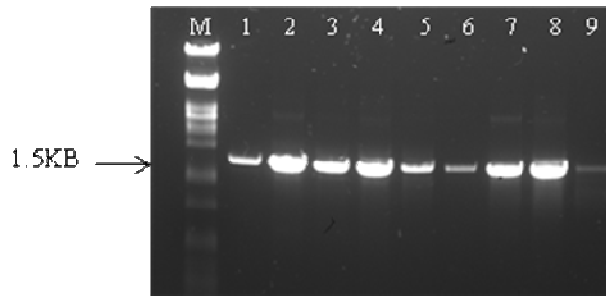

Figure 4.4: Purified16S rRNA gene PCR products. Lane M, DNA molecular weight marker (λ *Pst*I); Lanes 1 to 9, purified PCR products from 9 bacterial isolates.

Forty one fragments were successfully cloned into pGEM ® -T Easy vector and transformed into chemically competent *E. coli* GeneHogs cells. The resulting recombinant plasmids were digested with the restriction enzyme *Eco*RI (Fermentas) to verify the presence of an approximately 1.5 kb inserts prior to sequencing (Figure 4.5).

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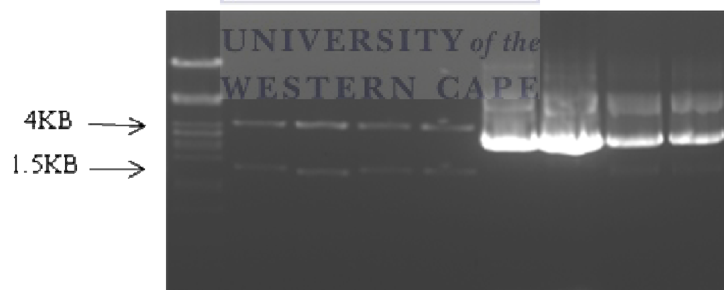

Figure 4.5: Complete restriction enzyme digestion by *Eco*RI of some recombinant plasmids. Lane M, DNA molecular weight marker (λ *Pst*I); Lanes 1 to 4, digested plasmids.The arrows indicate the pGEM ® -T Easy vector backbone (approximately 4 kb), and PCR inserts (approximately 1.5 kb), respectively. Lanes 5 to 8 represent undigested plasmids.

4.3 16S rRNA gene phylogenetic analysis of *S. rugosum* isolates

16S rRNA gene analysis is a powerful tool for the identification of microorganisms (Weisburg et al. 1991). Bacteria associated with the surface of *S. rugosum* were isolated by culture-based

methods. Comparative 16S rRNA gene analysis allowed bacterial identification of 41 isolates to the species level or otherwise revealed closest phylogenetic neighbours (Table 4.2). Approximately 98% of the 16S rRNA gene, close to 1.5 kb, was sequenced and strains with a sequence identity greater than 99% were considered members of the same species.

Table 4.2: Identities of the closest relatives of *S. rugosum* associated bacterial isolates based on 16S rRNA gene sequences obtained from a Blast search of the GenBank databases. The accession numbers of the sequences generated in this study are from KP204118 to KP204158.

Strain #	Nearest neighbour	Percentage identity	Nucleotide sequence accession number	Source
TCBS_1	*Vibrio* sp.D4058	99.73%	DQ480136.1	Seawater
	Vibrio cyclitrophicus	99.66%	AB682659.1	NBRC:107756
MA_27	*Vibrio* sp.BSw21405	99.80%	FJ748512.1	Seawater
	Vibrio splendidus isolate PB1-10rrnh	99.66%	EU091326.1	Fish symbiont
MA_13	*Vibrio* sp.V004	99.66%	DQ146970.1	Fish (*Latris lineata*)
	*Vibrio splendidus*strain 03/012	99.46%	AJ874367.1	Oyster (*Crassostrea gigas*)
TCBS_2	*Vibrio splendidus* isolate PB1-10rrnh	99.80%	EU091332.1	Fish symbiont
TCBS_5	*Vibrio splendidus* isolate PB1-10rrnh	99.66%	EU091332.1	Fish symbiont
TCBS_6	*Vibrio splendidus* isolate PB1-10rrnh	99.66%	EU091332.1	Fish symbiont
MA_18	*Vibrio splendidus* isolate PB1-10rrnh	99.73%	EU091332.1	Fish symbiont
MA_24	*Vibrio splendidus* isolate PB1-10rrnh	99.73%	EU091332.1	Fish symbiont
TCBS_4	*Vibrio splendidus* LGP32 strain	99.73%	NR_074953.1	Strain (LGP32)
MA_29	*Vibrio comitans*	99.66%	AB681693.1	NBRC:102081

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MA_23	*Vibrio comitans*	98.91%	AB681693.1	NBRC:102081
MA_16	*Vibrio comitans*	99.18%	AB681689.1	NBRC:12985
MA_8	*Vibrio comitans*	98.98%	AB681689.1	NBRC:102077
NSA_10	*Vibrio celticus* strain Rd215	99.73%	FN582229.1	Clam (*Venerupis pullastra*)
MA_17	*Pseudoalteromonas carrageenovora*	100%	AB680359.1	NBRC:12985
MA_6	*Pseudoalteromonas carrageenovora*	99.79%	AB680359.1	NBRC:12985
MA_28	*Pseudoalteromonas carrageenovora*	99.66%	AB680359.1	NBRC:12985
MA_5	*Pseudoalteromonas espejiana*	99.93%	AB681520.1	NBRC:101667
MA_10	*Pseudoalteromonas espejiana*	99.79%	AB681520.1	NBRC:101667
MA_7	*Pseudoalteromonas* sp. STAB201	99.79%	JF825439.1	Marine biofilms
	Pseudoalteromonas carrageenovora	99.73%	AB680359.1	NBRC:12985
MA_2	*Pseudoalteromonas* BSs20138	99.79%	EU365489.1	Marine sediments
	Pseudoalteromonas carrageenovora	99.66%	AB680359.1	NBRC:12985
MA_11	Uncultured proteobacterium	99.24%	JQ218664.1	Brown algae (*S. japonica*)
	Pseudoalteromonas sp.	98.62%	EF089559.1	Marine sediments
	Pseudoalteromonas carrageenovora	98.42%	NR_113605.1	NBRC:12985
NSA_1	*Alteromonas* sp. U70	99.52%	AJ832999.1	Seawater
	Alteromonas stellipolaris strain LMG 21861T	99.38%	AJ295715.2	Seawater
NSA_9	*Alteromonas* sp. DH12	99.38%	FJ404749.1	Seawater
	Alteromonas stellipolaris strain LMG 21861T	99.17%	AJ295715.2	Seawater
NSA_12	Uncultured proteobacterium	99.66%	JQ218606.1	Brown algae (*S. japonica*)
	Psychromonas arctica	98.91%	EF101549.1	Brown algae (*U. pinnatifida*)
NSA_23	Uncultured bacterium	99.66%	HE576021.1	Metal working fluid
	Pseudomonas poae RE*1-1-14 strain	99.59%	NR_102514	Endorhiza sugar beet

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Isolate	Closest match	Similarity	Accession no.	Source
MA_26	Uncultured bacterium	99.79%	HE576021.1	Metal working fluid
	Pseudomonas poae RE*1-1-14 strain	99.73%	NR_102514	Endorhiza sugar beet
MA_25	*Pseudomonas poae* strain LS172	99.79%	FJ937922.1	Sponge (*Gelliodes carnosa*)
NA_1	*Pseudomonas poae* strain LS172	99.86%	FJ937922.1	Sponge (*Gelliodes carnosa*)
	Pseudomnas fluorescens	99.73%	EU360313	Milk
MA_3	*Neptumonas* sp.S21	98.90%	JN226744.1	Coastal seawater
	Neptunomonas naphthovorans	98.08%	NR_114018.1	NBRC 101991
MA_12	*Cobetia amphilecti*	99.59%	AB646236.1	Sponge (*Amphilectus digitatus*)
NSA_5	Marine bacterium Mst85	98.97%	AJ400705.1	Marine sediments
NSA_6	Marine bacterium Mst85	99.25%	AJ400705.1	Marine sediments
NSA_7	Marine bacterium Mst85	98.97%	AJ400705.1	Marine sediments
NSA_4	*Shewanella* sp. STAB101	96.85%	JF825437.1	Marine biofilms
NSA_2	*Sphingomonas* sp. W2.10-2	96.59%	JX458462.1	Deep mineral water qualifier
NSA_11	*Sulfitobacter* sp. SCSWA07	96.72%	FJ461425.1	Seawater
NSA_8	*Polaribacter* sp. TC5	99.64%	KF472182.1	Seawater
	Polaribacter dokdonesis isolate AP36	99.42%	HE584783.1	Abalone (*Haliotis diversicolor*)
NSA_3	*Polaribacter* sp. WP25	98.70%	KC878325.1	Pen shell (*Atrina pectinata*)
	Polaribacter irgensii clone SE99	97.55%	AY771779.1	Arctic bacteria
MA_9	*Bacillus* sp. 3428BRRJ	99.93%	FJ215791.2	Pharma product contaminant
	Bacillus jeotgali	99.46%	NR025060	Fermented seafood
MA_20	*Staphylococcus saprophyticus* subsp. *saprophyticus* strain JUN-9	99.66%	KF228927.1	Sewage water

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Note: NBRC refer to NITE Biological Resource Centre.

Closest relatives of the majority of isolates from the surface of *S. rugosum* were originally identified from marine samples (Table 4.2). To evaluate the taxonomy of the *S. rugosum* isolates, a phylogenetic tree was constructed using 27 representative *Splacnidium* isolates, as well as their closest relatives as obtained from GenBank. Results are presented in Figures 4.6 and 4.7. The majority (21 isolates) were closest related to members of the phylum Gamma-Proteobacteria including *Vibrio* and *Pseudoalteromonas* (Figure 4.6). The remaining six isolates clustered with the phyla Alpha-Proteobacteria, Firmicutes and Bacteriodetes (Figure 4.7).

Bacteria belonging to the phylum Gamma-Proteobacteria were the most abundant. This finding is consistence with previous studies investigating bacterial associations of *Laminaria saccharina* (Wiese et al. 2009) and *Laminaria digitata* (Salaun et al. 2010). Gamma-Proteobacteria isolates included in the phylogenetic analysis are representatives of the genera *Vibrio* (TCBS_1, TCBS_2, MA_27, NSA_10, MA_13, TCBS_4 and MA_29), *Pseudoalteromonas* (MA_11, MA_7, MA_6, MA_2 and MA_5), *Alteromonas* (NSA_1 and NSA_9), *Pseudomonas* (MA_26 and NA_1), *Psychromonas* (NSA_12), *Neptumonas* (MA_3), *Cobetia* (MA_12), as well as an unspecified marine bacterium (NSA_6).

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Vibrio was the dominant genus identified in this study with the majority of the *Vibrio* isolates closest related to *Vibrio splendidus* (Table 4.2). Previous studies confirmed that this species is the dominant *Vibrio* species in coastal marine sediments, seawater and bivalves in temperate climates (Lambert et al. 1998; Sobecky et al. 1998; Beaz-Hidalgo et al. 2010). Other *Vibrio* species identified include *Vibrio celticus* and *Vibrio comitans*. Furthermore, the *S. rugosum* isolates clustered closely with species such as *Vibrio pacinii* (Hugh et al. 1964), *Vibrio gallicus*

(Sawabe et al. 2004), *Vibrio litoralis* (Nam et al. 2007), and the recently described novel species *Vibrio aestivus* and *Vibrio quintilis* (Lucena et al. 2012) (Figure 4.6).

Of the numerous members of the genus *Pseudoalteromonas* present on the surface of *S. rugosum*, all but for one isolate (MA_11) were closely related to either *P. carrageenovora* or *P. espejiana* (>99% sequence identity). Both species are agarolytic, producing extracellular ß-agarase (Akagawa-Matsushita et al. 1992; Uchida et al. 1997; Guibet et al. 2007). Isolate MA_11 shared less that 99% identity with *P. carrageenovora* and its closest relative was isolated from brown algae (*Saccharina japonica*) (Balakirev et al. 2012). *Pseudoalteromonas* is commonly found in marine habitats in association with eukaryotic hosts (Holmström and Kjelleberg1999) and algal surfaces (Bowman 2007). *Pseudoalteromonas* species express a wide range of biological activities, including anti-bacterial, bacteriolytic, agarolytic and algicidal actions (Holmström and Kjelleberg 1999), while several strains have been shown to specifically prevent the settlement of common fouling organisms (Holmström and Kjelleberg 1999; Holmström et al. 2002).Therefore, it is possible that the *Pseudoalteromonas* species present on the surface of *S. rugosum* may protect the seaweed against fouling organisms. Isolates NSA_1 and NSA_9 were grouped tightly with marine isolates from the genus *Alteromonas* (Figure 4.7), members of which display algicidal activity (Su et al. 2010). *Pseudoalteromonas* and *Alteromonas* are indeed the most dominant genera amongst bacteria with confirmed algicidal activity (Skerratt et al. 2002; Lee et al. 2008). Such strains have potential biotechnological applications as algicidal properties are useful traits in protecting shellfish farms from toxic dinoflagellate blooms (Skerratt et al. 2002).

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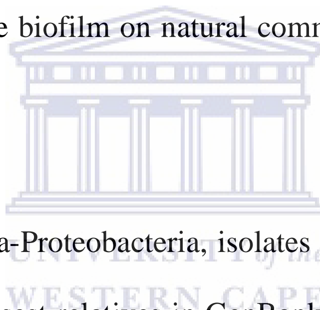
Isolate NSA_12 was related to *Psychromonas arctica* (98.91% identity) isolated from the algae *Undaria pinnatifida* (Korean, Miyok) (Lee et al. 2006). The closest relative of isolate MA_12 is a newly described species *Cobetia amphilecti* (98.91%) which was isolated from a marine sponge (*Amphilectus digitatus)* in the Gulf of Alaska (Romanenko et al. 2013). 16S rRNA gene analysis of isolate NA_1 was unable to differentiate between *Pseudomonaspoae* (99.86%% identity) and *P. fluorescence* (99.73% identity). As the threshold identity value delineating bacterial species is considered to be 97.5% (Wayne et al. 1987), amongst isolates in the Gamma-Proteobacteria phylum, NSA_4 is potentially a novel species as its 16S rRNA gene sequence shared less than 97 % identity with members of the *Shewanella* genus. Isolate NSA_4's closest relative was isolated from a marine biofilm on natural common minerals in seawater (Finnegan et al. 2011).



Similarly, within the phylum Alpha-Proteobacteria, isolates NSA_2 and NSA_11 were distantly related (<97% identity) to their closest relatives in GenBank (Figure 4.7) and possibly represent novel species. Isolate NSA_2 was closest related to a *Sphingomoas* species obtained from a deep mineral water qualifier (GenBank accession no. JX458462.1), while isolate NSA_11 was closest to a *Sulfitobacter* species (GenBank accession no. FJ461425.1). *Sulfitobacter* species have been reported to possess probiotic properties which may be useful as a treatment against infections caused by pathogenic bacteria such as *V. anguillarum*in fish (Sharifah and Eguchi 2011).

Isolates MA_9 and MA_20 were affiliated with the phylum Firmicutes (Figure 4.7) and were identified as *Bacillus jeotgali* and *Staphylococcus saprophyticus* subsp. *saprophyticus*, respectively. Finally, isolates NSA_8 and NSA_3 were affiliated with the phylum Bacteriodetes

within the genus *Polaribacter*. The closest relative of NSA_8 (*Polaribacter* sp. TC5) was obtained from Mediterranean Sea and this strain exhibited different abilities to form biofilms (GenBank accession no. KF472182.1), while the closest relative of NSA_3 (*Polaribacter* sp. WP25) was isolated from the intestines of a pen shell (*Atrinapectinata*) (GenBank accession no. KC878325.1). *Polaribacter* (Flavobacteria) is one of the major genera of Bacteriodetes found in the marine environment. Representatives of this group from aquatic habitats have been described as surface-associated bacteria, as they were found mostly in floating assemblages (Nold and Zwart 1998). As reviewed by Michel et al. (2006), Flavobacteria may produce carageenases and agarases, and are thus able to degrade algal compounds. Therefore, the algal isolates affiliated with the Bacteriodetes possibly represent opportunistic algae-degrading bacteria. *S. rugosum* is a known producer of fucoidan (Miller et al. 1996) and Flavobacteria have been shown to produce fucoidan hydrolysing secondary metabolites which may have biotechnological potential (Descamps et al. 2005;Urvantsevaet al. 2006). Future work should be conducted to screen these isolates for fucoidanase activity to determine if they have the capacity to degrade fucoidan polymers.

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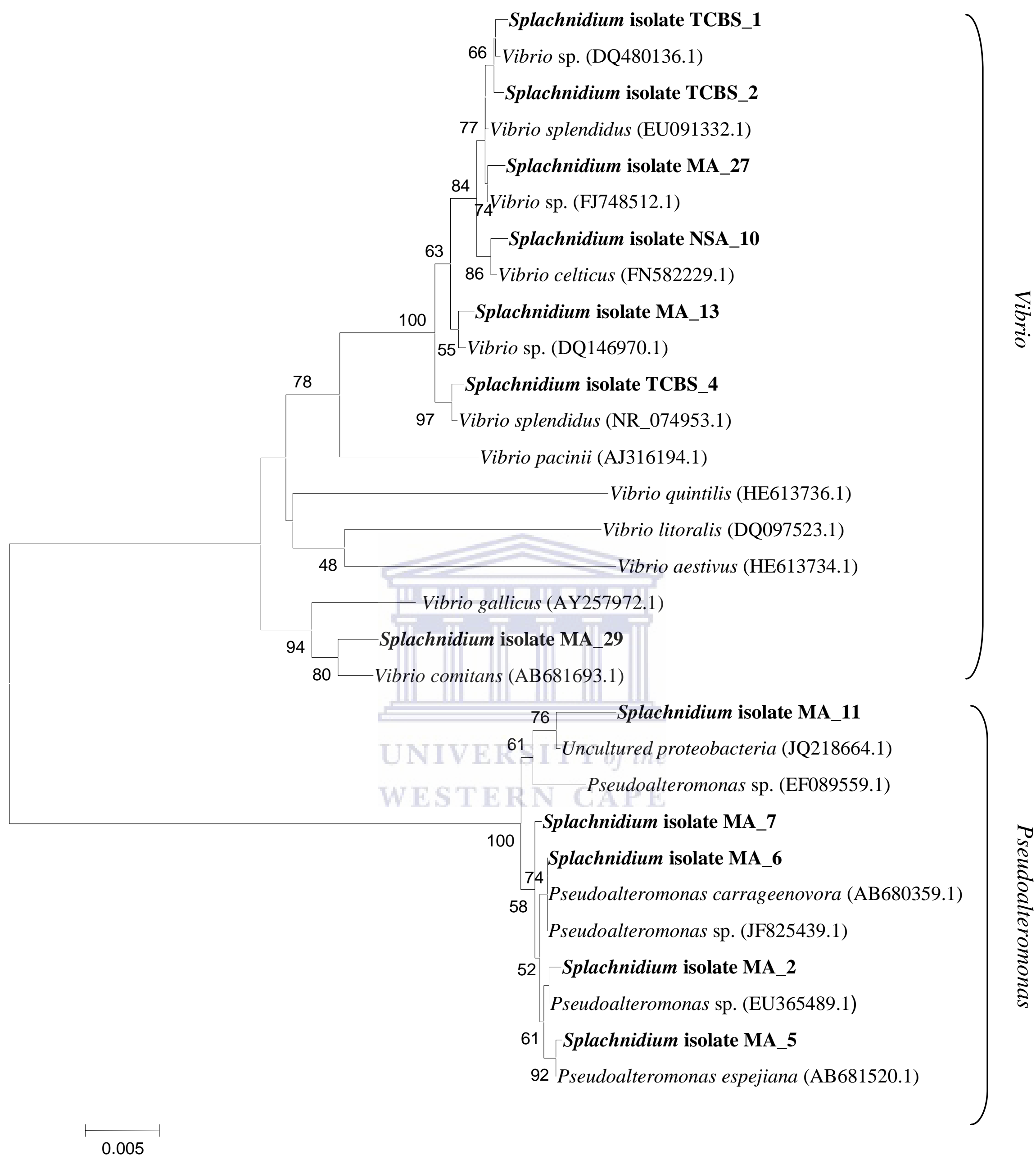

Figure 4.6: Neighbor-joining tree showing the 16S rRNA gene sequences phylogenetic relationships of *S.rugosum* isolates associated with *Vibrio* and *Pseudoalteromonas* species. The tree includes the *S. rugosum* isolates sequences (bold text) and the closest relatives determined by BLAST search. Bootstrap values are given in percentage (only values above 50 are shown) at branch nodes based on 1000 resembling. The scale bar indicates evolutionary distance. The number of nucleotides is 1468 base pairs. Bar 0.005 substitutions per nucleotide position. The accessions numbers of strains are given in parentheses.

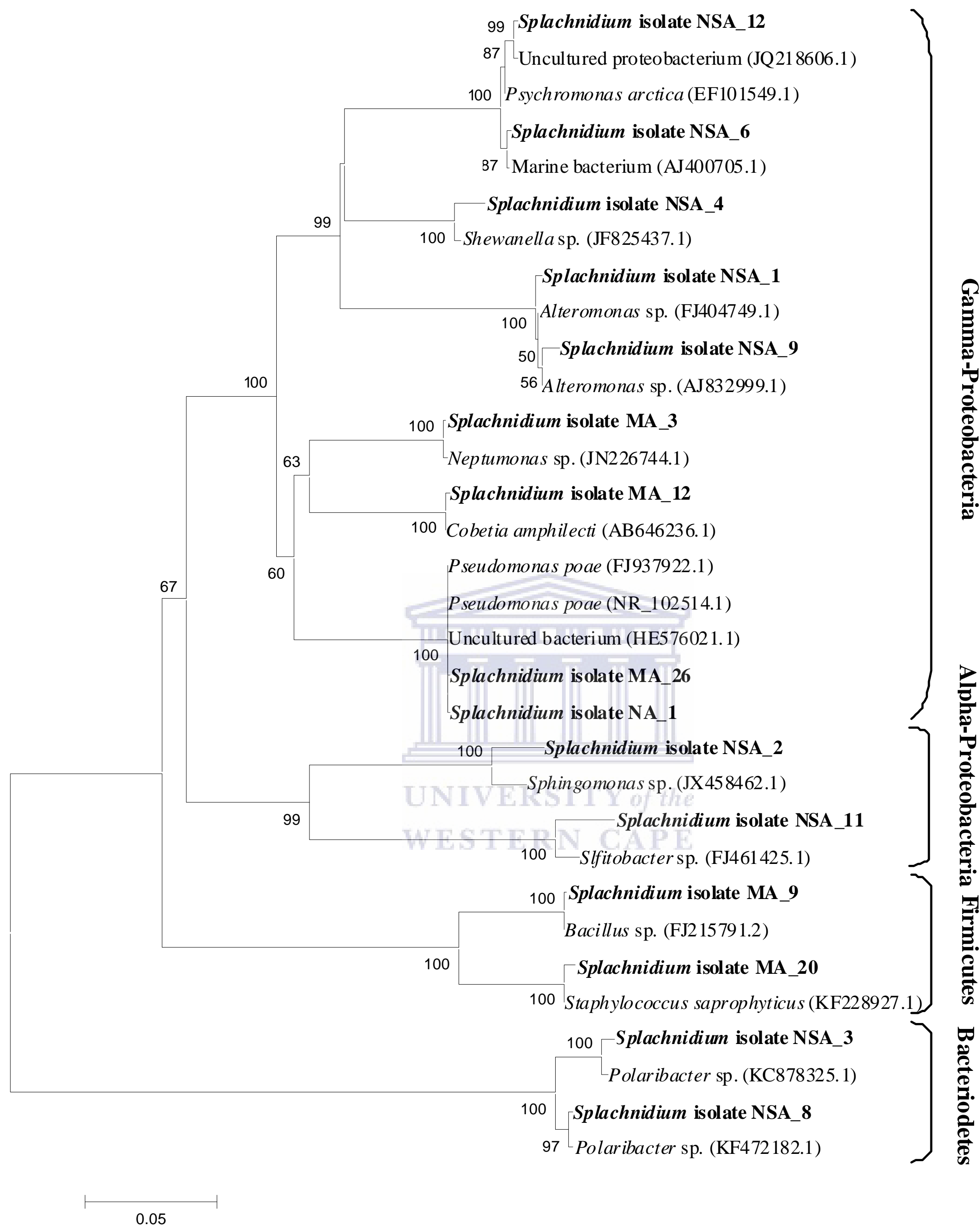

Figure 4.7: Neighbor-joining tree showing the 16S rRNA gene sequences phylogenetic relationships of *S.rugosum* strains associated with Gamma-Proteobacteria, Alpha-Proteobacteria, Bacteriodetes and Firmicutes. The tree includes the *S. rugosum* isolates sequences (bold text) and the closest relatives determined by BLAST search. Legend: Bootstrap values are given in percentage (only values above 50 are shown) at branch nodes based on 1000 resembling. The scale bar indicates evolutionary distance. The number of nucleotides is 1465 base pairs. Bar 0.05 substitutions per nucleotide position. The accessions numbers of strains are given in parentheses.

4.4 Screening of *S. rugosum* isolates for antimicrobial activity

An objective of this study was the screening of bacterial isolates associated with *S. rugosum* for antimicrobial activity. *S. rugosum* isolates (41, Table 4.3) were screened for antimicrobial activity against the bacterial pathogens *Escherichia coli* (1699), *Bacillus cereus*, *Staphylococcus epidermidis, Mycobacterium smegmatis* (LR22)*, Micrococcus luteus* and *Pseudomonas putida* (ATCC27853) using the antibiotic overlay assay technique (Bauer et al. 2005; Fyfe et al. 1984) Fifteen isolates (36%) displayed antimicrobial activity against one or more of the test strains. It is known that brown algae are colonized by bacteria which may exhibit antimicrobial activity (Mazure and Field 1980; Corre and Prieur 1990). Wiese et al. (2009) isolated and identified 210 bacterial strains associated with the brown macroalgae *Laminaria saccharina* (Baltic Sea, Germany). The majority belonged to the phyla Alpha-Proteobacteria, Gamma-Proteobacteria, Bacteriodetes, Firmicutes and Actinobacteria. It was observed that 50% of the isolates exhibited antimicrobial activity against at least one test microorganism from a panel comprised of Gram-negative and Gram-positive bacteria (Wiese et al. 2009). It has been shown that bacteria growing on a host surface as part of a complex microbial community may have enhanced chemical defence mechanisms in contrast with planktonic communities (Matz et al. 2008). Isolate NA_1 (*Pseudomanas* sp.) displayed a broad spectrum antimicrobial activity against all test stains, except *E. coli* (Table 4.3). The inhibition of Gram-positive bacteria by *S. rugosum*-associated isolates was more common than the inhibition of Gram-negative bacteria which is inconsistent with the results of a study on *L. saccharina* associated isolates (Wiese et al. 2009). It was interesting to observe that the inhibition of *M. smegmatis* by *S. rugosum*-associated isolates was more widespread than other bacterial test strains (Table 4.3). The genus *Mycobacterium* includes several human pathogens including *Mycobacterium leprae* which causes leprosy and

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Mycobacterium tuberculosis (MTB) which causes tuberculosis (TB). Potentially new antibiotic substances active against pathogenic organismsis of high clinical importance and antimicrobial compounds produced by *S. rugosum* isolates may have biotechnological potential.

Table 4.3: Screening *S. rugosum* isolates for antimicrobial activity

Isolate	Identified strains	Antimicrobial activity against test strains					
		E.coli Gram -	*B.cereus* Gram +	*S. epidermidis* Gram +	*M. smegmatis* Gram +	*M. luteus* Gram +	*P. putida* Gram-
MA_2	*Pseudoalteromonas* sp.	-	-	-	-	-	-
MA_3	*Neptumonas* sp.	-	-	-	-	-	-
MA_5	*P. espejiana*	-	-	-	-	-	-
MA_6	*P. carrageenovora*	-	-	-	-	-	+
MA_7	*Pseudoalteromonas* sp.	-	-	-	-	-	-
MA_8	*Vibrio comitans*	-	-	-	+	+	-
MA_9	*Bacillus* sp.	-	-	-	-	-	-
MA_10	*P. espejiana*	-	-	-	-	-	-
MA_11	*Proteobacterium*	-	-	-	+	-	-
MA_12	*Cobetia amphilecti*	-	-	-	-	-	-
MA_13	*Vibrio* sp.	-	-	-	-	+	-
MA_16	*Vibrio comitans*	-	-	-	+	+	-
MA_17	*P. carrageenovora*	-	-	-	-	-	-
MA_18	*Vibrio splendidus*	-	-	-	-	-	-
MA_20	*S.saprophyticus*	-	-	-	+	+	-
MA_23	*Vibrio comitans*	-	-	-	-	-	-
MA_24	*Vibrio splendidus*	-	-	-	-	++	-
MA_27	*Vibrio* sp.	-	-	-	-	-	-
MA_28	*P. carrageenovora*	-	-	-	-	-	-
MA_29	*Vibrio comitans*	-	-	-	-	-	-
NA_1	*Pseudomonas poae*	-	+++	+++	+++	+++	+++
NSA_3	*Polaribacter* sp.	-	-	-	+	-	-
NSA_8	*Polaribacter* sp.	-	-	-	+	+	-

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NSA_5	Marine bacterium	-	-	-	+	+	-
NSA_6	Marine bacterium	-	-	-	-	-	-
NSA_7	Marine bacterium	-	-	-	+	+	-
NSA_4	*Shewanella* sp.	-	-	-	-	-	-
NSA_1	*Alteromonas* sp.	-	-	-	-	-	-
NSA_2	*Sphinogomonas* sp.	-	-	-	-	-	-
MA_23	Uncultured bacterium	-	-	-	-	-	-
MA_25	*Pseudomonas poae*	-	-	-	-	-	-
MA_26	Uncultured bacterium	-	-	-	-	-	-
NSA_9	*Alteromonas* sp.	-	-	-	-	-	-
NSA_10	*Vibrio celticus*	-	-	-	-	++	++
NSA_11	*Sulfitobacter* sp.	-	-	-	+	-	-
NSA_12	*Psychromonas arctic*	-	-	-	+	-	-
TCBS_1	*Vibrio* sp.	-	-	-	-	-	-
TCBS_2	*Vibrio splendidus*	-	-	-	-	-	-
TCBS_4	*Vibrio splendidus*	-	-	-	-	-	-
TCBS_5	*Vibrio splendidus*	-	-	-	-	-	-
TCBS_6	*Vibrio splendidus*	-	-	-	-	-	-

Note: *P., Pseudoalteromonas*; *S., Staphylococcus;* (+), small zone of inhibition of approximately 2 mm; (++), medium zone of inhibition of approximately 6 mm and (+++), large zone of inhibition of approximately 10 mm.

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While most of the isolates in this study exhibited small inhibition zones (approximately 2 mm), isolate NSA_10 (*Vibrio celticus*) displayed an approximately 6 mm inhibition zone against *Pseudomonas putida* and *Micrococcus luteus* (Figure 4.8 a). *V. celticus* was first identified by Beaz-Hidalgoa et al. (2010) and was discovered from the clam *Venerupis pullastra.* This isolate displayed pathogenic activity against adult clams as shown by virulence assays. In addition, species within the *V. splendidus* clade have been shown to be associated with the mortality of a wide range of marine animals such as fish (Jensen et al. 2003). *Vibrio splendidus* isolate MA_24 displayed moderate activity against *M. luteus*. Other *V. splendidus* isolates identified from the

surface of *Splachnidium* (TCBS_2, TCBS_4, TCBS_5, TCBS_6 and MA_18) did not exhibit antimicrobial activity against the test strains (Table 4.3). This clearly indicates a strain-specific production of antimicrobial compounds.

Isolate NA_1 is closely related to *Pseudomonas poae* (99.86%) and *Pseudomnas fluorescens* (99.73%), and displayed broad spectrum antimicrobial activity with large inhibition zones (approximately 10 mm) against *M. smegmatis* (Figure 4.8 b), as well as *B. cereus, S. epidermidis, M. luteus* and *P. putida* (zones of inhibition not presented). It has been shown that *Pseudomonas* species produces a range of antimicrobial substances (Wiese et al. 2009; Berdy 2005). The most note worthy includes massetolide A, a potent surfactant with a broad spectrum antimicrobial activity, produced by *Pseudomonas fluorescens* (Gerad et al. 1997; de Bruijn et al. 2008). Massetolide A inhibits the growth of *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* (Gerad et al. 1997). The compound is a 9-amino-acid cyclic lipopeptide (CLP) linked to 3-hydroxydecanoic acid (Gerad et al. 1997). This peptide was first described by Gerad et al. (1997) who isolated the peptide from *Pseudomonas* cultures collected from the surface of leafy red algae collected in Masset Inlet, British Columbia, Canada. Considering the possibility that isolate NA_1 may be a producer of massetolide A, future studies include activity assays against *Mycobacterium tuberculosis*. Interestingly, isolate NA_1 produces a distinct lemon coloured fluorescent pigment which is diffused throughout the surface of the agar. Previous studies have demonstrated that antibiotic-producing marine bacteria are often pigmented (Lemos et al. 1985). In stark contrast, *Pseudomonas poae* isolate MA_25 produces no pigments and displayed no antimicrobial activity against test strains, Morover, MA_25 is less closely related to *P. poae* than NA_1.

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Isolate NSA_11 clustered with species of the genus *Sulfitobacter* and displayed a small inhibition zone against *M. smegmatis*. It's closest relative (96.72% identity) also shared low sequence identity (96.65%) to other *Sulfitobacter* species and was shown to possess probiotic properties with potential for treating fish infected with pathogenic bacteria such as *Vibrio anguillarum* (Sharifah and Eguchi 2011). Isolates NSA_4 and NSA_2 shared low sequence identities (<97% identity) to members of the genera *Shewanella and Shingomonas*, respectively, and did not display antimicrobial activity against the test strains.

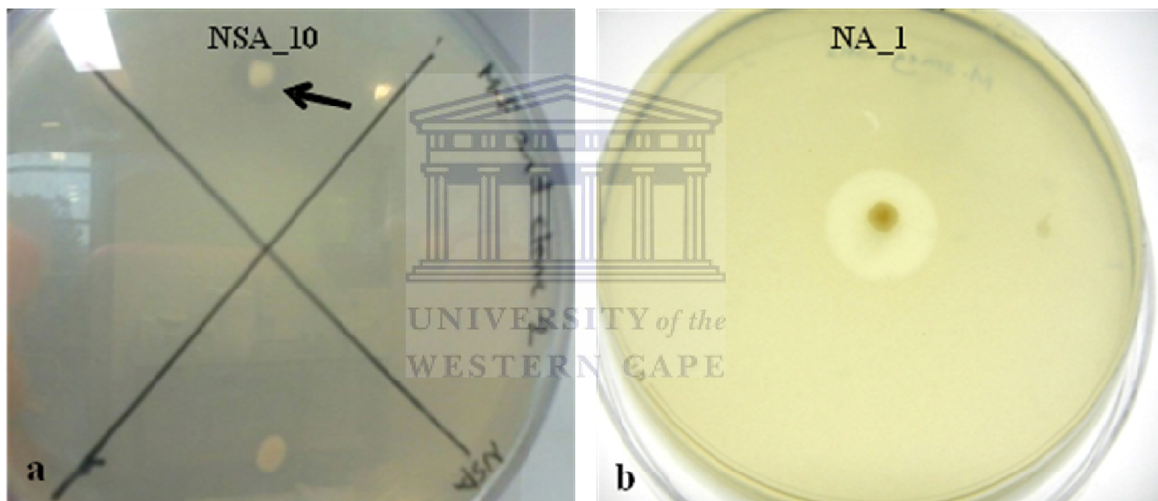

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Figure 4.8: Growth inhibition zones. (a) Isolate NSA_10 (*V. celticus*) exhibiting anti-microbial activity with medium inhibition zone (~6mm diameter; arrow) against *Micrococcus luteus*. (b) Isolate NA_1 (*P. poae*) exhibiting anti-microbial activity with large inhibition zone (~10mm diameter) against *Mycobacterium smegmatis*.

4.5 Growth parameters of antimicrobial producing isolate NA_1

Growth parameters of antimicrobial producing isolate NA_1 (*Pseudomonas* sp.) were determined hourly (three biological replicates) over a period of 24 hours. Isolate NA_1 displayed antimicrobial activity against *Bacillus cereus*, *Staphylococcus epidermidis, Mycobacterium*

smegmatis, Micrococcus luteus and *Pseudomonas putida*. The growth curve of isolate NA_1 is presented in figure 4.9. Mid log phase was attained at an OD_{600} nm of 1.5 and stationary phase reached at an OD_{600} nm of 2.3 after ten hours. Growth phase significantly influences secondary metabolites synthesis and hence interactions with other organisms (LeFlaive and Ten-Hage 2007). Some secondary metabolites attain maximal metabolites synthesis during the stationary phase, while others are maximally produced during the exponential phase (LeFlaive and Ten-Hage 2007). Microbial secondary metabolites are usually produced during the stationary phase (Namikoshi and Rinehart 1996), and it has been proposed that *Pseudomonas* species increase production of secondary metabolites at early stationary phase (Birgit et al. 2002; de Bruijn et al. 2008).

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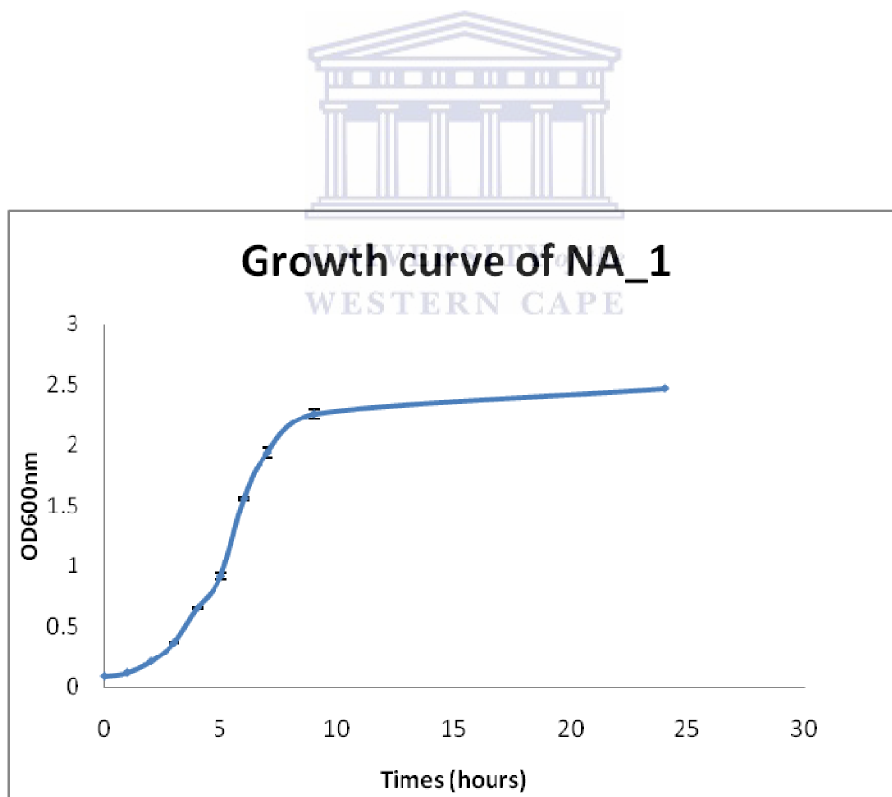
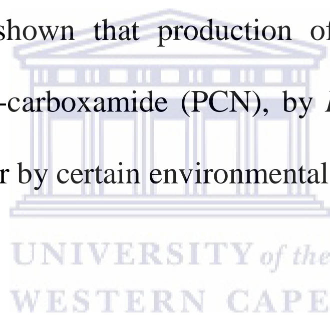


Figure 4.9: Growth curve of isolate NA_1 isolate (*Pseudomonas* sp.) in nutrient broth. Data represents the mean ± standard error (ŋ=3). Data shown are mean values of oneexperiment performed in triplicate. Standard errors are shown as bars.

The antimicrobial activity of cell free supernatant was monitored during growth of the producer (isolate NA_1) to ascertain if production was growth phase dependent. However, no inhibitions zones against *M. smegmatis* (LR22) were observed on TSB plates spotted with cell free supernatant. Activity appeared to be associated with the cellular fraction. These results suggest that the antimicrobial compound may be closely associated with the cell wall of the producer strain. It is also possible that the activity in supernatant was below the level of detection using the spot-on-lawn assays. Future studies may require activity assays with concentrated supernatant. Massetolide A, an antimicrobial compound produced by *Pseudomonas fluorescens,* was shown to be produced in the early exponential growth phase (de Bruijn 2008). On the other hand, Tjeerd et al. (2004) has shown that production of an antimicrobial compound, the secondary metabolite phenazine-1-carboxamide (PCN), by *Pseudomonas chlororaphis* may be induced by a high optical density or by certain environmental conditions (Tjeerd et al. 2004).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Chapter 5

General conclusion

Bacteria cultured from the surface of *S. rugosum* were grouped into four bacterial phyla based on 16S rRNA gene analysis, namely Gamma-Proteobacteria, Alpha-Proteobacteria, Firmicutes and Bacteroidetes. Of the 41 isolates identified, 85% were Gamma-Proteobacteria (Figure 5.1).

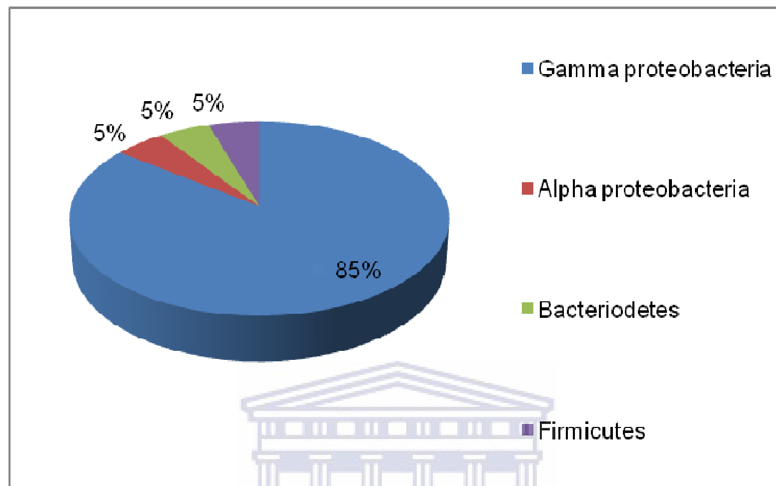

Figure 5.1: Classification of *S. rugosum* bacterial isolates into various phyla based on 16S rRNA phylogenetic analysis.

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This finding was not unexpected considering that bacteria belonging to the Gamma-Proteobacteria phylogenetic group are among the most known and readily cultivable microorganisms from the marine environment (Fuhrman and Hagström 2008). Similar results have been observed in other studies investigating bacterial associations with brown algae (Wiese et al. 2009; Salaun et al. 2010) and the red alga *Delisea pulchra* (Penesyan et al. 2009). Staufenberger et al. (2008) used a culture independent approach which involved the use of denaturing gradient gel electrophoresis and 16S rRNA gene clone libraries to investigate the bacterial community associated with the brown alga *Laminaria saccharina*. Gamma-Proteobacteria was found to be the dominant phylum and most of the phylotypes were related to

uncultured bacteria (Staufenberger et al. 2008). Marine bacteria in this phylogenetic group are generally aerobic or facultative anaerobes (Fuhrman and Hagström 2008). *S. rugosum* grows in the rocky shore environment which is abundant in oxygen due to wave action and various photosynthetic processes. Therefore, it is not surprising that such environments are dominated by aerobic bacteria.

Most of the *S. rugosum* isolates were predominantly *Vibrio* and *Pseudoalteromonas* species. *Vibrio splendidus* was the most abundant among *Vibrio* isolates, and other identified species included *Vibrio celticus* and *Vibrio comitans*. The identification of *Vibrio* bacteria was of major significance as they are involved in the mineralization of organic material in the sea and for causing disease (Reen et al. 2006). It has been demonstrated that *Vibrio* species are opportunistic pathogens of diseased *Porphyra* and *Laminaria* thalli (Wang et al. 2008). Thus, the existence of *Vibrio* on the surface of *S. rugosum* might have a negative impact on the host's health. *Pseudoalteromonas* species are commonly found in association with higher organisms such as marine macroalgae, and it has been suggested that this genus is a significant competitor for space and nutrients within the marine environment (Holmström et al. 2002). *Pseudoalteromonas* is often associated with the expression of a wide range of biological activities, including anti-bacterial, bacteriolytic, agarolytic and algicidal activities (Holmström and Kjelleberg 1999). It has also been shown that members of the genus *Pseudoalteromonas* show potential to be used as anti-fouling agents in aquaculture and for the control of toxic algal blooms (Holmström and Kjelleberg 1999). Several *Pseudoalteromonas* isolates have furthermore been shown to specifically prevent the settlement of common fouling organisms (Holmström et al. 2002) and the host organism may employ bacterially produced compounds for their own chemical defense

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against fouling (Holmström and Kjelleberg 1999). A good example of this is provided by studies conducted with *Ulva lactuca (*Egan et al. 2000; Holmstrom et al. 2002*)*. This green alga does not produce any secondary metabolites for protection against fouling organisms but has been reported to host antifouling producing *Pseudoalteromonas* species (Lemos et al. 1985; Egan et al. 2000). Thus, the presence of *Pseudoalteromonas* on the surface of *S. rugosum* might play a significant role in defenses against fouling microorganisms in harsh intertidal environments.

Isolates from the surface of *S. rugosum* were dominated by Gram-negative bacteria (Gamma-Proteobacteria, Alpha-Proteobacteria and Bacteriodetes). Several studies have reported a greater presence of Gram-negative bacteria associated with intertidal seaweeds (Kong and Chan 1979; Penesyan et al. 2009; Salaün et al. 2010). In the culture-independent study of Tujula et al. (2010), it was found that *Ulva australis* harbored a sub-population of bacteria which is consistently present in different samples. Results were based on DGGE-based spatial (between tidal pools) and temporal (between season) comparisons. This stable sub-population included bacteria belonging to the phyla Alpha-Proteobacteria and Bacteriodetes, and these bacteria are likely to play an important role in the function of the marine epiphytic microbial communities associated with *U. australis* (Tujula et al. 2010).

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Another culture-independent study showed that Bacteriodetes are among the most abundant bacteria associated with marine eukaryotes (Longord et al. 2007). Two *S. rugosum* isolates (NSA_8 and NSA_3) belonged to this phylum and were grouped into the class *Flavobacteria*. Marine Bacteriodetes isolates are often grouped into the classes *Flavobacteria* and *Sphingobacteria* (Lydell et al. 2004). These classes are also found in freshwater, soil and

sediments (Lydell et al. 2004). They are ubiquitous heterotrophs known for their ability to degrade complex lignocellulosic plant materials (Lydell et al. 2004). This feature may describe their abundance on surfaces of marine seaweeds, which can provide a constant source of nutrients for these organisms. *Flavobacteria* identified on *S. rugosum* could possibly be opportunistic algal degrading microorganisms which might be able to degrade important cell wall components such as fucoidan, the dominant polysaccharide present in *S. rugosum* cell walls (Miller et al. 1996). The presence of a substrate (carbon) source that can be used by surface associated bacteria is probably an important factor in shaping specific macroalgae-bacteria interactions.

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Three *S. rugosum* isolates shared low sequence identities (<97%) to their closest relatives in GenBank. All three isolates grouped with unidentified bacterial species, and may therefore be novel. A number of bacteria associated with other marine environments including brown algae, marine sponges and marine sediments were among the closest relatives of phylotypes associated with *S. rugosum*. As the use of universal primers combined with the conserved nature of the 16S rRNA gene generally restricts the number of closely related species which can be resolved (Dahllof et al. 2000), underestimation of diversity is likely. In addition, the inability to culture the majority of the bacteria from this environment, as well as experimental limitations such as DNA extraction efficiency and the presence of PCR inhibitors would affect the diversity detected. Among 49 bacterial isolates, the 16S rRNA gene of eight isolates could not be amplified. These included isolates that were highly pigmented on different growth media. The presence of inhibitors such as pigments (Monroe et al. 2103) may adversely affected PCR amplification.

Numerous *S. rugosum* isolates produced antimicrobial compounds, most notably isolate NA_1 (*Pseudomanas* species) which was active against test strains *Bacillus cereus*, *Staphylococcus epidermidis*, *Mycobacterium smegmatis* (LR22), *Micrococcus luteus* and *Pseudomonas putida* (ATCC27853). Another remarkable antimicrobial producer was the potentially novel *Sulfitobacter* species (isolate NSA_11, 96.72% identity to closest neighbor) which displayed significant activity against *M. smegmatis*. Marine macroalgae are potentially a rich source of biologically active compounds and numerous algae associated bacteria with antimicrobial activity have been identified by culture based methods (Boyd et al. 1999b; Burgess et al. 1999; Kanagasabhapathy et al. 2006, 2008; Penesyan et al. 2009; Wiese et al. 2009). Culture based methods have indeed been shown to deliver a high frequency of novel antimicrobials compared to alternative technologies (Li et al. 2005; McArthur 2008; Newton 2008). Metagenomic technologies, including sequence-driven analysis and functional screening of environmental clone libraries, are commanding tools in natural product discovery. However, these technologies have yielded limited results in the discovery of novel bioactive compounds such as antimicrobials (Schloss and Handelsman 2003; Handelsman 2005). The rates of attaining positive bioactive-producing clones in metagenomic libraries have been shown to be close to 2 in 113 700 clones screened (Lim et al. 2005), and even as low as 1 in 730 000 (Henne et al. 2000). possibly due to the difficulties in expressing foreign genes in a heterologous host (Penesyan et al. 2009). The increase in metagenomic data may however play an essential role in understanding the biochemical potential of uncultured microorganisms and may lead to the improvement of suitable cultivation strategies in the future (Handelsman 2004). Culture-based approaches remain the most commanding resource in discovering novel bioactives of bacterial origin.

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In conclusion, analysis of the phylogenetic affiliation of *S. rugosum* associated bacteria by 16S rRNA gene analysis has extended our understanding about these microorganisms, including their diversity and distribution, and added to a growing database of bacterial communities associated with brown algae. Potential novel bacterial species were isolated, as well as isolates with biotechnological potential such as producers of antimicrobial compounds active against human pathogens. Results presented may be useful in the design of future studies investigating bacteria-seaweed interactions, including beneficial (symbiotic) and detrimental (pathogenic) interactions.

Future work should be extended to biochemical characterization studies for the novel bacterial species.

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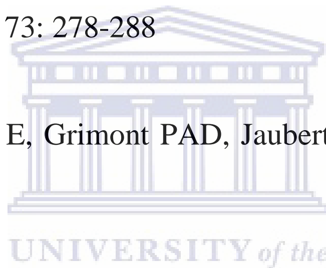

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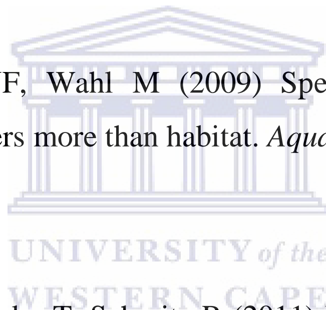

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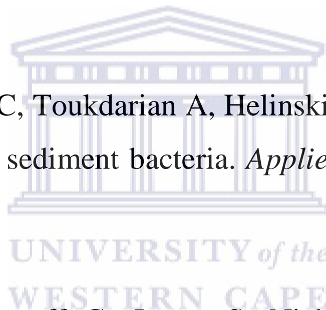
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