An Investigation into the Bacterial Diversity Associated with South African Latrunculid Sponges that Produce Bioactive Secondary Metabolites

A thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

Rhodes University

Tara Aisling Walmsley

February 2013

‘All are but parts of one stupendous whole,

whose body Nature is,

and God the soul’

TABLE OF CONTENTS

List of Figures vi

List of Tables xiii

List of Abbreviations xiv

Acknowledgements xvi

Abstract xviii

Chapter One 1

Literature Review 1

1.1 Sponge taxonomy 3

1.2 Sponges as the dominant source of biologically active marine natural products 4

1.2.1 Psammaplin A 5

1.2.2 Spermatinamine 6

1.2.3 Crambescidin 800 7

1.2.4 Candidaspongiolide 8

1.2.5 Bengamides 9

1.2.6 Halichondrin B 10

1.2.7 Pyrroloiminoquinones 12

1.3 Diversity of sponge-associated microorganisms 17

1.3.1 Sponge-associated microbial communities – a product of habitat or maternal instinct? 19

1.3.2 Sponge-bacteria symbiosis 20

1.4 The biotechnological potential of sponge associated microorganisms 23

1.4.1 Theonella swinhoei 25

1.4.2 Manzamine A 27

1.4.3 Genomic approaches for the production of secondary metabolites associated with sponge symbionts 29

1.5 The sponge genus *Tsitsikamma*, a source of biologically active pyrroloiminoquinone metabolites and novel bacterial communities 31

1.6 Hypothesis 39

1.7. Research Objectives 39

1.8 References 40

Chapter Two 53

Sponge Identification and Chemical Characterisation 53

2.1 Introduction 53

2.2 Methods and Materials 55

2.2.1 Sponge collection 55

2.2.2 Morphological identification of sponge specimens 56

2.2.3 Total genomic DNA extraction from sponge tissue 56

2.2.4 General PCR amplification protocol 57

2.2.5 Molecular identification and phylogenetic analysis of sponge species 57

2.2.6 Isolation of the major secondary metabolite from a batch collection of *T. favus* 58

2.2.7 NMR stack plot comparison of a crude extract of *T. favus* specimens Sp01, Sp02 and Sp03 60

2.2.8 NMR stack plot comparison and mass spectrometry analysis of a crude extract of *T. scurra* and *T. favus* 60

2.3 Results 61

2.3.1 Sponge taxonomy 61

2.3.2 Molecular Identification of sponges using the COX1 and 28S rRNA gene fragments 62

2.3.3 Isolation and characterisation of the major secondary metabolite in *Tsitsikamma favus* 65

2.3.4 NMR and LCMS analysis of the secondary metabolite profile of *T. favus* and *T. scurra* specimens 67

2.4 Discussion 70

2.5 References 73

Chapter Three 75

Diversity and population structure of *Tsitsikamma favus*- associated bacteria 75

3.1 Introduction 75

3.2 Materials and Methods 76

3.2.1 Extraction of total genomic DNA 76

3.2.2 Primers used in the 16S rRNA molecular analysis 76

3.2.3 DGGE analysis of bacterial 16S rRNA gene amplicons 77

3.2.4 Preparation and sequencing of 16S rRNA clonal libraries derived from *T. favus* 77

3.2.5 Phylogenetic analysis of the 16S rRNA clonal libraries of *T. favus* associated bacteria 78

3.2.6 454 pyrosequencing analysis of the *T. favus*-associated 16S rRNA amplicons 78

3.3 Results 80

3.3.1 DGGE analysis of sponge-associated bacterial diversity 80

3.3.2 16S rRNA clone library analysis of sponge-associated bacterial communities 82

3.3.3 Direct comparison of *T. favus* DGGE profiles with the dominant 16S rRNA gene clones 86

3.3.4 454 pyrosequencing analysis of the bacterial community associated with *T. favus* 88

3.4 Discussion 92

3.5 References 95

Chapter Four 99

Comparing the Bacterial Diversity associated with Algoa Bay Latrunculid Sponges 99

4.1 Introduction 99

4.2 Methods and Materials 100

4.2.1 Isolation of total genomic DNA followed by DGGE and NGS analysis of sponge-associated 16S rRNA amplicons 100

4.3 Results 102

4.3.1 Comparison of the bacterial communities associated with Algoa Bay Tsitsikamma and Latrunculia sponges using DGGE profiling 102

4.3.2 454 pyrosequencing analysis of the bacterial communities associated with Algoa Bay Latrunculid sponges 103

4.3.3 OTU based analysis of sponge-associated bacterial communities 108

4.4 Discussion 116

4.5 References 119

Chapter Five 122

Isolation and culture of sponge-associated bacteria 122

5.1 Introduction 122

5.2 Methods and Materials 124

5.2.1 Microbial strains and general culture conditions 124

5.2.2 Isolation of dominant *T. favus*-associated bacterial strains 125

5.2.3 Actinomycete isolation media and growth conditions 126

5.2.4 Screening of a crude *T. favus* extract for antimicrobial activity 127

5.2.5 Screening of actinomycete isolates for antimicrobial activity 127

5.3 Results 129

5.3.1 Antimicrobial activity of the crude sponge extract 129
5.3.2 Isolation and culture of *T. favus* associated bacteria.......................... 130
5.3.3 Isolation and culture of sponge-associated actinomycetes 137
5.3.4 Antimicrobial activity of sponge-associated actinomycetes................ 139
5.4 Discussion 141
5.5 References 143

Chapter Six 147
General discussion and future prospects 147
6.1 General Discussion... 147
6.2 Future Prospects 153
6.3 References 155

Appendices 158
Appendix A1. 158
Appendix A2. 158

LIST OF FIGURES

Figure 1.1 Chemical structure of psammaplin A and the clinically significant analogue NVP-LAQ824 (Quinoa and Crews, 1987; Pina et al., 2003) 5

Figure 1.2 Chemical structure of spermatinamine (Buchanan *et al.*, 2007) 6

Figure 1.3 Chemical structure of Crambescidin 800 (Jares-Erijman *et al.*, 1991) 7

Figure 1.4 Chemical structure of candidaspongiolide (Trisciuoglio *et al.*, 2008). 8

Figure 1.5 Chemical structure of bengamide A (Quinoa *et al.*, 1986) and its derivative LAF389 (Xu *et al.*, 2003) 9

Figure 1.6 Chemical structure of the halichondrin B analogue eribulin mesylate (Seletsky *et al.*, 2004) 10

Figure 1.7 Chemical structure of discorhabdin A (Perry *et al.*, 1988) and makaluvamine A (Barrows *et al.*, 1993) 13

Figure 1.8 Secondary metabolites isolated from the South African sponges **(A)** *Latrunculia bellae* (Antunes *et al.*, 2004), **(B)** *Strongylodesma algoaensis* (Antunes *et al.*, 2004), and **(C)** *Strongylodesma aliwaliensis* (Keyzers *et al.*, 2004, Keyzers *et al.*, 2005) 15

Figure 1.9 Diketopiperazines isolated from a sponge-associated *Micrococcus sp.* (Stierle *et al.,* 1988) 23

Figure 1.10 Secondary metabolites commonly isolated from *O. spongeliae* associated with *L. herbacea* (Unson *et al.,* 1994; Flowers *et al.,* 1998) 24

Figure 1.11 Chemical structure of swinholide A (Fusetani *et al.,* 1990) and scytophycin C (Carmeli *et al.,* 1990) 25

Figure 1.12 Chemical structure of Manzamine A (Sakai *et al.*, 2002) and tetracyclic ABCE ring substructure (Li *et al.,* 1998) 27

Figure 1.13 Chemical structure of pederin (Sakemi *et al.,* 1988), onnamide A (Kellner & Dettner, 1995), and discodermolide (Gunasekera *et al.,* 1990). 29

Figure 1.14 Secondary metabolites isolated from the South African marine sponge *Tsitsikamma pedunculata* (Antunes *et al.*, 2004) and *Tsitsikamma favus* (Hooper *et al.*, 1996; Antunes *et al.*, 2005). 33

Figure 1.15 Pyrroloiminoquinones from unrelated sources – tsitsikammamine A (marine sponge, Antunes *et al.*, 2005), wakayin (ascidian, Copp *et al.,* 2002), makaluvamine A (sponge and myxomycete, Ishibashi *et al.*, 2001), ammosamide A and B (*Streptomyces*, Hughes *et al.,* 2009), mycenarubin A (mushroom, Peters & Spiteller, 2007a) and lymphostin (*Streptomyces* and *Salinospora*, Nagata *et al.*, 1997 and Miyanaga *et al.,* 2011). 35

Figure 1.16 Putative biosynthetic pathway for the synthesis of pyrroloiminoquinones with tryptophan as a precursor (taken from Antunes *et al.* 2005). 37

Figure 2.1 Schematic representation of spicule structure used to describe the discorhabd spicules of sponges belonging to the family *Latrunculiidae* (taken from Kelly & Samaai, 2002) 53

Figure 2.2 Freshly collected Algoa Bay specimens identified as *Tsitsikamma favus* (**A**), *Tsitsikamma scurra* (with yellow encrusting sponge) (**B**) and *Latrunculia sp.* (**C**). 61

Figure 2.3 Neighbour-joining phylogenetic tree based on *COX1* and 28S rRNA sequences of *T. favus*, *T. scurra, Latrunculia sp.,* and the yellow encrusting sponge; as well as relevant outlier sponges. Genbank accession numbers are indicated in brackets and bootstrap values (100 replicates) are indicated at the nodes. The scale bar represents 1% sequence divergence. 64

Figure 2.4 NMR spectra of the major secondary metabolite from *T. favus*. **(Top)** ^{1}H NMR spectra (δH 2.8 ppm – 7.5 ppm) and **(Bottom)** HMBC spectra (δH 6.7 ppm – 7.5 ppm and δC 180 ppm – 110 ppm). The spectra of the major secondary metabolite isolated from

a batch of *T. favus* specimens collected from Evans Peak, Algoa Bay in May 2009 (pink) were compared with those of tsitsikammamine B isolated from a previous collection (Tsitsikamma Marine Reserve) of *T. favus* by Antunes *et al.* (2004) (blue)......... 66

Figure 2.5 A section (δH 6.7 ppm - 7.8 ppm) of the ^{1}H NMR spectra (600 MHz, MeOD) of the 25% acetone fraction of a crude methanol extract of *T. favus* specimens Sp01 (black), Sp02 (blue) and Sp03 (pink), and *T. scurra* (green). 68

Figure 2.6 Discorhabdin compounds identified in *T. scurra* using ESI LCMS with the M + 1 ion bromine isotopic cluster on the right................ 69

Figure 3.1 Schematic representation of the 16S rDNA gene fragment with the known variable regions and primers used during this study indicated... 76

Figure 3.2 DGGE analyses of the 16S rRNA gene sequences of bacterial communities associated with individual *T. favus* specimens. (**A**) Comparison of DGGE profiles of 16S rRNA gene amplicons derived from duplicate (a and b) DNA samples extracted from *T. favus* specimens Sp01, Sp02 and Sp03, collected in 2009 and from seawater squeezed from specimen 2 (Sp02*). (**B**) Comparison of 16S rRNA gene sequence diversity of bacterial communities associated with *T. favus* specimens collected in 2009 (Sp01, Sp02 and Sp03) and 2010 (Sp04, Sp05, Sp13, Sp14, Sp15 and Sp17). The relative positions of conserved bands are indicated on the right (i-v)... 80

Figure 3.3 The complete 16S rRNA gene sequences from the clone libraries of Sp02 and Sp02* were used to generate a Neighbour-joining phylogenetic tree represented by 8 different phyla. The Proteobacteria were the most dominant of the phyla and are displayed as a separate tree (**A**) with the other 7 phyla grouped together in tree (**B)**. Bootstrap values (100 replicates) are indicated by numbers at the nodes. The scale bar represents 10% sequence divergence. The above ARB phylogram was taken from Walmsley

et al. (2012) with permission from the editor of Marine Biotechnology (see appendix A2) and is the original work of Fan Zhang. 85

Figure 3.4 Comparison of DGGE profile of dominant species in clone libraries (Sp02-9, Sp02-3, Sp02-2, Sp02sw-5 and Sp02-1) with microbial diversity associate with *T. favus* specimens Sp01 (1), Sp02 (2) and Sp03 (3). The relative positions of conserved bands i, ii, iii and v are indicated on the right 86

Figure 3.5 Rarefaction analysis, with distance values of 0.01, 0.03, 0.05 and 0.1 of 16S rRNA amplicons from *T. favus* specimen Sp02 (as determined by Sanger sequencing of a clonal library as well as by 454-pyrosequencing, (**A**) and (**B**) respectively) and *T. favus* specimen Sp05 (as determined by 454-pyrosequencing (**C**)) (Cole *et al.,* 2009). 88

Figure 3.6 Analysis of the 16S rRNA diversity associated with *T. favus* specimens Sp02 and Sp05. Relative percentages of the total number of 16S rRNA sequence reads for each of the dominant (**A**) and less well-represented (**B**) bacterial phyla, as well as for the phylum Protoebacteria (**C**); as determined by 454-pyrosequencing (Pyroseq Sp02 and Pyroseq Sp05) and Sanger sequencing (Sanger Sp02); are represented 90

Figure 4.1 DGGE analysis of the 16S rRNA gene sequences of bacterial communities associated with the Algoa Bay sponge specimens. Sp01, Sp02 and Sp03 are *T. favus* specimens; Sp06 is the *Latrunculia sp.*; Sp07, Sp09 and Sp11 are *T. scurra* specimens; and Sp08, Sp10 and Sp12 are specimens of the yellow encrusting sponge associated with Sp07, Sp09 and Sp11 respectively. Unique bands within a sponge species are indicated with a '*' 102

Figure 4.2 Rarefaction analyses of 16S rRNA gene amplicons from *T. favus* specimens Sp02 and Sp16, *T. scurra* specimens Sp07 and Sp27, *Latrunculia sp.* specimen Sp06 and yellow encrusting sponge specimen Sp08. The horizontal axis represents the number of

sequences sampled and the vertical axis the number of phylotypes respectively. 105

Figure 4.3 Phylogenetic classification of bacterial diversity associated with *T. favus* (Sp02, Sp16), *T. scurra* (Sp07, Sp27), yellow encrusting sponge (Sp08) and *Latrunculia sp*. (Sp06) specimens as indicated by 16S rRNA gene sequences. Relative percentages of the total number of 16S rRNA sequence reads for each of the dominant (**A**) and less well-represented (**C**) bacterial phyla as well as the relative percentages of the 16S rRNA sequence reads assigned to the phylum Proteobacteria for each of the represented proteobacterial classes (**B**). 107

Figure 4.4 Relative abundance of the top 10 OTUs within each sponge specimen. 110

Figure 4.5 Neighbour-joining phylogenetic tree based on 16S rRNA sequences of the top 10 OTUs assigned to *T. favus*, *T. scurra*, *Latrunculia sp.,* and the yellow encrusting sponge. Genbank accession numbers are indicated in brackets and bootstrap values (100 replicates) are indicated at the nodes. The scale bar represents 10% sequence divergence. 111

Figure 4.6 Heatmap of OTUs assigned to *T. favus* (Sp02 and Sp16), *T. scurra* (Sp07 and Sp27), *Latrunculia sp.* (Sp06) and the yellow encrusting sponge (Sp08) at a distance value of 0.03. 113

Figure 4.7 Venn diagram showing the degree of OTU overlap between (**A**) T. favus specimens Sp02 and Sp16, (**B**) T. scurra. specimens Sp07 and Sp27 and (**C**) Algoa Bay sponge species *T. favus*, *T. scurra*, *Latrunculia sp.* and the yellow encrusting sponge; at a distance value of 0.03. 114

Figure 5.1 The effect of a crude methanol extract of *T. favus* on the growth of a range of Gram-positive and Gram-negative bacteria. Row 1 (clockwise starting from top left quadrant): *S. aureus* (Sa), *S. marcescens* (Sm), *Erwinia* (E), *K. pneumonia* (Kp); Row 2 (clockwise starting from top left quadrant): *B. subtilis* (Bs), *S. sonnei*

(Ss), *E. coli* (Ec), *P. aeruginosa* (Pa). Row 3 (clockwise starting from top left quadrant): UCT-05-011 #3 (#3), UCT-05-011 3d (3d), *P. putida* strain (RUKM3s) (Pp), *E. coli* (Ec)............................... 129

Figure 5.2 DGGE analysis of culturable *T. favus* associated bacteria**.** **(A)** Comparison of DGGE profiles of dominant species in clone libraries (Sp02-9, Sp02-3, Sp02-2, Sp02sw-5 and Sp02-1) with microbial diversity present in *T. favus* specimens Sp01 (1), Sp02 (2) and Sp03 (3). The relative positions of conserved bands i, ii, iii and v are indicated on the right. **(B)** DGGE profile of 'culturable' bacteria in *T. favus* on different solid and liquid media (Sp26BM – MA2216 broth, Sp26I – ISP2 solid medium and Sp26M – MA2216 solid medium) with dominant *T. favus* associated bands indicated on the left. Sp26 refers to *T. favus* specimen Sp26............................... 131

Figure 5.3 **(A)** Colonies of *P. ascidiaceicola* growing on MA2216 plates. **(B)** ^{1}H NMR of 25% acetone fraction of *P. ascidaiceicola* (purple) and *T. favus* (black).. 133

Figure 5.4 DGGE profile of colonies picked from MA2216*. Each DGGE well represents 8 individual colonies all picked from the same plate (as described in section 5.2.2). Only the lanes that were loaded with colonies from MA2216* are indicated because they alone contained a band corresponding to Sp02-1. 134

Figure 5.5 An 'Sp02-1' colony after 4 days **(A)** and 10 days **(B)** of growth on MA2216* at 28 °C. The image on the right is of the colony itself and the image on the left is a Gram-stain of the colony. 135

Figure 5.6 DGGE profiles of 6 individual colonies (lanes 1 to 6) that were picked after re-streaking and monitoring of the colonies containing a band corresponding to 'Sp02-1' in the DGGE profile displayed in Fig. 5.4. Lane 7 – DGGE profile of bacteria associated with *T. favus* specimen Sp02 (Sp02), lane 8 – DGGE profile of the *Staphylococcus sp*. identified ('Staph") with a '*' next to the most dominant band, lane 9 – DGGE profile of *T. favus* clone Sp02-1

(Sp02-1) and lane 10 – DGGE profile of the dominant *T. favus* associated clones (Clones). 136

LIST OF TABLES

Table 3.1: Distribution of *T. favus*-associated 16S rRNA gene sequences within the major bacterial phylogenetic groups.............................. 83

Table 4.1: Assignment of sequencing reads at various stages in the molecular analysis of bacterial communities associated with Algoa Bay Latrunculid sponges. 104

Table 4.2: Sequence reads assigned to the top 20 OTUs represented within the Latrunculid sponges. 109

Table 5.1: Diversity of Actinobacteria isolated from Algoa Bay sponges 138

Table 5.2: Antibiotic activity of Algoa Bay actinomycetes against *M. aurum*. The numbers indicate the zone of inhibition in millimetres on different actinomycete growth media as described in section 5.2.5 (taken from Matongo & Meyers, 2011). 140

LIST OF ABBREVIATIONS

ASW	sterile artificial seawater
bp	base pair
CAN	candidaspongiolide
COX1	mitochondrial cytochrome c oxidase subunit I gene
CQ	chloroquine
CTAB	cetyltrimethylammonium bromide
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EF1α	nuclear protein elongation factor 1-α
ESI	electron spray ionisation
FISH	fluorescence *in situ* hybridization
gDNA	genomic DNA
^{1}H	proton
HDAC	histone diacetylase
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HP20	polyaromatic adsorbent resin
HMA	high microbial abundance
Icmt	isoprenylcysteine methyltransferase
IC_{50}	half maximal inhibitory concentration
ITS	internal transcribed spacer
LCMS	liquid chromatography mass spectrometry
LMA	low microbial abundance
MeOD	deuterated methanol
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
NA	nutrient agar
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
nt	nucleotide
OTU	operational taxonomic unit

PCR	polymerase chain reaction
PKS	polyketide synthase
PSDVB	polystyrene-divinylbenzene
rDNA	ribosomal DNA
RDP	ribosomal database project
RNA	ribonucleic acid
ROV	remotely operated vehicle
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
SCUBA	self-contained underwater breathing apparatus
SOP	standard operating procedure
TE	tris-ethylenediaminetetraacetic acid
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TRFLP	terminal restriction fragment length polymorphism
UPLC	ultra-high performance liquid chromatrography

ACKNOWLEDGEMENTS

"One of the ironies of courage, and the reason why we prize it so highly, is that we find it easier to be brave for someone else than we do for ourselves alone."

To all those I hold close to my heart… your encouragement and unwavering faith, your belief that I was stronger than I ever felt, have made my dream a reality. I cannot put into words the gratitude that I feel, but I shall try.

Firstly, to my husband Ryan who has listened to me stress and cry and doubt myself for the last five years, thank you for always encouraging me and for always being able to make me laugh. You (and of course sweet Twab Sanjeev Walmsley) are my source of joy every day. Also, your skills at formatting have impressed me to no end. To my Mom (Aisling Beckerling) and my Dad (Andrew Beckerling), you are the most wonderful parents possible. All my life you have given me everything that I needed. You have always had so much faith in me and believed that I am capable of anything I put my mind to. Without you I would never even have attempted such an endeavour. I also knew that I wouldn't hear the end of it if I didn't finish what I had started… and that motivated me in more ways than you can imagine (nervous laugh). To my brother (Dylan Beckerling), you came to Rhodes when I started my Honours degree. That was a tough year but you were always there for me. We laughed and we cried. We played squash (me badly) and we swam in the pool. It was so comforting having you there throughout most of my post graduate studies. You have always been my closest friend, and some things really never change. Gigi, Grandpa, Granny and Papa; you were always so proud of us grandchildren and made us feel so loved. Thank you for only speaking kind words into my life. Such things will shape a person forever. To all the cousins, aunties and uncles who make any family gathering a joyful one, my only regret is that I don't see you more often.

Of course no scientific study is complete without a laboratory gang, a home away from home. Adriano, James, Jane, Mpho, Jen, Gareth, Ritah and Gwynneth… thank you for changing the radio from Algoa to 5fm, thank you for doing your lab duty every week when it was your turn, thank you for being conscientious and not

contaminating the communal kits, thank you for awkward birthday celebrations in the computer room despite the fact that it was cramped and devoid of ample seating, thank you for laughing and being understanding when things got tough and stressful. I miss you guys in the work place. To Val Hodgson, thank you for watching over us. You kept Lab 417 running like a well-oiled machine which made doing research a pleasure. With you in charge things worked… and it is always better when things work. To the tearoom regulars Margot, Sagran and Joan, thanks for the 10:30 and 3:30 daily breaks. They kept me going towards the end. To the chemistry crowd in S2/S3 who were always there to welcome me back when the time came. I started as a chemist and ended as a microbiologist but there was always a desk and a cupboard and a bottle of deuterated methanol waiting for me when I returned.

To my supervisors Prof Dorrington and Prof Davies-Coleman, two very different people with two very different approaches. I learnt so much from both of you. Prof D-C you encouraged me right from the start. Even when I was just a little undergrad going through the motions, you were there encouraging me. You persuaded me to do my Honours degree and then persuaded me to start a rather daunting Masters degree. It was that Masters degree that took me across the road to Prof Dorrington. I then became the odd one out… a chemist in a microbiology lab and a microbiologist in a chemistry lab. Prof Dorrington you taught me so much about everything. You taught me to stand up for myself, you taught me to take criticism, you taught me to work hard, you taught me and taught me and taught me. You pour so much effort into your students. I miss walking into your flower filled office and chatting about all the possibilities that awaited us in the lab.

A final thanks to everyone else who played a role in making this thesis a reality. Thanks to my collaborators Prof Russell Hill, Fan Zhang, Dr Paul Meyers and Tarisayi Matongo. Thanks to the technical support staff and the Rhodes research office who always went out of their way to assist. Finally, thanks to NRF, DAAD, the 1820 British Settler's fund and Rhodes University for funding my studies.

ABSTRACT

Algoa Bay Latrunculid sponges are well known for their production of cytotoxic pyrroloiminoquinones with speculation that these secondary metabolites may have a microbial origin. This study describes a thorough investigation into the bacterial community associated with *Tsitsikamma favus*, *Tsitsikamma scurra* a newly described *Latrunculia sp*. and a yellow encrusting sponge associated with *T. scurra.* Molecular and chemical characterisation were used in conjunction with traditional taxonomy in identification of the sponge specimens. The 28S rRNA and *COX1* analysis confirmed the traditional taxonomy with *T. favus* and *T. scurra* being very closely related. Chemical analysis revealed that *T. favus* and *T. scurra* shared the discorhabdins 2,4-debromo-3-dihydrodiscorhabdin C, 7,8-dehydro-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V in common with each other and *Tsitsikamma pedunculata* indicating that these pyrroloiminoquinones are common to *Tsitsikamma* sponges in general.

The bacterial community associated with *T. favus* was explored using 16S rRNA molecular techniques including DGGE, clonal libraries of full length 16S rRNA genes, as well as 454 pyrosequencing. DGGE analysis revealed that the bacterial community associated with *T. favus* appeared to be highly conserved, which was confirmed by both the clone library and 454 pyrosequencing, with the Betaproteobacteria as the most dominant class. Further exploration into *T. favus,* as well as *T. scurra*, *Latrunculia sp*. and the yellow encrusting sponge indicated that the bacterial populations associated with each of these sponge species were conserved and species specific. OTU analysis to the species level revealed that *T. favus* and *T. scurra* shared an abundant Spirochaete species in common while the most abundant species in the *Latrunculia sp.* and the yellow encrusting sponge belonged to the class Betaproteobacteria. The exclusivity of the tsitsikammamines to *T. favus* precipitated attempts to culture the *T. favus* associated bacteria, with a focus on the dominant betaproteobacterium as indicated by the 16S rRNA clone library. Actinobacteria associated with the Algoa Bay sponge specimens were also cultured and the actinobacterial isolates were sent for screening against *Mycobacterium aurum* with two *Kocuria kristinae* isolates and a *Streptomyces albdioflavus* isolate showing good antimycobacterial activity.

Chapter One

LITERATURE REVIEW

The first medicinally important natural products were obtained from plants and include morphine and aspirin, which are still economically important (reviewed in Cragg *et al.*, 1999). The isolation of penicillin from the *Penicillium* fungus in 1928 was a major break-through in the treatment of infectious diseases, and catalysed research into the use of microorganisms for the production of bioactive compounds (Wainright, 1990). A comprehensive review of natural products by Newman & Cragg (2007) highlights the importance of natural products in the development of new drugs. However, the terrestrial environment (plants, invertebrates, and microorganisms) has been extensively explored, creating the need for a new source of novel bioactive metabolites. The chemistry of natural products includes isolation and characterization of bioactive compounds from natural sources; structural modification for optimization of activity, absorption and chemo-therapeutic efficacy; as well as total and semi-synthesis for determining structure-activity relationships and clinical trials (Liang & Fang, 2006). The above-mentioned chemistry can be time-consuming and expensive and thus, despite the success of natural product drug discovery, pharmaceutical companies started moving towards combinatorial chemistry as a source of new drugs in the 1980s. Since then, most of the research done on developing new drugs has been focused in this area. However, the results have been disappointing with only one clinically-approved drug, the antitumor compound, sorafenib, resulting from this method of chemical discovery (Blunt *et al.,* 2011; Newman & Cragg, 2007). The significance of natural product research for the discovery of new drugs is undisputable as natural products and their derivatives are and always have been the most prolific source of medicinally significant compounds (Blunt *et al.*, 2011; Newman & Cragg, 2007).

The marine environment comprises two thirds of the earth's surface providing a major, underexplored, biologically diverse ecosystem. The cone snail toxin (also known as Prialt), the first marine-derived drug, was approved in 2004 for the treatment of severe neuropathic pain (Newman & Cragg, 2007). A second marine

natural product success story that is currently used for the treatment of soft tissue sarcomas is trabectedin (marketed by PharmaMar under the trade name Yondelis), which was first extracted from the ascidian, *Ecteinascidia turbinate,* in 1969. Low yields, limited success in aquaculture, and complex chemical synthesis made production of trabectedin very difficult and clinical trials were hampered as a result (Rinehart, 2000). A breakthrough then came in the form of cyanosafracin B, a chemical analogue to trabectedin, produced by the marine bacterium *Pseudomonas fluorescens* (Cuevas *et al*., 2000). Cyanosafracin B could be produced in large quantities under standard industrial fermentation conditions and then chemically modified by semi-synthesis to trabectedin in a relatively cost-effective way, which made manufacturing of the drug for the treatment of soft tissue sarcomas possible (Cuevas *et al.*, 2000; Molinski *et al.*, 2009).

Marine organisms are currently regarded as the most promising source of unique bioactive molecules, producing a rich diversity of secondary metabolites that have shown significant activity in anti-tumour, anti-inflammatory, analgesia, immunomodulation, allergy, and anti-viral assays (Blunt *et al.,* 2011; Hill & Fenical, 2010; Newman & Cragg, 2007). It has been estimated that less than 1% of the earth's surface (i.e. the narrow ocean fringe and deep sea vents) are home to the majority of the earth's species and are thus the most species-rich and biologically productive regions of the world. The intense concentration of species coexisting in such a limited habitat makes them highly competitive and complex, and therefore a high percentage of species have developed chemical defence systems against predation or overgrowth by competing species, as well as to subdue mobile prey for ingestion. These chemicals are generally secondary metabolites and need to be potent enough to overcome the dilution effects of seawater. They include well known chemical classes such as terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids, and a number of biogenesis metabolites (Simmons *et al.,* 2005). A unique feature of secondary metabolites from marine organisms is the relatively common utilization of covalently bound halogen atoms, mainly chlorine and bromine, probably due to their availability in sea water (Simmons *et al*., 2005). Sponges, microorganisms, coelenterates, and tunicates are the primary source of bioactive metabolites in the marine environment, with sponges being at the forefront of this field (Blunt *et al*., 2011).

1.1 Sponge taxonomy

As mentioned above, the marine environment is a prolific source of novel bioactive compounds with the phylum Porifera as the major contributor. Porifera are considered to be the most primitive of the metazoans, dating back to the Pre-Cambrian period some 600 million years ago (Taylor *et al.*, 2007b). Sponges are defined as sedentary, filter-feeding organisms with a complex arrangement of aquiferous channels, but with no distinct tissue layers or organs. Each independent cell has a unique and specific function and its position within the sponge network is determined accordingly (Carte, 1996).

Although sponges are extremely diverse in terms of size, shape and colour; they tend to share a basic body plan comprised of several different cell layers. The pinacoderm (outer surface) is comprised of epithelial cells known as pinacocytes which extend along the interior canals of the sponge via ostia (pores) on the sponge surface (Wilkinson, 1978a; Wilkinson, 1978b; Wilkinson, 1978c). Inside the sponge are channels that are lined with specialised flagellated cells (choanocytes) that are responsible for the unidirectional pumping of water as well as the trapping of food particles (Borchiellini *et al.*, 2001). The food particles (including bacteria, microalgae, and fungi) are transferred to the mesohyl where they are digested by the archaeocytes via phagocytosis. Once filtered, water is expelled from the sponge via the osculum. The mesohyl of most sponges also contains a dense community of microorganisms that are not digested by the neighbouring sponge archaeocytes. The presence of these presumed symbionts implies either recognition of different microbial types by the sponge cells or shielding of symbiont cells to prevent consumption (Wilkinson, 1978a; Wilkinson, 1978b; Wilkinson, 1978c). The structural integrity of sponges is provided primarily by the presence of siliceous or calcareous spicules, although collagenous tissues (for example spongin) also play a role in providing structural support and allow for the differentiation of sponges into unique shapes and sizes (Simpson, 1984).

The spicules are diverse and unique to sponge species and are currently the most reliable method of sponge taxonomy. The phylum Porifera is a paraphyletic grouping with three major sublineages: the Hexactinellida, Calcarea, and

Demospongiae which comprise the majority of existing species (Borchiellini *et al.*, 2001). The Demospongiae contain siliceous spicules that can be divided into megascleres and microscleres. The megascleres are quite basic in shape and are usually monaxonic or tetraxonic, while the microscleres are more elaborate in shape and can be polyaxial or monaxone. It is the shape and size of these megascleres and microscleres that is used to identity sponges (Hooper & Van Soest, 2002). According to Hooper & Van Soest (2002) the Class Demonspongiae contains three subclasses namely Homoscleromorpha, Tetractinomorpha and Ceractinomorpha. The Homoscleromorpha are defined as Demospongiae with cinctoblastula larvae and viviparous reproduction. The skeleton consists of tertraxonic siliceous spicules arranged around choanocyte chambers. The Tetractinomorpha are defined as Demospongiae with parenchymella or blastula larvae and mostly oviparous reproduction. The megascleres are monaxonic and tetraxonic while the microscleres are asterose forms and derivatives. The Ceractinomorpha are defined as Demospongiae with parenchymella larvae and mostly viviparous sexual reproduction. They generally have both a spicule skeleton and well developed spongin fibres. The Ceractinomorpha contain the order Poecilosclerida, which is of particular interest to us as it contains the family *Latrunculiidae* that in turn contains the genera *Tsitsikamma*, *Latrunculia* and *Strongylodesma*. All three of the before-mentioned genera are found along the South African coastline and will be further discussed throughout the course of this study, with particular focus on the genus *Tsitsikamma*.

1.2 Sponges as the dominant source of biologically active marine natural products

The sessile nature of marine sponges makes them vulnerable to predation, competition and disease; forcing them to rely heavily on chemical defence as a means of survival. This dependence on chemical defence makes sponges a rich source of bioactive compounds, many of which have potential applications in the pharmaceutical industry (Newman & Cragg, 2007; Blunt *et al.*, 2011; Hill & Fenical, 2010) with more novel bioactive metabolites being discovered in Porifera each year than any other marine phylum. In a recent review Blunt *et al.* (2011)

noted that 287 new compounds were isolated from sponges in 2009 alone, with similar numbers of new compounds arising from sponges in previous years.

The first sponge marine natural products were isolated in the 1950's by Bergman and his co-workers from the Caribbean sponge *Cryptotethia crypta* (Carte, 1996; Bergmann & Feeney, 1950). The major metabolite spongouridine has potent antitumour activity and inspired the chemical synthesis of a new class of arabinosyl nucleoside analogs (Carte, 1996; Roberts & Dekker, 1967; Walwick *et al.*, 1959). The most significant example of these is arabinosyl cytosine, which is classified as an 'antimetabolite' and is currently in clinical use for the treatment of different types of leukaemia and non-Hodgkin's lymphoma under the trade names AraC, Cytarabine and Cytosar-U (Kadia *et al.,* 2012). A number of structurally diverse sponge natural products with different modes of biological activity are further discussed.

1.2.1 Psammaplin A

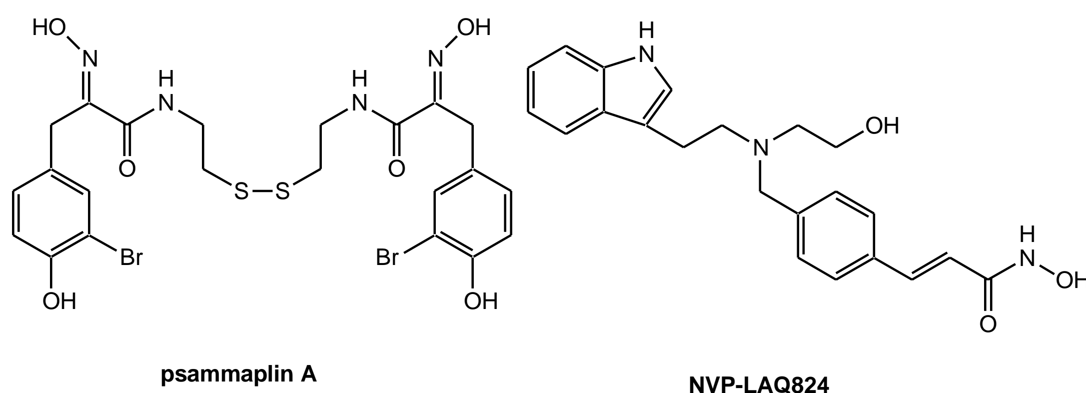


Figure 1.1 Chemical structure of psammaplin A and the clinically significant analogue NVP-LAQ824 (Quinoa and Crews, 1987; Pina et al., 2003).

Psammaplin A is a symmetrical bromotyrosine-derived disulfide natural product (Fig. 1.1) that was first isolated from *Psammaplysilla* sponge found off the coast of Tonga in 1987 (Quinoa & Crews, 1987). Psammaplin A exhibits *in vitro* antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA). Elucidation of the structure of psammaplin A led to the development of a combinatorial chemistry strategy for the construction of heterodimeric disulfide analogues, resulting in the construction of a 3 828 membered library from 88

homodimeric disulfides (Nicolau *et al.,* 2001). These psammaplin A analogues were screened directly against various Gram-positive bacterial strains, leading to the discovery of a series of potent antibacterial agents active against MRSA strains (MIC <1 μg/ml) (Nicolaou *et al.*, 2001). Novartis screened the analogues, together with the microbial products trapoxin B and trichostatin A (due to their structural similarity to the psammaplins), for activity as histone diacetylase (HDAC) inhibitors, and psammaplin A showed very promising activity. Using chemical rationales described by Pina *et al.* (2003), the compound NVP-LAQ824 (Fig. 1.1) was developed from the aforementioned structures, and is in Phase I clinical trials against hematologic malignancies (Newman & Cragg, 2004; Simmons *et al.*, 2005; Pina *et al.*, 2003).

1.2.2 Spermatinamine

Figure 1.2 Chemical structure of spermatinamine (Buchanan *et al.*, 2007).

Spermatinamine (Fig. 1.2) is a novel polyamine alkaloid that was isolated from an Australian Verongida sponge, *Pseudoceratina sp.* (Buchanan *et al.*, 2007). It contains a newly-discovered bromotyrosyl-spermine-bromotyrosyl sequence that is a rare example of a spermine alkaloid from a marine sponge, and other than the synthetic compound cysmethynil, is the only reported small molecule inhibitor of

isoprenylcysteine methyltransferase (Icmt) (Buchanan *et al.*, 2007). Icmt catalyzes the carboxyl methylation of CAAX proteins, which perform a number of important functions in oncogenesis such as targeting of the proteins to the plasma membrane and the subsequent induction of malignancy. Therefore the inhibition of Icmt provides an attractive and novel anticancer target as it has been shown to retard the transformation of cells to a malignant state (Buchanan *et al.,* 2007). Spermatinamine is the first natural product inhibitor of Icmt with an IC_{50} value of 1.9 µM, however it did not display the required drug-like pharmacokinetic properties (Buchanan *et al.*, 2007). The complete synthesis of spermatinamine (García *et al.*, 2009) has allowed for further activity studies and the possible development of novel analogues with more favourable biological activity.

1.2.3 Crambescidin 800

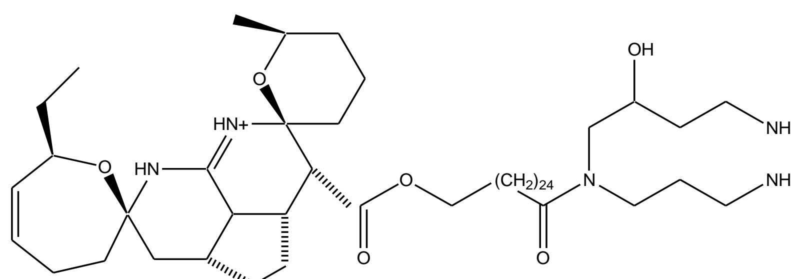


Figure 1.3 Chemical structure of Crambescidin 800 (Jares-Erijman *et al.*, 1991).

The Crambescidins, isolated from the Mediterranean red encrusting sponge, *Crambe crambe*, by bioassay-guided extraction, are a family of complex pentacyclic guanidines linked by a linear ω-hydroxy fatty acid to a hydroxyspermidine. They show activity against the *Herpes simplex* virus type 1 (HSV-1) and are cytotoxic to L1210 murine leukemia cells (Jares-Erijman *et al.*, 1991). Crambescidin 800 (Fig. 1.3) exhibited *in vitro* activity against chloroquine (CQ)-resistant FCR3 (IC_{50} = 240 nM) and CQ-sensitive 3D7 (IC_{50} = 160 nM) *Plasmodium falciparum* strains. The mode of action is unpublished, but similar compounds have been shown to be strong Ca^{2+} channel blockers (Gademann & Kobylinska, 2009; Lazaro *et al.*, 2006).

1.2.4 Candidaspongiolide

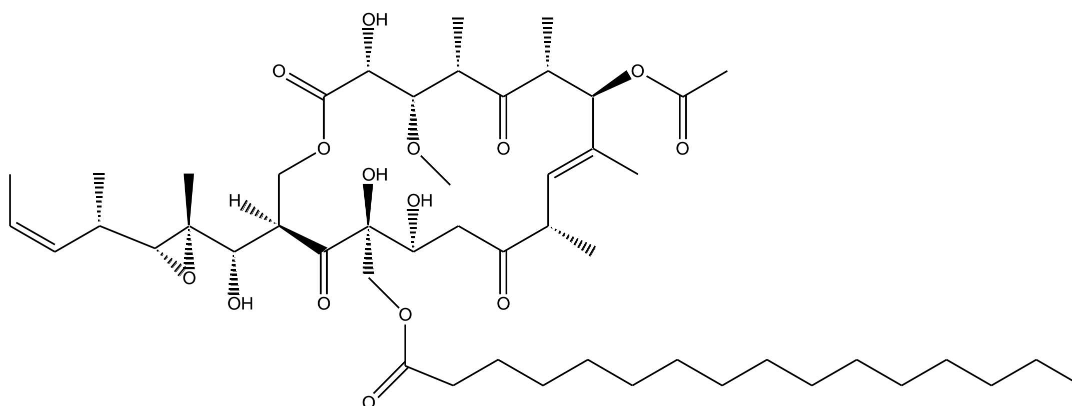


Figure 1.4 Chemical structure of candidaspongiolide (Trisciuoglio *et al.*, 2008).

The novel polyketides known collectively as candidaspongiolides were first isolated from *Candidaspongia flabellata* collected off the coast of Australia (Meragelman *et al.,* 2007). The candidaspongiolide (CAN) shown above (Fig. 1.4) is the active component of a mixture that was shown to be potently cytotoxic (mean IC_{50} = 14 ng/ml) in the National Cancer Institute's human disease oriented 60-cell-line primary antitumor screen (Meragelman *et al.*, 2007, Trisciuoglio *et al.*, 2008). The medicinal significance of CAN is that it triggers PKR/eIF2α/caspase 12-dependent apoptosis as well as inhibiting protein synthesis in cancer cells, while only inhibiting protein synthesis in normal cells (Trisciuoglio *et al.*, 2008). The candidaspongiolides are a complex mixture of acyl esters that are structurally related to tedanolide (isolated from the marine sponge *Tedania ignis*, see section 1.3) (Schmitz *et al.*, 1984). The presence of compounds with the same macrolide nucleus from unrelated sponges in different geographical locations, and the branched nature of the aliphatic esters, points to microorganisms as the source of these compounds (Meragelman *et al.*, 2007).

1.2.5 Bengamides

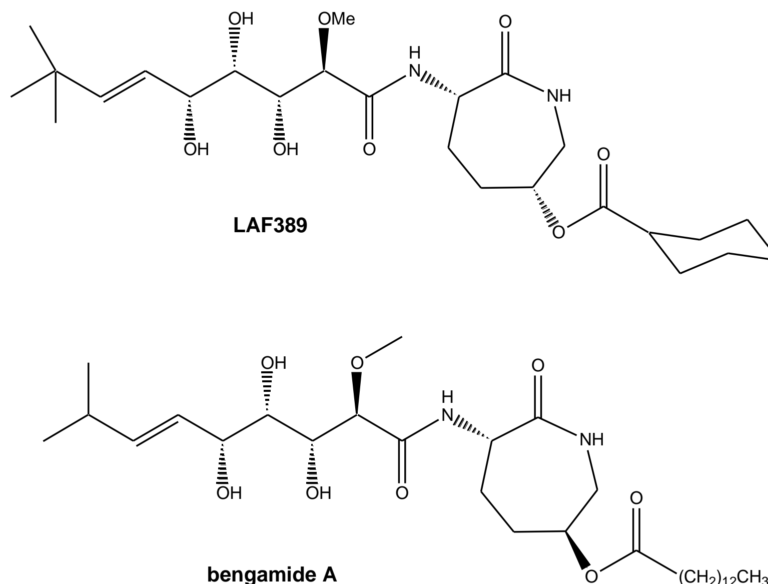


Figure 1.5 Chemical structure of bengamide A (Quinoa *et al.*, 1986) and its derivative LAF389 (Xu *et al.*, 2003).

Bengamide A and B (Fig. 1.5) were first isolated from marine sponges of the genus *Jaspis* (Quinoa *et al.*, 1986) and were reported to be antihelminthic compounds that showed promising antibiotic and cytotoxic activities. Their pattern of activity in the National Cancer Institute's 60-cell-line screen was unique compared with other chemotherapeutic agents, but their further development as therapeutic agents has been hampered by limited availability from natural resources, complexity of synthesis, and poor solubility. Despite this, the bengamide derivative LAF389 (Fig. 1.5) is in phase I clinical trials for the treatment of solid tumours (Singh *et al.*, 2008). Ring-opening of the caprolactam in bengamides has given rise to analogues with simplified structures, improved aqueous solubility and potent cytotoxicity against human breast cancer cells, overcoming some of the factors hindering their development as antitumor agents (Tai *et al.*, 2011).

Bengamides are inhibitors of methionine aminopeptidases (Newman & Cragg, 2004, Towbin *et al.*, 2003) hence their cytotoxicity and this property has been exploited in the development of novel anti-tubercular agents (Lu *et al.*, 2011). Interestingly, there is a patent that relates to bengamide derivatives that are

produced during fermentation by the microorganism *Myxococcus virescens* (Hoffman *et al.,* 2006), lending further support to the notion that microorganisms may be important in the production of sponge-derived secondary metabolites.

1.2.6 Halichondrin B

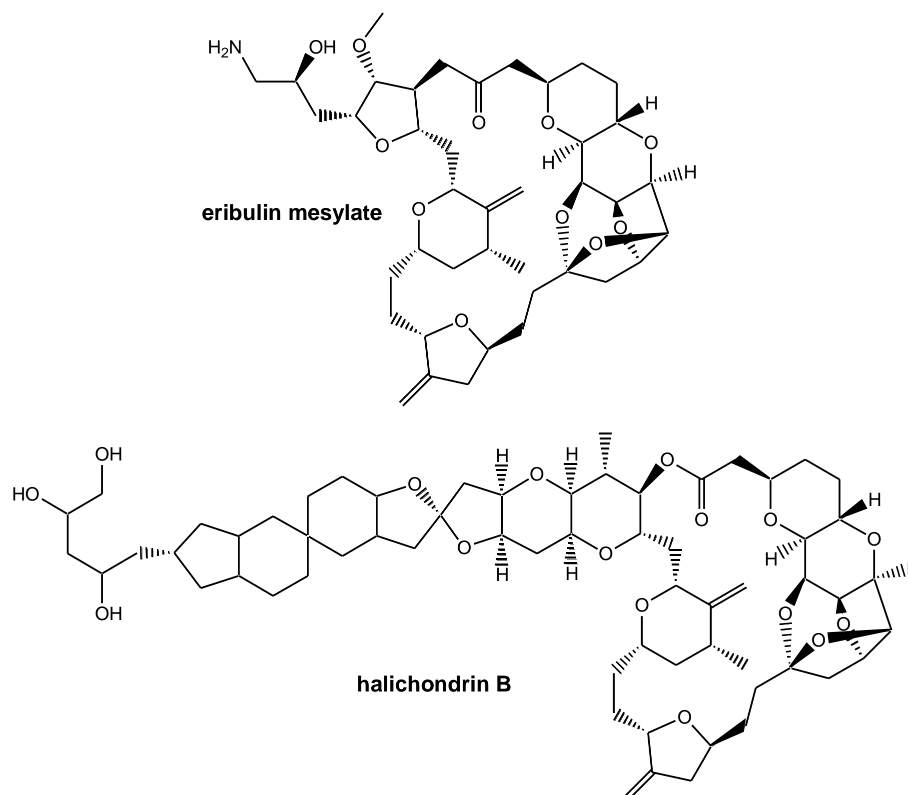


Figure 1.6 Chemical structure of the halichondrin B analogue eribulin mesylate (Seletsky *et al.*, 2004).

Anti-cancer activity dominates for the secondary metabolites isolated from marine sponges, and there are currently a number of sponge metabolites in pre-clinical or clinical trials as potential anti-cancer treatments (Blunt *et al.*, 2011). The most successful of these to date is halichondrin B. Initial work on the marine sponge *Halichondria okadai* yielded the cytotoxic polyether okadaic acid (Tachibana *et al.*, 1981) which was shown to be responsible for diarrhetic shellfish poisoning and was later found to be produced by a number of dinoflagellate species (Yasumoto *et al.*, 1980, Morton *et al.*, 1998, Faulkner, 1991). Further natural product isolation studies on *H. okadai*, guided by a bioassay against B-16 melanoma cells, resulted in the isolation of the first halichondrins (Uemura *et al.*, 1985). Initial interest in this

sponge and its associated natural products was due to the remarkable *in vivo* antitumor activity of the crude sponge extracts, a characteristic that was subsequently used to isolate halichondrin B and numerous natural analogues from various unrelated sponges including *H. okadai*, *Lissodendoryx sp., Phakellia carteri*, and *Axanella sp.* (Faulkner, 2000, Hirata & Uemura, 1986).

Halichondrin B was shown to be the most potent of the halichondrins against P-388 leukemia, B-16 melanoma and L-1210 leukemia *in vivo* (Faulkner, 2000, Hirata & Uemura, 1986). The discovery of these complex structures in different sponge species strongly suggested a microbial origin for the characteristic halichondrin skeletal structure, a theory that was supported by the earlier discovery of a protistan origin for okadaic acid (Simmons *et al.*, 2005). The low yield of the halichondrins (Hirata & Uemura processed 600 kg of the sponge *Halichondria okadai* and only obtained 12.5 mg of halichondrin B, a yield of only 0.00002%), coupled with the difficulty of obtaining deep-water sponges and the environmental impact of harvesting vast amounts of sponge material from the ocean, resulted in a supply problem that severely hampered initial progress.

Lissodendoryx sp. is a rare, deep water marine sponge (80 to 100 m) found exclusively off the Kaikoura Peninsula (Dumdei *et al.*, 1998). An extensive environmental survey using a remotely operated vehicle (ROV) and a benthic camera, established that the 'sponge field' was only 5 km^2 in size with an estimated total biomass of 290 tonnes thus reinforcing the conclusion that halichondrin B could never be supplied on a commercial scale by collection from the wild. However, based on the results of the survey a permit was obtained for the collection of 1 tonne of biomass that yielded 310 mg of halichondrin B, which was sufficient for initial preclinical trials only (Dumdei *et al.*, 1998). Investigations into the bioactivity of halichondrin B revealed that it bound tubulin at a site close to the vinca site thus altering depolymerization with no effect on colchicine binding (Bai *et al.*, 1991), inhibiting the formation of an intra-chain cross-link between two sulfhydryl groups in beta-tubulin. However, halichondrin B had no effect on alkylation of tubulin sulfhydryl groups by iodoacetamide (unlike vinblastin) and enhanced the exposure of hydrophobic areas on the tubulin molecule (Jordan *et al.*, 2005).

In the meantime, largescale aquaculture of *Lissodendoryx sp.* was initiated (Dumdei & Blunt, 1996) with maintenance of biosynthetic ability, albeit at a lower yield, that was considered a sustainable and commercially viable method for the production of halichondrin B. Synthetic organic chemists were also participating in the endeavour to produce halichondrin B, and total synthesis was eventually achieved via a 90 step reaction (Aicher *et al.*, 1992). This synthetic work was expanded by scientists at the Eisai Research Institute who produced a range of structurally stable halichondrin B analogues with equavalent bioactivity (Seletsky *et al.*, 2004). Of those analogues, eribulin mesylate (E7389) had the best activity in pre-clinical models of diseases in which microtubule inhibitors already have a therapeutic role, such as breast cancer. Eribulin mesylate (Fig. 1.6) a non-taxane, completely synthetic microtubule inhibitor, has recently been approved by the U.S. Food and Drug Administration as a third-line treatment for metastatic breast cancer and is marketed as Eribulin or Easia (Jain & Vahdat, 2011).

1.2.7 Pyrroloiminoquinones

Pyrroloiminoquinones belong to a larger group of chemical compounds known as the alkaloids, which are a group of nitrogen containing secondary metabolites derived from amino acids or purines (Dewick, 2001). The principle structural feature of these alkaloids is the planar iminoquinone core that can intercalate with DNA and cleave the DNA double helix or inhibit the action of topoisomerase II (Ding *et al.*, 1999). Consequently almost all the pyrroloiminoquinone metabolites isolated to date have exhibited significant cytotoxicity making them of particular interest to both natural product and synthetic chemists. Included in this family of secondary metabolites are the batzellines, isobatzellines, damirones, discorhabdins, prianosins, epinadrins, makaluvamines, tsitsikammamines, veiutamine and wakayin. The isolation, chemical structures and bioactivity of marine pyrroloiminoquinones has been reviewed by Antunes *et al.* (2005) and a brief overview of the discorhabdins and makaluvamines is presented here.

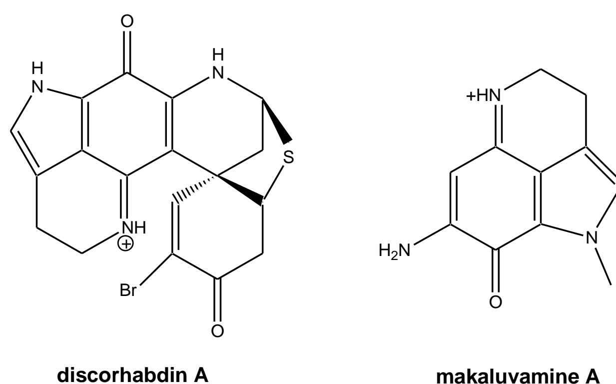


Figure 1.7 Chemical structure of discorhabdin A (Perry *et al.*, 1988) and makaluvamine A (Barrows *et al.*, 1993).

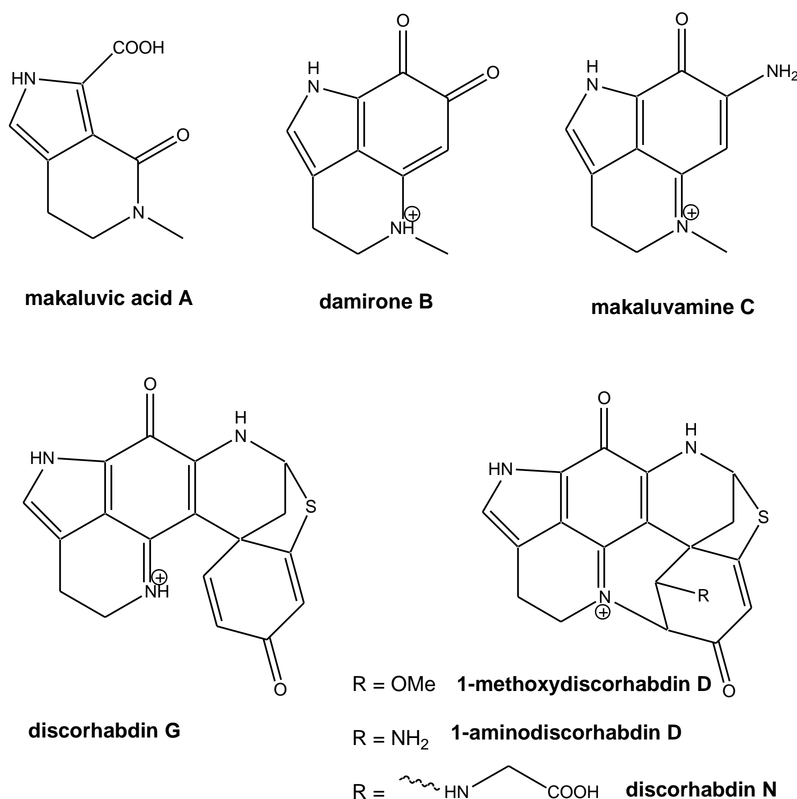
The name discorhabdin, derived from the characteristic discorhabd microscleres used to identify many Latrunculid sponges, is used to describe the cytotoxic green and brown pigments isolated primarily from the family *Latrunculiidae*. Discorhabdin C, isolated as the major compound from sponges of the genus *Latrunculia* in 1986, was the first of the discorhabdins to be discovered (Perry *et al.*, 1986). Discorhabdin A (Fig. 1.7) was first isolated from three different species of *Latrunculia* sponges collected off the coast of New Zealand, and was later also isolated from a Latrunculid sponge, *Strongylodesma algoaensis*, collected off the coast of South Africa (Perry *et al.*, 1988; Antunes *et al.*, 2004). The DNA reductive cleavage activity of certain pyrroloiminoquinones is mediated by redox chemistry, and a thorough investigation of discorhabdin A showed that it was able to cause extensive DNA single stranded scission (Barrows *et al.*, 1993). Discorhabdin A showed good cytotoxicity against human colon tumour cancer (HCT-116) cells (IC_{50} = 0.007 µM), murine leukaemia (L1210) cells (IC_{50} = 37 ng/ml), murine leukaemia (L5178) cells (IC_{50} = 14 ng/ml) and has also been shown to have strong antimicrobial activity (Antunes *et al.*, 2004; Perry *et al.*, 1988).

Makaluvamines A - G were originally discovered in a sponge of the genus *Zyzzya* using bioassay-guided isolation against the human colon carcinoma cell line HCT-116 and their cytotoxic activity was further investigated, with makaluvamine A (Fig. 1.7) and F being the most active (Barrows *et al.*, 1993). Makaluvamines H – M (Schmidt *et al.*, 1995) and N, O and P were isolated from the sponge *Zyzzya fuliginosa* (Venables *et al.*, 1997; Casapullo *et al.*, 2001). The makaluvamines were further investigated as anticancer drug candidates due to their ability to

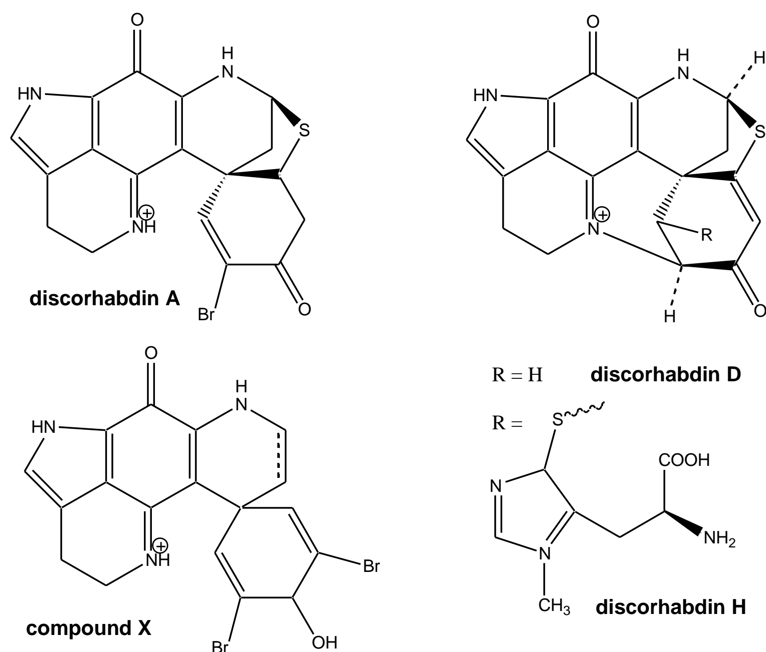
induce dose-dependent DNA cleavage via the inhibition of topoisomerase II (Ding *et al.*, 1999). Synthetic strategies led to the makaluvamine analogues BA-TPQ and FBA-TPQ, which were the most active against breast cancer causing decreased cancer cell growth, apoptosis and cell cycle arrest at sub-micromolar concentrations (Schumacher *et al.*, 2011). Makaluvamines have subsequently been isolated from a number of *Zyzzya* and *Latrunculiidae* sponges and were considered strictly sponge metabolites until makaluvamine A was isolated from a laboratory culture of the myxomycete *Didymium bahiense* (Ishibashi *et al.*, 2001).

The sponge family *Latrunculiidae* are a rich source of these bioactive secondary metabolites, and have therefore attracted considerable interest from researchers around the world. Of particular interest to us are the Latrunculid sponges found off the coast of South Africa, and their associated chemistry, some of which is described below.

A



B



C

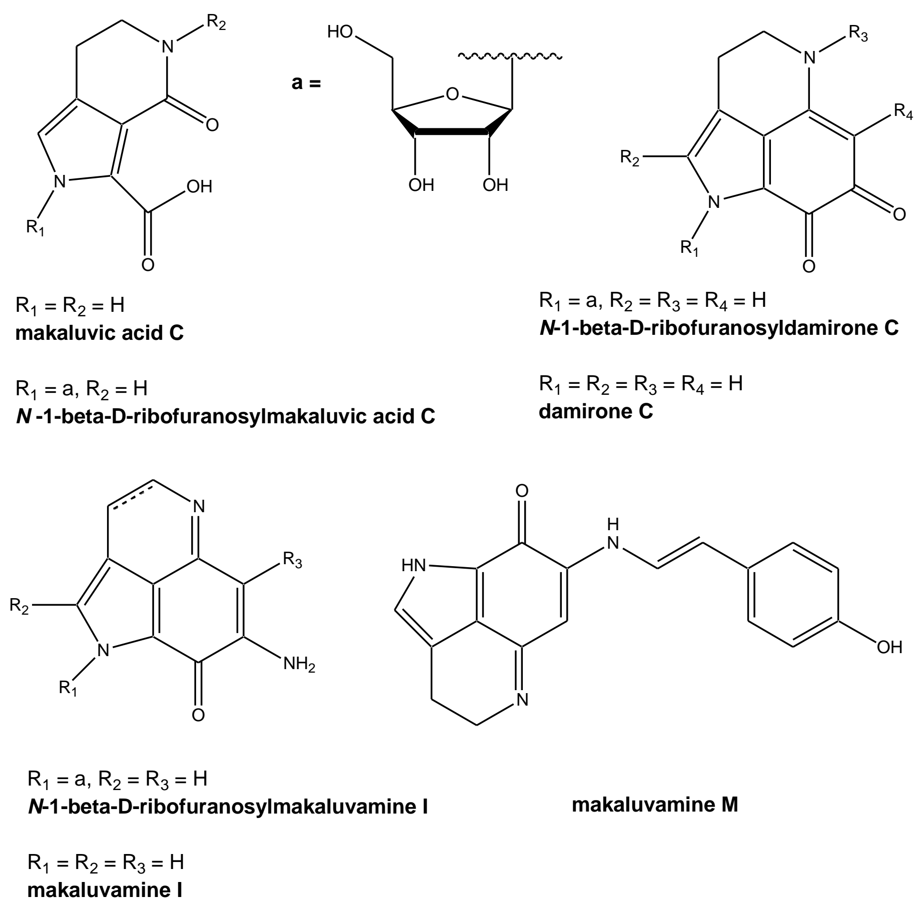


Figure 1.8 Secondary metabolites isolated from the South African sponges **(A)** *Latrunculia bellae* (Antunes *et al.*, 2004), **(B)** *Strongylodesma algoaensis* (Antunes *et al.*, 2004), and **(C)** *Strongylodesma aliwaliensis* (Keyzers *et al.*, 2004, Keyzers *et al.*, 2005).

Latrunculia bellae, a dark green encrusting sponge, was discovered in Algoa Bay at a depth of approximately 24 m. It yielded the new discorhabdins 1-methoxydiscorhabdin D and 1-aminodiscorhabdin D as well as five known metabolites, damirone B, makaluvicacid A, makaluvamine C, and discorhabdins G* and N (Fig. 1.8A). Also found in Algoa Bay was *Strongylodesma algoaensis*, a shallow water Latrunculid sponge from which discorhabdins A, D and H and a compound that is yet to be named (referred to as compound X in Fig. 1.8B) were isolated (Antunes *et al.*, 2004). *Strongylodesma aliwaliensis* was collected by SCUBA from the Aliwal Shoal, an extensive subtropical reef system off the east coast of South Africa. The first investigation of *S. aliwaliensis* revealed two novel pyrroloquinoline metabolites, *N*-1-β-D-ribofuranosyldamirone C and *N*-1-β-D-ribofuranosylmakaluvamine I, as well as three known metabolites namely damirone C, makaluvamine I, and makaluvamine M (Fig. 1.8B) (Keyzers *et al.*, 2004). The polar aqueous eluent, from HP20 chromatography of the crude extract of *S. aliwaliensis*, was a deep red-brown colour, indicative of pyrroloquinoline metabolites, which are highly pigmented (Keyzers *et al.,* 2005). This fraction contained two new pyrroloquinoline metabolites makaluvic acid C and *N*-1-β-D-ribofuranosylmakaluvic acid C (Fig. 1.8C). Damirone C, *N*-1-β-D-ribofuranosyldamirone C, and *N*-1-β-D-ribofuranosylmakaluvic acid C exhibited moderate cytotoxicity against oesophageal cancer cells (IC_{50} = 56, 38 and 61 μM respectively), while Makaluvamine M and *N*-1-β-D-ribofuranosylmakaluvamine I both exhibited good activity against oesophageal cancer cells (IC_{50} = 0.7 and 1.6 μM respectively) (Keyzers *et al.*, 2005).

As was well-illustrated in the case of halichondrin B, the major obstacle preventing the completion of clinical studies and the development of highly effective novel drugs is the so-called “supply problem” (Mohamed *et al.*, 2008). Obtaining sufficient quantities of secondary metabolites from marine sponges is near impossible as yields from chemical extractions are often low, and there are limited amounts of available biomass (Mohamed *et al.*, 2008). A possible solution to this problem is large-scale aquaculture but this is not always sustainable or commercially feasible. Large-scale sponge aquaculture (Gerçe *et al.,* 2009; Mohammed *et al.*, 2008; Bergman *et al.* 2011b) has often resulted in changes in bacterial and secondary metabolite profiles over time. However, certain sponges

such as *Negombata magnifica* (Bergman *et al.,* 2011b) and *Diacarnus erythraenus* (Bergman *et al.,* 2011a) show promise as sponge aquaculture candidates due to the stability of their secondary metabolite production and microbial communities. Chemical synthesis of novel analogues of the secondary metabolites that show promising biological activity (for example the synthesis of novel pyrroloiminoquinone derivatives that show good cytotoxicity) (Beneteau, 2001; Antunes *et al*., 2005) is also an option. However, due to the stereochemical complexity of the marine compounds, chemical synthesis is proving to be extremely difficult and expensive (Wang, 2006; Kennedy *et al*., 2007) and an alternative method of production needs to be considered if research in this field is to be truly successful (Kennedy *et al*., 2007).

The presence of bacteria in marine invertebrates has been known for many years and was not considered of great significance until natural product chemists remarked on the close similarity of compounds isolated from completely different taxa, suggesting a common potentially microbial origin. A noteworthy example was the observation by Perry *et al.* (1988) of the similarities between the structures of mycalamides A & B from *Mycale hentscheli* collected in Dunedin Harbor, South Island, New Zealand, and pederine, a toxin originally isolated from the *Paederus* beetle in South America. Similarly makaluvamine A, a secondary metabolite commonly found in marine sponges, has also been isolated from a laboratory culture of the myxomycete *Didymium bahiense* (Ishibashi *et al*., 2001).

1.3 Diversity of sponge-associated microorganisms

The increased interest in sponges over the past decade stems not only from their pivotal role as a source of bioactive secondary metabolites, but also from the fact that they are known to form close associations with a wide variety of microorganisms (Taylor *et al*., 2007b). The average marine sponge filters up to 24 m^3 (24 000 litres) of seawater per kg of sponge per day, and each millilitre of seawater contains between 1×10^6 and 5×10^6 bacteria (Wang, 2006). As a result, an astonishing number of bacteria (between 2.4×10^{13} and 1.2×10^{14}) pass through every kilogram of sponge every day. It is therefore not surprising that microbes can represent up to 60% of the sponge tissue volume (Vacelet, 1977;

Weisz *et al.*, 2007). Many of these microbes are transferred into the mesohyl tissue where they are ingested by archaeocytes and consequently digested by the sponge (Kennedy *et al.*, 2007). In addition, it is well known that sponges harbour large and diverse microbial communities.

Microbial distribution within sponges varies between species, with the majority of microorganisms occurring extra-cellularly in the mesohyl matrix, while in some cases bacteria are found within sponge bacteriocytes and even in the sponge nuclei (Friedrich *et al.*, 2001, Vacelet & Donadey, 1977, Hentschel *et al.*, 2006). These bacterial communities are remarkably diverse and surprisingly well conserved within members of the same species, with increasing evidence that the sponge-specific microbes were selected for over time and are conserved through vertical transfer to the gametes (Wang, 2006; Turque *et al.*, 2008; Radwan *et al.*, 2010; Webster *et al.*, 2010; Muscholl-Silberhorn *et al.*, 2008; Enticknap *et al.*, 2006). Most bacterial species living within marine sponges are thought to be involved in symbiotic relationships with their host. The sponge provides a niche and nutrients for the microbes while the microbes are proposed to play a role in the processing of waste products, transfer of nutrients, and production of secondary metabolites for chemical defence (Turque *et al.*, 2008; Wang, 2006; Webster & Blackall, 2008; Taylor *et al.*, 2007a).

It is estimated that only a small percentage of the sponge-associated microbial consortium is culturable making culture-independent characterization techniques of importance in obtaining a true understanding of the sponge-associated microbial diversity. The most accurate and widely used techniques involve DNA fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP), coupled with 16S rRNA sequence analysis (Taylor *et al.*, 2007a; Newman & Hill, 2006; Webster *et al.*, 2001; Enticknap *et al.*, 2006; Lee *et al.*, 2009). The 16S rRNA gene fragment is a large polynucleotide of approximately 1 500 bases that functions as part of the small subunit of the prokaryote ribosome (the eukaryote counterpart is 18S rRNA). The 16S rRNA sequence is highly conserved amongst species and can be used to obtain evolutionary information (Weisburg *et al.*, 1991). Most of the 16S rRNA data available for sponge derived sequences is from bulk screening of sponge

samples, so the majority of the sequences represent uncultivated and unclassified species (Taylor *et al.*, 2007b). A comprehensive phylogenetic analysis by Simister *et al.* (2012) on the 7 546 publicly available sponge derived 16S and 18S rRNA sequences revealed a number of sponge specific sequence clusters which were most well represented within the Chloroflexi, Cyanobacteria, Poribacteria, Betaproteobacteria and Acidobacteria (Simister *et al.,* 2012).

1.3.1 Sponge-associated microbial communities – a product of habitat or maternal instinct?

The function of the non-transient sponge-associated bacterial communities and how they are transferred from generation to generation has been addressed by investigating an alphaproteobacterial symbiont common to many marine sponges. In a study by Webster *et al.* (2001) on the sponge *Rhopaloides odorabile*, the alphaproteobacterium NW001 was shown to have a probiotic function because all the sponge specimens containing this symbiont were healthy, while the two sponge specimens that lacked NW001 were infected with a disease-causing bacterium (Webster *et al.*, 2001). Fluorescence *in situ* hybridization (FISH) with probes specific to NW001 showed the presence of this bacterium in the larvae of the sponge *Mycale laxissima* and that the bacteria were densely associated with the larvae rather than being evenly distributed throughout the mesohyl. This data demonstrated the transfer of a bacterial sponge-symbiont through to the gametes (Enticknap *et al.*, 2006).

There is a general consensus that sponges harbour diverse microbial populations, many of which are sponge-specific and are seldom obtained from the surrounding seawater (Jiang *et al.*, 2007; Grozdanov & Hentschel, 2007; Wang, 2006). A comprehensive study of the marine sponges *Myxilla incrustans*, *Haliclona rufescens*, and *Halichondria panacea* from San Juan Island revealed that the bacterial communities found in the sponges were significantly different to and less diverse than those found in the water column from the same site (Lee *et al.*, 2009). A combination of DGGE and TRFLP analysis was used to compare the bacterial communities of sponges within the same species collected at different geographical sites to each other and to the associated water column. The results

illustrated that although the sponge and bacterial community associations were sponge species-specific in some instances, as was the case for *M. incrustans* and *H. rufescens*, this was not always found to be true. *H. panacea* had different microbial profiles when collected at different sites indicating that the associated bacterial community was not sponge-species specific and was largely influenced by its geographical location (Lee *et al.*, 2009).

In a more recent study by Gerce *et al.* (2011), bacterial communities associated with the surfaces were compared to those present in the mesohyl of several Mediterranean sponge species (*Agelas oroides, Chondrosia reniformis, Petrosia ficiformis, Geodia sp., Tethya sp., Axinella polypoides, Dysidea avara,* and *Oscarella lobularis*) as well as with other reference surfaces and bulk seawater. Both high microbial abundance (HMA) sponges, defined as sponges that contain a high number of microbes, and low microbial abundance sponges (LMA), which contain a low number of microbes, were investigated. Bacterial communities associated with the mesohyls of sponges with high microbial abundance (*A. oroides, C. reniformis, P. ficiformis, Geodia sp*.), differed from those on the sponge surface and from those on reference surfaces, indicating that these microbial communities are conserved within the sponge. In contrast, the bacterial community associated with the mesohyl of low microbial abundance sponges (*A. polypoides*, *D. avara* and *O. lobularis*) reflected that on the sponge surface and on reference surfaces indicating a transient bacterial community that arose due to sponge filter activity. Bacterial communities within the mesohyl of HMA sponges of the same species showed a close relationship to each other and seemed to be conserved and sponge species-specific (Gerçe *et al.*, 2011).

1.3.2 Sponge-bacteria symbiosis

The newly described bacterial phylum Poribacteria are found almost exclusively in sponges and were described as a sponge-specific candidate phylum (Fieseler *et al.*, 2004, Taylor *et al.*, 2007b). However, Webster *et al.* (2010) used 16S rRNA gene tag sequencing to detect members of the rare seawater biosphere and showed that Poribacteria were indeed present in the seawater column, albeit at a very low abundance. Bacterial-symbionts that are rare in the seawater biosphere

but prolific in the sponge tissue are now being referred to as seed organisms and their association with their host may have evolved far more recently than previously thought (Webster *et al.*, 2010). The Poribacteria are one of only two prokaryotic lineages that show cell compartmentalization, the other being the Planctomycetes (Lindsay *et al.,* 2001). Fieseler *et al.* (2004) screened the sponges *Aplysina aerophoba*, *Aplysina fistularis*, *Aplysina insularis*, *Aplysina lacunose*, *Verongula gigantean*, and *Smenosponiga aurea* for the presence of Poribacteria. A number of Poribacteria-specific FISH probes were designed and the fluorescence patterns obtained showed that the Poribacteria were abundant within sponge tissue. Siegl *et al.* (2011) used fluorescence-activated cell sorting and phi29 polymerase-mediated whole-genome amplification to isolate approximately 70% of the genome of a member of the Poribacteria. This study (together with that done by Fieseler *et al.* in 2006) provided the first detailed insight into the functional properties and lifestyle of a potentially ancient bacterial sponge symbiont.

From the genome sequence information it was extrapolated that the Poribacteria are Gram-negative mixotrophic bacteria capable of aerobic and anaerobic respiration, with the absence of chemotaxis and flagellar genes (Siegl *et al.,* 2011). The poribacterial genome contained two polyketide synthase (PKS) genes that are frequently found in sponge metagenomic libraries. PKSs are a family of enzymes that produce a large group of secondary metabolites known as polyketides. The discovery of sponge-associated PKS genes in a symbiotic poribacterium potentially links the production of secondary metabolites (thought to be produced by the host sponge) to symbiotic bacteria rather than to the sponge itself (Siegl *et al.*, 2011, Fieseler *et al.*, 2006). In a study by Hocmuth *et al.* (2010) the symbiont-rich sponge, *Cacospongia mycofijiensis*, a source of tubulin-inhibiting fijianolides, was analysed for PKS genes using large-scale sequencing. This study revealed a highly diverse set of PKS genes that clustered with a unique set of PKSs found exclusively in Poribacteria and which are proposed to be involved in methyl-branched fatty acid synthesis. There is increasing evidence to support the hypothesis that many sponge derived natural products are produced by sponge-symbionts and that further insight into the metabolic activity of these symbionts is well worth the effort, as highlighted in a review by Webster & Taylor (2012).

In a recent pioneering study by Kamke *et al.* (2010) the 16S rRNA gene profiles for two sponges, *Ancorina alata* (a high microbial abundance sponge) and a *Polymastia sp.* (a low microbial abundance sponge), both collected in north-eastern New Zealand, were compared. The 16S rDNA gene is present in all bacterial species and provides an indication of the total bacterial diversity present in the sponge, whereas cellular concentrations of 16S rRNA are generally correlated with growth rate and activity (DeLong *et al*., 1989;, Poulsen *et al*., 1993). Hence the rRNA itself can yield useful information about which community members are active. The authors amplified both the DNA gene sequence coding for the 16S ribosomal RNA as well as the actively transcribed ribosomal RNA using the polymerase chain reaction (PCR) and the reverse transcriptase PCR (RT-PCR) respectively, constructed clone libraries and sequenced the inserts in pGEM-T easy recombinant plasmids.

Analysis of the data revealed the presence of a highly diverse bacterial community in *Ancorina alata*, and a much lower diversity in *Polymastia sp*. There was substantial overlap between the 16S rDNA gene fragments and the transcribed 16S ribosomal RNA evident at both the phylum and phylotype levels, indicating that the majority of the more dominant sponge-associated bacteria in both the sponges investigated were physiologically active. This active fraction included uncultivated, sponge-specific lineages within Actinobacteria, Chloroflexi, Spirochaeta and Gemmatimonadetes (Kamke *et al*., 2010). There was also overlap between the microbial consortium found in *A. alata* with that found in other sponges. This finding was not unexpected as there have been numerous reports on distantly related sponges as well as sponges found in different geographical locations sharing a subset of their microbial community (Radwan *et al.,* 2010; Montalvo & Hill, 2011; Schmitt *et al.,* 2012). This sponge associated microbial community is also usually absent or inconsequential in the surrounding water column (Jiang *et al*., 2007; Grozdanov & Hentschel, 2007; Taylor *et al*., 2007a; Wang, 2006).

1.4 The biotechnological potential of sponge associated microorganisms

There is general consensus that sponges harbour diverse microbial populations, many of which are sponge-specific and are seldom obtained from the surrounding seawater (Jiang *et al.*, 2007; Grozdanov & Hentschel, 2007; Wang, 2006). The sponge provides a niche and nutrients for the microbes while the microbes are proposed to play a role in the processing of waste products, transfer of nutrients, and production of secondary metabolites for chemical defence (Turque *et al.*, 2008; Wang, 2006; Webster & Blackall, 2008; Taylor *et al.*, 2007a). It is this production of secondary metabolites by sponge-associated microbes that is of particular interest in the biotechnological application of sponge symbionts in the production of pharmacologically significant compounds. A comprehensive review by Thomas *et al.,* (2010) outlines the production of clinically important bioactive compounds by sponge-microbe associations. Selected examples will be further discussed to illustrate the history and potential of this emerging field of science.

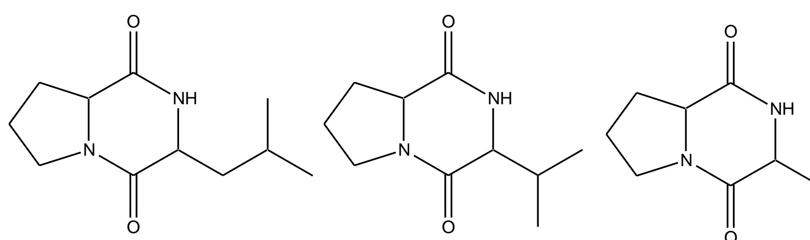


Figure 1.9 Diketopiperazines isolated from a sponge-associated *Micrococcus sp.* (Stierle *et al.,* 1988).

Tedania ignis is commonly known as the fire sponge due to its ability to cause severe skin rashes on contact. Three diketopiperazines originally isolated from this marine sponge (Fig. 1.9), were later found to be produced by a *Micrococcus sp.* isolated from the same sponge (Stierle *et al.*, 1988). This was the first discovery of a ‘sponge’ compound being produced by a sponge-associated bacterium and helped to strengthen the hypothesis that sponge-associated marine microorganisms could be the source of many bioactive sponge natural products. Diketopiperazines are the smallest possible cyclic peptides. They have a wide range of biological activities including antitumor, antifungal, antibacterial and antiviral properties. Their small structure and simple design coupled with their

chiral nature makes diketopiperazines very popular precursors in drug design (Martins & Carvalho, 2007).

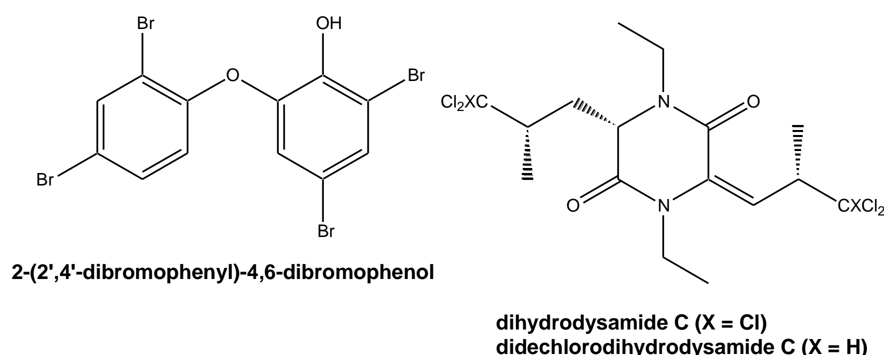


Figure 1.10 Secondary metabolites commonly isolated from *O. spongeliae* associated with *L. herbacea* (Unson *et al.,* 1994; Flowers *et al.,* 1998).

The tropical marine shallow water sponge *Lamellodysidea herbacea* (formerly *Dysidea herbacea*), which is common throughout the Indo-Pacific, is associated with the intercellular filamentous non-heterocystous cyanobacterium *Oscillatoria spongeliae* (first described by Schulze*,* 1879). These cyanobacterial symbionts have been reported to be responsible for the production of a wide array of secondary metabolites including the chlorinated diketopiperazines, dihydrodysamide C and didechlorodihydrodysamide C (Fig. 1.10), both isolated from *O. spongeliae* associated with *L. herbacea* collected off the Great Barrier Reef, Australia (Flowers *et al.*, 1998); and 2-(2',4'-dibromophenyl)-4,6-dibromophenol (Fig. 1.10), collected off the coast of Caroline Island, Palau (Unson *et al.,* 1994). This investigation into the cellular origin of 'sponge' metabolites has been used as a model system that incorporates various microscopy techniques, centrifugation and chemical extraction to isolate the cellular source (be it different sponge cells or microbial symbionts) of extracted secondary metabolites. This model is often used to describe and study sponge-microbe associations (Unson *et al.,* 1994; Flowers *et al.,* 1998; Thomas *et al.,* 2010).

1.4.1 Theonella swinhoei

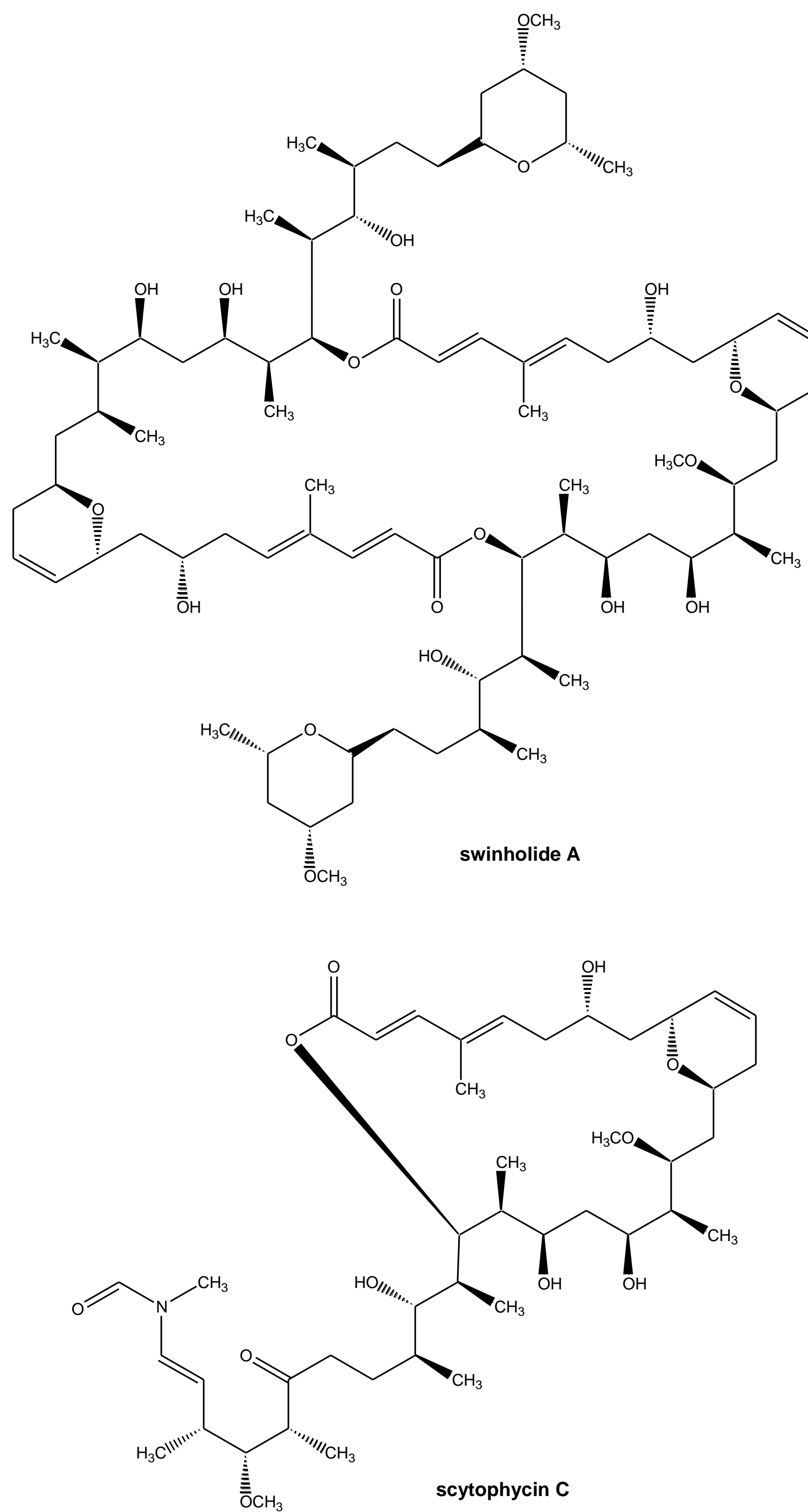


Figure 1.11 Chemical structure of swinholide A (Fusetani *et al.,* 1990) and scytophycin C (Carmeli *et al.,* 1990).

Theonella swinhoei is classified within the order Lithistid, an artificial assemblage of species grouped together because their skeletons contain fused or interlocked spicules called desmas. T. *swinhoei* is one of the most comprehensively studied marine sponges to date (Kho *et al.*, 1981; Bewley & Faulkner, 1998; Fusetani *et al.*, 1990, Qureshi & Faulkner, 2000; Ratnayake *et al.*, 2006; Festa *et al.*, 2011). The first compounds isolated from *T. swinhoei* were two new 4-methylene sterols, conicasterol and theonellasterol (Kho *et al.*, 1981), followed by the isolation of theonellin, a novel bisabolene-type sesquiterpenoid with a conjugated diene unit, and its corresponding isothiocyanate and formamide (Nakamura *et al.*, 1984). During the period 1984 to 1989 a number of secondary metabolites possessing potent bioactivities and novel chemical features were isolated from *T. swinhoei* for example swinholide A, bistheonellides, onnamide A, theonellamide F and S, and theonellapeptolides (Fusetani *et al.*, 1990). In 1990 two novel cyclic peptides, cyclotheonamides A and B, were isolated and have since become important model compounds for serine protease inhibitors. Further examination of *T. swinhoei* resulted in the isolation of six new peptides related to the cyclotheonamides: pseudotheonamides A_1, A_2, B_2, C, D and dihydrocyclotheonamide A (Nakao *et al.*, 1999, Fusetani *et al.*, 1990). The number of bioactive compounds isolated from *T. swinhoei* is remarkable (Fusetani & Matsunaga, 1993) and is still on the increase with numerous novel compounds isolated since 2000 for example 7α-hydroxytheonellasterol (Qureshi & Faulkner, 2000), theopapuamide (Ratnayake *et al.*, 2006), koshikamide B (Araki *et al.*, 2008), and solomonsterols A and B (Festa *et al.*, 2011).

The initial interest in the microbiology of *T. swinhoei* was sparked by the complexity of its associated chemical structures as well as the discovery of ‘symbiotic’ blue-green algae (filamentous cyanobacteria) in the sponge tissue (Fusetani & Matsunaga, 1993). The structural similarity of swinholide A to scytophycin C (isolated from the terrestrial cyanobacterium *Scytonema pseudohofmanni*) (Fig. 1.11) led to the assumption that the filamentous cyanobacterium *Aphanocapsa feldmanni* (present in high numbers in *T. swinhoei*) was responsible for the production of swinholide A (Carmeli *et al.,* 1990; Kitagawa *et al.*, 1990).

It was finally shown that swinholide A was limited to the mixed population of unicellular heterotrophic bacteria and the anti-fungal cyclic peptide theopaluaumide was associated with the filamentous heterotrophic bacterial fraction (Bewley *et al.*, 1996). Subsequent application of molecular approaches identified this filamentous bacterium as a previously unknown deltaproteobacterium with close association to the myxococcales and designated as *Entotheonella palauensis* (Schmidt *et al.*, 2000). Interestingly, 16S rRNA sequences that showed 98% identity to that of the filamentous alphaproteobacterium *E. palauensis* were detected in *T. swinhoei* specimens containing the closely related metabolites theonegramide and theonellamide F. These 16S rRNA sequences were absent in sponges with different metabolites (Piel, 2004) and therefore are proposed to be chemical markers for symbiosis of *E. palauensis* with sponges (Thomas *et al.*, 2010). The discovery of sponge-associated microbes that produce supposed 'sponge' metabolites is on the increase (this topic is comprehensively reviewed by Thomas *et al.*, 2010) and provides the opportunity for a biotechnology approach to the production of pharmaceutically relevant sponge-associated natural products.

1.4.2 Manzamine A

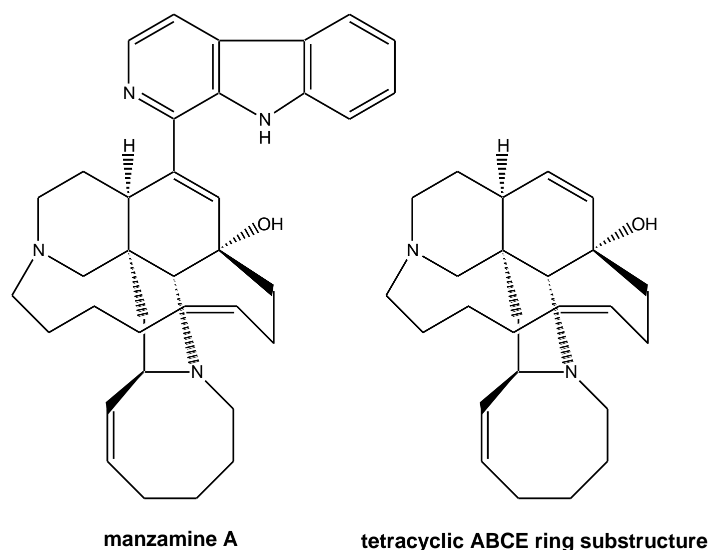


Figure 1.12 Chemical structure of Manzamine A (Sakai *et al.*, 2002) and tetracyclic ABCE ring substructure (Li *et al.,* 1998).

Manzamine A (Fig. 1.12) was isolated by Sakai & Higa in 1986 from a deep water Indonesian sponge *Acanthostrongylophora sp.* collected off the coast of Manzamo, Okinawa. Initial interest in this compound was due to its inhibition of P388 mouse leukaemia cells (IC_{50} = 0.07 µg/ml) (Sakai *et al.*, 2002). In later research manzamine A showed the most promising antimalarial activity within the class of manzamine alkaloids, which are characterised by a unique polycyclic ring system coupled with a β-carboline moiety. Manzamine A showed improved potency against the malaria parasite, *Plasmodium falciparum*, *in vitro* and *in vivo* compared to the clinically used drugs chloroquine and artemisinin; and it also showed activity against *Mycobacterium tuberculosis*. However, the major drawback was the toxicity associated with higher dosing schedules. Further research into the structure-activity relationship of manzamine A was therefore necessary to better understand the functional role of each moiety of this complex antimalarial compound (Wahba *et al.*, 2009). Due to the chemical complexity of manzamine A, synthetic studies are often focused on the synthesis of a number of “core ring structures” making up the manzamine A molecule (for example the tetracyclic ABCE substructure I) (Li *et al.*, 1998) as the total synthesis of manzamine A is complex and expensive. The discovery of a means of producing manzamine A fairly easily and in high yields would therefore contribute significantly to research into the use of derivatives of this compound as pharmaceutically viable options for the treatment of resistant malarial strains (Hamann *et al*., 2005)

Recently, Hamann *et al*. (2005) were able to successfully produce manzamine A by fermentation of the sponge-associated actinomycete, *Micromonospora sp*. strain M42, isolated from the Indonesian sponge *Acanthostronglyophora*. The use of this sponge-associated bacterium for the production of manzamine A has subsequently been patented enabling the synthesis of a number of manzamine derivatives with multiple pharmaceutical applications (Hamann *et al.*, 2005).

1.4.3 Genomic approaches for the production of secondary metabolites associated with sponge symbionts

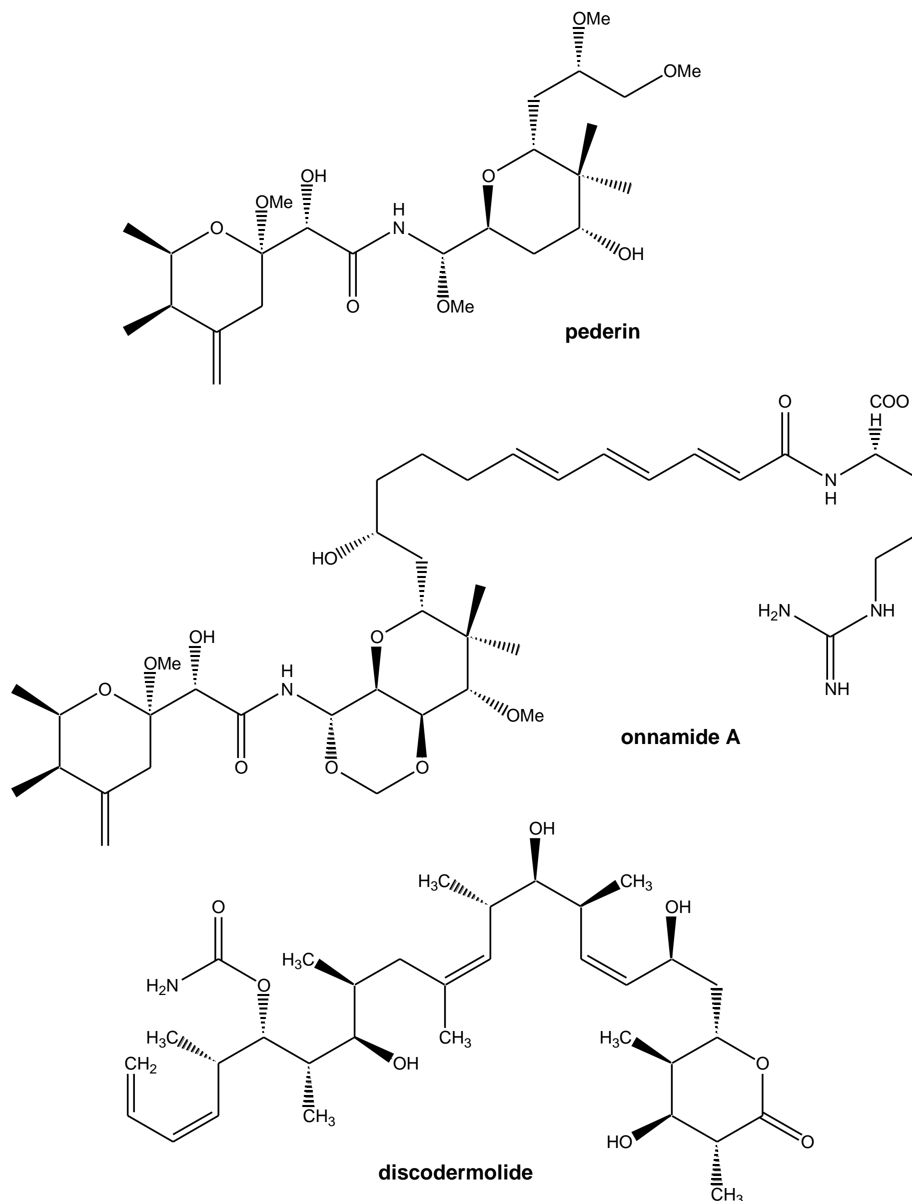


Figure 1.13 Chemical structure of pederin (Sakemi *et al.,* 1988), onnamide A (Kellner & Dettner, 1995), and discodermolide (Gunasekera *et al.,* 1990).

Many bacteria associated with sponges have not been cultured despite considerable effort .An alternative strategy, such as genomic approaches aimed at isolating the biosynthetic genes and expressing them in surrogate hosts, is necessary. This involves the construction and screening of metagenomic libraries, in which large pieces of DNA isolated from mixed populations without prior cultivation are cloned and screened for targeted genes. Genes encoding putative

enzymes involved in pederin, bryostatin, and onnamide (Fig. 1.13) biosynthesis have been cloned from metagenomic libraries of a beetle, a bryozoan, and a sponge, respectively (Schirmer *et al.*, 2005).

Of the 109 bacterial isolates cultured from four South China Sea sponges; namely *Steletta tenuis*, *Halichondria rugosa*, *Dysidea avara* and *Craniella australiensis*; thirteen Firmicutes and two Proteobacteria have been shown to contain non-ribosomal peptide synthetase (NRPS) adenylation (A) domain genes (Zhang *et al.,* 2008). Antimicrobial bioassays confirmed that all the bacteria containing the NRPS genes had antimicrobial properties, and most of them indicated activity against fungi, Gram-positive and Gram-negative bacteria. This combined molecular and bioassay strategy provides a useful tool in the isolation of sponge-associated bacteria that have the potential to synthesize bioactive compounds (Zhang *et al.*, 2008). The isolation of an uncultured *Pseudomonas* sp. as the most likely producer of the antitumor polyketide pederin in *Paederus fuscipes* beetles precipitated an investigation into the metagenome of the marine sponge *T. swinhoei*, which is the source of the onnamides and theopederins, a group of polyketides that structurally resemble pederin (Piel *et al.*, 2004). Sequence features of the isolated PKS genes indicated that they were of prokaryotic origin and were predicted to be responsible for the biosynthesis of almost the entire portion of onnamide that is correlated with antitumor activity (Piel *et al.,* 2004).

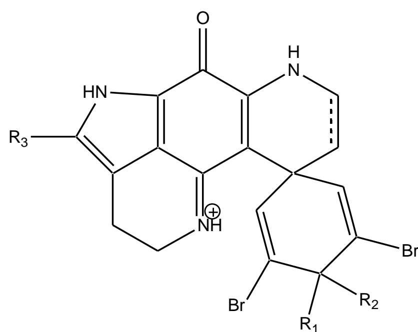
The sponge *Discodermia dissoluta* produces the promising antitumour compound discodermolide (Fig. 1.13), which causes extensive rearrangement of the microtubule skeleton and is able to arrest breast carcinoma cells during the G2- and M-phase at a concentration of 10 nM making it 100 times more effective than taxol (where a concentration of 1 μM is required to illicit the same response) (Gunasekera *et al.,* 1990; ter Haar *et al.,* 1996). In 1998 Novartis began phase I clinical trials despite the difficulty in obtaining sufficient quantities of this compound. Harvesting of the compound from *D. dissoluta* was unfeasible due to low yields and the instability of the compound in the presence of light. Chemical synthesis, which involved multiple steps with low yields, was therefore the only option at the time and the entire quantity of discodermolide used in the phase I clinical trials was obtained in this way. An alternate biosynthetic method was

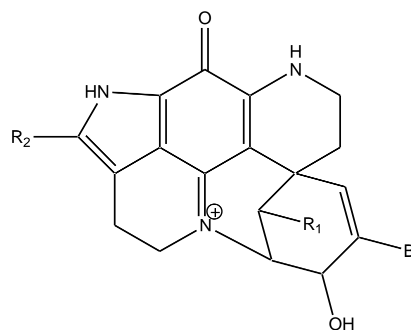
suggested on the basis that the structure of discodermolide is consistent with biosynthesis by a bacterial type I modular PKS, although this has not been exclusively shown (Schirmer *et al.*, 2005). The toxicity of discodermolide during the phase I clinical trials prevented further investigation into this compound as an anti-cancer therapy, although analogues with lower toxicity are still being investigated using both synthetic and biosynthetic approaches (Mickel, 2004).

1.5 The sponge genus *Tsitsikamma*, a source of biologically active pyrroloiminoquinone metabolites and novel bacterial communities

The genus *Tsitsikamma* belongs to the family *Latrunculiidae*. The most distinguishing morphological feature of the family *Latrunculiidae* are its microscleres, also known as discorhabds, which occur in a number of different forms (Kelly & Samaai, 2002). They can either have two whorls of spines or two or three discs on a straight or spined axial rod; and they can have one swollen spined end, both ends spined or both ends smooth. Latrunculid sponges sometimes have what is known as chessman spicules that are often aggregated into a dense ectosomal crust (Kelly & Samaai, 2002). Southern hemisphere *Latrunculiidae* sponges are commonly found in cold water environments off the coast of Antarctica, New Zealand, Australia, Tasmania and South Africa. They are abundant off the temperate southeastern coast of South Africa and are often found on sheltered rocky reefs down to a depth of 50 m. They are of particular interest due to their production of bioactive alkaloid pigments which possess a characteristic pyrroloiminoquinone core (Antunes *et al.*, 2004; Antunes *et al.*, 2005).

Tsitsikamma pedunculata





$R_1 = R_2 = O$, $R_3 = Br$
14-bromodiscorhabdin C

$R_1 = H$, $R_2 = OH$, $R_3 = Br$
14-bromo-3-dihydrodiscorhabdin C

$R_1 = H$, $R_2 = OH$, $R_3 = H$
3-dihydrodiscorhabdin C

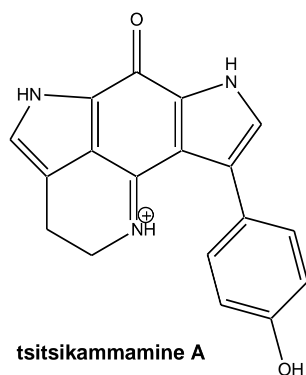
$R_1 = H$, $R_2 = OH$, $R_3 = H$
7,8-dehydro-3-dihydrodiscorhabdin C

$R_1 = H$, $R_2 = OH$, $R_3 = Br$
14-bromo-3-dihydro-7,8-dehydrodiscorhabdin C

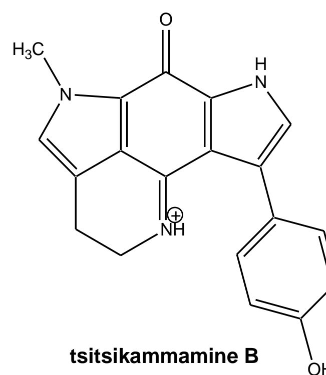
$R_1 = R_2 = H$
discorhabdin V

$R_1 = OH$, $R_2 = Br$
14-bromo-1-hydroxydiscorhabdin V

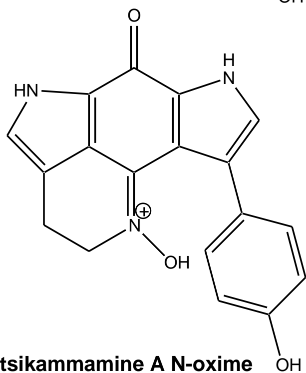
Tsitsikamma favus



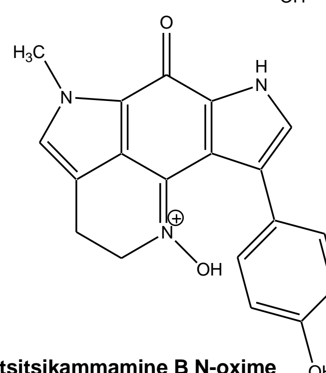
tsitsikammamine A



tsitsikammamine B



tsitsikammamine A N-oxime



tsitsikammamine B N-oxime

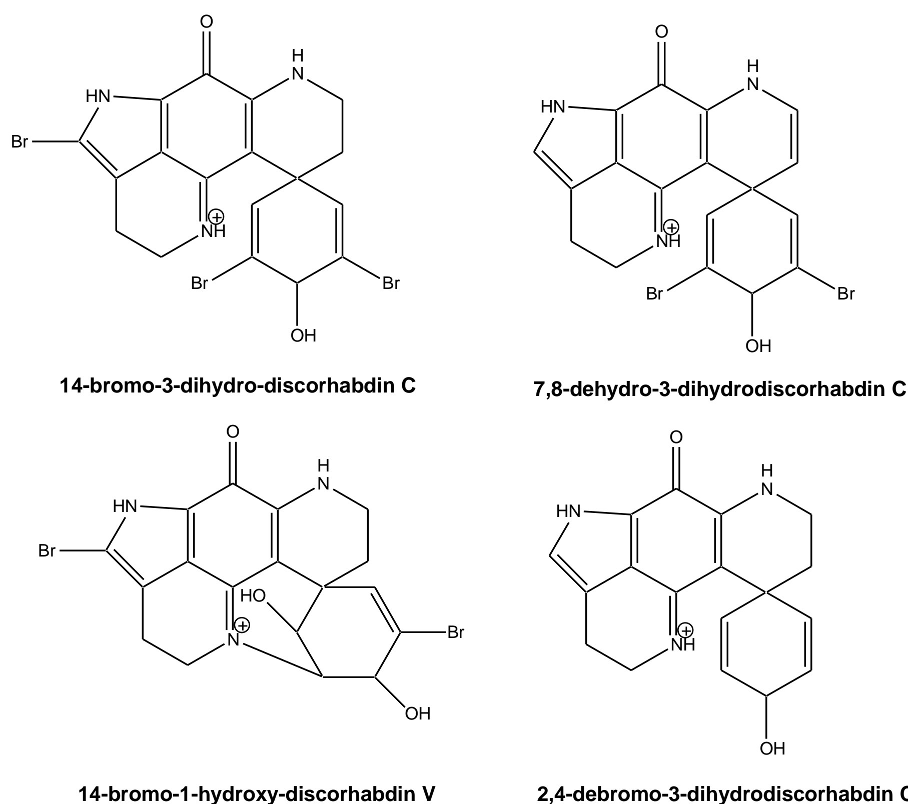


Figure 1.14 Secondary metabolites isolated from the South African marine sponge *Tsitsikamma pedunculata* (Antunes *et al.*, 2004) and *Tsitsikamma favus* (Hooper *et al.*, 1996; Antunes *et al.*, 2005).

The genus *Tsitsikamma* is endemic to the south-eastern coast of South Africa and is currently comprised of three species, namely *T. pedunculata*, *T. favus* and *T. scurra*. *T. scurra* has not been well studied and there is no published information available on this sponge. *T. pedunculata*, an unusual, stalked, golf ball-shaped sponge, was first collected in Algoa Bay in late 1999. *T. pedunculata* yielded the known compounds 14-bromodiscorhabdin C, 14-bromo-3-dihydrodiscorhabdin C, and 3-dihydrodiscorhabdin C. Further analysis also identified four novel, minor metabolites; 3-dihydro-7,8-dehydrodiscorhabdin C, 14-bromo-3-dihydro-7,8-dehydrodiscorhabdin C, discorhabdin V, and 14-bromo-1-hydroxydiscorhabdin V (Fig. 1.14) (Antunes *et al.,* 2004). Discorhabdins are well known for their general cytotoxicity and a previous investigation of discorhabdin A showed that it was able to cause extensive DNA single stranded scission (Barrows *et al.*, 1993). In an investigation by Antunes *et al.* (2004) the discorhabdins isolated from *T. pedunculata* all showed varying levels of cytotoxicity when screened against the common human colon tumor cell line HCT-116.

T. favus was first collected in 1994 by SCUBA at a depth of approximately 20 m in the Tsitsikamma marine reserve and a detailed description of the sponge was reported by Kelly & Samaai (2002). The secondary metabolites of *Tsitsikamma favus* were first investigated by Hooper *et al* (1996) and three major pyrroloiminoquinones, namely tsitsikammamine A, tsitsikammamine B, and 14-bromo-3-dihydro-discorhabdin C, were isolated (Fig. 1.14). Due to improvements in chromatographic and spectroscopic techniques, *T. favus* was subsequently re-investigated with the focus on characterising minor pyrroloiminoquinone metabolites and the new pyrroloiminoquinones tsitsikammamine N-oxime, tsitsikammamine B N-oxime, 7,8-dehydro-3-dihydrodiscorhabdin C, 14-bromo-1-hydroxy-discorhabdin S, and 2,4-debromo-3-dihydrodiscorhabdin C were isolated (Antunes *et al.,* 2004) (Fig. 1.14). The two major compounds isolated from *T. favus*, tsitsikammamine A and tsitsikammamine B, which are both bis-pyrroloiminoquinone secondary metabolites, are unique to *T. favus* and have not been isolated from any other sponge of the genus *Tsitsikamma* (Antunes *et al.*, 2004). Both tsitsikammamine A and tsitsikammamine B performed well in the HCT-116 colon tumor cell line screen, showing high levels of cytotoxicity. Their structural similarity to the topoisomerase I inhibitor wakayin led to further studies into their ability to cleave DNA through topoisomerase I inhibition. Both tsitsikammamine A and B were shown to have topoisomerase I inhibition and DNA intercalation properties equal to that of wakayin. Interestingly, the tsitsikammamine N18-oxmies were inactive in the HCT-116 cytotoxicity screen indicating that the molecular structure of the pyrroloiminoquinone compounds is of paramount importance to their activity (Antunes *et al.*, 2004).

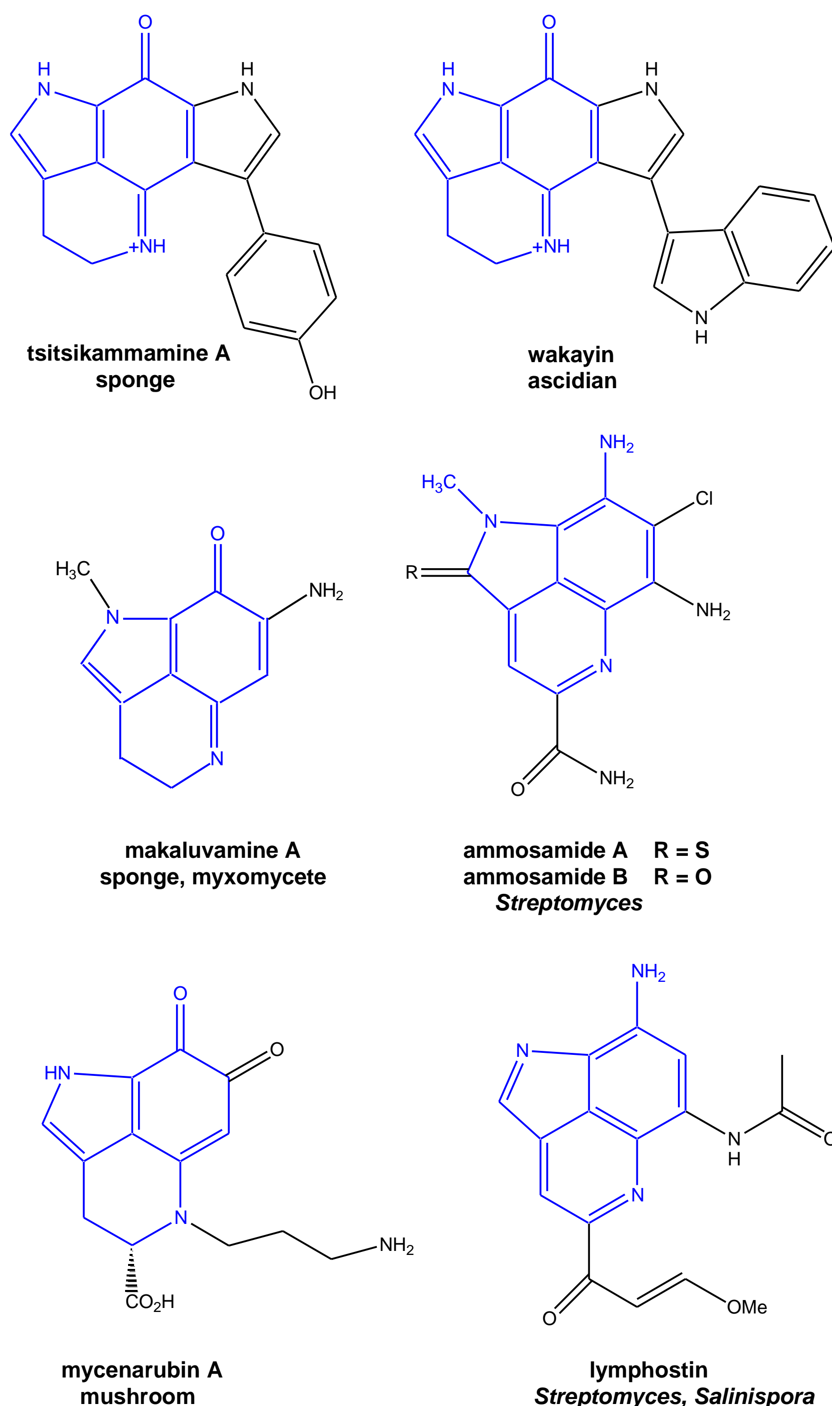


Figure 1.15 Pyrroloiminoquinones from unrelated sources – tsitsikammamine A (marine sponge, Antunes *et al*., 2005), wakayin (ascidian, Copp *et al.,* 2002), makaluvamine A (sponge and myxomycete, Ishibashi *et al*., 2001), ammosamide A and B (*Streptomyces*, Hughes *et al.,* 2009), mycenarubin A (mushroom, Peters & Spiteller, 2007a) and lymphostin (*Streptomyces* and *Salinospora*, Nagata *et al*., 1997 and Miyanaga *et al.,* 2011).

Wakayin (Fig. 1.15), isolated from a Fijian ascidian *Clavelina sp.*, is a related bis-pyrroloiminoquinone with structural similarities to the tsitsikammamines (Copp *et al.*, 2002) that also inhibits topoisomerase I catalysed relaxation of DNA and is a good DNA intercalator (Kokoshka *et al.*, 1996). Wakayin is one of a number of pyrroloiminoquinone secondary metabolites isolated from a phylum other than Porifera. The other non-sponge pyrroloiminoquinones are all isolated from microbial sources and include makaluvamine A, isolated from the myxomycete *Didymium bahiense* (Ishibashi *et al.,* 2001) (discussed in section 1.2.7); ammosamide A and B, isolated from the *Streptomyces* strain CNR-698 (Hughes *et al.,* 2009); lymphostin, isolated from both the *Streptomyces* sp. KY11783 (Nagata *et al.,* 1997) and a *Salinispora* spp. (Miyanaga *et al.,* 2011) and the mycenarubins and sanguinones, isolated from mushrooms of the genus *Mycena* (Peters & Spiteller, 2007a; Peters & Spiteller, 2007b).

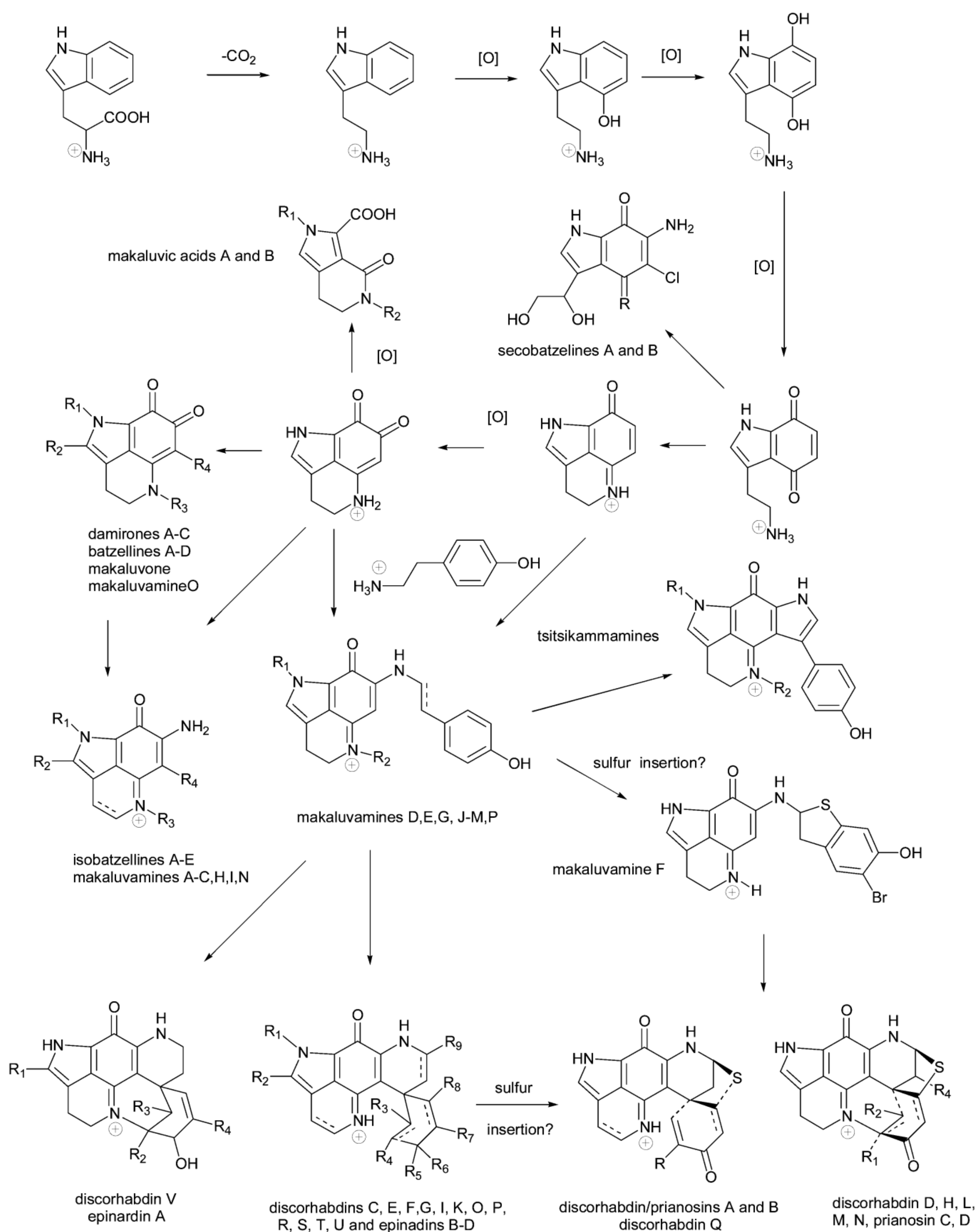


Figure 1.16 Putative biosynthetic pathway for the synthesis of pyrroloiminoquinones with tryptophan as a precursor (taken from Antunes *et al.*, 2005).

Antunes *et al.* (2005) proposed a putative biosynthetic pathway for the pyrroloiminoquinones (Fig. 1.16) that starts with tryptophan as a precursor, which is then decarboxylated by the enzyme tryptophan decarboxylase to form tryptamine. Tryptamine is modified by the appropriate functionalization and oxidation to give rise to the pyrroloiminoquinone backbone. Tyramine, a derivative of phenylalanine, is then added to give rise to the makaluvamines, which are then modified in various ways to give rise to the discorhabdins and tsitsikammamines (Lill *et al.*, 1995; Urban *et al.,* 2005; Antunes *et al.,* 2005). Miyanaga *et al.,* (2011) elucidated the biosynthetic pathway for the production of lymphostin in the marine actinobacteria *Salinispora tropica* and *Salinispora arenicola*, using tryptophan as a precursor. Genome sequencing of *S. tropica* and *S. arenicola* revealed a unique hybrid NRPS-PKS locus that has been assigned to the production of the pyrrolo[4,3,2-*de*]quinoline core characteristic of pyrroloiminoquinone metabolites. *T. favus* and *T. pedunculata* both have the discorhabdins 7,8-dehydro-3-dihydrodiscorhabdin C, 14-bromo-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V in common. According to the biosynthetic pathway, proposed by Antunes *et al.* (2005) all of the secondary metabolites isolated from the *Tsitsikamma* sponges have makaluvamines, which have been shown to have a microbial source (Ishibashi *et al.,* 2001), as an intermediate.

1.6 Hypothesis

The pyrroloiminoquinone core and/or many of the intermediates and/or the final secondary metabolites associated with sponges within the *Tsitsikamma* genus are produced by one or a group of closely related microbial symbionts.

1.7. Research Objectives

1. To collect and characterise *Tsitsikamma* sponge specimens using taxonomic, chemotaxonomic and molecular based techniques.

2. To characterise the diversity and population structure of microbial consortia associated with sponge species from the genus *Tstsikamma* and to compare them with those of other Latrunculid sponge species.

3. To isolate and culture sponge-associated bacteria and screen for secondary metabolite production.

1.8 References

Aicher, T.D., Buszek, K.R., Fang, F.G., Forsyth, C.J., Jung, S.H., Kishi, Y., Matelich, M.C., Scola, P.M., Spero, D.M. & Yoon, S.K. (1992). Total synthesis of halichondrin B and norhalichondrin B. *Journal of the American Chemical Society,* 114: 3162-3164.

Antunes, E.M., Beukes, D.R., Kelly, M., Samaai, T., Barrows, L.R., Marshall, K.M., Sincich, C. & Davies-Coleman, M.T. (2004). Cytotoxic pyrroloiminoquinones from four new species of South African Latrunculid sponges. *Journal of Natural Products,* 67: 1268-1276.

Antunes, E.M., Copp, B.R., Davies-Coleman, M.T. & Samaai, T. (2005). Pyrroloiminoquinones and related metabolites from marine sponges. *Natural Products Reports,* 22: 62-72.

Araki, T., Matsunaga, S., Nakao, Y., Furihata, K., West, L., Faulkner, D.J. & Fusetani, N. (2008). Koshikamide B, a cytotoxic peptide lactone from a marine sponge *Theonella* sp. *The Journal of Organic Chemistry,* 73: 7889-7894.

Bai, R.L., Paull, K.D., Herald, C.L., Malspeis, L., Pettit, G.R. & Hamel, E. (1991). Halichondrin B and homohalichondrin B, marine natural products binding in the vinca domain of tubulin. Discovery of tubulin-based mechanism of action by analysis of differential cytotoxicity data. *Journal of Biological Chemistry,* 266: 15882-15889.

Baker, D.D., Chu, M., Oza, U. & Rajgarhia, V. (2007). The value of natural products to future pharmaceutical discovery. *Natural Products Reports,* 24: 1225 - 1244.

Barrows, L.R., Radisky, D.C., Copp, B.R., Swaffar, D.S., Kramer, R.A., Warters, R.L. & Ireland, C.M. (1993). Makaluvamines, marine natural products, are active anti-cancer agents and DNA topo II inhibitors. *Anticancer Drug Design,* 8: 333 - 347.

Beneteau, V., Besson, T. (2001). Synthesis of novel pentacyclic pyrrolothiazolobenzoquinolinones, analogs of natural marine alkaloids. *Tetrahedron Letters,* 42: 2673-2676.

Bergman, O., Haber, M., Mayzel, B., Anderson, M., Shpigel, M., Hill, R. & Ilan, M. (2011a). Marine-based cultivation of *Diacarnus* sponges and the bacterial community composition of wild and maricultured sponges and their larvae. *Marine Biotechnology*: 1-14.

Bergman, O., Mayzel, B., Anderson, M.A., Shpigel, M., Hill, R.T. & Ilan, M. (2011b). Examination of marine-based cultivation of three Demosponges for acquiring bioactive marine natural products. *Marine Drugs,* 9: 2201-2219.

Bergmann, W. & Feeney, R.J. (1950). The isolation of a new thymine pentoside from sponges. *Journal of the American Chemical Society,* 72: 2809-2810.

Bewley, C.A. & Faulkner, D.J. (1998). Lithistid sponges: Star performers or hosts to the stars. *Angewandte Chemie International Edition,* 37: 2162-2178.

Bewley, C.A., Holland, N.D. & Faulkner, D.J. (1996). Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Cellular and Molecular Life Sciences,* 52: 716-722.

Blunt, J.W., Copp, B.R., Munro, M.H.G., Northcote, P.T. & Prinsep, M.R. (2011). Marine natural products. *Natural Product Reports,* 28: 196-268.

Borchiellini, C., Manuel, M., Alivon, E., Boury-Esnault, N., Vacelet, J. & Parco, Y.L. (2001). Sponge paraphyly and the origin of Metazoa. *Journal of Evolutionary Biology,* 14: 171-179.

Buchanan, M.S., Carroll, A.R., Fechner, G.A., Boyle, A., Simpson, M.M., Addepalli, R., Avery, V.M., Hooper, J.N.A., Su, N., Chen, H. & Quinn, R.J. (2007). Spermatinamine, the first natural product inhibitor of isoprenylcysteine carboxyl methyltransferase, a new cancer target. *Bioorganic & Medicinal Chemistry Letters,* 17: 6860-6863.

Carmeli, S., Moore, R.E. & Patterson, G.M.L. (1990). Tolytoxin and new scytophycins from three species of *Scytonema*. *Journal of Natural Products*, 53:1533-1542.

Carte, B.K. (1996). Biomedical Potential of Marine Natural Products. *BioScience,* 46: 271-286.

Casapullo, A., Cutignano, A., Bruno, I., Bifulco, G., Debitus, C., Gomez-Paloma, L. & Riccio, R. (2001). Makaluvamine P, a new cytotoxic pyrroloiminoquinone from *Zyzzya fuliginosa*. *Journal of Natural Products,* 64: 1354-1356.

Copp, B.R., Ireland, C.M. & Barrows, L.R. (2002). Wakayin: a novel cytotoxic pyrroloiminoquinone alkaloid from the ascidian *Clavelina* species. *The Journal of Organic Chemistry,* 56: 4596-4597.

Cragg, G.M., Boyd, M.R., Khanna, R., Kneller, R., Mays, T.D., Mazan, K.D., Newman, D.J. & Sausville, E.A. (1999). International collaboration in drug discovery and development: The NCI experience. *Pure and Applied Chemistry,* 71: 1619-1633.

Cuevas, C., Parez, M., Marta-N, M.A.J., Chicharro, J.L., Fernaindez-Rivas, C., Flores, M.A., Francesch, A.S., Gallego, P., Zarzuelo, M.A., De La Calle, F., Garca, J.S., Polanco, C.N., Rodriguez, I. & Manzanares, I. (2000). Synthesis of ecteinascidin ET-743 and phthalascidin Pt-650 from cyanosafracin B. *Organic Letters,* 2: 2545-2548.

Delong, E.F., Wickham, G.S. & Pace, N.R. (1989). Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. *Science,* 243: 1360-1363.

Dewick, P.M. (2001). In Medicinal natural products: a biosynthetic approach (Chichester, John Wiley & Sons Ltd), pp. 291-403.

Ding, Q., Chichak, K. & Lown, J.W. (1999). Pyrroloquinoline and pyridoacridine alkaloids from marine sources. *Current Medicinal Chemistry,* 6: 1-27.

Donadio, S., Staver, M.J., Mcalpine, J.B., Swanson, S.J. & Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. *Science,* 252: 675-679.

Dumdei, E.J. & Blunt, J.W. (1996). Sponge aquaculture for drug production. *Aquaculture Updates,* 16: 5-6.

Dumdei, E.J., Blunt, J.W., Munro, M.H.G., Battershill, C.N. & Page, M.J. (1998). The whys and whats of sponge chemistry: why chemists extract sponges and what problems does this cause? IN Watanabe, Y. & Fusetani, N. (Eds.) *Sponge Sciences Multidisciplinary Perspectives.* Tokyo: Springer.

Enticknap, J.J., Kelly, M., Peraud, O. & Hill, R.T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied Environmental Microbiology,* 72: 3724-3732.

Faulkner, D.J. (1991). Marine natural products. *Natural Product Reports,* 8: 97-147.

Faulkner, D.J. (2000). Marine pharmacology. *Antonie van Leeuwenhoek,* 77: 135-145.

Festa, C., De Marino, S., D'auria, M.V., Bifulco, G., Renga, B., Fiorucci, S., Petek, S. & Zampella, A. (2011). Solomonsterols A and B from *Theonella swinhoei*. The first example of C-24 and C-23 sulfated sterols from a Marine source endowed with a PXR agonistic Activity. *Journal of Medicinal Chemistry,* 54: 401-405.

Fieseler, L., Horn, M., Wagner, M. & Hentschel, U. (2004). Discovery of the novel candidate phylum "Poribacteria" in marine sponges. *Applied and Environmental Microbioogyl,* 70: 3724-3732.

Fieseler, L., Quaiser, A., Schleper, C. & Hentschel, U. (2006). Analysis of the first genome fragment from the marine sponge-associated, novel candidate phylum Poribacteria by environmental genomics. *Environmental Microbiology,* 8: 612-624.

Flowers, A.E., Garson, M.J., Webb, R.I., Dumdei, E.J. & Charan, R.D. (1998). Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). *Cell and Tissue Research,* 292: 597-607.

Friedrich, A.B., Fischer, I., Proksch, P., Hacker, J. & Hentschel, U. (2001). Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiology Ecology,* 38: 105-115.

Fusetani, N. & Matsunaga, S. (1993). Bioactive sponge peptides. *Chemical Reviews,* 93: 1793-1806.

Fusetani, N., Matsunaga, S., Matsumoto, H. & Takebayashi, Y. (1990). Bioactive marine metabolites. 33. Cyclotheonamides, potent thrombin inhibitors, from a marine sponge *Theonella sp. Journal of the American Chemical Society,* 112: 7053-7054.

Gademann, K. & Kobylinska, J. (2009). Antimalarial natural products of marine and freshwater origin. *The Chemical Record,* 9: 187-198.

García, J., Pereira, R. & De Lera, A.R. (2009). Total synthesis of the natural isoprenylcysteine carboxyl methyltransferase inhibitor spermatinamine. *Tetrahedron Letters,* 50: 5028-5030.

Gerçe, B., Schwartz, T., Voigt, M., Rühle, S., Kirchen, S., Putz, A., Proksch, P., Obst, U., Syldatk, C. & Hausmann, R. (2009). Morphological, bacterial, and secondary metabolite changes of *Aplysina aerophoba* upon long-term maintenance under artificial conditions. *Microbial Ecology,* 58: 865-878.

Gerçe, B., Schwartz, T., Syldatk, C. & Hausmann, R. (2011). Differences between bacterial communities associated with the surface or tissue of mediterranean sponge species. *Microbial Ecology*: 1-14.

Grozdanov, L. & Hentschel, U. (2007). An environmental genomics perspective on the diversity and function of marine sponge-associated microbiota. *Current Opinion in Microbiology,* 10:215 - 220.

Gunasekera, S.P., Gunasekera, M., Longley, R.E. & Schulte, G.K. (1990). Discodermolide: a new bioactive polyhydroxylated lactone from the marine sponge *Discodermia dissoluta*. *The Journal of Organic Chemistry,* 55: 4912-4915.

Hamann, M.T., Rao, K.V. & Peng, J. (2005). Methods of treating disease through the administration of a manzamine analogue or derivative IN Greenberg Traurig. Met Life Building, P.A., New York, United States

Haygood, M.G., Schmidt, E.M., Davidson, S.K. & Faulkner, D.J. (1999). Microbial symbionts of marine invertebrates: Opportunities for microbial biotechnology. *Journal of Molecular Microbiology and Biotechnology,* 1: 33-43.

Hentschel, U., Usher, K.M. & Taylor, M.W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiology Ecology,* 55: 167-177.

Hill, R.T. & Fenical, W. (2010). Pharmaceuticals from marine natural products: surge or ebb? *Current Opinion in Biotechnology,* 21: 777-779.

Hirata, Y. & Uemura, D. (1986). Halichondrins – antitumor polyether macrolides from a marine sponge. *Pure and Applied Chemistry,* 58: 701 - 710.

Hochmuth, T., Niederkruger, H., Gernert, C., Siegl, A., Taudien, S., Platzer, M., Crews, P., Hentschel, U. & Piel, J. (2010). Linking chemical and microbial diversity in marine sponges: Possible role for poribacteria as producers of methyl-branched fatty acids. *Chembiochem,* 11: 2572-2578.

Hoffman, H.H.-R., S.; Kurz, M.; Tietgen, H. (2006). Bengamide derivatives, process for preparing them, and their use. IN 7153846, U.S.P. (Ed.) *Free Patents Online.* Sanofi-Aventis Deutschland GmbH (Frankfurt am Main, DE).

Hooper, G.J., Davies-Coleman, M.T., Kelly-Borges, M. & Coetzee, P.S. (1996). New alkaloids from a South African Latrunculid sponge. *Tetrahedron Letters,* 37: 7135-7138.

Hooper, J.N.A & van Soest, R. W. M. (Eds.) (2002). Systema Porifera: A guide to the classification of sponges.

Hughes CC, MacMillan JB, Gaudêncio SP, Jensen PR, Fenical W (2009). The ammosamides: structures of cell cycle modulators from a marine-derived *Streptomyces* species. *Angewandte Chemie International Edition* 2009, 48:725-727.

Ishibashi, M., Iwasaki, T., Imai, S., Sakamoto, S., Yamaguchi, K. & Ito, A. (2001). Laboratory culture of the myxomycetes: Formation of fruiting bodies of *Didymium bahiense* and its plasmodial production of makaluvamine A. *Journal of Natural Products,* 64: 108-110.

Jain, S. & Vahdat, L.T. (2011). Eribulin mesylate. *Clinical Cancer Research*, 17:6615-6622.

Jares-Erijman, E.A., Sakai, R. & Rinehart, K.L. (1991). Crambescidins: new antiviral and cytotoxic compounds from the sponge *Crambe crambe*. *The Journal of Organic Chemistry,* 56: 5712-5715.

Jiang, S., Sun, W., Chen, M., Dai, S., Zhang, L., Liu, Y., Lee, K. & Li, X. (2007). Diversity of culturable actinobacteria isolated from marine sponge *Haliclona sp. Antonie van Leeuwenhoek,* 92: 405-416.

Jordan, M.A., Kamath, K., Manna, T., Okouneva, T., Miller, H.P., Davis, C., Littlefield, B.A. & Wilson, L. (2005). The primary antimitotic mechanism of action of the synthetic halichondrin E7389 is suppression of microtubule growth. *Molecular Cancer Therapeutics,* 4: 1086-1095.

Kadia, T.M., Kantarjian, H., Kornblau, S., Borthakur, G., Faderl, S., Freireich, E.J., Luthra, R., Garcia-Manero, G., Pierce, S., Cortes, J. & Ravandi, F. (2012). Clinical and proteomic characterisation of acute myeloid leukemia with mutated RAS. *Cancer*, 118: 5550-5559.

Kamke, J., Taylor, M.W. & Schmitt, S. (2010). Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs. 16S rRNA gene comparisons. *ISME J,* 4: 498-508.

Kellner, R.L.L. & Dettner, K. (1995). Allocation of pederin during lifetime of Paederus rove beetles (Coleoptera: Staphylinidae): Evidence for polymorphism of hemolymph toxin. *Journal of Chemical Ecology,* 21: 1719-1733.

Kelly, M. & Samaai, T. (2002). Family Latrunculiidae Topsent, 1922. IN Hooper, J.N.A. & Van Soest, R.W.M. (Eds.) *System Porifera: A guide to the Classification of Sponges.* New York: Kluwer/Plenum Academic.

Kennedy, J., Baker, P., Piper, C., Cotter, P., Walsh, M., Mooij, M., Bourke, M., Rea, M., O'connor, P., Ross, R., Hill, C., O'gara, F., Marchesi, J. & Dobson, A. (2008). Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish Waters. *Marine Biotechnology*.

Kennedy, J., Marchesi, J.R. & Dobson, A.D. (2007). Metagenomic approaches to exploit the biotechnological potential of the microbial consortia of marine sponges. *Applied Microbiology and Biotechnology,* 75: 11 - 20.

Keyzers, R.A., Arendse, C.E., Hendricks, D.T., Samaai, T. & Davies-Coleman, M.T. (2005). Makaluvic acids from the South African Latrunculid sponge *Strongylodesma aliwaliensis*. *Journal of Natural Products,* 68: 506-510.

Keyzers, R.A., Samaai, T. & Davies-Coleman, M.T. (2004). Novel pyrroloquinoline ribosides from the South African Latrunculid sponge *Strongylodesma aliwaliensis*. *Tetrahedron Letters,* 45: 9415-9418.

Kho, E., Imagawa, D.K., Rohmer, M., Kashman, Y. & Djerassi, C. (1981). Sterols in marine invertebrates. 22. Isolation and structure elucidation of conicasterol and theonellasterol, two new 4-methylene sterols from the Red Sea sponges *Theonella conica* and *Theonella swinhoei*. *The Journal of Organic Chemistry,* 46: 1836-1839.

Khosla, C., Gokhale, R.S., Jacobsen, J.R. & Cane, D.E. (1999). Tolerance and specificity of polyketide synthases. *Annual Review of Biochemistry,* 68: 219-253.

Kitagawa, I., Kobayashi, M., Katori, T., Yamashita, M., Tanaka, J., Doi, M. & Ishida, T. (1990). Absolute stereostructure of swinholide A, a potent cytotoxic macrolide from the Okinawan marine sponge *Theonella swinhoei. Journal of the American Chemical Society,* 112: 3710-3712.

Kokoshka, J.M., Capson, T.L., Holden, J.A., Ireland, C.M. & Barrows, L.R. (1996). Differences in the topoisomerase I cleavage complexes formed by camptothecin and wakayin, a DNA-intercalating marine natural product. *Anti-Cancer Drugs,* 7: 758-765.

Lazaro, J.E.H., Nitcheu, J., Mahmoudi, N., Ibana, J.A., Mangalindan, G.C., Black, G.P., Howard-Jones, A.G., Moore, C.G., Thomas, D.A., Mazier, D., Ireland, C.M., Concepcion, G.P., Murphy, P.J. & Diquet, B. (2006). Antimalarial cctivity of crambescidin 800 and synthetic analogues against liver and blood stage of *Plasmodium sp. The Journal of Antibiotics,* 59: 583-590.

Lee, O.O., Wong, Y.H. & Qian, P.-Y. (2009). Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. *Applied Environmental Microbiology,* 75: 3513-3521.

Li, S., Kosemura, S. & Yamamura, S. (1994). Synthetic studies on manzamine A: construction of a key bicyclic AD ring subunit I. *Tetrahedron Letters,* 35: 8217-8220.

Li, S., Yamamura, S., Hosomi, H. & Ohba, S. (1998). Synthetis studies on manzamine A: Construction of the tetracyclic ABCE ring substructure I. *Tetrahedron Letters,* 39: 2601-2604.

Liang, X. & Fang, W. (2006). *Medicinal chemistry of bioactive natural products,* Hoboken, New Jersey: John Wiley & sons.

Lill, R. E., Major, D. A., Blunt, J. W., Munro, M. H. G. (1995) Studies on the biosynthesis of of discorhabdin B in the New Zealand sponge *Latrunculia sp. B. Journal of Natural Products*, 58: 306-311.

Lindsay, M., Webb, R., Strous, M., Jetten, M., Butler, M., Forde, R. & Fuerst, J. (2001). Cell compartmentalisation in Planctomycetes: novel types of structural organisation for the bacterial cell. *Archives of Microbiology,* 175: 413-429.

Lu, J.-P., Yuan, X.-H., Yuan, H., Wang, W.-L., Wan, B., Franzblau, S.G. & Ye, Q.-Z. (2011). Inhibition of *Mycobacterium tuberculosis* methionine aminopeptidases by bengamide derivatives. *ChemMedChem,* 6: 1041-1048.

Matcher, G.F., Dorrington, R.A., Henninger, T.O., Froneman, P.W. (2011). Insights into the bacterial diversity in a freshwater-deprived permanently open Eastern Cape estuary, using 16S rRNA pyrosequencing analysis. *Water SA* 37:381–390

Martins, M.B. & Carvalho, I. (2007). Diketopiperazines: biological activity and synthesis. *Tetrahedron,* 63: 9923-9932.

Menzella, H.G., Reid, R., Carney, J.R., Chandran, S.S., Reisinger, S.J., Patel, K.G., Hopwood, D.A. & Santi, D.V. (2005). Combinatorial polyketide biosynthesis by de novo design and rearrangement of modular polyketide synthase genes. *Nature Biotechnology,* 23: 1171-1176.

Meragelman, T.L., Willis, R.H., Woldemichael, G.M., Heaton, A., Murphy, P.T., Snader, K.M., Newman, D.J., Van Soest, R., Boyd, M.R., Cardellina, J.H. & Mckee, T.C. (2007). Candidaspongiolides, distinctive analogues of tedanolide from sponges of the genus *Candidaspongia*. *Journal of Natural Products,* 70: 1133-1138.

Mickel, S.J. (2004). Toward a commercial synthesis of (+)-discodermolide. *Common opinion in drug discovery development,* 7: 869-881.

Miyanaga, A., Janso, J.E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustaì•Quio, A.S., Fielding, E.N., Carter, G.T., Jensen, P.R., Feng, X., Leighton, M., Koehn, F.E. & Moore, B.S. (2011). Discovery and assembly-line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in *Salinispora* bacteria. *Journal of the American Chemical Society,* 133: 13311-13313.

Mohamed, N.M., Enticknap, J.J., Lohr, J.E., Mcintosh, S.M. & Hill, R.T. (2008). Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. *Applied and Environmental Microbiology,* 74: 1209-1222.

Molinski, T.F., Dalisay, D.S., Lievens, S.L. & Saludes, J.P. (2009). Drug development from marine natural products. *Nature Reviews Drug Discovery,* 8: 69-85.

Montalvo, N.F. & Hill, R.T. (2011). Sponge-associated bacteria are strictly maintained in two closely-related but geographically distant sponge hosts. *Applied and Environmental Microbiology*.

Morton, S.L., Moeller, P.D.R., Young, K.A. & Lanoue, B. (1998). Okadaic acid production from the marine dinoflagellate *Prorocentrum belizeanum* Faust isolated from the Belizean coral reef ecosystem. *Toxicon,* 36: 201-206.

Muscholl-Silberhorn, A., Thiel, V. & Imhoff, J. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microbial Ecology,* 55: 94-106.

Nagata, H., Ochiai, K., Aotani, Y., Ando, K., Yoshida, M., Takahashi, I., Tamaoki, T. J. (1997). Lymphostin (LK6-A), a novel immunosuppressant from *Streptomyces sp.* KY11783: Taxonomy of the producing organism, fermentation, isolation and biological activities. *The Journal of Antibiotics*, 50: 537–542.

Nakamura, H., Kobayashi, J.I., Ohizumi, Y., Mitsubishi, K. & Hirata, Y. (1984). Novel bisabolene-type sesquiterpenoids with a conjugated diene isolated from the okinawan sea sponge *Theonella swinhoei*. *Tetrahedron Letters,* 25: 5401-5404.

Nakao, Y., Masuda, A., Matsunaga, S. & Fusetani, N. (1999). Pseudotheonamides, serine protease inhibitors from the marine sponge *Theonella swinhoei*. *Journal of the American Chemical Society,* 121: 2425-2431.

Newman, D.J. & Cragg, G.M. (2004). Marine natural products and related compounds in clinical and advanced preclinical trials *Journal of Natural Products,* 67: 1216-1238.

Newman, D.J. & Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 Years. *Journal of Natural Products,* 70: 461-477.

Newman, D.J. & Hill, R.T. (2006). New drugs from marine microbes: the tide is turning. *Journal of Industrial Microbiology and Biotechnolology,* 33: 539 - 544.

Nicolaou, K.C., Hughes, R., Pfefferkorn, J.A., Barluenga, S. & Roecker, A.J. (2001). Combinatorial synthesis through disulfide exchange: Discovery of potent psammaplin A type antibacterial agents active against Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Chemistry – A European Journal,* 7: 4280-4295.

Perry, N.B., Blunt, J.W., Mccombs, J.D. & Munro, M.H.G. (1986). Discorhabdin C, a highly cytotoxic pigment from a sponge of the genus *Latrunculia*. *The Journal of Organic Chemistry,* 51: 5476-5478.

Perry, N.B., Blunt, J.W. & Munro, M.H.G. (1988). Cytotoxic pigments from New Zealand sponges of the genus *Latrunculia* : discorhabdins A, B and C. *Tetrahedron,* 44: 1727-1734.

Peters, S. & Spiteller, P. (2007a) Mycenarubins A and B, red pyrroloquinoline alkaloids from the mushroom *Mycena rosea. European Journal of Organic Chemistry*, 2007: 1571-1576

Peters, S. & Spiteller, P. (2007b). Sanguinones A and B, blue pyrroloquinoline alkaloids from the fruiting bodies of the mushroom *Mycena sanguinolenta*. *Journal of Natural Products,* 70: 1274-1277.

Piel, J. (2004). Metabolites from symbiotic bacteria. *Natural Product Reports,* 21: 519-538.

Piel, J.R., Hui, D., Wen, G., Butzke, D., Platzer, M., Fusetani, N. & Matsunaga, S. (2004). Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *PNAS,* 101: 16222-16227.

Pina, I.C., Gautschi, J.T., Wang, G.-Y.-S., Sanders, M.L., Schmitz, F.J., France, D., Cornell-Kennon, S., Sambucetti, L.C., Remiszewski, S.W., Perez, L.B., Bair, K.W. & Crews, P. (2003). Psammaplins from the sponge *Pseudoceratina purpurea*: Inhibition of both histone deacetylase and DNA methyltransferase. *The Journal of Organic Chemistry,* 68: 3866-3873.

Poulsen, L.K., Ballard, G. & Stahl, D.A. (1993). Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. *Applied Environmental Microbiology,* 59: 1354-1360.

Quinoa, E., Adamczeski, M., Crews, P. & Bakus, G.J. (1986). Bengamides, heterocyclic anthelmintics from a *Jaspidae* marine sponge. *The Journal of Organic Chemistry,* 51: 4494-4497.

Quinoa, E. & Crews, P. (1987). Phenolic constituents of *Psammaplysilla*. *Tetrahedron Letters,* 28: 3229-3232.

Qureshi, A. & Faulkner, D.J. (2000). 7α-Hydroxytheonellasterol, a cytotoxic 4-methylene sterol from the Philippines sponge *Theonella swinhoei*. *Journal of Natural Products,* 63: 841-842.

Radwan, M., Hanora, A., Zan, J., Mohamed, N., Abo-Elmatty, D., Abou-El-Ela, S. & Hill, R. (2010). Bacterial community analyses of two Red Sea sponges. *Marine Biotechnology,* 12: 350-360.

Ratnayake, A.S., Bugni, T.S., Feng, X., Harper, M.K., Skalicky, J.J., Mohammed, K.A., Andjelic, C.D., Barrows, L.R. & Ireland, C.M. (2006). Theopapuamide, a cyclic depsipeptide from a Papua New Guinea Lithistid sponge *Theonella swinhoei*. *Journal of Natural Products,* 69: 1582-1586.

Rinehart, K.L. (2000). Antitumor compounds from tunicates. *Medicinal Research Reviews,* 20: 1-27.

Roberts, W.K. & Dekker, C.A. (1967). Convenient synthesis of 1-(D-arabinofuranosyl)cytosine (cytosine-D-arabinofuranoside). *The Journal of Organic Chemistry,* 32: 816-817.

Sakai, R., Higa, T., Jefford, C.W. & Bernardinelli, G. (2002). Manzamine A, a novel antitumor alkaloid from a sponge. *Journal of the American Chemical Society,* 108: 6404-6405.

Sakemi, S., Ichiba, T., Kohmoto, S., Saucy, G. & Higa, T. (1988). Isolation and structure elucidation of onnamide A, a new bioactive metabolite of a marine sponge, *Theonella sp*. *Journal of the American Chemical Society,* 110: 4851-4853.

Schirmer, A., Gadkari, R., Reeves, C.D., Ibrahim, F., Delong, E.F. & Hutchinson, C.R. (2005). Metagenomic analysis reveals diverse polyketide synthase gene clusters in microorganisms associated with the marine sponge *Discodermia dissoluta*. *Applied Environmental Microbiology,* 71: 4840-4849.

Schmidt, E.W., Harper, M.K. & Faulkner, D.J. (1995). Makaluvamines H-M and damirone C from the Pohnpeian sponge *Zyzzya fuliginosa*. *Journal of Natural Products,* 58: 1861-1867.

Schmidt, E.W., Obraztsova, A.Y., Davidson, S.K., Faulkner, D.J. & Haygood, M.G. (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as the novel proteobacterium candidate *Entotheonella palauensis*. *Marine Biology,* 136: 969-977.

Schmidtz, F.J., Vanderah, D.J., Hollenbeak, K.H., Enwall, C.E.L., Gopichand, Y., Sengupta, P.K., Hossain, M.B. & Van Der Helm, D. (1984). Metabolites from the marine sponge *Tedania ignis*. A new atisanediol and several

known diketopiperazines. *The Journal of Organic Chemistry,* 48: 3941-3945.

Schmitt, S., Hentschel, U. & Taylor, M. (2012). Deep sequencing reveals diversity and community structure of complex microbiota in five Mediterranean sponges. *Hydrobiologia,* 687: 341-351.

Schmitz, F.J., Gunasekera, S.P., Yalamanchili, G., Hossain, M.B. & Van Der Helm, D. (1984). Tedanolide: a potent cytotoxic macrolide from the Caribbean sponge Tedania ignis. *Journal of the American Chemical Society,* 106: 7251-7252.

Schulze FE (1879) Untersuchungen t~ber den Bau und die Entwicklung der Spongien. Sechste Mittheilung. Die Gattung *Spongelia.* Z wiss Zool 32:117-157

Schumacher, M., Kelkel, M., Dicato, M. & Diederich, M. (2011). A survey of marine natural compounds and their derivatives with anticancer activity reported in 2010. *Molecules,* 16: 5629-5646.

Seletsky, B.M., Wang, Y., Hawkins, L.D., Palme, M.H., Habgood, G.J., Dipietro, L.V., Towle, M.J., Salvato, K.A., Wels, B.F., Aalfs, K.K., Kishi, Y., Littlefield, B.A. & Yu, M.J. (2004). Structurally simplified macrolactone analogues of halichondrin B. *Bioorganic & Medicinal Chemistry Letters,* 14: 5547-5550.

Siegl, A., Kamke, J., Hochmuth, T., Piel, J., Richter, M., Liang, C., Dandekar, T. & Hentschel, U. (2011). Single-cell genomics reveals the lifestyle of Poribacteria, a candidate phylum symbiotically associated with marine sponges. *ISME J,* 5: 61-70.

Simister, R.L., Deines, P., Botte, E.S., Webster, N.S. & Taylor, M.W. (2012). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environmental Microbiology*, 14:517-524.

Simmons, T.L., Andrianasolo, E., Mcphail, K., Flatt, P. & Gerwick, W.H. (2005). Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics,* 4: 333-342.

Simpson, T.L. (1984). The cell biology of sponges. New York: Springer-Verlag.

Singh, R., Sharma, M., Joshi, P. & Rawat, D.S. (2008). Clinical status of anti-cancer agents derived from marine sources. *Anti-Cancer Agents in Medicinal Chemistry,* 8: 603-617.

Stierle, A.C., Cardellina, J.H. & Singleton, F.L. (1988). A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis*. *Experientia,* 44: 1021.

Tachibana, K., Scheuer, P.J., Tsukitani, Y., Kikuchi, H., Van Engen, D., Clardy, J., Gopichand, Y. & Schmitz, F.J. (1981). Okadaic acid, a cytotoxic polyether from two marine sponges of the genus *Halichondria*. *Journal of the American Chemical Society,* 103: 2469-2471.

Tai, W.Y., Zhang, R.T., Ma, Y.M., Gu, M., Liu, G., Li, J. & Nan, F.J. (2011). Design, synthesis, and biological evaluation of ring-opened bengamide analogues. *ChemMedChem,* 6: 1555-1558.

Taylor, M.W., Hill, R.T., Piel, J., Thacker, R.W. & Hentschel, U. (2007a). Soaking it up: the complex lives of marine sponges and their microbial associates. *ISME J,* 1: 187-190.

Taylor, M.W., Radax, R., Steger, D. & Wagner, M. (2007b). Sponge-associated microorganisms: Evolution, Ecology, and Biotechnological potential. *Microbiology and Molecular Biology Reviews,* 71: 295-347.

Ter Haar, E., Kowalski, R.J., Hamel, E., Lin, C.M., Longley, R.E., Gunasekera, S.P., Rosenkranz, H.S. & Day, B.W. (1996). Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than Taxol. *Biochemistry,* 35: 243-250.

Thomas, T.R.A., Kavlekar, D.P. & Lokabharathi, P.A. (2010). Marine drugs from sponge-microbe associations - A Review. *Marine Drugs,* 8: 1417-1468.

Towbin, H., Bair, K.W., Decaprio, J.A., Eck, M.J., Kim, S., Kinder, F.R., Morollo, A., Mueller, D.R., Schindler, P., Song, H.K., Van Oostrum, J., Versace, R.W., Voshol, H., Wood, J., Zabludoff, S. & Phillips, P.E. (2003). Proteomics-based target identification. *Journal of Biological Chemistry,* 278: 52964-52971.

Trisciuoglio, D., Uranchimeg, B., Cardellina, J.H., Meragelman, T.L., Matsunaga, S., Fusetani, N., Del Bufalo, D., Shoemaker, R.H. & Melillo, G. (2008). Induction of apoptosis in human cancer cells by candidaspongiolide, a novel sponge polyketide. *Journal of the National Cancer Institute,* 100: 1233-1246.

Turque, A., Cardoso, A., Silveira, C., Vieira, R., Freitas, F., Albano, R., Gonzalez, A., Paranhos, R., Muricy, G. & Martins, O. (2008). Bacterial communities of the marine sponges *Hymeniacidon heliophila* and *Polymastia janeirensis* and their environment in Rio de Janeiro, Brazil. *Marine Biology,* 155: 135-146.

Uemura, D., Takahashi, K., Yamamoto, T., Katayama, C., Tanaka, J., Okumura, Y. & Hirata, Y. (1985). Norhalichondrin A: an antitumor polyether macrolide from a marine sponge. *Journal of the American Chemical Society,* 107: 4796-4798.

Unson, M.D., Holland, N.D. & Faulkner, D.J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Marine Biology,* 119: 1-11.

Urban, S., Hickford, S. J. H., Blunt, J. W., Munro, M. H. G. (2000). Bioactive marine alkaloids. *Current Organic Chemistry*, 4:765–805

Vacelet, J. & Donadey, C. (1977). Electron microscope study of the association between some sponges and bacteria. *Journal of Experimental Marine Biology and Ecology,* 30: 301-314.

Venables, D.A., Concepcian, G.P., Matsumoto, S.S., Barrows, L.R. & Ireland, C.M. (1997). Makaluvamine N: a new pyrroloiminoquinone from *Zyzzya fuliginosa*. *Journal of Natural Products,* 60: 408-410.

Wahba, A.E., Peng, J., Kudrimoti, S., Tekwani, B.L. & Hamann, M.T. (2009). Structure-activity relationship studies of manzamine A: Amidation of positions 6 and 8 of the [beta]-carboline moiety. *Bioorganic & Medicinal Chemistry,* 17: 7775-7782.

Wainright, M. (1990). *Miracle cure,* Cambridge: Basil Blackwell Inc.

Walmsley, T.A., Matcher, G.M., Zhang, F., Hill, R.T., Davies-Coleman, M.T., Dorrington, R.A. (2012). Diversity of bacterial communities associated with the Indian Ocean sponge *Tsitsikamma favus* that contains the bioactive pyrroloiminoquinones, tsitsikammamine A and B. *Marine Biotechnology,* 14:681-691.

Walwick, E.R., Roberts, W.K. & Dekker, C.A. (1959). Proceedings of the Chemical Society. March 1959. *Proceedings of the Chemical Society*: 73-108.

Wang, G. (2006). Diversity and biotechnological potential of the sponge-associated microbial consortia. *Journal of Industrial Microbiology and Biotechnology,* 33: 545-551.

Webster, N.S. & Blackall, L.L. (2008). What do we really know about sponge-microbial symbioses. *ISME J,* 3: 1-3.

Webster, N.S. & Taylor, M.W. (2012). Marine sponges and their microbial symbionts: love and other relationships. *Environmental Microbiology,* 14: 335-346.

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology,* 12: 2070-2082.

Webster, N.S., Wilson, K.J., Blackall, L.L. & Hill, R.T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology,* 67: 434-444.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173: 697-703.

Weisz, J.B., Hentschel, U., Lindquist, N. & Martens, C.S. (2007) *Marine Biology,* 152: 475-483.

Wilkinson, C.R. (1978a). Microbial associations in sponges. I. Ecology, physiology and microbial populations of coral reef sponges. *Marine Biology,* 49: 161-167.

Wilkinson, C.R. (1978b). Microbial associations in sponges. II. Numerical analysis of sponge and water bacterial populations. *Marine Biology,* 49: 169-176.

Wilkinson, C.R. (1978c). Microbial associations in sponges. III. Ultrastructure of the in situ associations in coral reef sponges. *Marine Biology,* 49: 177-185.

Xu, D.D., Waykole, L., Calienni, J.V., Ciszewski, L., Lee, G.T., Liu, W., Szewczyk, J., Vargas, K., Prasad, K., Repicìœ, O. & Blacklock, T.J. (2003). An expedient synthesis of LAF389, a bengamide B analogue. *Organic Process Research & Development,* 7: 856-865.

Yasumoto, T., Oshima, Y., Sugawara, W., Fukuyo, T., Oguri, H., Igarashi, T. & Fujita, N. (1980). Identification of *Dinophysis fortii* as the causative organism of diarrhetic shellfish poisoning. *Nippon Suisan Gakkaishi,* 46: 1405 - 1411.

Zhang, W., Li, Z., Miao, X. & Zhang, F. (2008). The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China Sea sponges. *Marine Biotechnology*.

Chapter Two

SPONGE IDENTIFICATION AND CHEMICAL CHARACTERISATION

2.1 Introduction

Taxonomic identification of sponges is based on their physical morphology (size, shape, colour, distribution and shape of the oscular fistules and aerolate porefields) as well as the type of spicules found within the sponge tissue (Kelly & Samaai, 2002). All species of *Latrunculiidae* possess aniso(acantho)discorhabds or "chessman" spicules (Fig. 2.1) that have a straight shaft with four whorls of spines that frequently coalesce to form a full or partial disc. Isochia(acantho)discorhabds, with a stout straight shaft bearing three whorls of terminally acanthose truncate tubercles, characterise species of *Tsitsikamma* (Kelly & Samaai, 2002).

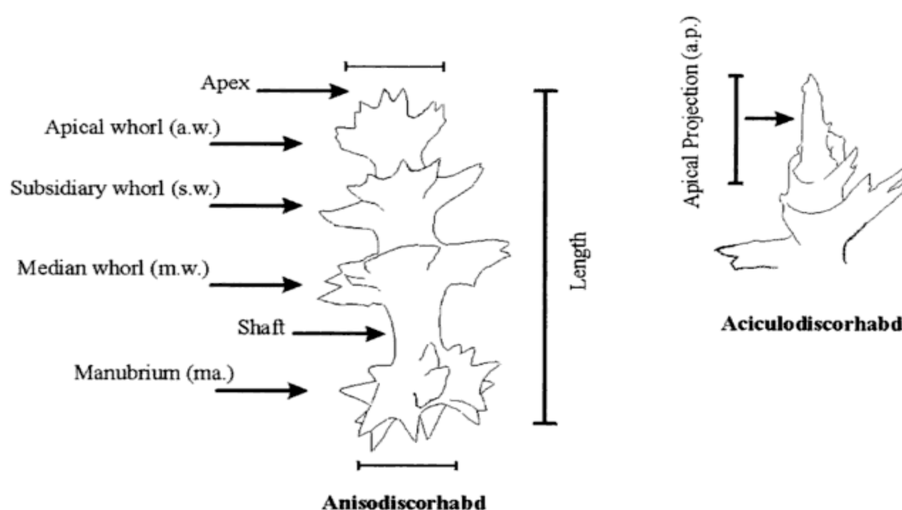


Figure 2.1 Schematic representation of spicule structure used to describe the discorhabd spicules of sponges belonging to the family *Latrunculiidae* (taken from Kelly & Samaai, 2002)

Alternately, chemotaxonomic markers can be used for identifying sponges. However, this method cannot be used in isolation as many sponges from different families share similar chemistry, and it should therefore rather be used as a means of confirming the initial taxonomic identification based on morphology (Antunes *et al.,* 2005; Urban *et al.,* 2000). In the case of *Tsitsikamma favus* however, the tsitsikammamines are considered taxonomic markers of this species

as they have, to date, only been isolated from this species of sponge (Antunes *et al.*, 2005). The previous isolation and characterisation of *T. favus* natural products was conducted on a batch collection of sponge specimens harvested from the Tsitsikamma Marine Reserve, off the south-eastern coast of South Africa (Hooper *et al.,* 1996; Antunes *et al.*, 2004). This protected wildlife area has unique biodiversity coupled with a turbulent and unpredictable coastline. Its protected status coupled with the temperamental diving conditions make collection of samples in this area logistically difficult. Consequently, the *T. favus* specimens collected for this study were from Evans peak in Algoa Bay. Collection of sponge specimens from this area is still very weather dependent but easier to access than those found in the Tsitsikamma Marine Reserve.

An emerging field in sponge taxonomy is the use of genetic markers to distinguish between sponge species. This is still a relatively new and underexplored field and there is limited data available on databases such as Genbank and the Sponge Barcoding Project. The most common locus used for molecular identification is a 710 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (*COX1*), which can be PCR amplified using primers derived from conserved regions (Folmer *et al.*, 1994). The *COX1* is one of the most highly conserved protein-coding genes in the mitochondrial genome of animals (Folmer *et al.*, 1994; Brown, 1985). A number of other 'barcoding' regions have also been suggested, including the cytoplasmic 28S and 18S rRNA gene fragments, the nuclear protein elongation factor 1-α (EF1α) and the ribosomal internal transcribed spacer (ITS) regions (Erpenbeck *et al.,* 2006a; Worheide *et al.,* 2004). The aim of the research described in this chapter was to identify the sponge specimens collected in Algoa Bay using both traditional taxonomy and molecular based techniques, as well as to investigate the secondary metabolite profiles of *T. favus* and *T. scurra* specimens.

2.2 Methods and Materials

2.2.1 Sponge collection

All sponge specimens were collected by SCUBA in Algoa Bay, South Africa and morphologically identified by Dr Shirley Parker-Nance (Centre for African Conservation Ecology, Nelson Mandela Metropolitan University). *T. favus* specimens Sp01, Sp02 and Sp03 as well as other *T. favus* sponges used for chemical analysis were collected within 1 m of each other at a depth of approximately 30 m at Evans Peak (33°50.578S; 25°48.988E) in May 2009. The batch collection of sponges for chemical analysis was also collected at the same geographical location and at the same time. Specimens Sp04 - Sp17 were collected from the same location in May 2010. Specimens Sp18, Sp21 and Sp22 and specimens Sp19, Sp23 and Sp24 were collected from Evans Peak and Phillips Reef (33°58.4245S; 25°40.869E) respectively in August 2010, while specimens Sp25, Sp26 and Sp27 were collected in April 2011 at Evans Peak. All sponges were collected in separate ziplock bags together with seawater from their immediate vicinity, were maintained at 4˚C and processed within 3 hrs of collection.

The sponges were processed separately so as to prevent cross-contamination. All sponge specimens were rinsed in sterile artificial seawater (ASW) (24.6 g NaCl, 0.67 g KCl, 1.36 g $CaCl_2.2H_2O$, 6.29 g $MgSO_4.7H_2O$, 4.66 g $MgCl_2.6H_2O$ and 0.18 g $NaHCO_3$ made up to 1 litre with double-distilled water) to remove any transient microorganisms associated with the surrounding seawater. Specimen Sp02 was gently squeezed and the liquid obtained in this way (seawater squeezed from the sponge) will further be referred to as Sp02*. Ideally, seawater surrounding the sponges should have been collected and filtered but this was not possible at the time. Where two sponges were found growing in close association with one another (as was the case for *T. scurra* and its associated yellow encrusting sponge) the yellow sponge was peeled off the surface of *T. scurra* leaving little to no yellow sponge residue behind. A wedge (extending from the centre of the sponge to the perimeter) was then aseptically cut from each of the sponge specimens and ground with a mortar and pestle in 5 ml ASW for 2 min to release the sponge-associated microorganisms. Approximately 1.5 ml aliquots of

dissociated sponge tissue were pelleted by centrifugation at 16,000 x g for 5 min and the pellet stored at -80 °C until later being used for DNA extractions. Glycerol stocks were prepared by adding 750 µl dissociated sponge tissue to 750 µl 2 x MA2216 broth (Difco):glycerol (50:50) and stored at -80 °C. The freshly collected sponges were then further processed for the isolation of culturable sponge-associated microorganisms (see Chapter 5). The remaining sections of the sponge specimens were then stored at -20 °C.

2.2.2 Morphological identification of sponge specimens

The sponge specimens were identified by Dr Shirley Parker-Nance and Dr Toufiek Samaai (Oceans and Coast, South African Department of Environmental and Water Affairs). Taxonomic identification of sponges was based on their physical morphology (size, shape, colour, distribution and shape of the oscular fistules and aerolate porefields) as well as the type of spicules found within the sponge tissue (Kelly & Samaai, 2002).

2.2.3 Total genomic DNA extraction from sponge tissue

A number of DNA isolation techniques were tried, namely the CTAB method (Ausubel *et al.*, 1999), the lysozyme method (Yeates *et al.*, 1998), the Fungal/Bacterial DNA miniprep (Zymo Research) and the guanidinium thiocyanate method (modified protocol from Pitcher *et al.,* 1989). The guanidinium thiocyanate method consistently produced more DNA product with less shearing and RNA contamination and was therefore used as the primary method of DNA isolation. The precise protocol used is described as follows: Frozen sponge pellets (from 1.5 ml dissociated sponge tissue) were resuspended in 350 µl Tris-EDTA (TE) buffer (pH 8.0) with 200 µl acid-washed sterile glass beads. The glass bead and sponge tissue suspension was then vortexed for six 30 sec cycles, with 30 sec on ice between each cycle. The glass beads were allowed to settle, the supernatant was transferred to a sterile 1.5 ml microfuge tube, and 300 µl guanidinium thiocyanate buffer was added (60 g guanidinium thiocyanate in 20 ml 100 mM Tris-EDTA made up to 100 ml with double-distilled water). A 200 µl volume of a 10 M ammonium acetate solution was added, followed by an equal volume of phenol

(equilibrated to pH 8.0 using TE buffer). After centrifugation (16,000 x g) for 10 min the aqueous layer was transferred to a sterile 1.5 ml microfuge tube. A half volume of chloroform:isoamylalcohol (24:1) was added, mixed and the two phases separated by centrifugation (16,000 x g) for 10 min and the aqueous phase was then transferred to a sterile 1.5 ml microfuge tube. This step was repeated, after which a half volume of ice-cold isopropanol was added to precipitate the nucleic acids at -20 °C for 2 h. The nucleic acids were pelleted by centrifugation (16,000 x g) for 5 min, washed with ice-cold 70% ethanol, re-pelleted by centrifugation (16,000 x g) for 5 min, dried and resuspended in TE buffer (pH 8.0). The resuspended nucleic acids were treated with RNase A at 37 °C for 1 h and the DNA was ethanol-precipitated, dried and resuspended in TE buffer (pH 8.0).

2.2.4 General PCR amplification protocol

PCR reactions were performed using 0.3 µM of each primer, 10 ng DNA, and 1 unit KAPA Taq (KAPA Biosystems) in a 50 µl reaction volume, unless otherwise stated. KAPA Taq does not have proofreading capabilities, but according to the manufacturer, will incorporate an average of 4.0×10^4 nucleotides before an error occurs.

2.2.5 Molecular identification and phylogenetic analysis of sponge species

The L*COX1*490 forward primer (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 reverse primer (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') were used to amplify the standard 650 nt *COX1* gene fragment (Folmer *et al.*, 1994). The L*COX1*490 forward primer and C1Npor2760 reverse primer (5'-TCT AGG TAA TCC AGC TAA ACC-3') (Erpenbeck *et al.*, 2006b; Herbert & Gregory, 2005; Poppe *et al.,* 2010) were used for PCR amplification of the extended 1250 nt *COX1* gene fragment. DMSO (8% of total volume) and $MgCl_2$ (final concentration of 2 mM) were added to optimize the PCR amplification using the following thermal cycling parameters: 95 °C for 1 min; 30 cycles at 95 °C for 45 sec, 45 °C for 45 sec, and 72 °C for 2 min; followed by 72 °C for 5 min. PCR fragments were ligated into pGEM-T Easy (Promega) and the nucleotide sequence of the *COX1* gene fragments was determined by Sanger sequencing.

The PCR primers RD3A (5'-GAC CCG TCT TGA AAC ACG A-3') and RD5B2 (5'-ACA CAC TCC TTA GCG GA-3') (McCormack & Kelly, 2002) were used to amplify the 650 bp D3 – D5 fragment of the 28S rRNA gene. PCR amplification was performed with KAPA Taq and additional DMSO (8% of total volume) and $MgCl_2$ (final concentration of 2 mM), using the following thermal cycling parameters: 95 °C for 5 min; 35 cycles at 94 °C for 30 sec, 45 °C for 20 sec, and 72 °C for 1 min; followed by 72 °C for 10 min. PCR fragments were ligated into pGEM-T Easy (Promega), and the nucleotide sequence of the 28S rRNA gene fragments was determined by Sanger sequencing.

Selected sponge specimens were subjected to PCR amplification by all three of the above mentioned primer pairs (in triplicate). Where PCR amplification was successful the PCR fragments were ligated into pGEM-T Easy and 5 clones from each sponge specimen were sent for sequencing. The resultant nucleotide sequences for each sponge specimen were aligned and checked for single nucleotide polymorphisms. All the available sponge sequences (some of the clones sequenced contained PCR fragments from eukaryotic sponge inhabitants) were used in the phylogenetic analysis conducted in MEGA5 (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004).

2.2.6 Isolation of the major secondary metabolite from a batch collection of T. favus.

Combined frozen sponge tissue (from several sponges collected at the same time and location as Sp01, Sp02 and Sp03) was cut into 1 cm^3 blocks and lyophilised for 2 days, yielding approximately 188 g of dry sponge tissue. A litre of methanol was added to the lyophilised tissue and allowed to soak for 3 days. The methanol

extract was then filtered through cotton wool to remove sponge fragments, precipitated salt, and sand. Sponge tissue was re-extracted with 1 litre of methanol for a further 2 days. The two methanol extracts were combined and concentrated *in vacuo* to yield 7.6 g of lyophilised crude sponge extract. Approximately half (3.6 g) of this lyophilised sponge extract was dissolved in 130 ml of methanol and cyclic-loaded onto a PSDVB (HP20, 150 ml) column, which was then flushed with 450 ml of de-ionised water and eluted with 450 ml volumes of 25%, 40%, 60%, 80% and 100% acetone (in de-ionised water). The fractions were back-loaded onto a PSDVB (HP20, 50 ml) column. The 25% and 40% fractions were eluted with 150 ml methanol while the 60%, 80% and 100% fractions were eluted with 150 ml acetone. All fractions were concentrated *in vacuo* and analysed using 600 MHz proton Nuclear Magnetic Resonance (NMR). All NMR spectra were acquired on a Bruker 600 MHz Avance II spectrometer. Deuterated methanol (MeOD) was used as the solvent.

A C_{18} reversed phase silica column (LiChroprep RP-18, 40-63 µm, Merck) was used to perform flash chromatography on the 25% acetone PSDVB fraction (62.8 mg). After the 25% fraction was loaded, the C_{18} column was eluted with an increasing percentage of methanol (in double-distilled water supplemented with 0.05% trifluoroacetic acid). The chromatography was done primarily based on the colour differences of the eluted fractions which were visible as yellow, green and red bands on the column. Analytical normal phase thin layer chromatography (TLC) performed on DC-Plastiekfolien Kieselgel 60 RP-18 F254 plates was used for the analysis of the fractions which were viewed under UV light (254 nm). Fractions with similar TLC patterns were combined, concentrated *in vacuo* and analysed by 600 MHz proton NMR.

The fractions eluted with 25% methanol were further purified by reversed phase high performance liquid chromatography (HPLC) using a Spectra-Physics Spectra-Series P100 isocratic pump and a Waters 410 Differential Refractometer. A mixture of double-distilled water (supplemented with 0.05% trifluoroacetic acid) and chromatography grade methanol was used as the mobile phase and separation by reversed phase HPLC yielded a pure compound (1.6 mg). Proton

(^{1}H) NMR and Heteronuclear Multiple Bond Correlation (HMBC) NMR spectroscopy techniques were used in the chemical analysis.

2.2.7 NMR stack plot comparison of a crude extract of T. favus specimens Sp01, Sp02 and Sp03

An approximately 50 cm^3 portion of frozen sponge material from T. *favus* specimens Sp01, Sp02 and Sp03 were cut into 1 cm^3 blocks and lyophilised to yield 4.2 g, 4.4 g and 4.5 g of dry tissue respectively. The lyophilised sponge tissue was then extracted with 30 ml methanol overnight. The methanol extracts were cyclic-loaded onto PSDVB (HP20, 7 ml) columns, which were flushed with 30 ml of de-ionised water and eluted with 30 ml volumes of 25% acetone (in de-ionised water). The 25% acetone fractions were back-loaded onto PSDVB (HP20, 5 ml) columns and eluted with acetone before being concentrated *in vacou* to yield 8.6 mg, 6.4 mg, and 7.1 mg of crude sponge extract from Sp01, Sp02 and Sp03 respectively. NMR spectra were acquired as described above and were stacked one on top of the other in order to directly compare the secondary metabolite profiles of the three sponge specimens.

2.2.8 NMR stack plot comparison and mass spectrometry analysis of a crude extract of T. scurra and T. favus

Pooled frozen sponge tissue from three *T. scurra* sponges (collected in May 2010 and April 2011) was cut into 1 cm^3 blocks and lyophilised for two days to yield 67 g of dry tissue. The lyophilised tissue was soaked in 300 ml of methanol for three days after which the methanol extract was filtered through cotton wool to remove sponge debris. Sponge tissue was re-extracted with 300 ml of methanol for a further day. The two methanol extracts were combined and cyclic-loaded onto a PSDVB (HP20, 50 ml) column, which was then flushed with 150 ml of de-ionised water and eluted with 150 ml volumes of 25%, 40%, 60%, 80% and 100% acetone (in de-ionised water). The fractions were back-loaded onto a PSDVB (HP20, 20 ml) column. The 25% and 40% fractions were eluted with 60 ml methanol while the 60%, 80% and 100% fractions were eluted with 60 ml acetone. All fractions were concentrated *in vacuo* to yield 16.1 mg, 14.1 mg, 45.5 mg, 288 mg, and

185.7 mg respectively. NMR spectra were acquired as described above. The 25%, 40% and 60% fractions were separated on a Waters ultra-high performance liquid chromatographic (UPLC) system using a Waters Ethylene Bridged Hybrid (BEH) C_{18} 2.1 x 50 mm column at a flow rate of 0.4 ml/min with a water (with 0.1% formic acid) to acetonitrile gradient. The conditions employed for the analysis of pyrroloiminoquinones by liquid chromatography mass spectrometry (LCMS) using Electron Spray Ionisation (ESI) were: positive ion mode, capillary voltage 3 kV, cone voltage 15 V and Leucine enkaphelin as the lock mass.

2.3 Results

2.3.1 Sponge taxonomy

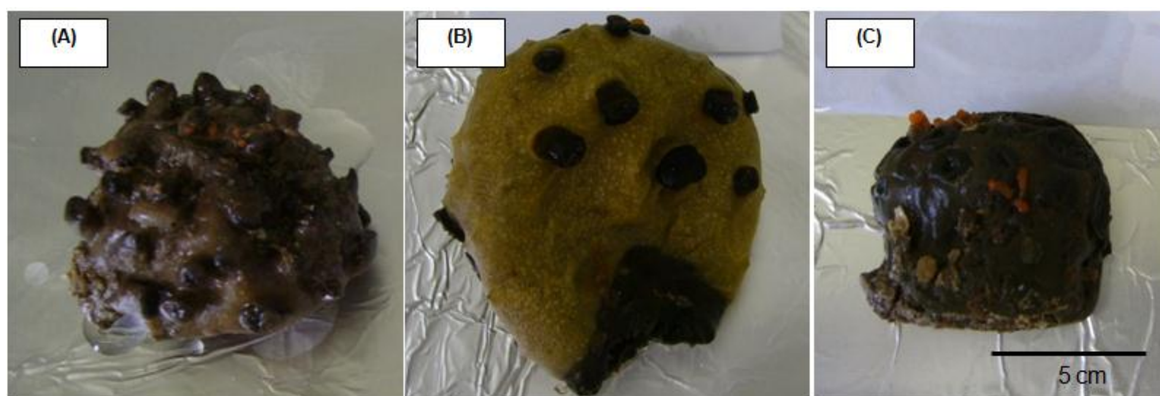


Figure 2.2 Freshly collected Algoa Bay specimens identified as *Tsitsikamma favus* (**A**), *Tsitsikamma scurra* (with yellow encrusting sponge) (**B**) and *Latrunculia sp.* (**C**).

Specimens Sp01, Sp02, Sp03 and Sp16 were identified as *T. favus* (Fig. 2.2A). Specimen Sp06 was identified as a new species within the genus *Latrunculia* (*Latrunculia sp.*) (Fig. 2.2C). Specimens Sp07, Sp09 and Sp11 were identified as *T. scurra* (Fig. 2.2B). Specimens Sp08, Sp10 and Sp12 are the yellow encrusting sponge found growing on the surface of *T. scurra* specimens Sp07, Sp09 and Sp11, respectively (Fig. 2.2B). Every *T. scurra* specimen collected had an associated yellow encrusting sponge growing on its surface.

2.3.2 Molecular Identification of sponges using the COX1 and 28S rRNA gene fragments

Multiple specimens of each species of sponge were collected (except for the *Latrunculia sp*. for which only a single specimen was available) and gDNA from all these sponge specimens was subjected to PCR amplification using both the standard (Folmer *et al.*, 1994) and the extended (Erpenbeck *et al.*, 2006b) *COX1* fragment as well as the 28S rRNA fragment (Erpenbeck *et al.* 2006a; Heim *et al.*, 2007; Worheide *et al.*, 2007). Where PCR amplification was successful, amplicons were cloned and used for DNA sequence analysis. The resultant nucleotide sequences were aligned using ClustalW2 to determine the number of single nucleotide polymorphisms between individual samples within the same species and between species. Two high fidelity Taq polymerases (Roche Applied Science, KAPA Biosystems) were used but neither produced PCR products despite multiple attempts at optimising the reactions. Consequently, KAPA Taq (KAPA Biosystems) was used for all PCR amplifications, even though it does not have proofreading capabilities. A possible explanation for difficulties in obtaining PCR products for the sponge DNA with the barcoding primers (despite optimization) may be that there were chemical compounds within the sponge samples that inhibited the PCR process and that the KAPA Taq was least sensitive to these compounds. The PCR protocols for amplification of the standard COX1 fragment, the extended COX1 fragment and the 28S rRNA fragment were all optimized with all three Taq polymerases mentioned previously. The *COX1* extended fragment was the least successful of the barcoding fragments (with limited success in obtaining a PCR product) while the 28S rRNA fragment had the highest success rate.

Initial alignment of the standard *COX1* fragment of three *T. favus* and three *T. scurra* specimens showed three polymorphisms; namely C/T, A/G and T/A. However, the standard *COX1* fragments showed no consistent differences in the 657 bp sequence and the observed single nucleotide polymorphisms could have been as a result of PCR errors. This fragment was therefore not useful in delineating between *T. favus* and *T. scurra.* The standard Folmer *COX1* fragment is the most widely used of the currently available genetic markers. However it has

been observed that the reduced mitochondrial substitution rates common to the phylum Porifera may prevent separation of specimens below the genus level (Erpenbeck *et al.,* 2006b; Folmer *et al.,* 1994; Worheide *et al.*, 2007). As a result, the extended *COX1* fragment has been proposed because it incorporates an additional 453 bp partition extending from the internal loop 3 to the transmembrane domain 11 (I3-M11 partition). Transversions are the dominant substitution types in the I3-M11 partition, which indicates a more progressive stage in character evolution and provides informative characters after shorter divergence times (Erpenbeck *et al.*, 2006b).

After several attempts, extended *COX1* fragments were obtained for three specimens of *T. favus* and two specimens of *T. scurra* all of which were aligned. As was the case for the standard *COX1* fragment, no consistent differences between *T. favus* and *T. scurra* were observed throughout the entire 1245 bp extended *COX1* sequence. Five SNPs; namely C/T, T/A, T/C, T/A and A/G; and two deletions were observed within the multiple alignment of the *T. favus* and *T. scurra* sequences. Again, all of these SNPs may have been due to PCR error. The standard *COX1* fragment was therefore used in the phylogenetic studies (Fig. 2.3) because there is more data available on this fragment in the Genbank and Sponge Barcoding Project databases. The 28S rRNA gene fragment proved to be the most successful of the three barcoding fragments discussed, both due to its higher PCR amplification success rate and its ability to distinguish between the closely related *T. favus* and *T. scurra.* A multiple sequence alignment of three *T. favus* and three *T. scurra* specimens shows three conserved SNPs (from all three specimens of *T. favus* to all three specimens of *T. scurra*), namely A/G, T/C, and T/G, thus enabling us to distinguish between these two species of *Tsitsikamma* sponges. Three random SNPs, namely T/A, A/G and A/G, were also observed and may have been due to PCR error. The standard *COX1* and 28S rRNA sequences (for *T. favus*, *T. scurra*, *Latrunculia sp.*, and the yellow encrusting sponge) were used to construct phylogenetic trees (in MEGA5) using the Neighbour joining method. Outlier sponges were chosen as the most relevant sequences available in the Genbank database.

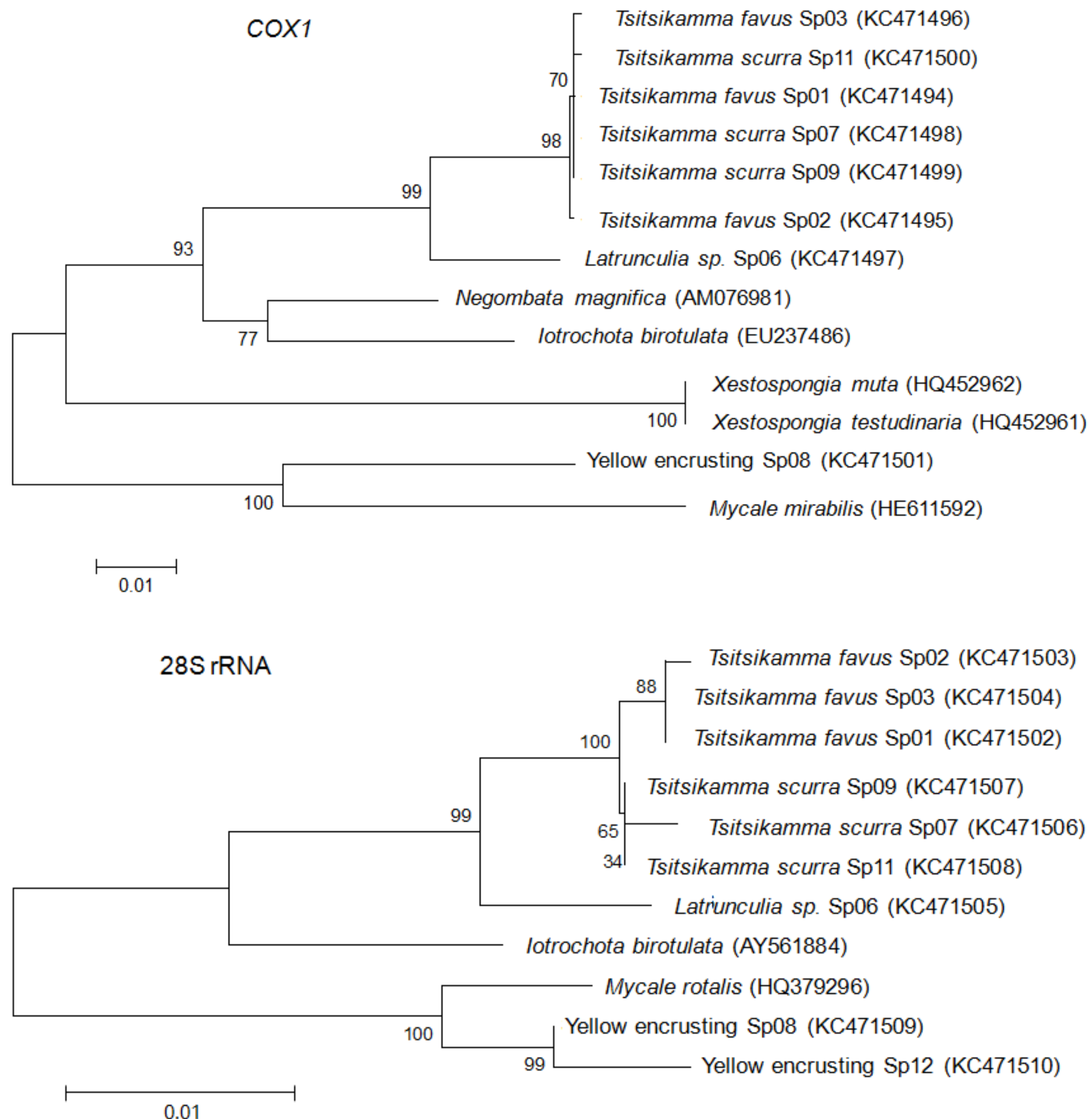


Figure 2.3 Neighbour-joining phylogenetic tree based on *COX1* and 28S rRNA sequences of *T. favus*, *T. scurra, Latrunculia sp.,* and the yellow encrusting sponge; as well as relevant outlier sponges. Genbank accession numbers are indicated in brackets and bootstrap values (100 replicates) are indicated at the nodes. The scale bar represents 1% sequence divergence.

The *COX1* phylogenetic tree shows clustering of *T. favus* and *T. scurra.* which was expected as the multiple sequence alignment showed no consistent differences between their nucleotide sequences. The *Latrunculia sp.* is also grouped close to the *Tsitsikamma* sponges (both the genus *Tsitsikamma* and the genus *Latrunculia* belong to the family *Latrunculiidae*). It is followed by *Negombata magnifica* which was chosen as an outlier species because it is the

closest match to the *Tsitsikamma* favus *COX1* sequence in the NCBI BLAST database. It belongs to the family *Podospongiidae*, which together with the family *Acarnidae*, is closely related to the family *Latrunculiidae* (which includes the genus *Tsitsikamma*) due to their very similar microscleres (Antunes *et al.,* 2005). Interestingly, *N. magnifica* is one of the most prolific producers of secondary metabolites belonging to the latrunculin family of compounds, which is named after its original discovery in sponges of the genus *Latrunculia* (El Sayed *et al.,* 2006). The yellow encrusting sponge groups with species belonging to the genus *Mycale*. *Mycale mirabilis* (88% identity) and *Mycale rotalis* (97% identity) were the closest matches to the yellow encrusting sponge in the BLAST database for the *COX1* and 28S rRNA gene fragments respectively. The genus *Mycale* belongs to the family *Mycaliidae*, which is not closely related to the family *Latrunculiidae*.

Both the 28S rRNA and *COX1* phylogenetic analyses show a close relationship between *T. favus* and *T. scurra* thus confirming the morphological IDs. The drawback of the *COX1* marker is that it was not able to differentiate between the closely related *T. favus* and *T. scurra,* even when the extended fragment was used. Although genetic markers offer a promising alternative to traditional taxonomy, they are not yet reliable enough to do so, given the difficulties involved in differentiating species to below the genus level. In addition, limited data is available on Porifera in databases such Genbank so increased research is certainly required in this field before genetic markers are routinely used in sponge taxonomy. Further insight into the chemistry of the two *Tsitsikamma* species may provide additional confirmation with regards to the taxonomy of these two sponge species, as well as insight into the relationship between sponge species and their associated secondary metabolite production.

2.3.3 Isolation and characterisation of the major secondary metabolite in Tsitsikamma favus

A batch collection of sponges (collected at Evans Peak, Algoa Bay at the same time and location as Sp01, Sp02 and Sp03) was lyophilised and extracted with methanol. The crude extract obtained in this way was separated using a series of chromatography techniques coupled with ^{1}H and HMBC NMR spectroscopy to

characterise the major secondary metabolite(s). The NMR profiles were compared with those obtained from purified tsitsikammamine B isolated from *T. favus* that was collected in the Tsitsikamma National Park in a previous study (Antunes *et al.*, 2004).

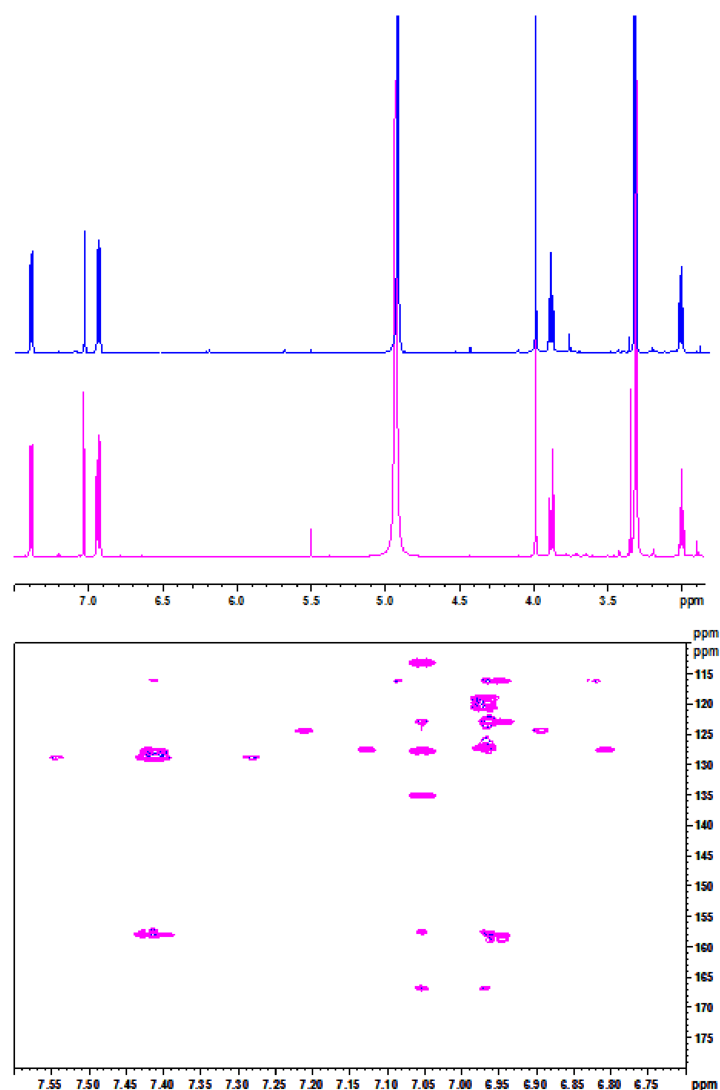


Figure 2.4 NMR spectra of the major secondary metabolite from *T. favus*. **(Top)** ^{1}H NMR spectra (δH 2.8 ppm – 7.5 ppm) and **(Bottom)** HMBC spectra (δH 6.7 ppm – 7.5 ppm and δC 180 ppm – 110 ppm). The spectra of the major secondary metabolite isolated from a batch of *T. favus* specimens collected from Evans Peak, Algoa Bay in May 2009 (pink) were compared with those of tsitsikammamine B isolated from a previous collection (Tsitsikamma Marine Reserve) of *T. favus* by Antunes *et al.* (2004) (blue).

The ^{1}H NMR and HMBC spectra of the major compound isolated from the May 2009 collection of *T. favus* in Algoa Bay were consistent with those obtained for tsitsikammamine B isolated from the *T. favus* collected in the Tsitsikamma

National Park by Antunes *et al*. (2004) (Fig. 2.4), presenting strong evidence that the major compound isolated from the May 2009 collection of *T. favus* is tsitsikammamine B. This finding is consistent with the theory that tsitsikammamine B is consistently the major compound isolated from *T. favus* (Hooper *et al*., 1996; Antunes *et al*., 2004;), which together with the fact that, to date, it has only ever been isolated from *T. favus*, puts it in good stead to be used as a chemotaxonomic marker for this sponge species (Antunes *et al*., 2005). The identification of tsitsikammamine B as the major secondary metabolite from the May 2009 batch collection of *T. favus* sponges collected in Algoa Bay, also confirmed that even in different geographical locations, tsitsikammamine B is consistently the major compound being produced by this sponge species

2.3.4 NMR and LCMS analysis of the secondary metabolite profile of T. favus and T. scurra specimens

There have been few natural product studies comparing the secondary metabolite profiles of individual sponges of the same species found growing alongside one another, and no such study has previously been conducted on *T. favus.* The secondary metabolite profile of individual sponge specimens within the same species was expected to vary slightly due to differences in individual sponge metabolism, possibly in response to pathogens or predators. Although the types of compounds produced are expected to remain constant, the ratios of these compounds to one another may vary slightly depending on what that individual sponge specimen has recently been exposed to.

Accordingly a study was conducted comparing major secondary metabolites of three individual *T. favus* specimens (Sp01, Sp02 and Sp03). The lyophilised sponge material was extracted with 30 ml methanol, and the methanol extracts were then separated by HP20 reversed-phase chromatography. The 25% acetone fraction of each sponge was analysed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra were examined for the signals between δH 6.7 and 7.8 ppm, which is the region where the signature peaks indicating the presence of tsitsikammamines and other pyrroloiminoquinones (e.g. discorhabdins) are expected to occur. The upfield multiplets of the tsitsikammamines centred at δH 3.0 and 3.9 are obscured

by the methylene envelope arising from primary and other metabolites present in the crude 25% acetone fraction and the upfield region was therefore not included in the ^{1}H NMR stack plot comparison. A comparison of the spectra showed that the signature compounds isolated from the three *T. favus* specimens were similar but that each sponge contained differing ratios of these compounds (Fig 2.5). As previously mentioned, the varying amounts of compounds between sponges of the same species may be in response to pathogens or predators. Alternatively, these variations may reflect differences in the relative abundance of the sponge-associated bacterial communities if sponge-associated bacteria are responsible for the production of the pyrroloiminoquinones in *T. favus*.

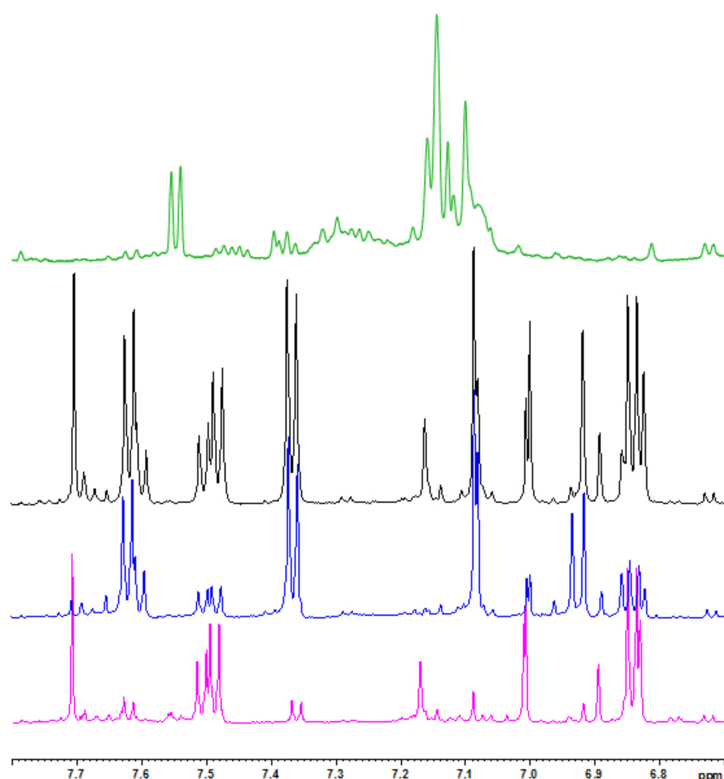


Figure 2.5 A section (δH 6.7 ppm - 7.8 ppm) of the ^{1}H NMR spectra (600 MHz, MeOD) of the 25% acetone fraction of a crude methanol extract of *T. favus* specimens Sp01 (black), Sp02 (blue) and Sp03 (pink), and *T. scurra* (green).

Next, major secondary metabolites of *T. scurra* were investigated. The ^{1}H NMR spectrum of *T. scurra* (green) is significantly different and does not resemble that of the three *T. favus* specimens (Figure 2.5) thus indicating that it potentially contained a different suite of secondary metabolites. Due to the significance of the

tsitsikammamines as chemotaxonomic markers for *T. favus*, *T. scurra* was further investigated for the presence of tsitsikammamines using LCMS. LCMS is an ideal, quick and exceptionally powerful analytical method due to its ability to achieve extremely low detection limits and to discriminate between peaks that co-elute during HPLC, and is therefore used extensively for metabolite profiling and identification (Brugger *et al.,* 1997). The basic imine nitrogen present in all pyrroloiminoquinones is protonated with the addition of TFA to give the pseudo (M + 1) molecular ion, which is easily detected by electron spray ionisation (ESI) LCMS, while the presence of bromine gives rise to a distinct molecular ion isotope pattern which is directly proportional to the number of bromines present within the compound.

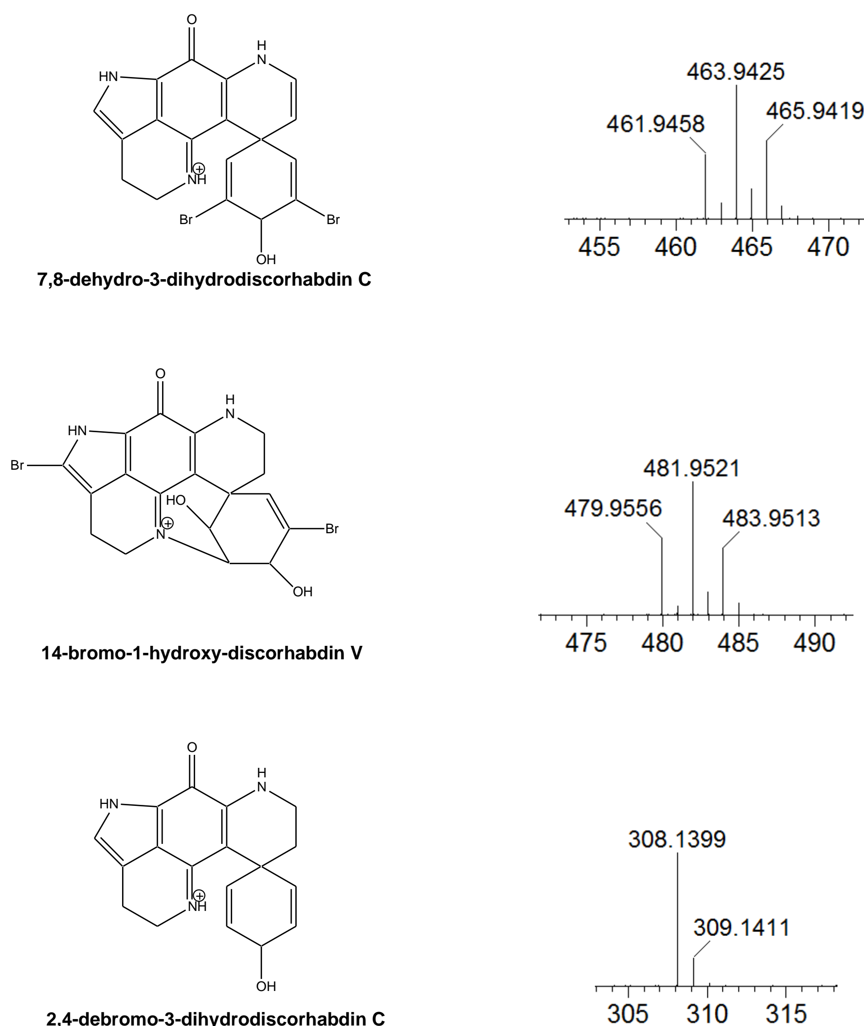


Figure 2.6 Discorhabdin compounds identified in *T. scurra* using ESI LCMS with the M + 1 ion bromine isotopic cluster on the right.

The crude HP20 fractions of *T. scurra* were subjected to ESI LCMS with the aim of detecting pseudomolecular ions corresponding to some of the pyrroloiminoquinones previously found in *T. favus* and *T. pedunculata*. Three known compounds were detected from *T. scurra* (Fig. 2.6); namely 7,8-dehydro-3-dihydrodiscorhabdin C, 14-bromo-1-hydroxy-discorhabdin V and 2,4-debromo-3-dihydrodiscorhabdin C (with calculated M + 1 masses of 461.9453, 479.9558, and 308.1399 corresponding almost exactly to the M + 1 ions detected, respectively). The M + 1 ion isotopic cluster (on the right) shows the presence of 2 bromine atoms, 2 bromine atoms, and 0 bromine atoms respectively. Tsitsikammamine B, consistently detected as the major compound in *T. favus*, was not present in any of the *T. scurra* fractions, confirming the exclusivity of this compound to *T. favus*. All three known compounds detected in *T. scurra* were common to *T. favus* and *T. pedunculata*, and are therefore common to all of the *Tsitsikamma* sponges studied to date (Antunes *et al.*, 2004; Antunes *et al.*, 2005).

2.4 Discussion

The objective of this chapter was to identify and investigate Algoa Bay *Latrunculid* sponges using traditional spicule taxonomy, chemotaxonomy and molecular taxonomy techniques. The chemical analysis of three specimens of *T. favus* reconfirmed the observation by Antunes *et al.* (2004) that tsitsikammamine B is consistently the major compound isolated from *T. favus*. However, the chemical profiles of the three specimens were not identical, with slight variations in the ratios of the secondary metabolites present which could be attributed to predation or infection. A comparison of the secondary metabolites associated with *T. favus* and *T. scurra* indicated a different chemical profile. Further ESI LCMS analysis indicated that *T. scurra* did not contain tsitsikammamines thus reinforcing the hypothesis that these compounds are produced strictly by *T. favus*. The three discorhabdins 2,4-debromo-3-dihydrodiscorhabdin C, 7,8-dehydro-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V; detected in *T. scurra* have previously been isolated from *T. favus* and *T. pedunculata* (Antunes *et al.,* 2003; Antunes *et al.*, 2004; Antunes *et al.*, 2005) and, to date, have been found exclusively in association with the sponge genus *Tsitsikamma* (Antunes *et*

al., 2004; Antunes *et al.,* 2005; Gribble *et al.,* 2012). These compounds may therefore be considered good chemotaxonomic markers for *Tsitsikamma* species.

There was good correlation between the morphological and molecular taxonomy, showing a very a close relationship between *T. favus* and *T. scurra.* The *Latrunculia sp.* was more closely related to the *Tsitsikamma* sponges than the outlier sponge *Negombata magnifica*. However, the sponge barcoding fragments used were not entirely reliable, especially the *COX1* gene which, even when using the extended fragment, was unable to distinguish between the *Tsitsikamma* sponge species. As a result this study points to a need to use more than one of the sponge 'barcode' sequences to distinguish between species in the same genus. In addition, the data available on the Sponge Barcoding and Genbank databases is limited, with a number of different barcoding fragments represented throughout the limited data. A possible solution to this would be for all sponge taxonomists to use the same two or three barcoding fragments when identifying sponges and that this data should be uploaded into either the Sponge Barcoding Project or the Genbank databases. As it stands, the limited information available with regards to sponge genetic markers, coupled with the inconsistency of the barcoding fragments, makes it very difficult to use this method in isolation for identification of sponge specimens.

The sponges *Xestospongia muta* and *Xestospongia testudinaria* were used as outliers in the *COX1* phylogenetic tree due to a recent study by Montalvo & Hill (2011) where these sponges were classified as two different species based on their taxonomy but were shown to be identical according to the *COX1* sequences. The study shows that the two sponges could actually be extremely closely related and may share a common ancestor despite having been classified as different species due to differences in their geographical distribution. An in depth study of the sponge-associated microbial community showed that these two species of sponge had very similar microbial communities but with distinct differences, which may be due to their lengthy geographical separation since the closing of the Isthmus of Panama at least 3 million years ago (Montalvo & Hill, 2011).

This study has shown that *T. favus* and *T. scurra* are taxonomically very similar based upon their *COX1* and 28S rRNA gene sequence. However they may be associated with different conserved populations of bacteria, which may have been responsible for the observed differences in their secondary metabolite profiles. The diversity and population structure of bacteria associated with *T. favus* and *T. scurra* as compared with other sponges is the subject of Chapters three and four.

2.5 References

Antunes, E.M. (2003). Pyrroloiminoquinone metabolites from South African Latrunculid sponges. *Chemistry.* Grahamstown: Rhodes University.

Antunes, E.M., Beukes, D.R., Kelly, M., Samaai, T., Barrows, L.R., Marshall, K.M., Sincich, C. & Davies-Coleman, M.T. (2004). Cytotoxic pyrroloiminoquinones from four new species of South African Latrunculid sponges. *Journal of Natural Products,* 67: 1268-1276.

Antunes, E.M., Copp, B.R., Davies-Coleman, M.T. & Samaai, T. (2005). Pyrroloiminoquinones and related metabolites from marine sponges. *Natural Products Reports,* 22: 62-72.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1999). *Current Protocols in Molecular Biology,* New York: Greene Publishing Associates and Wiley-Interscience.

Brown, W.M. (1985). The mitochondrial genome of animals. IN Macintyre, R.J. (Ed.) *Molecular Evolutionary Genetics.* New York: Plenum Press.

Brugger, B., Erben, G., Sandhoff, R., Wieland, F.T., Lehmann, W.D. (1997). Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *PNAS*, 94:2339-2344.

El Sayed, K.A., Youssef, D.T.A. & Marchetti, D. (2006). Bioactive natural and semisynthetic latrunculins. *Journal of Natural Products,* 69: 219-223.

Erpenbeck, D., Breeuwer, J.A.J., Parra-Velandia, F.J. & Van Soest, R.W.M. (2006a). Speculation with spiculation? Three independent gene fragments and biochemical characters versus morphology in demosponge higher classification. *Molecular Phylogenetics and Evolution,* 38: 293-305.

Erpenbeck, D., Hooper, J.N.A. & Wörheide, G. (2006b). *COX1* phylogenies in diploblasts and the 'Barcoding of Life'— are we sequencing a suboptimal partition? *Molecular Ecology Notes,* 6: 550-553.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39: 783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology,* 3: 294-299.

Gribble, G.W., van Pee, K-H., Vetter, W. (2012). *The Alkaloids.* Academic Press.

Hebert, P.D.N. & Gregory, T.R. (2005). The promise of DNA barcoding for taxonomy. *Systematic Biology,* 54: 852-859.

Heim, I., Nickel, M. & Brummer, F. (2007). Molecular markers for species discrimination in poriferans: a case study on species of the genus *Aplysina. Porifera Research: Biodiversity, Innovation and Sustainability*: 361 - 371.

Hooper, G.J., Davies-Coleman, M.T., Kelly-Borges, M. & Coetzee, P.S. (1996). New alkaloids from a South African Latrunculid sponge. *Tetrahedron Letters,* 37: 7135-7138

Kelly, M. & Samaai, T. (2002). Family *Latrunculiidae* Topsent, 1922. IN Hooper, J.N.A. & Van Soest, R.W.M. (Eds.) *System Porifera: A guide to the Classification of Sponges.* New York: Kluwer/Plenum Academic.

Lavrov, D.V., Wang, X. & Kelly, M. (2008). Reconstructing ordinal relationships in the Demospongiae using mitochondrial genomic data. *Molecular Phylogenetics and Evolution,* 49: 111-124.

McCormack, G.P. & Kelly, M. (2002). New indications of the phylogenetic affinity of *Spongosorites suberitoides* (Porifera, Demospongiae) as revealed by 28S ribosomal DNA. *Journal of Natural History,* 36: 1009-1021.

Montalvo, N.F. & Hill, R.T. (2011). Sponge-Associated bacteria are strictly maintained in two closely-related but geographically distant sponge hosts. *Applied and Environmental Microbiology*.

Pitcher, D.G., Saunders, N.A. & Owen, R.J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Letter of Applied Microbiology,* 8: 151-156.

Poppe, J., Sutcliffe, P., Hooper, J.N.A., Worheide, G. & Erpenbeck, D. (2010). COI barcoding reveals new clades and radiation patterns of Indo-Pacific sponges of the family *Irciniidae* (Demospongiae: Dictyoceratida). *PLoS ONE,* 5: e9950.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 4: 406-425.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA),* 101: 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). Molecular evolutionary genetics analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony methods. . *Molecular Biology and Evolution,* In Press.

Urban, S., Hickford, S. J. H., Blunt, J. W., Munro, M. H. G. (2000). Bioactive Marine Alkaloids. *Curr Org Chem*, 4:765–805

West, L.M. (2001). The isolation of secondary metabolites from New Zealand marine sponges. *Chemistry.* Wellington: Victoria University of Wellington.

Worheide, G., Erpenbeck, D. & Menke, C. (2007). The Sponge Barcoding Project: aiding in the identification and description of poriferan taxa. *Porifera Research: Biodiversity, Innovation and Sustainability*: 123 - 128.

Worheide, G., Nichols, S.A. & Goldberg, J. (2004). Intragenomic variation of the rDNA internal transcribed spacers in sponges (Phylum Porifera): implications for phylogenetic studies. *Molecular Phylogenetics and Evolution,* 33: 816-830.

Yeates, C., Gillings, M., Davison, A., Altavilla, N. & Veal, D. (1998). Methods for microbial DNA extraction from soil for PCR amplification. *Biological Procedures Online,* 1: 40-47

Chapter Three

DIVERSITY AND POPULATION STRUCTURE OF *TSITSIKAMMA FAVUS*- ASSOCIATED BACTERIA

3.1 Introduction

A study into the secondary metabolites associated with *Tsitsikamma favus* (Chapter Two) revealed that tsitsikammamine B is consistently the major compound isolated. Additionally, it appears that tsitsikammamine B is restricted to *T. favus* as it was not detected in *T. scurra* and has not previously been detected in *T. pedunculata* (Antunes *et al.,* 2004; Antunes *et al*., 2005). The secondary metabolite signatures of three specimens of *T. favus* indicated that the chemistry was conserved, albeit with differences in the ratios of the relevant compounds. The apparent exclusivity of the tsitsikammamines to *T. favus* and the consistency of secondary metabolite production between individual *T. favus* specimens instigated an investigation into the bacterial communities associated with *T. favus* using 16S rRNA molecular techniques. Molecular techniques such as 16S rDNA gene amplification and subsequent sequencing have greatly improved our ability to study the complex communities of sponge-associated bacteria (Wang, 2006). The 16S rRNA techniques involve PCR amplification of the DNA coding for the 16S rRNA gene fragment, which is highly conserved in bacteria due to the fundamental role of 16S rRNA in protein synthesis (Rosella-Mora & Amann, 2001), and yet exhibits adequate differentiation between bacterial species to allow for clear identification (Brunk *et al*., 1996). Analysis of the DNA sequence of this gene fragment provides an accurate means of species identification and can be used for further phylogenetic studies (Lane, 1991).

The aim of the research described in this chapter was to characterise the diversity and population structure of bacteria associated with *T. favus*, focusing on 16S rRNA sequence diversity, using three different approaches. Denaturing gradient gel electrophoresis (DGGE), clonal libraries of full length 16S rRNA genes as well as next generation sequencing (NGS) analysis were used to compare the microbial populations associated with *T. favus* specimens collected over two years (May 2009 and May 2010) from Evan's peak, Algoa Bay. Data reported in this

chapter has been published in Walmsley, T.A., Matcher, G.M., Zhang, F., Hill, R.T., Davies-Coleman, M.T., Dorrington, R.A. (2012). Diversity of Bacterial Communities Associated with the Indian Ocean Sponge Tsitsikamma favus That Contains the Bioactive Pyrroloiminoquinones, Tsitsikammamine A and B. *Marine Biotechnology,* 14:681-691 (Appendix A2).

3.2 Materials and Methods

3.2.1 Extraction of total genomic DNA

Genomic DNA was isolated using the guanidium thiocyanate method as described in section 2.2.3.

3.2.2 Primers used in the 16S rRNA molecular analysis

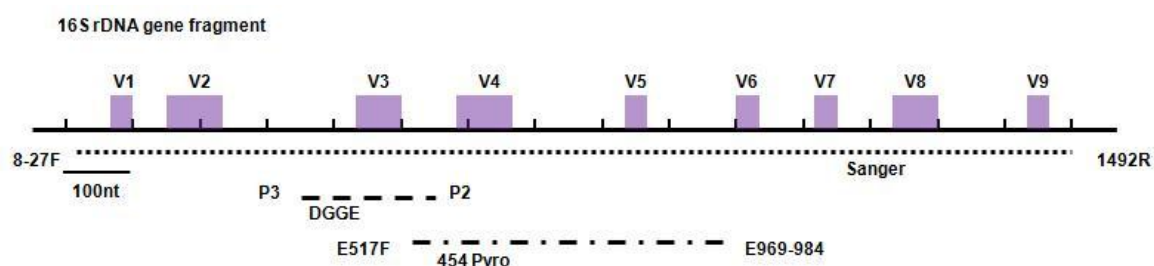


Figure 3.1 Schematic representation of the 16S rDNA gene fragment with the known variable regions and primers used during this study indicated.

The 1.5 kb full length 16S rRNA gene fragment, PCR amplified using the universal primer pair 8-27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane, 1991), was used in the construction of the clonal library and was sequenced using Sanger sequencing. The PCR primers P2 (5'-ATT ACC GCG GCT GCT GG-3') and P3 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') which correspond to positions 341 and 534 in the 16S rRNA sequence of *E. coli*, were used to amplify the 194 bp V3 variable region of the bacterial 16S rRNA gene for DGGE analysis (Enticknap *et al*., 2006; Muyzer *et al.,* 1993). For 454 pyrosequencing analysis the PCR primer pair E517F (5'-CAG CAG CCG CGG TAA-3') and E969-984 (5'-GTA AGG TTC YTC GCG T-3') was used to amplify the

470 bp hypervariable regions V4 and V5 of the 16S rRNA gene. This primer pair was shown to have a high coverage rate in almost all bacterial phyla (Wang *et al.,* 2007, Wang & Qian, 2009).

3.2.3 DGGE analysis of bacterial 16S rRNA gene amplicons

The PCR primers P2 and P3 were used to amplify the 194 bp region of the bacterial 16S rRNA gene for DGGE analysis (Enticknap *et al.,* 2006; Muyzer *et al.,* 1993). PCR amplification was performed with 10 ng gDNA, 0.4 µM of each primer, 1 unit KAPA Taq, and additional DMSO (8% of total volume) and $MgCl_2$ (final concentration of 2 mM) in a final volume of 50 µl; using the following thermal cycling parameters: 94 °C for 5min; 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; followed by 72 °C for 5 min. DGGE was performed using the Dcode Universal Mutation Detection System (Bio-Rad) and PCR samples were resolved using polyacrylamide gel containing 8% Acrylamide:Bisacrylamide (37.5:1) and a linear gradient of the denaturants (formamide and urea) ranging from 40% to 70%. Electrophoresis was performed in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM Na_2EDTA, pH 8.0) at 60 °C with a constant voltage of 60 V applied to the submerged gel for 16 h. DNA fragments were visualised by UV light after staining with syber green (Roche).

3.2.4 Preparation and sequencing of 16S rRNA clonal libraries derived from T. favus

The 8-27f and 1492 universal primers (Lane, 1991) were used for PCR amplification of the full length 1.5 kb 16S rRNA gene fragment as described in section 2.2.4 using KAPA Taq and the following thermal cycling parameters: 94 °C for 5 min; 25 cycles at 92 °C for 30 sec, 48 °C for 2 min, and 72 °C for 90 sec, followed by 72 °C for 5 min. The amplicons were ligated into the pGEM-T Easy vector after which the sequence of the inserts was determined by Sanger sequencing. Unique clone sequences were deposited in GenBank (Accession numbers HQ241757 - HQ241817, HQ287901, JF930155, JF930156, JF930157 and JN001720).

3.2.5 Phylogenetic analysis of the 16S rRNA clonal libraries of T. favus associated bacteria

Initial analysis of edited 16S rRNA gene sequences from two clone libraries derived from *T. favus* specimen Sp02 and squeezed water from Sp02 (Sp02*) was performed using the BLASTN tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to assist in the selection of appropriate reference sequences. This database was supplemented with relevant environmental sequences submitted to GenBank, selected according to the top BLASTN hits of the clone sequences. The selected sponge-associated bacterial clones and their nearest relative matches were aligned using the ARB alignment tool (Ludwig *et al.*, 2004). Multiple alignments were verified and improved manually using the ARB editor tool, the aligned sequences were analysed using the ARB neighbour-joining distance matrix method and chimeric clones were excluded. Neighbour-joining trees (Jukes-Cantor correction) (Saitou & Nei, 1987) were constructed based on 1 342 nucleotide alignment positions and the accuracy of the inferred tree topologies was evaluated after 100 bootstrap replicates of the neighbour-joining data using PHYLIP (Felsenstein, 2004).

3.2.6 454 pyrosequencing analysis of the T. favus-associated 16S rRNA amplicons

The 454 pyrosequencing analysis was performed essentially as described in Matcher *et al.* (2011). Genomic DNA was isolated from *T. favus* isolate Sp02 (collected May 2009) as well as *T. favus* isolate Sp05 collected a year later (May 2010) from the same location, using the guanidium thiocyanate method as described in section 2.2.3. PCR amplification of the hypervariable regions V4 and V5 of the 16S rRNA gene was achieved via a two step regime using the primer pair E517F and E969-984 (Wang *et al.*, 2007, Wang & Qian, 2009). Multiplex Identifier tags derived from the manufacturer's manual (Roche) were used to track the amplicons from each of the two samples, thus facilitating the assignment of each sequence generated to the correct sample. The primary PCR amplification consisted of 5-10 ng gDNA, 1 X PCR buffer (containing $MgCl_2$), 300 µM dNTPs, 0.3 µM of each template specific primer and 0.5 units KAPAHiFi Hotstart DNA

Polymerase (KAPA Biosystems) in a final volume of 25 µl. KAPA HiFi Hotstart is an antibody based hotstart formulation with 100x lower error rates than wild-type Taq. Initial enzyme activation and DNA denaturation was performed at 95 °C for 5 min followed by thermal cycling parameters of: 5 cycles at 98 °C for 45 sec, 45 °C for 30 sec and 72 °C for 45 sec; and 10 cycle at 98 °C for 45 sec, 50 °C for 30 sec and 72 °C for 45 sec. A final extension was done at 72 °C for 5 min. The resultant 468 nt PCR products were gel purified using the Zymo Gel DNA Recovery kit (Zymo Research).

Approximately 2 ng of the PCR product was then subjected to a second PCR amplification using FLX fusion primers (consisting of sequence specific nucleotides, a multiplex identifier tag and template-specific nucleotides), as described below. Each sample was amplified with a primer set containing a different multiplex tag. The secondary PCR amplification consisted of 2 ng template, 0.4 µM forward and reverse primers, 300 µM dNTPs, 1 X PCR Buffer and 0.5 units KAPAHiFi Hotstart DNA Polymerase in a final volume of 25 µl. Initial enzyme activation and DNA denaturation was done at 95 °C for 5 min, followed by thermal cycling parameters of: 5 cycles at 98 °C for 20 sec, 52 °C for 45 sec and 72 °C for 1 min; and 15 cycles at 98 °C for 20 sec, 65 °C for 45 sec and 72 °C for 1 min. A final extension was done at 72 °C for 5 min. The resultant 538 nt PCR products were gel purified as before. The resultant amplicons were then pooled in equal amounts and subjected to emulsion PCR before sequencing using a 454 Genomesequencer FLX instrument (Roche Applied Science). Sequence reads were quality filtered using standard software provided by 454 Life Sciences and MOTHUR (http://www.mothur.org, Schloss *et al.*, 2009), cured for primer/tag sequences, and chimeras and all reads less than 100 nt were removed from the dataset. The remaining 12 986 reads were analyzed using software available on the Ribosomal Database Project (RDP). The RDP Classifier assigns 16S rRNA sequences using a Naïve Bayesian rRNA classifier algorithm (Cole *et al.*, 2009; Wang *et al.*, 2007). The default bootstrap confidence threshold was applied to all sequence reads longer than 250 nt. For reads <250 nt a bootstrap cut-off of 50% was applied (Claesson *et al.,* 2009). The rarefaction calculations were carried out using the rarefaction analysis tool on the RDP Pyrosequencing Pipeline and curves generated for 0.01, 0.03, 0.05 and 0.1 distance values (Cole *et al.*, 2009).

3.3 Results

3.3.1 DGGE analysis of sponge-associated bacterial diversity

Initially DGGE was used as a means of rapidly and visually assessing the degree of variation within the bacterial population as well as the extent to which the bacterial community was conserved. The PCR primers P2 and P3 were used to amplify the 194 bp V3 variable region of the bacterial 16S rRNA gene for DGGE analysis (Enticknap *et al.,* 2006; Muyzer *et al*., 1993) of *T. favus* and the resultant DGGE profiles are displayed in Fig. 3.2. Only a small section (194 bp) of the 16S rRNA gene was used in the DGGE analysis which may have limited the taxonomic resolution.

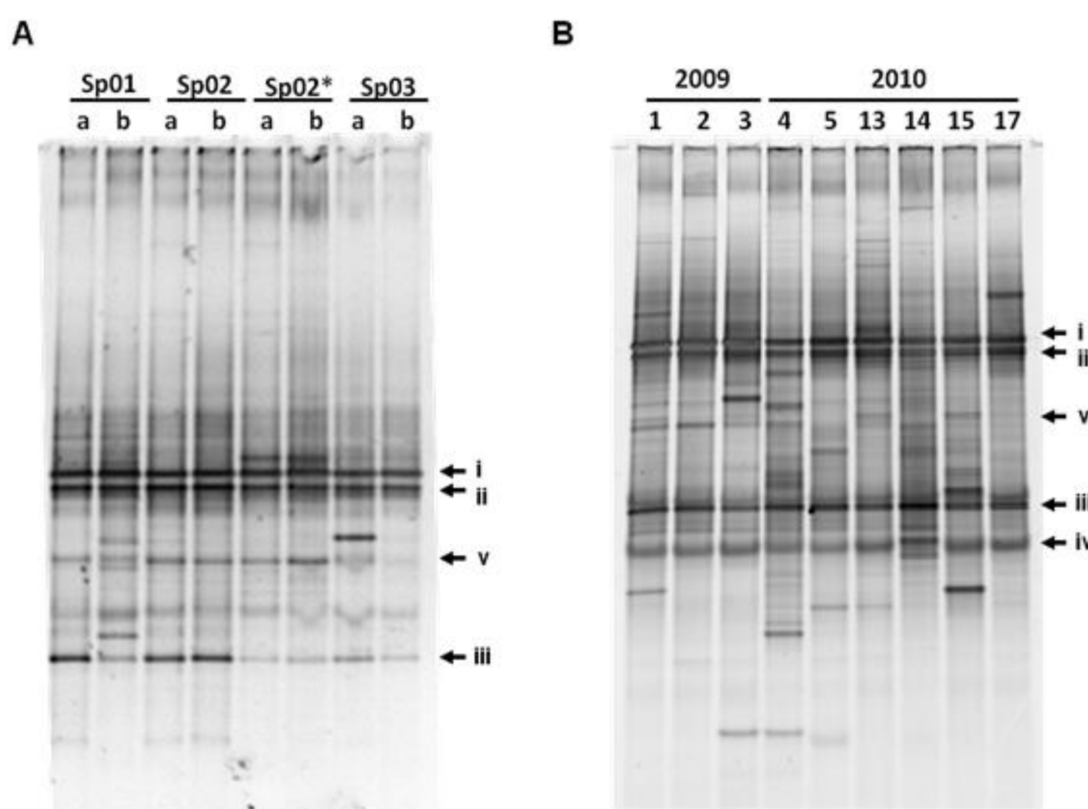


Figure 3.2 DGGE analyses of the 16S rRNA gene sequences of bacterial communities associated with individual *T. favus* specimens. (**A**) Comparison of DGGE profiles of 16S rRNA gene amplicons derived from duplicate (a and b) DNA samples extracted from *T. favus* specimens Sp01, Sp02 and Sp03, collected in 2009 and from seawater squeezed from specimen 2 (Sp02*). (**B**) Comparison of 16S rRNA gene sequence diversity of bacterial communities associated with *T. favus* specimens collected in 2009 (Sp01, Sp02 and Sp03) and 2010 (Sp04, Sp05, Sp13, Sp14, Sp15 and Sp17). The relative positions of conserved bands are indicated on the right (i-v).

The diversity and conservation of the sponge associated bacterial community in *T. favus* was investigated by extracting gDNA from each of three individual specimens (biological replicates Sp01, Sp02 and Sp03) collected from within 1 m^2 of each other in May 2009. The difference between the stable and the more transient bacterial community was also taken into account by comparing the "squeezed" water from specimen Sp02 (referred to as Sp02*) with the above mentioned specimens (where the gDNA was extracted from sponge material that had been thoroughly ground with a mortar and pestle as described in section 2.2.1). For each sponge specimen described above, gDNA was isolated from two different frozen sponge pellets (technical replicates a and b) to ensure that any differences seen were not due to technical faults (either in the processing of the sponge, the gDNA isolation or the PCR amplification).

The DGGE profiles showed the presence of a diverse bacterial community in all three *T. favus* specimens, with relatively conserved banding patterns between duplicate DNA samples for each sponge specimen (Fig. 3.2A lanes labelled a vs. b). This confirmed the reproducibility of the DNA isolation and PCR amplification protocols. Comparison of the DGGE profiles of different *T. favus* specimens (Sp01, Sp02, Sp03) showed that they were very similar to each other (conserved bands i, ii, iii and v) suggesting conservation of the bacterial community across different specimens of *T. favus*. The similarity between the profiles obtained from DNA extracted from sponge tissue and the water squeezed from the sponge (Fig. 3.2A, Sp02 and Sp02*) suggested that the bacteria isolated were likely to be associated extracellularly with the sponge or that the squeezing process was too forceful and disrupted the sponge cells.

Next the DGGE profiles of Sp01, Sp02 and Sp03 were compared with those of Sp04, Sp05, Sp13, Sp14, Sp15 and Sp17, collected from the same location a year later. Three conserved DNA bands (Figure 3.2B, labelled i – iii) were present in all nine *T. favus* specimens. A fourth apparently conserved band (Figure 3.2B, labelled iv) was not reproducibly detected in subsequent DGGE analyses of the same samples and this band was probably an artefact generated during PCR amplification. A fifth band (labelled "v") was observed in *T. favus* specimens Sp01, Sp02, Sp03, Sp04 and Sp14 as well as in Sp02*, but was not conserved in

all the other sponge specimens (Fig. 3.2A and 3.2B). The presence of conserved DGGE bands between all of the *T. favus* specimens suggests that the bacterial community associated with *T. favus* is conserved and stable.

3.3.2 16S rRNA clone library analysis of sponge-associated bacterial communities

The limitation of DGGE analysis is that it involves the use of a small 16S rRNA gene fragment (193 bp) and only shows average changes in the GC content of the sequence which may provide little more than an estimate as to the total microbial diversity. A more accurate way of assessing microbial diversity within a population is to construct 16S rRNA clone libraries. Genomic DNA extracted from each of the specimens Sp01, Sp02, Sp03 and Sp02* collected in May 2009 was used to amplify the full 16S rRNA gene sequence, giving rise to four clone libraries. The complete 16S rRNA gene sequence (1.5 kb) of a total of 164 clones was acquired from two clone libraries obtained from Sp02 and Sp02*, while 173 partial sequences were obtained for clones from the Sp01 and Sp03 library. All 80 sequences from the Sp01 library were represented by sequences obtained for Sp02 and Sp02*, while the full sequences for 15 unique clones from the Sp03 library were obtained. The relative abundance of the clones is represented in Table 3.1, and a phylogenetic tree was constructed using the unique 16S rRNA sequences from Sp02 and Sp02* together with their closest relevant BLAST Hit as reference sequences (Fig 3.3). The Sp02* sequences are referred to as Sp02sw in the ARB phylogram.

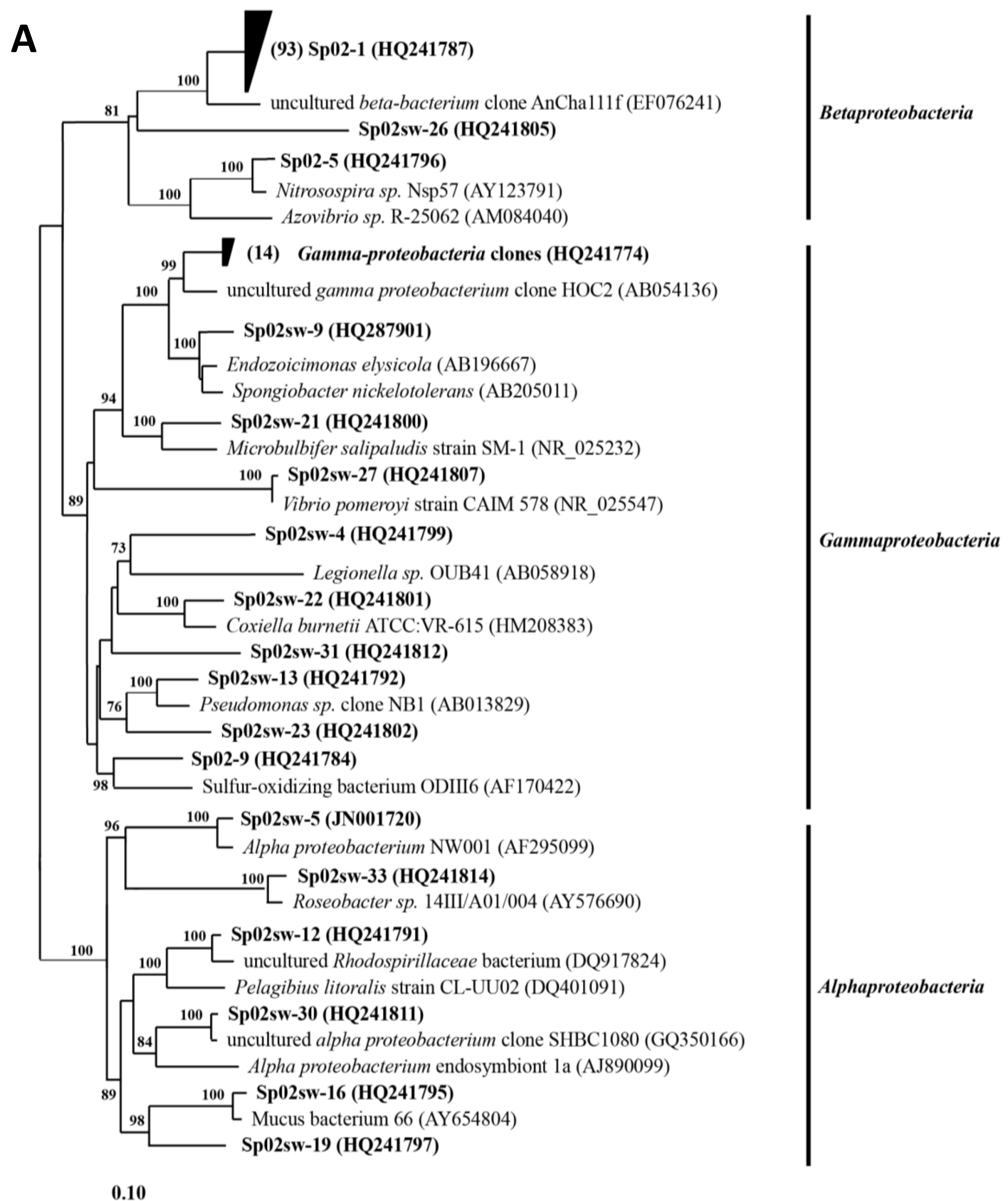
Table 3.1: Distribution of T. favus-associated 16S rRNA gene sequences within the major bacterial phylogenetic groups

Major Phyla	Sp01		Sp02		Sp03		Sp02*	
	No.	%	No.	%	No.	%	No.	%
Proteobacteria	75	94%	73	84%	70	75%	59	77%
Alphaproteobacteria[1]	3	4%	1	1%	1	1%	8	10%
Betaproteobacteria[2]	70	88%	66	76%	58	62%	31	40%
Deltaproteobacteria[3]	0	0%	2	2%	0	0%	1	1%
Gammaproteobacteria[4]	2	3%	4	5%	11	12%	19	25%
Unclassified bacteria[5]	0	0%	2	2%	3	3%	1	1%
Other phyla	5	6%	14	16%	20	22%	17	22%
Bacteroidetes[6]	0	0%	1	1%	1	1%	2	3%
Acidobacteria[7]	0	0%	2	2%	1	1%	0	0%
Chlamydiae[8]	0	0%	0	0%	2	2%	0	0%
Firmicutes[9]	0	0%	0	0%	0	0%	2	3%
Planctomycetes[10]	0	0%	2	2%	1	1%	5	6%
Verrucomicrobia[11]	0	0%	0	0%	2	2%	1	1%
Actinobacteria[12]	0	0%	0	0%	0	0%	2	3%
Spirochaeta[13]	5	6%	9	10%	13	14%	5	6%
Total	80		87		93		77	

Accession numbers associated with major phyla: [1]HQ241777, JN001720, HQ241791, HQ241795, HQ241797, HQ241811, HQ241814, HQ241767; [2]HQ241787, HQ241813, HQ241817, HQ241790, HQ241779, HQ241796, HQ241805; [3]HQ241778, HQ241810, HQ241780; [4]HQ241786, HQ241784, HQ241799, HQ287901, HQ241792, HQ241800, HQ241801, Q241802, HQ241807, HQ241809, HQ241812, JF930155, HQ241758, HQ241759, HQ241762, HQ241765, HQ241768 ; [5]HQ241785, HQ241782, HQ241816, HQ241760, HQ241766, HQ241769; [6]HQ241776, HQ241793, HQ241803, HQ241764; [7]HQ241757; [8]JF930156; [9]HQ241806; [10]HQ241781, HQ241808, HQ241783, HQ241815, JF930157, HQ241789; [11]HQ241804, HQ241761, HQ241763; [12]HQ241794, HQ241798; [13]HQ241788

Analysis of the 16S rRNA sequences revealed the Proteobacteria as the most dominant phylotype (more than 75% of the clones in each sponge library) in all three sponges and also in water squeezed from Sp02 (Table 3.1). The dominant species, representing 75% of all clones obtained from the three sponges, was a Betaproteobacteria species (Genbank Accession Number HQ241787), which was also present in Sp02* albeit at a lower level (40% of clones in the library). The other dominant Proteobacteria were the Gammaproteobacteria and Alphaproteobacteria which were also present in significant numbers, but at a much lower level than the Betaproteobacteria (Table 3.1). The only other phylum that

dominated within the 16S library was the Spirochaeta. More species diversity was observed in Sp02*, which contained microbial species not found in Sp02 (Table 3.1)). This could be due to the fact that Sp02* represent seawater squeezed from Sp02 and that some of the more transient bacteria were lost during this squeezing process and were therefore no longer present in specimen Sp02 when it was processed.



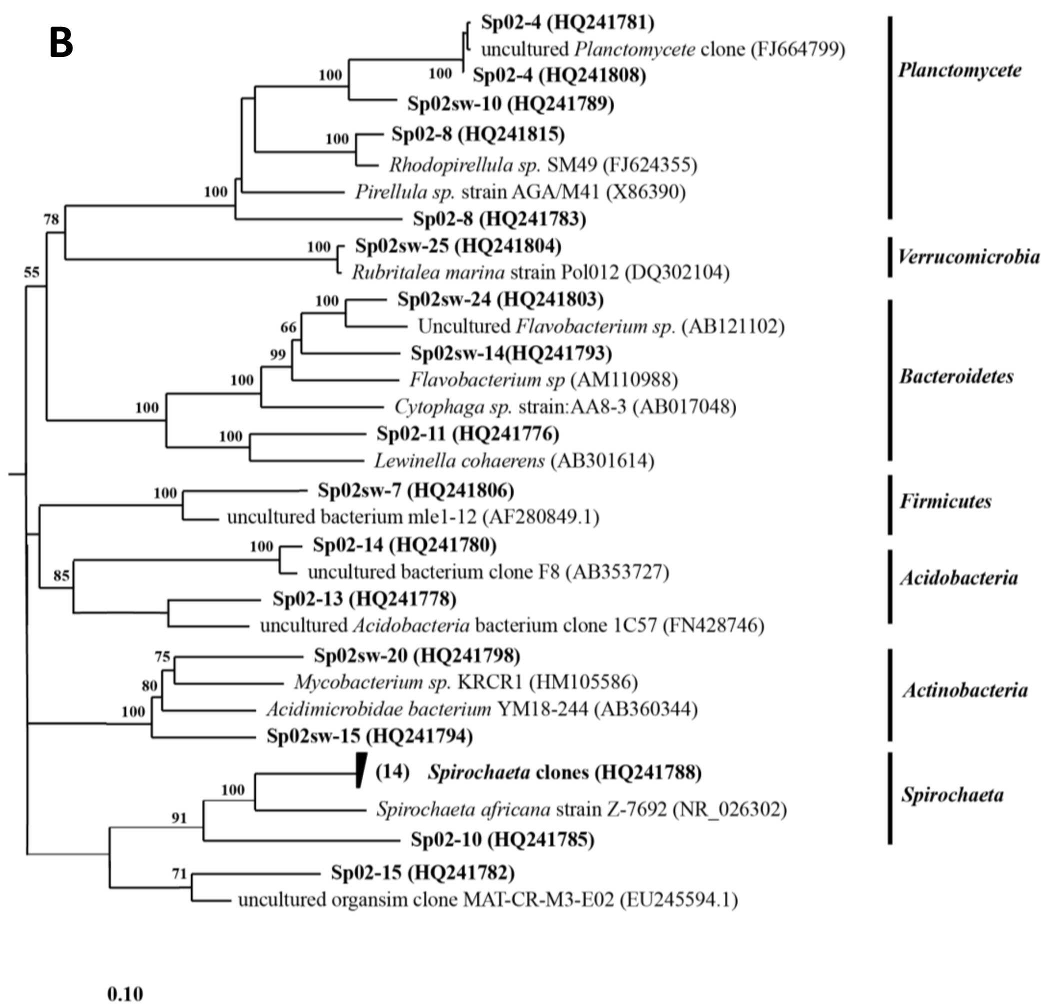


Figure 3.3 The complete 16S rRNA gene sequences from the clone libraries of Sp02 and Sp02* were used to generate a Neighbour-joining phylogenetic tree represented by 8 different phyla. The Proteobacteria were the most dominant of the phyla and are displayed as a separate tree (**A**) with the other 7 phyla grouped together in tree (**B)**. Bootstrap values (100 replicates) are indicated by numbers at the nodes. The scale bar represents 10% sequence divergence. The above ARB phylogram was taken from Walmsley *et al.* (2012) with permission from the editor of Marine Biotechnology (see appendix A2) and is the original work of Fan Zhang.

This dominant betaproteobacterial clone (represented by the sequence Sp02-1) is most closely related to an unclassified betaproteobacterium previously identified in the Mediterranean sponge *Tethya aurantium* (Thiel *et al.*, 2007) and is also related to a *Nitrosospira sp*. and an *Azovibrio sp.* (Fig. 3.3A). The Spirochaeta clones (represented by the sequence Sp02-3) that dominated the non-proteobacterial phyla (Fig. 3.3B) are most closely related to a *Spirochaeta africana* strain isolated from Soda Lakes in the East African Rift (Zhilana *et al.*, 1996).

3.3.3 Direct comparison of T. favus DGGE profiles with the dominant 16S rRNA gene clones

The DGGE studies together with the 16S rRNA clone library analysis strongly suggested that the bacterial community associated with *T. favus* is highly conserved and stable over time. An important question was whether the bacterial species that gave rise to the distinct DGGE banding patterns correspond to the dominant clones in the 16S rRNA clone library. To answer this question, the five dominant 16S rRNA clones (Sp02-1, Sp02-9, Sp02-3, Sp02-2 and Sp02sw-5) were amplified using the 16S rRNA DGGE primer pair and directly compared to the DGGE finger prints of *T. favus* specimens Sp01, Sp02 and Sp03.

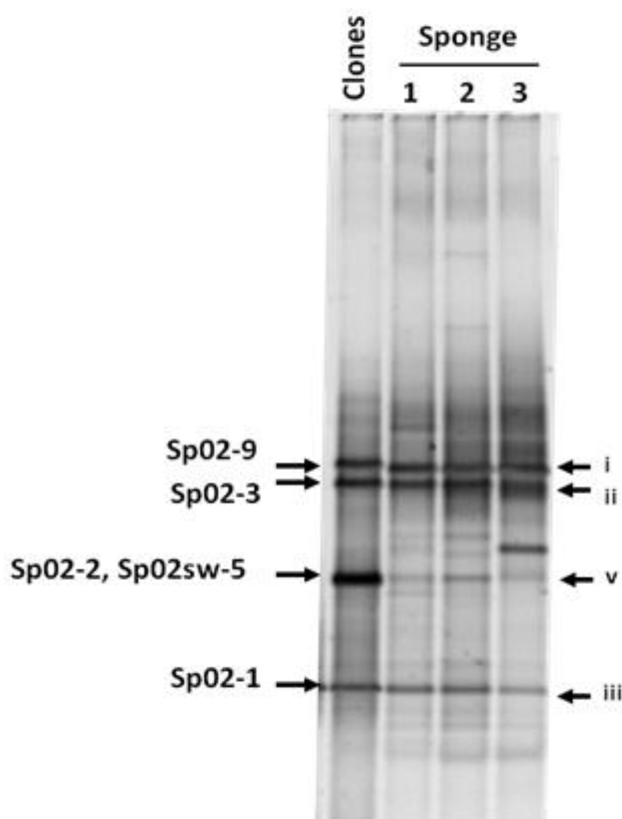


Figure 3.4 Comparison of DGGE profile of dominant species in clone libraries (Sp02-9, Sp02-3, Sp02-2, Sp02sw-5 and Sp02-1) with microbial diversity associate with *T. favus* specimens Sp01 (1), Sp02 (2) and Sp03 (3). The relative positions of conserved bands i, ii, iii and v are indicated on the right

Clone Sp02-9 (HQ241784) co-migrated with the major, conserved band i (Fig. 3.4). Similarly, based on their relative migration, bands ii and iii are likely to be represented by clones Sp02-3 (HQ241788) and the dominant betaptroteobacterial species, Sp02-1 (HQ241787), respectively (Fig. 3.4). Band v co-migrated with the bands from clones Sp02-2 (HQ241786) and/or Sp02sw-5 (JN001720) (Fig. 3.4). The only way to confirm that the dominant bands in the DGGE profile are in fact represented by the dominant clones would be to excise the DGGE bands and sequence them. It is interesting to note the relative faintness of the DGGE band corresponding to the dominant betaproteobacterial clone Sp02-1, suggesting that it may not be as dominant as indicated by the 16S clone library analysis.

Both DGGE fingerprinting and 16S rRNA clonal library studies limit the depth to which a population can be studied. As a general rule, the more dominant bacterial species in a given population will be represented by the DGGE profile (as was shown above) and in a 16S rRNA clone library. This is mostly as a result of statistical chance. The more dominant a bacterial species, the greater the chance it will be successfully PCR amplified and ligated into the cloning vector and therefore the greater the chance it will be represented in the clone library. The rare, less well represented bacterial phylotypes are therefore not identified using the above techniques and an alternate, high throughput method such as NGS is required.

3.3.4 454 pyrosequencing analysis of the bacterial community associated with T. favus

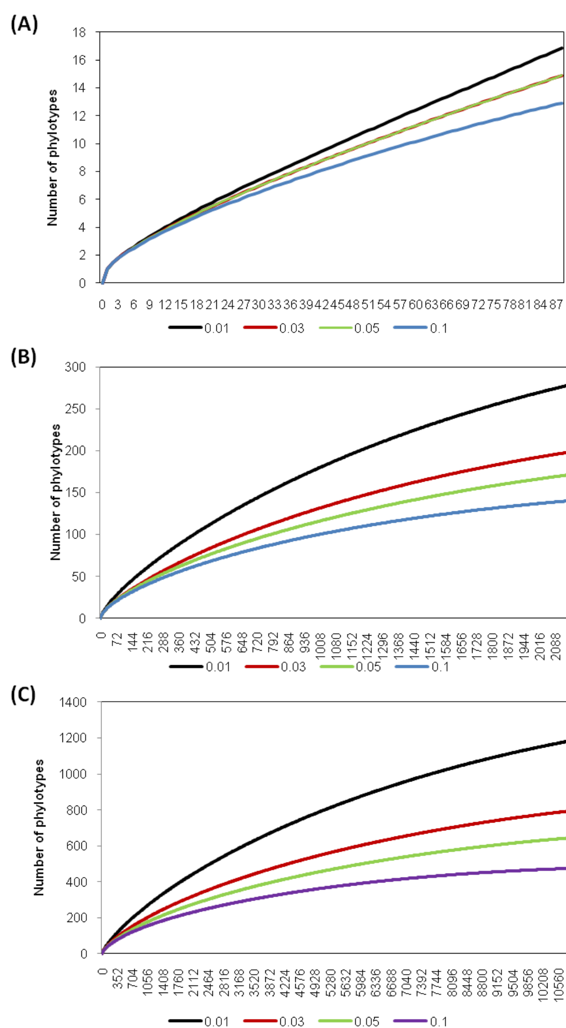


Figure 3.5 Rarefaction analysis, with distance values of 0.01, 0.03, 0.05 and 0.1 of 16S rRNA amplicons from *T. favus* specimen Sp02 (as determined by Sanger sequencing of a clonal library as well as by 454-pyrosequencing, (**A**) and (**B**) respectively) and *T. favus* specimen Sp05 (as determined by 454-pyrosequencing (**C**)) (Cole *et al.,* 2009).

For the rarefaction analysis (Fig. 3.5) distance values of 0.01, 0.03, 0.05 and 0.1 were chosen as it is generally accepted that species, genera and families/classes differ from one another by 3, 5 and 10% respectively, whereas 1% is a more stringent and therefore more accurate representation of species level (Stackebrandt and Goebel, 1994; Hugenholtz *et al*., 1998; Sait *et al*., 2002). The primary aim of rarefaction analysis is to gauge whether or not the sample size is large enough to ensure that the diversity within a particular population is assessed

holistically. The respective rarefaction curves are plotted as the number of unique phylotypes vs. the number of individuals present in the sequence data set. If the curve tends towards the horizontal it can be assumed that the population has been sampled to completion because the likelihood of detecting a new phylotype is rare (Hughes & Hellman, 2005). Both the degree of diversity within a given population and the relative sample size will determine the rarefaction output.

Rarefaction curves were plotted for both the Sanger and 454 pyrosequencing data obtained for Sp02 (Fig. 3.5 A and B) and the pyrosequencing data for Sp05 (Fig. 3.5C). With regards to the NGS data sets, the species level rarefaction curve for Sp05 indicates that the bacterial population associated with Sp05 was sampled almost to completion (Fig. 3.5C) whereas the rarefaction curve for Sp02 is less horizontal indicating that the bacterial population associated with Sp02 is not as well represented. The rarefaction analysis of the NGS data sets for both Sp02 and Sp05 indicate that the bacterial phylotypes within *T. favus* are well represented and could therefore be assessed over time. In contrast, the rarefaction curve of the Sanger sequencing data for Sp02 is quite steep indicating that a relatively low percentage of the associated bacterial population was sampled which is expected due to the much smaller dataset associated with Sanger sequencing (Table 3.1, Fig. 3.5A) vs. NGS (Fig. 3.5B).

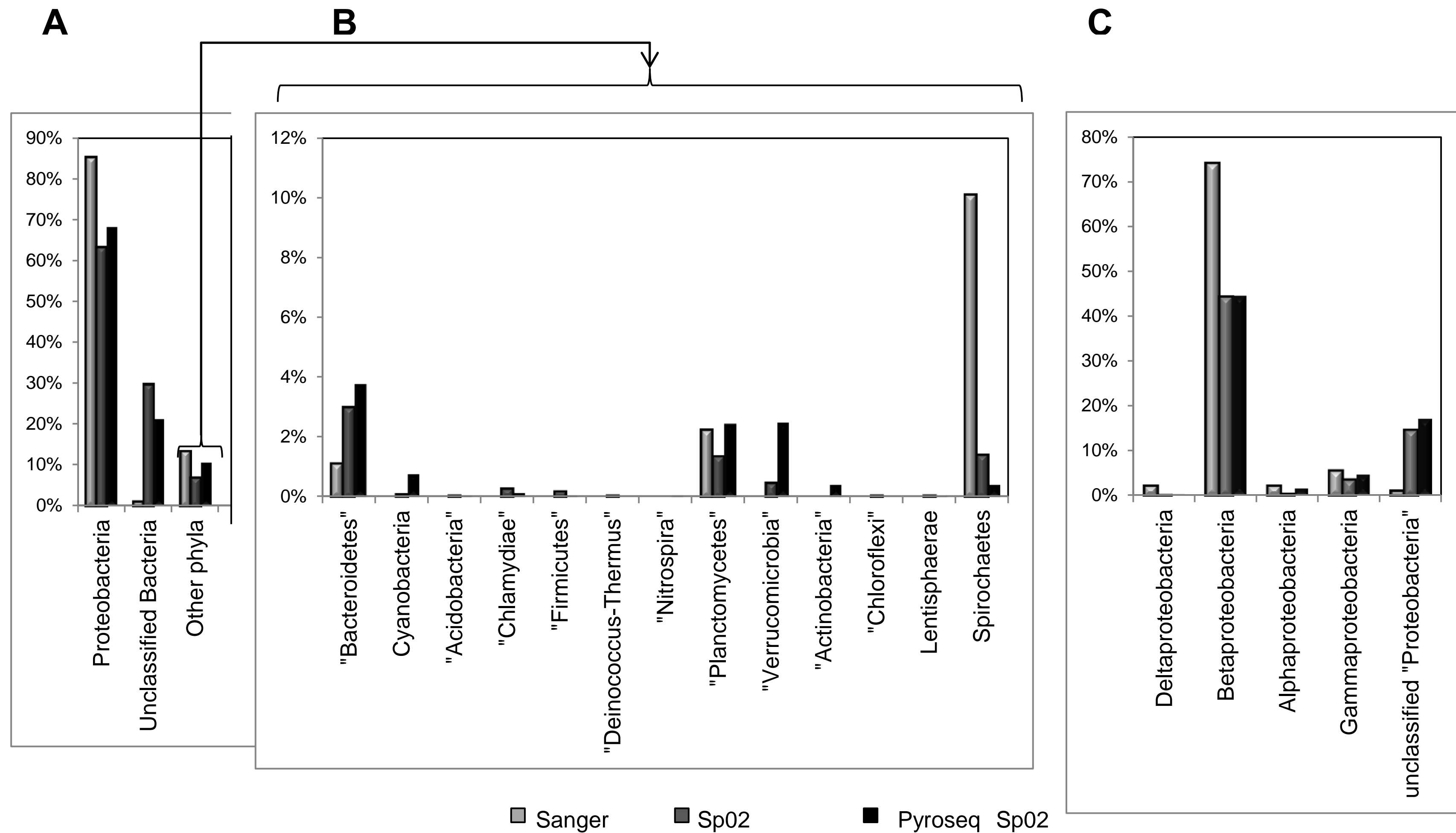


Figure 3.6 Analysis of the 16S rRNA diversity associated with *T. favus* specimens Sp02 and Sp05. Relative percentages of the total number of 16S rRNA sequence reads for each of the dominant (**A**) and less well-represented (**B**) bacterial phyla, as well as for the phylum Protoebacteria (**C**); as determined by 454-pyrosequencing (Pyroseq Sp02 and Pyroseq Sp05) and Sanger sequencing (Sanger Sp02); are represented.

The pyrosequencing reads were classified using the RDP, which revealed that the *T. favus* specimens Sp02 and Sp05 had very similar bacterial populations in terms of phylotypes despite minor variations in the total percentage of sequencing reads assigned to each phylum (Fig. 3.6). The percentage of the total number of clone library sequencing reads assigned to the dominant phyla corresponded to that obtained via pyrosequencing with Betaproteobacteria dominating. The other proteobacterial phyla and Bacteroidete*s,* Planctomycetes, Verrucomicrobiae and Spirochaetes were also represented but at a lower level (Fig. 3.6 B and C). However, bacteria belonging to the phyla Cyanobacteria, Acidobacteria, Chlamydiae, Firmicutes, Deinococcus-Thermus, Nitrospira, Actinobacteria, Verrucomicrobia, Chloroflexi and Lentisphaera*e* were only detected in the pyrosequencing analysis (Fig. 3.6B), possibly due to their relatively low abundance within the sponge-associated bacterial population which does not necessarily diminish their importance in the health and functioning of the sponge.

There are, however, significant differences between the results obtained from the clone library versus those obtained from pyrosequencing, specifically with respect to Verrucomicrobia and Spirochaetes as well as the unclassified bacteria and unclassified Proteobacteria (Fig. 3.6 B and C). The difference in Verrucomicrobi*a* and Spirochaetes abundances in the pyrosequencing versus the clone library analysis may be due to the use of different primer sets for each application resulting in primer bias (Fig. 3.1) in the amplification of certain 16S rRNA genes. The high number of unclassified sequence reads assigned to the pyrosequencing data versus the clone library could be attributed to: (a) the shorter sequence length (100 – 450 bp) compared to that obtained from Sanger sequencing (1.5 kb), which allows for greater ease in sequence identification or (b) the larger dataset obtained from pyrosequencing in comparison to the clone library thus increasing the potential of sequencing previously uncharacterized 16S rRNA sequences.

3.4 Discussion

The bacterial diversity associated with the marine sponge *Tsitsikamma favus* was thoroughly investigated not only because of the interesting secondary metabolite production associated with this sponge, but also as a means of establishing reliable methodologies for future investigations of a similar nature. Three different approaches were used to analyse the 16S rDNA gene fragment; namely DGGE, clonal libraries of full length 16S rRNA genes as well as 454 pyrosequencing. DGGE provides insight into the diversity of the associated bacteria based on GC content and is useful when screening for diversity. The clonal library consists of 1.5 kb sequence reads thus providing useful phylogenetic information but only the more dominant species are sequenced. Conversely, the 454 pyrosequencing analysis provides a shorter sequence read (100 – 480 bp) but a better view of the population (due to the large number of sequence reads generated) and allows sampling of even the rare species. 454 pyrosequencing, unlike other NGS methods such as Illumina and ABI SOLiD, gives longer sequence reads and therefore a better measure of the population structure (Ansorge, 2009).

Three sponge specimens collected at the same time in the same location (Sp01, Sp02 and Sp03; collected May 2009) were studied using 16S rRNA Sanger sequencing and were shown to have very similar bacterial diversity profiles, with a unique betaproteobacterium contributing to 70% of the sequences in each ot the *T. favus* clone libraries. This betaproteobacterial species (represented by the sequence Sp02-1) is most closely related (94% identity) to an unclassified betaproteobacterium previously identified in the Mediterranean sponge *Tethya aurantium* (Thiel *et al.*, 2007). It is also related to a *Nitrosospira sp.* and an *Azovibrio sp.* Both the *Nitrosospira* and *Azovibrio* genera are involved in nitrogen fixation which is important in oligotrophic marine environments with low nitrogen levels (Purkhold *et al.*, 2003), and both denitrification and aerobic ammonium oxidation (Anammox) have been demonstrated to occur in marine sponges (Mohamed *et al.*, 2010; Hoffmann *et al.*, 2009; Schläppy *et al.*, 2010). The phylum Spirochaeta was the most dominant of the non-proteobacterial phyla.

Further studies on sponges collected a year apart (Sp02 vs. Sp05; collected May 2009 and May 2010 respectively) using 454 pyrosequencing yielded very similar results. The DGGE banding patterns of the bacteria associated with *T. favus* were also conserved and became the most reliable way of distinguishing between even closely related species. DNA fingerprinting techniques such as DGGE are widely used in sponge diversity studies and are especially useful in monitoring changes in diversity (Mohamed *et al.,* 2008; Webster *et al.,* 2001; Webster, 2007). In sponge species where the microbial community is highly conserved, the characteristic DGGE banding pattern could tentatively be used to distinguish between sponge species as well as qualitatively assess their associated microbial diversity. The use of different primer sets (for DGGE, 16S rRNA Sanger sequencing and 454 pyrosequencing) as well as multiple samples (collected at the same time and a year apart) gave similar results showing both technical and biological reproducibility. It is very important to confirm the aforementioned qualities in a dataset as studies of this nature mean very little if the accuracy and reproducibility of the data is questionable. With this in mind, all future studies (described in later chapters within this thesis) were carried out using the same protocols as described in this chapter. However, it must be noted that despite the dominance of the betaproteobacterial clone Sp02-1 in the clone library, the corresponding band in the DGGE analysis was less intense than those of the other dominant clones, indicating a lower abundance of this isolate. This could be as a result of primer bias, with the primers used in the clone library analysis having a higher affinity for the betaproteobacterial sequence than the primers used in the DGGE analysis.

The microbial community associated with *T. favus* does not follow the general diversity profiles found in other sponges reported in the literature. The dominance of the class Betaproteobacteria within the genus *Tsitsikamma* is unique as it is usually the Alpha-, Gamma- and Delta- Proteobacteria that dominate within the Proteobacteria, while the Betaprotebacteria are relatively uncommon and typically occur at low levels in sponge populations (Muscholl-Silberhorn *et al.,* 2008; Taylor *et al.,* 2007; Webster *et al.,* 2010; White *et al.*, 2012). An exception to this was a recent study on the Brazilian sponge *Arenosclera brasiliensis* which contained a high abundance of Betaproteobacteria, especially in comparison to the

surrounding water column (Trindade-Silva *et al.,* 2012). The dominance of the Spirochaeta relative to the other non-proteobacterial phyla (Fig. 3.2B) is also noteworthy as Spirochaeta are repeatedly found in marine sponges. A recent study by Neulinger *et al.* (2010) showed high abundance and metabolic activity of two novel Spirochaeta associated with the mesohyl of the host sponge *Clathrina clathrus*. The bacterial phyla Planctomycetes, Bacteroidetes, Acidobacteria and Actinobacteria are also of interest as their closest relatives are not usually associated with marine sponges (Fig. 3.3B). These findings suggest that the microbial flora associated with *T. favus* is not typical of sponges characterised to date (Li and Liu, 2006; Turque *et al.*, 2008; Gernert *et al.*, 2005; Lee *et al.*, 2010; Radwan *et al.*, 2010; Hardoim *et al.*, 2009; Wang *et al.*, 2009; Sipkema and Blanch, 2010; Webster *et al.*, 2010).

It would be interesting to determine whether these dominant bacterial species are also conserved in other members of the *Tsitsikamma* family or whether they are unique to *T. favus*. Further insight into the bacterial diversity profile of related sponges isolated from the same geographical region would provide a useful means of further assessing the specificity of the *T. favus* associated microbial population, and is discussed in Chapter Four.

3.5 References

Ansorge, W.J. (2009). Next-generation DNA sequencing techniques. *New Biotechnology,* 25: 195-203.

Antunes, E.M., Beukes, D.R., Kelly, M., Samaai, T., Barrows, L.R., Marshall, K.M., Sincich, C. & Davies-Coleman, M.T. (2004). Cytotoxic pyrroloiminoquinones from four new Species of South African Latrunculid sponges. *Journal of Natural Products,* 67: 1268-1276.

Antunes, E.M., Copp, B.R., Davies-Coleman, M.T. & Samaai, T. (2005). Pyrroloiminoquinones and related metabolites from marine sponges. *Natural Products Reports,* 22: 62-72.

Brunk, C.F., Avaniss-Aghajani, E. & Brunk, C.A. (1996). A computer analysis of primer and probe hybridisation potential with bacterial small-subunit rRNA sequences. *Applied and Environmental Microbiology,* 62: 872-879.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J.R., Smidt, H., De Vos. W.M., Ross, R.P. and O'Toole, P.W. (2009). Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. *PLoS ONE* 4(8):e6669.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrel, D.M., Marsh, T., Garrity, G.M. and Tiedje, J.M. (2009). The Ribosomal Database Project: Improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*, 37 (Database Issue) D141-D145. URL: http://rdp.cme.msu.edu/classifier/classifier.jsp; http://pyro.cme.msu.edu/.

Enticknap, J.J., Kelly, M., Peraud, O., and Hill, R.T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae, *Applied Environmental Microbiology*, 72:3724-3732.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39: 783-791

Gernert, C., Glöckner, F., Krohne, G. & Hentschel, U. (2005). Microbial diversity of the freshwater sponge *Spongilla lacustris. Microbial Ecology,* 50: 206-212.

Hardoim, C.C.P., Costa, R., Araujo, F.V., Hajdu, E., Peixoto, R., Lins, U., Rosado, A.S. & Van Elsas, J.D. (2009). Diversity of bacteria in the marine sponge *Aplysina fulva* in Brazilian coastal waters. *Applied Environmental Microbiology,* 75: 3331-3343.

Hoffmann, F., Radax, R., Woebken, D., Holtappels, M., Lavik, G., Rapp, H.T., Schläppy, M. L., Schleper, C. & Kuypers, M.M.M. (2009). Complex nitrogen cycling in the sponge *Geodia barretti. Environmental Microbiology,* 11: 2228-2243.

Hughes, J.B. & Hellmann, J.J. (2005). The application of rarefaction techniques to molecular inventories of microbial diversity. In: Leadbetter, J.E. (Eds.) *Methods in Enzymology,* 397:292-308.

Hugenholtz, P., Goebel, B.M. & Pace, N.R. (1998). Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology*, 180:4765-4774.

Hentschel, U., Fieseler, L., Wherl, A., Gernert, C., Steinert, M., Hacker, J. & Horn, M. (2003). Microbial diversity of marine sponges. IN Weg, M. (Ed.) *Sponges (Porifera).* New York: Springer Berlin Heidelberg.

Huse, S.M., Dethlefsen, L., Huber, J.A., Welch, D.M., Relman, D.A. & Sogin, M.L. (2008). Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. *PLoS Genetics,* 4(11):e1000255.

Lane, D.J. (1991). 16S/23S rRNA sequencing. IN Stackebrandt, E. & Goodfellow, M. (Eds.) Nucleic acid techniques in bacterial systematics. Hoboken, N.J.: John Wiley and Sons.

Lee, O.O., Wang, Y., Yang, J., Lafi, F.F., Al-Suwailem, A. & Qian, P.-Y. (2010). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J 5: 650-664.

Li, Z.Y. & Liu, Y. (2006). Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis. *Letters in Applied Microbiology* 43: 410-416.

Matcher, G.F., Dorrington, R.A., Henninger, T.O., Froneman, P.W. (2011). Insights into the bacterial diversity in a freshwater-deprived permanently open Eastern Cape estuary, using 16S rRNA pyrosequencing analysis. *Water SA* 37:381–390

Miyanaga, A., Janso, J.E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustaì•Quio, A.S., Fielding, E.N., Carter, G.T., Jensen, P.R., Feng, X., Leighton, M., Koehn, F.E. & Moore, B.S. (2011). Discovery and assembly-line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in *Salinispora* bacteria. *Journal of the American Chemical Society,* 133: 13311-13313.

Mohamed, N.M., Saito, K., Tal, Y. & Hill, R.T. (2010). Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges. *ISME J*, 4: 38-48.

Muscholl-Silberhorn, A., Thiel, V. & Imhoff, J. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microbial Ecology,* 55: 94-106.

Muyzer, G., De Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59: 695-700.

Neulinger, S.C., Stohr, R., Thiel, V., Schmaljohann, R., Imhoff, J.F. (2010). New phylogenetic lineages of the Spirochaetes phylum associated with *Clathrina* species (Porifera). *The Journal of Microbiology*, 48: 411-418.

Purkhold, U., Wagner, M., Timmermann, G., Pommerening-Roser, A. & Koops, H.-P. (2003). 16S rRNA and amoA-based phylogeny of 12 novel

betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the *nitrosomonads*. *International Journal of Systematic Evolutionary Microbiology*, 53: 1485-1494.

Radwan, M., Hanora, A., Zan, J., Mohamed, N., Abo-Elmatty, D., Abou-El-Ela, S. & Hill, R. (2010). Bacterial community analyses of two Red Sea sponges. *Marine Biotechnology*, 12: 350-360.

Rosella-Mora, R. & Amann, R.I. (2001). The species concept for prokaryotes. *FEMS Microbiology Reviews,* 25: 39-67.

Sait, M., Hugenholtz, P. & Janssen, P.H. (2002). Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environmental Microbiology*, 4:654-666.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 4: 406-425.

Schläppy, M.-L., Schöttner, S., Lavik, G., Kuypers, M., De Beer, D. & Hoffmann, F. (2010). Evidence of nitrification and denitrification in high and low microbial abundance sponges. *Marine Biology*, 157: 593-602.

Schloss, P. D., *et al.* (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75: 7537–7541.

Sipkema, D. & Blanch, H.W. (2010). Spatial distribution of bacteria associated with the marine sponge *Tethya californiana*. *Marine Biology,* 157: 627-638.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. and Herndl, G.J. (2006) Microbial diversity in the deep sea and the unexplored ‘rare biosphere’. *PNAS U.S.A.*, 103:12115-12120.

Stackebrant, E. & Goebel, B.M. (1994). A place for DNA-DNA reassociation and 16S rRNA sequence-analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology*, 44:846-849.

Taylor, M.W., Radax, R., Steger, D. & Wagner, M. (2007). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews,* 71: 295-347.

Trindade-Silva, A.E., Rua, C., Silva, G.G.Z., Dutilh, B.E., Moreira, A.P.B. (2012) Taxonomic and functional microbial signatures of the endemic marine sponge *Arenosclera brasiliensis*. *PLoS ONE* 7(7): e39905.

Turque, A., Cardoso, A., Silveira, C., Vieira, R., Freitas, F., Albano, R., Gonzalez, A., Paranhos, R., Muricy, G. & Martins, O. (2008). Bacterial communities of the marine sponges *Hymeniacidon heliophil* and *Polymastia janeirensis*, and their environment in Rio de Janeiro, Brazil. *Marine Biology*, 155: 135-146.

Walmsley T, Matcher G, Zhang F, Hill R, Davies-Coleman M, Dorrington R (2012). Diversity of bacterial communities associated with the Indian Ocean sponge *Tsitsikamma favus* That contains the bioactive pyrroloiminoquinones, tsitsikammamine A and B. *Marine Biotechnology,* 14:681-691.

Wang, G. (2006). Diversity and biotechnological potential of the sponge-associated microbial consortia. *Journal of Industrial Microbiology and Biotechnology,* 33: 545-551.

Wang, Y. & Qian, P. (2009) Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* 4(10): e7401.

Wang, G., Yoon, S.-H. & Lefait, E. (2007). Microbial communities associated with the invasive Hawaiian sponge *Mycale armata*. *ISME J*, 3: 374-377.

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology,* 12: 2070-2082.

White, J.R., Patel, J., Ottesen, A., Arce, G., Blackwelder, P. (2012). Pyrosequencing of bacterial symbionts within *Axinella corrugata* sponges: diversity and seasonal variability. *PLoS ONE* 7(6): e38204.

Zhilina,T.N., Zavarzin,G.A., Rainey,F., Kevbrin,V.V., Kostrikina,N.A., Lysenko,A.M. (1996). *Spirochaeta alkalica sp. nov.*, *Spirochaeta africana sp. nov.*, and *Spirochaeta asiatica sp. nov.,* alkaliphilic anaerobes from the Continental Soda Lakes in Central Asia and the East African Rift. I*nternational Journal of Systematic Bacteriology,* 46:305-312.

Chapter Four

COMPARING THE BACTERIAL DIVERSITY ASSOCIATED WITH ALGOA BAY LATRUNCULID SPONGES

4.1 Introduction

The genus *Tsitsikamma* is currently comprised of three species, namely *T. favus*, *T. scurra* and *T. pedunculata*. The original aim of this research project was to investigate whether there was conservation in the bacterial diversity, correlating with pyrroloiminoquinone production associated with these three species. Unfortunately only *T. favus* and *T. scurra*, but not *T. pedunculata,* were collected during the course of this study. However the collection of a new species of *Latrunculia*, which like the genus *Tsitsikamma*, belongs to the family *Latrunculiidae*, provided the opportunity to compare the microbial diversity of a species from a different genus but within the family *Latrunculiidae*. An interesting observation of *T. scurra* was that it was almost always collected with a yellow encrusting sponge covering its surface. A similar yellow encrusting sponge has been observed to be associated with *T. pedunculata* specimens in Algoa Bay (Shirley Parker-Nance, personal communication). As previously discussed (Chapter 2) analysis of the *COX1* and 28S rRNA gene sequences (Fig. 2.3) showed that the yellow encrusting sponge found on *T. scurra* belonged to the family *Mycaliidae*, which is not closely related to the family *Latrunculiidae* (Fig. 2.3). Thus the yellow encrusting sponge was selected to serve as a useful outlier in the comparative study. This chapter describes the characterisation of the microbial diversity associated with three Latrunculid sponges all collected from Evan's peak, Algoa Bay using DGGE and 454 pyrosequencing analyses of 16S rRNA gene sequences.

4.2 Methods and Materials

4.2.1 Isolation of total genomic DNA followed by DGGE and NGS analysis of sponge-associated 16S rRNA amplicons

Genomic DNA was isolated using the guanidinium thiocyanate method as described in section 2.3.3. DGGE analysis was performed as described in section 3.2.2. The sequence reads were generated using the GS FLX Titanium Sequencer (454 Life Sciences, Roche) as described in section 3.2.5. Sequence reads were quality filtered using standard software provided by 454 Life Sciences and MOTHUR (http://www.mothur.org; v.1.28.0; Schloss *et al.*, 2009.), cured for primer/tag sequences and all reads less than 100 nt were removed from the dataset. The Ribosomal Database Project (RDP) Classifier was then used to analyse the remaining 72 170 reads as well as to generate rarefaction curves at distance values of 0.1, 0.05 and 0.03.

The sequence reads were then further quality filtered to remove all reads less than 200 nt, chimeras (using the UChime method) as well as Mitochondrial and Chloroplast sequences. The remaining 62 963 sequences were then clustered as Operational Taxonomic Units (OTUs), at a distance value of 0.03 which is generally where speciation occurs, using the OTU analysis standard operating procedure (SOP) provided by MOTHUR (http://www.mothur.org; v.1.28.0; Schloss *et al.*, 2009). The SOP for OTU analysis involves the formation of group files (with each group representing sequences from each sponge specimen) and the sequence reads were then assigned to OTUs with their attached group extension which enables one to determine the source of that sequence. The top 10 OTUs and relevant *T. favus* clones (refer to section 3.3.3) together with relevant environmental sequences submitted to GenBank, selected according to the top BLASTN hits (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the clone sequences, were used in the phylogenetic analysis conducted in MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the

phylogenetic tree. The evolutionary distances were computed based on 410 nucleotide alignment positions using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

OTUs represented by single sequences were removed from the dataset (leaving a total of 15 570 sequences) before Venn diagrams were used to illustrate community diversity and overlap using the Chao1 estimator and Inverse Simpson index (Chao, 2005; Simpson, 1949). Heatmaps were constructed using a log2 scale in MOTHUR according to the Yue & Clayton theta structural diversity measure (Yue & Clayton, 2005). Both the Venn diagrams and heatmaps were calculated at a distance value of 0.03.

4.3 Results

4.3.1 Comparison of the bacterial communities associated with Algoa Bay Tsitsikamma and Latrunculia sponges using DGGE profiling

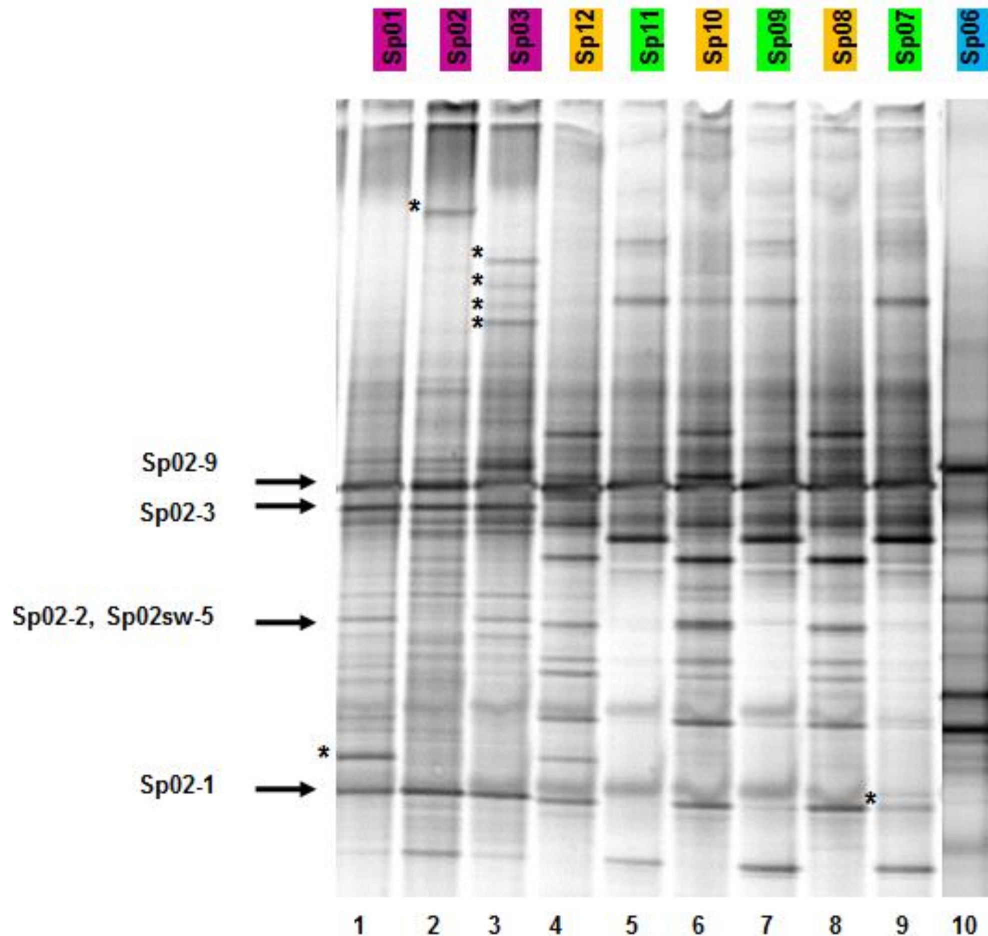


Figure 4.1 DGGE analysis of the 16S rRNA gene sequences of bacterial communities associated with the Algoa Bay sponge specimens. Sp01, Sp02 and Sp03 are *T. favus* specimens; Sp06 is the *Latrunculia sp.*; Sp07, Sp09 and Sp11 are *T. scurra* specimens; and Sp08, Sp10 and Sp12 are specimens of the yellow encrusting sponge associated with Sp07, Sp09 and Sp11 respectively. Unique bands within a sponge species are indicated with a '*'.

The DGGE profiles of the sponges collected from Algoa Bay during the course of this study were compared to those of *T. favus*. As observed previously (Chapter 3), four conserved bands were observed in the DGGE profiles of three *T. favus* specimens, Sp01, Sp02 and Sp03 (Fig. 4.1, lanes 1, 2 and 3). The three *T. favus* specimens also contained bands that were unique to each specimen (indicated with a *). *T. scurra* specimens Sp07, Sp09 and Sp11 had 6 bands in common (Fig.

4.1, lanes 9, 7 and 5), with a single unique band present in Sp07 (lane 9) that corresponds to a conserved band within the associated yellow encrusting sponges (Sp08, Sp10, Sp`12; lanes 8, 6 and 4 respectively). The three specimens of the yellow encrusting sponge (Sp08, Sp10 and Sp12) appeared to have highly conserved bacterial species diversity, with 12 DGGE bands in common (Fig. 4.1, lanes 8, 6 and 4). The *Latrunculia sp.* (Sp06) had a unique DGGE profile of its own (Fig. 4.1, lane 10). The band represented by *T. favus* isolate Sp02-9 appeared to be common throughout the sponge specimens, with the exception of the L*atrunuclia sp*. (Sp06). This raised the possibility that *T. favus* and *T. scurra* potentially contained a common dominant bacterial species with a relatively high GC content equivalent to that of isolate Sp02-9. *T. favus* (lanes 1, 2, and 3) also appears to share a band, with a GC content corresponding to that of clone Sp02-1, with the yellow encrusting sponge (lanes 8, 6 and 4). The single band shared between *T. scurra* Sp07 and the yellow encrusting sponges was possibly due to cross-contamination. Given that DGGE separates DNA fragments based on their GC content, the common DGGE bands could be derived from the same bacterial species or from a species that happens to have a 16S rRNA gene sequence of the same GC content.

4.3.2 454 pyrosequencing analysis of the bacterial communities associated with Algoa Bay Latrunculid sponges

The identity and population structure of the microbial species associated with *T. favus, T. scurra*, *Latrunculia sp.* and yellow encrusting sponge specimens were investigated using the 454 pyrosequencing of 16S rRNA amplicons. The raw sequence reads were quality-filtered (as described in section 4.2.1.) and are referred to in Table 4.1.

Table 4.1: Assignment of sequencing reads at various stages in the molecular analysis of bacterial communities associated with Algoa Bay Latrunculid sponges.

	Number of reads						
	Sp02	Sp16	Sp07	Sp27	Sp06	Sp08	**Total**
After first quality filter	1893	7435	14990	12948	11032	23872	**72170**
After OTU quality filter	1071	3936	13747	11256	10456	22497	**62963**
After removing single OTUs	306	2235	2143	2921	2665	5300	**15570**

* OTUs were assigned at the species level (a distance value of 3%)

The sequence data set for Sp02 used for analysis in Chapter 3 was generated by a commercial sequencing company and the quantity of 454 sequence reads was not as high as that of the data sets generated subsequently for Sp16, Sp07, Sp27, Sp06 and Sp08. All 72 170 sequence reads that remained after the initial quality filtering were analysed using the RDP classifier to estimate the relative abundance of sequences assigned to each phylum. They were also used in the rarefaction analysis as an indication of the degree to which the bacterial community was sampled.

Further curation of the sequence reads for OTU analysis (as described in the OTU standard operating protocol for use in MOTHUR (http://www.mothur.org) resulted in the removal of approximately 10 000 reads from the dataset. The remaining 62 963 sequence reads were then classified to OTUs at a distance of 0.03, which is generally accepted to account for speciation. For the generation of the Venn diagrams and heat maps, the OTUs represented by single sequence reads were removed from the dataset.

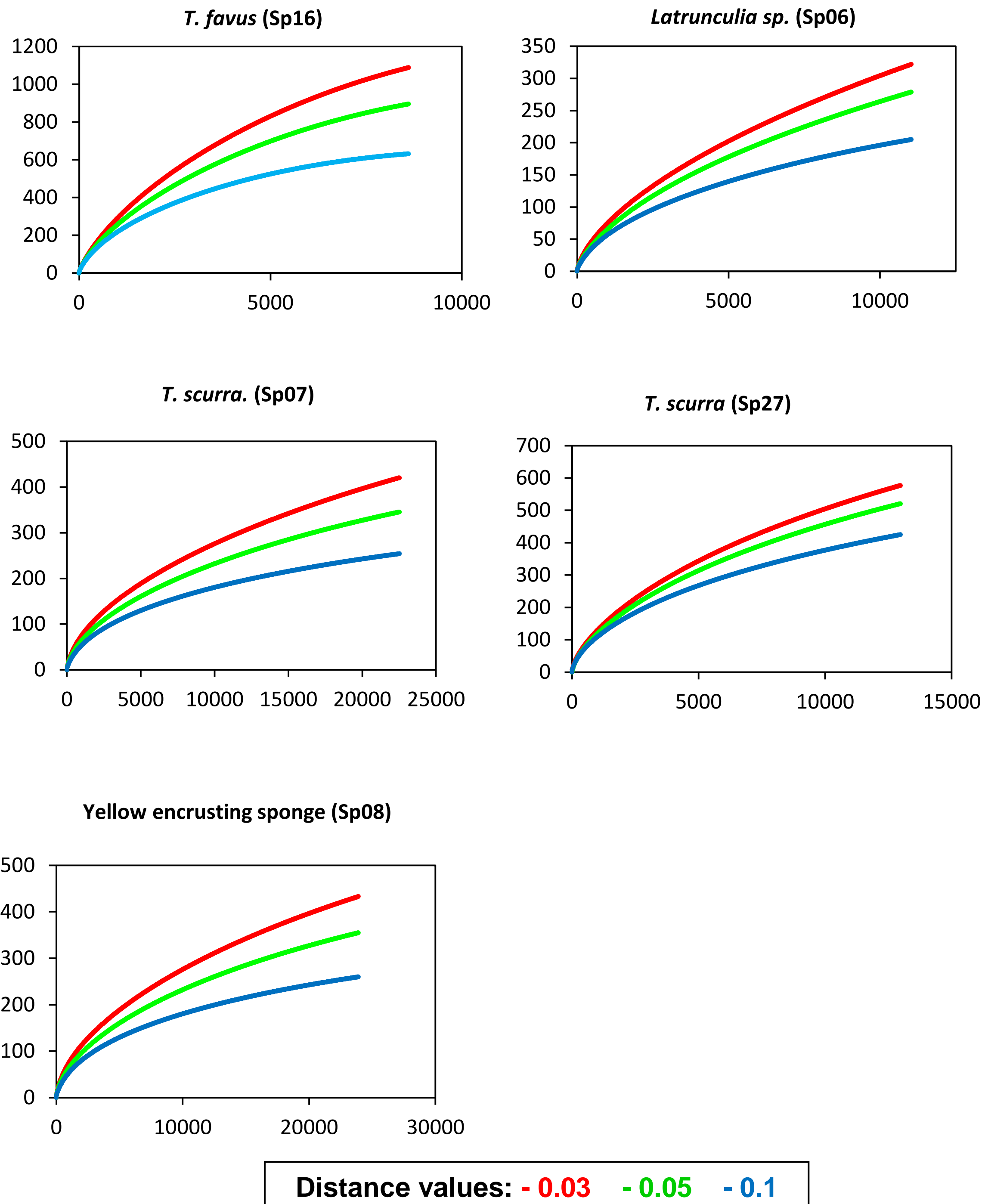


Figure 4.2 Rarefaction analyses of 16S rRNA gene amplicons from *T. favus* specimens Sp02 and Sp16, *T. scurra* specimens Sp07 and Sp27, *Latrunculia sp.* specimen Sp06 and yellow encrusting sponge specimen Sp08. The horizontal axis represents the number of sequences sampled and the vertical axis the number of phylotypes respectively.

As described in section 3.3.5, rarefaction analysis is useful for determining the degree to which a given population has been sampled. The closer the resultant rarefaction curve is to the horizontal, the more complete the sampling of the population. The results of rarefaction analysis at the species level (a distance value of 0.03) suggest that the sequence data set for *T. favus* specimen Sp16, *T. scurra* specimens Sp07 and Sp27, and the yellow encrusting sponge specimen Sp08 all appear to have been sampled almost to completion despite the differences in the relative sample sizes (Fig. 4.2, Table 4.1). The rarefaction curves for *Latrunculia sp.* specimen Sp06 and *T. favus* specimen Sp02 (see Fig. 3.5B) are slightly steeper, indicating that the associated bacterial populations may not have been sampled to the same degree of completion as the other sponge specimens. These data sets would therefore be reliable for identifying dominant OTUs, but the conclusions about the diversity of rare OTUs would be less reliable. With this in mind, it was decided to remove “singleton” OTUs for comparative analyses.

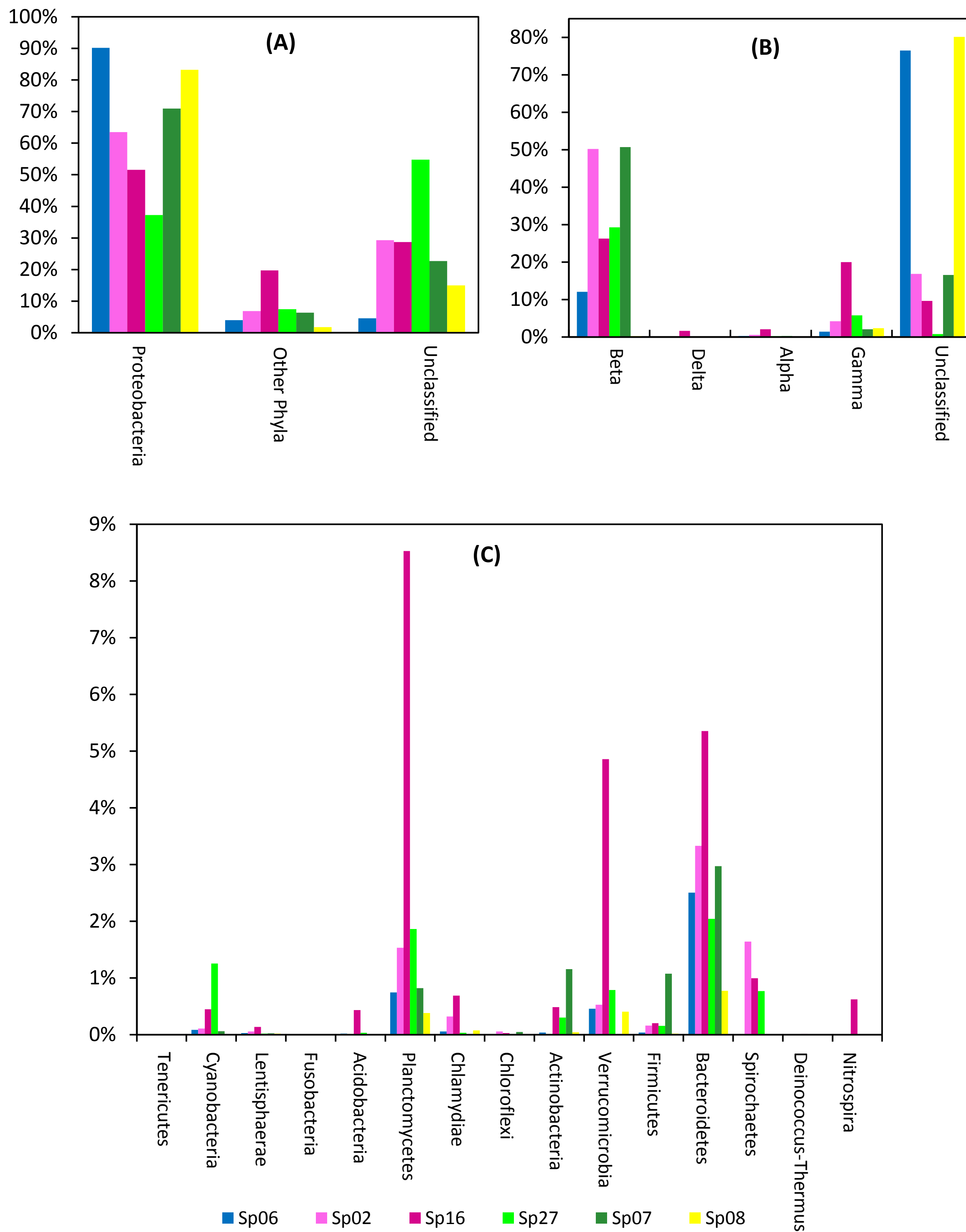


Figure 4.3 Phylogenetic classification of bacterial diversity associated with *T. favus* (Sp02, Sp16), *T. scurra* (Sp07, Sp27), yellow encrusting sponge (Sp08) and *Latrunculia sp.* (Sp06) specimens as indicated by 16S rRNA gene sequences. Relative percentages of the total number of 16S rRNA sequence reads for each of the dominant (**A**) and less well-represented (**C**) bacterial phyla as well as the relative percentages of the 16S rRNA sequence reads assigned to the phylum Proteobacteria for each of the represented proteobacterial classes (**B**).

According to the RDP classification, the majority of the sequence reads obtained from all four sponge species were assigned to the phylum Proteobacteria, with the exception of *T. scurra* specimen Sp27, which was dominated by unclassified, bacterial species. Within the Proteobacteria, Betaproteobacteria dominated *T. favus* and *T. scurra,* and unclassified Proteobacteria were dominant in the *Latrunculia sp.* and the yellow encrusting sponge (Fig. 4.3 A and B). Bacterial species belonging to the Gammaproteobacteria were also significant (20% abundance) in *T. favus* specimen Sp16. The abundance of sequences assigned to bacterial phyla other than the Proteobacteria was low (Fig. 4.3C), with the Planctomycetes, Bacteroidetes and Verrucomicoribia most well-represented amongst all the sponge specimens sampled, especially *T. favus* specimen Sp16. The Spirochaetes, Firmicutes, Actinobacteria and Cyanobacteria were also quite well represented relative to the other non-proteobacterial phyla (Fig 4.3C).

The RDP Classifier was used to group the sequence reads to the phylum and class (for the Proteobacteria) level. The classification of sequences according to taxonomic rankings does not necessarily provide a complete representation of the potential genetic diversity present in a given environment (Wooley *et al*., 2010). If the reference database does not contain a corresponding sequence to match the query sequence then the query sequence may not be classified at all and will be referred to as “unclassified bacteria” (Wooley *et al.,* 2010). Generally this “unclassified bacteria” group is overlooked despite the fact that it may contain unique and potentially dominant bacterial species. These limitations can be overcome using OTU based methods, which group like sequences together as the same OTU.

4.3.3 OTU based analysis of sponge-associated bacterial communities

Altogether 62 963 reads were assigned to OTUs at a distance value of 3% as sequences with >97% identity are typically assigned to the same species (Table 4.1). The top 20 OTUs, representing species that were most dominant within individual sponge specimens as well as those that were most dominant amongst all the sponge specimens sampled are listed in Table 4.2, along with the number of reads assigned to each OTU within each sponge specimen.

Table 4.2: Sequence reads assigned to the top 20 OTUs represented within the Latrunculid sponges.

	T. favus				*T. scurra*				*Latrunculia sp.*		Yellow	
	Sp02		**Sp16**		**Sp07**		**Sp27**		**Sp06**		**Sp08**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
OTU001	0	0.00	0	0.00	270	1.96	1	0.01	0	0.00	1824	8.11
OTU002	20	1.87	69	1.75	10	0.07	6	0.05	1989	19.02	0	0.00
OTU003	136	12.70	676	17.17	707	5.14	536	4.76	3	0.03	3	0.01
OTU004	0	0.00	0	0.00	60	0.44	2	0.02	0	0.00	1687	7.50
OTU005	9	0.84	125	3.18	89	0.65	145	1.29	28	0.27	40	0.18
OTU006	0	0.00	6	0.15	29	0.21	294	2.61	0	0.00	7	0.03
OTU007	3	0.28	0	0.00	44	0.32	269	2.39	0	0.00	0	0.00
OTU008	0	0.00	0	0.00	5	0.04	0	0.00	1	0.01	295	1.31
OTU009	1	0.09	37	0.94	114	0.83	39	0.35	30	0.29	46	0.20
OTU010	1	0.09	20	0.51	45	0.33	41	0.36	56	0.54	88	0.39
OTU011	0	0.00	3	0.08	18	0.13	145	1.29	26	0.25	13	0.06
OTU012	0	0.00	177	4.50	9	0.07	2	0.02	2	0.02	7	0.03
OTU013	0	0.00	0	0.00	11	0.08	76	0.68	0	0.00	181	0.80
OTU014	0	0.00	0	0.00	1	0.01	0	0.00	2	0.02	185	0.82
OTU015	1	0.09	5	0.13	7	0.05	66	0.59	1	0.01	0	0.00
OTU016	0	0.00	0	0.00	2	0.01	132	1.17	35	0.33	24	0.11
OTU017	0	0.00	33	0.84	15	0.11	60	0.53	10	0.10	17	0.08
OTU018	1	0.09	0	0.00	12	0.09	126	1.12	0	0.00	0	0.00
OTU019	0	0.00	0	0.00	15	0.11	86	0.76	0	0.00	1	0.00
OTU020	0	0.00	0	0.00	0	0.00	25	0.22	108	1.03	0	0.00
Total	**1071**	**16.1**	**3936**	**29.2**	**13747**	**10.6**	**11256**	**18.2**	**10456**	**21.9**	**22497**	**19.6**

The two specimens of *T. favus* (Sp02 and Sp16) show a similar distribution of dominant OTUs with OTU003, OTU005 and OTU002 accounting for 12.7%, 0.8% and 1.9% of the reads for Sp02 and 17.2%, 3.2% and 1.8% of the reads for Sp16, respectively. In contrast, *T. scurra* specimens Sp07 and Sp27 have differences in the relative abundance of OTUs with OTU001 and OTU003 being the most abundant in Sp07 at 2% and 5.1%, respectively and OTU003 (4.8%), OTU006 (2.6%) and OTU007 (2.4%) being the most abundant in Sp27. The most abundant species in the yellow encrusting sponge (Sp08) found growing in close association with *T. scurra* (Sp07) were OTU001 (at 8.1%) and OTU004 (7.5%). The differences in the relative abundance of OTUs between Sp07 and Sp27 could be attributed to cross contamination with the yellow encrusting sponge. *T. scurra* specimen Sp07 shared the high abundance of OTU001 in common with its associated yellow encrusting sponge Sp08, whereas Sp27 only had a single

sequence read assigned to OTU001 (Table 4.2). This correlates well with the DGGE banding patterns observed in Fig. 4.1 which showed a band in Sp07 that was not visible in Sp27 but corresponded to a band in Sp08. Alternatively, *T. scurra* sponges may not exhibit the conservation of species observed in *T. favus.*

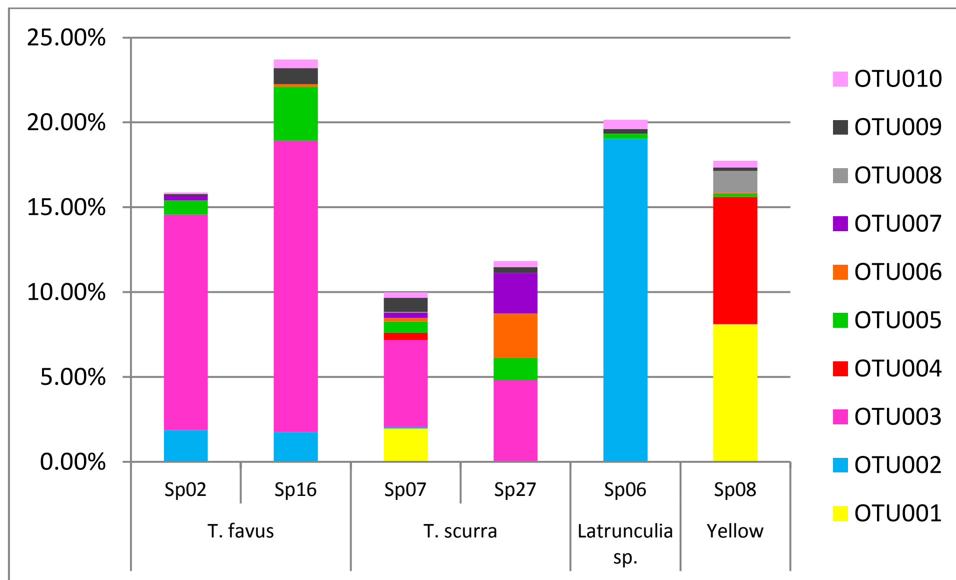


Figure 4.4 Relative abundance of the top 10 OTUs within each sponge specimen.

The most notable similarity between *T. favus* and *T. scurra* is the relative abundance of OTU003 which represents only 0.03% and 0.01% of the *Latrunculia sp.* and yellow encrusting sponge reads, respectively. OTU002 represents just over 19% of the total number of reads in the *Latrunculia sp.*, in contrast to 1.8% and 0.05% in *T. favus* and *T. scurra* respectively, being completely absent from the yellow encrusting sponge. The majority of the sequence reads for each sponge specimen were assigned to the top 10 OTUs (Table 4.2; Fig. 4.4). The percentage abundance of the top 10 OTUs is presented in Figure 4.4 as a visual representation of the similarities between the sponge specimens with regard to the occurrence of abundant species.

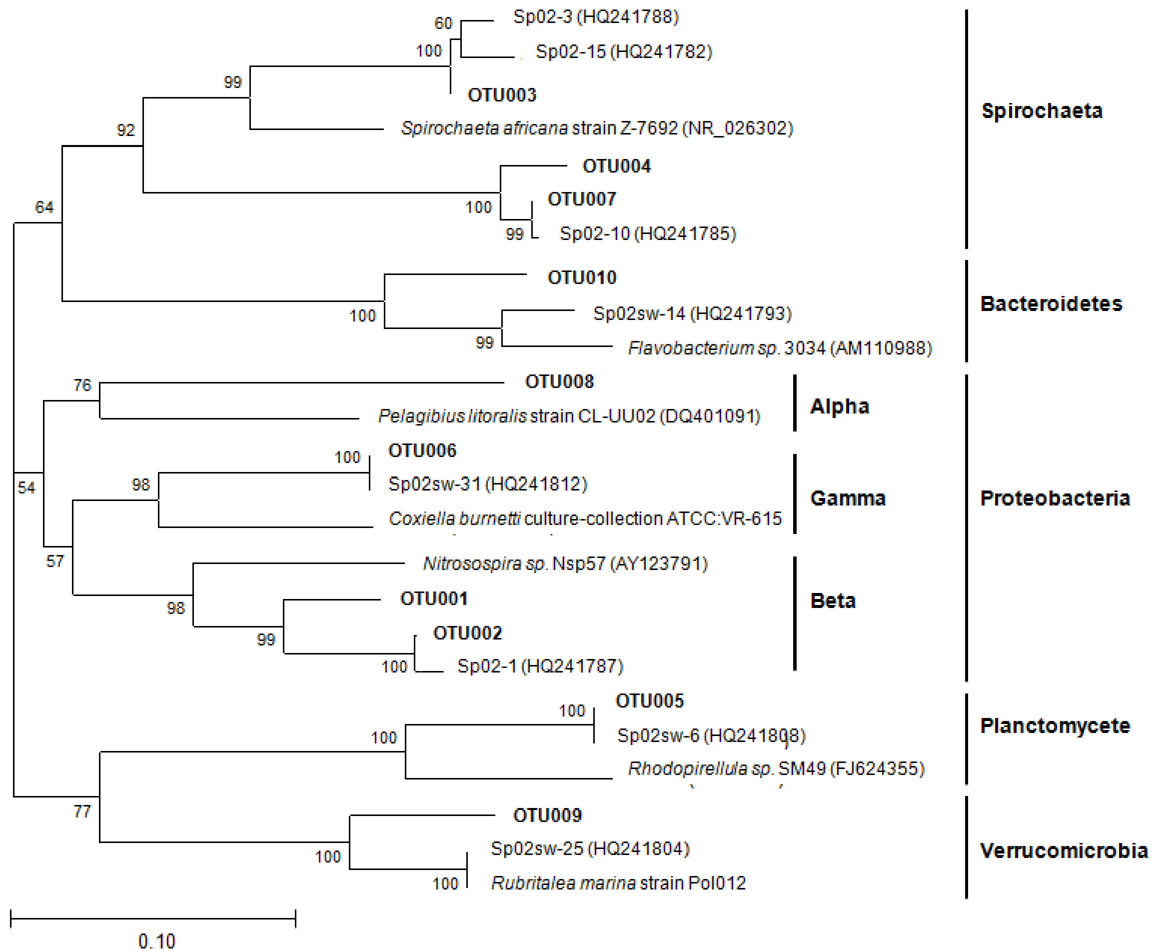


Figure 4.5 Neighbour-joining phylogenetic tree based on 16S rRNA sequences of the top 10 OTUs assigned to *T. favus*, *T. scurra*, *Latrunculia sp.,* and the yellow encrusting sponge. Genbank accession numbers are indicated in brackets and bootstrap values (100 replicates) are indicated at the nodes. The scale bar represents 10% sequence divergence.

A phylogenetic analysis comparing the top 10 OTUs with dominant OTUs from the *T. favus* clone library and relevant sequences showing homology to sponge OTUs, was conducted, and the results are presented as a Neighbour-joining phylogenetic tree (Fig. 4.5). OTU003, which was dominant in both *T. favus* and *T. scurra* was classified as an unclassified bacterium using the MOTHUR alignment tool and the Silva database as the reference alignment. However, in this phylogenetic tree OTU003 appeared to cluster with sequences within the phylum Spirochaeta and is closely related to the *T. favus* clones Sp02-3 and Sp02-15. OTU004 (7.5% in the yellow encrusting sponge) and OTU007 (2.39% in *T. scurra* specimen Sp27) also grouped within the Spirochaetes.

OTU005, assigned to the phylum Planctomycete, is conserved within all the sponge specimens within the genus *Tsitsikamma* and corresponds to *T. favus* clone Sp02sw-6. OTU001 (8.11% in the yellow encrusting sponge and 1.96% in *T. scurra* Sp07) and OTU002 (19.02% in *Latrunculia sp*. and 1.87% and 1.75% in *T. favus* specimens Sp02 and Sp16 respectively) were assigned to the phylum Betaproteobacteria. OTU002 is closely related, but not identical, to *T. favus* clone Sp02-1, which, according to the 16S rRNA clone library analysis (see section 3.3.3) was the most dominant bacterial species in *T. favus*. OTU002 and Sp02-1 likely represent different strains of the same betaproteobacterial species. The dominant OTU (OTU001) in the yellow encrusting sponge was assigned to the class Betaproteobacteria but groups separately to the dominant *T. favus* clone (Sp02-1) and OTU002.

The above OTU analysis provided insight into the most abundant species present within the Latrunculid sponges. OTU analysis can also provide insight into the degree of similarity between specimens as well as an estimate of the species richness associated with a specimen, and this is visually represented by means of a heatmap (Fig. 4.6) and Venn diagram (Fig. 4.7) respectively.

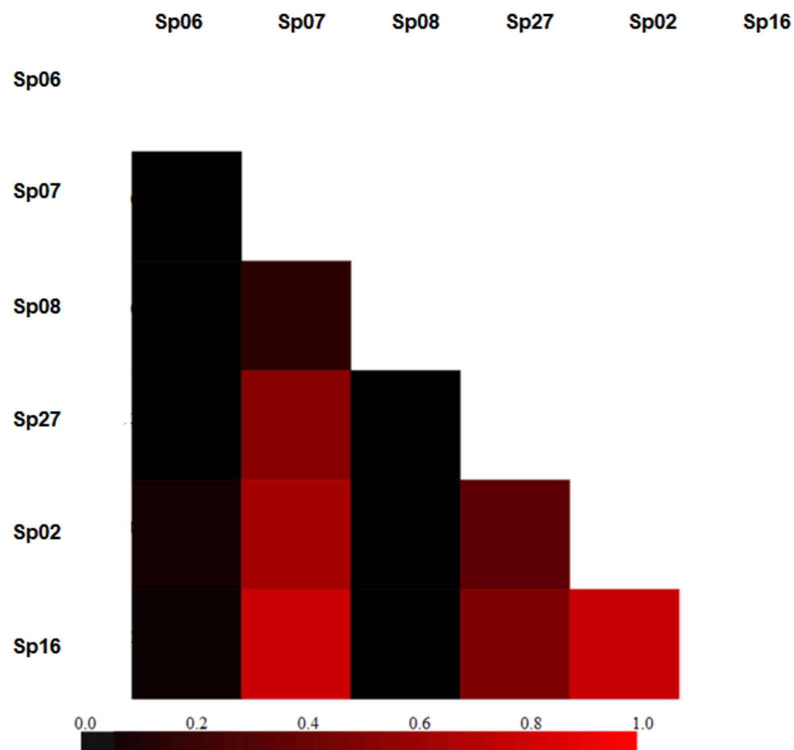


Figure 4.6 Heatmap of OTUs assigned to *T. favus* (Sp02 and Sp16), *T. scurra* (Sp07 and Sp27), *Latrunculia sp.* (Sp06) and the yellow encrusting sponge (Sp08) at a distance value of 0.03.

A heatmap was generated using the Yue & Clayton theta structural diversity measure to calculate the dissimilarity between datasets at a species level. The scale bar indicates a gradual change from 0 (black) to 1 (red), with 0 indicating 0% similarity and 1 indicating 100% similarity (Fig. 4.6). *T. favus* specimens Sp02 and Sp16 appeared to be very similar whereas *T. scurra* specimens Sp07 and Sp27 were less similar potentially due to the before mentioned cross-contamination between Sp07 and its yellow encrusting sponge Sp08. This was further confirmed by the increase in percentage similarity between Sp07 and Sp08 (yellow encrusting sponge) than between Sp27 and Sp08. Both *T. favus* specimens showed relatively high percentage similarity to both *T. scurra* specimens. This reconfirmed the previous assumptions, based on the percentage abundance of the top 20 (Table 4.2) and top 10 (Fig. 4.4) OTUs, that the two sponge species within the genus *Tsitsikamma* shared a similar distribution of associated bacteria. The *Latrunculia sp.* and the yellow encrusting sponge showed little similarity to the *T. scurra* sponges. However, the *Latrunculia sp*. is more similar to *T. favus* than to *T. scurra* whereas the opposite was true for the yellow encrusting sponge.

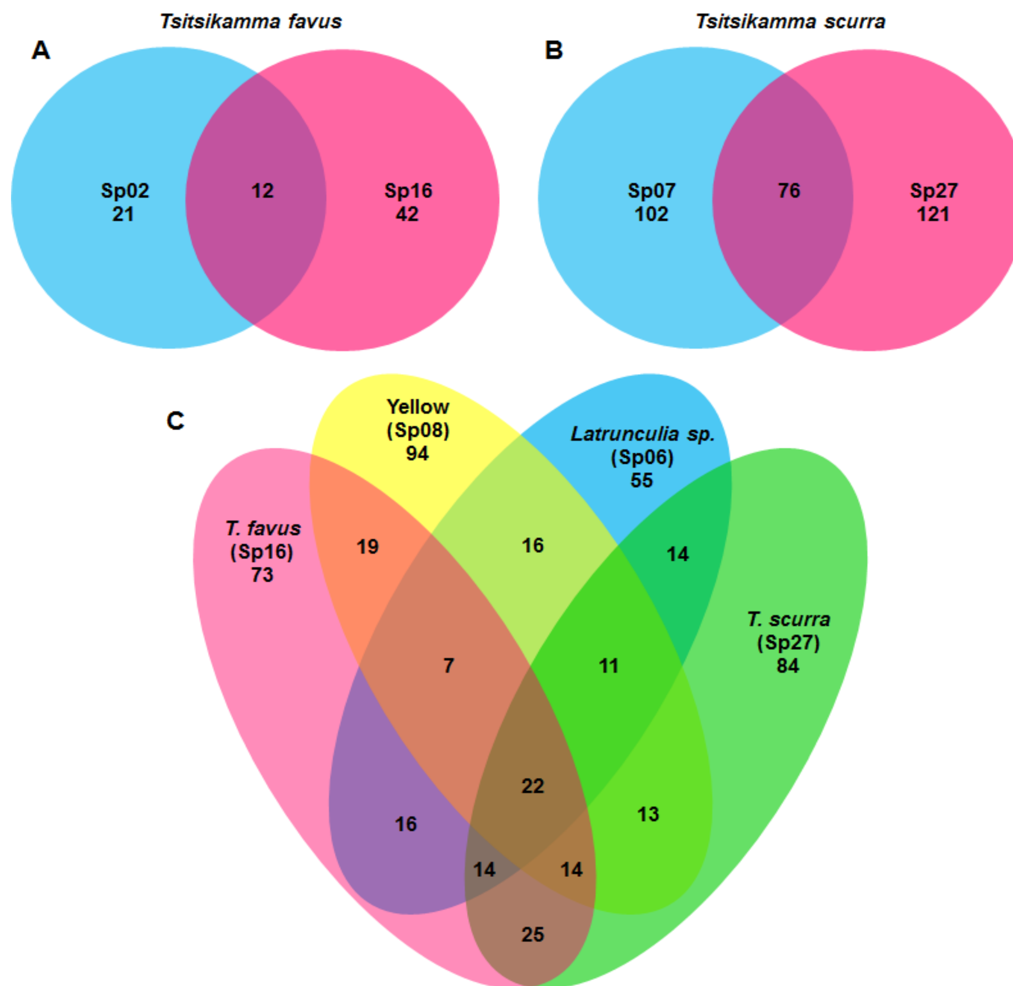


Figure 4.7 Venn diagram showing the degree of OTU overlap between (**A**) T. favus specimens Sp02 and Sp16, (**B**) T. scurra. specimens Sp07 and Sp27 and (**C**) Algoa Bay sponge species *T. favus*, *T. scurra*, *Latrunculia sp.* and the yellow encrusting sponge; at a distance value of 0.03.

To generate Venn diagrams, OTUs representative of single sequences were removed to decrease the influence of potentially transient bacteria. The dataset was also sub-sampled to 'normalize' the datasets which is advised as often the datasets vary quite substantially in size. *T. favus* specimens Sp02 and Sp16 have 12 shared OTUs (Fig. 4.7A) and 21 and 42 unique OTUs respectively. Despite the relatively high number of unique OTUs, the heatmap (Fig. 4.6) implies that the two *T. favus* specimens are highly similar which is not unexpected as the 12 shared OTUs could represent a significant portion of the sequence reads. *T. scurra* specimens Sp07 and Sp27 have 76 shared OTUs with 102 and 121 unique OTUs

respectively (Fig. 4.7B). However, the heatmap (Fig. 4.6) suggests that these two sponge specimens are less similar to each other than the two *T. favus* specimens potentially due to contamination from Sp08. There is also discrepancy between the total number of OTUs assigned to the *T. favus* specimens (33 and 55 for Sp02 and Sp16 respectively) and the *T. scurra* specimens (178 and 197 for Sp07 and Sp27 respectively) which can be attributed to the larger data set obtained for the *T. scurra* specimens (see Table 4.1).

Due to the small dataset generated for Sp02 and the potential cross-contamination observed in Sp07, the four-way Venn diagram, depicting common and unique OTUs, (Fig. 4.7C) was generated using the datasets obtained for *T. favus* specimen Sp16, *T. scurra* Sp27, *Latrunculia sp.* Sp06 and yellow encrusting sponge Sp08. From a total of 477 OTUs, 22 OTUs were shared amongst all four sponge specimens. Each individual sponge specimen was represented by approximately the same number of OTUs (155, 196, 197 and 190 for each of Sp06, Sp08, Sp27 and Sp16 respectively) indicating similar degrees of diversity richness. *T. favus* and *T. scurra* shared 75 OTUs in common and 36 in common with *Latrunculia sp.*; while *T. scurra* and the yellow encrusting sponge shared 60 OTUs in common. Venn analysis is a good estimator of diversity richness within specimens and gives an indication of the number of shared OTUs. However, not all the OTUs are necessarily conserved within a particular sponge species (which is indicated by the low number of overlapping OTUs within individual specimens of the same species, Fig. 4.7 A and B). This was potentially due to transient bacteria in the surrounding water column which, despite thoroughly rinsing the sponges with ASW, cannot be completely excluded from the 16S rRNA datasets. However, taking the results of the heatmap analysis into consideration, it can be assumed that these transient bacteria represent only a small percentage of the total sponge-associated bacterial community.

4.4 Discussion

The objective of the research described in this chapter was to compare the bacterial diversity associated with Algoa Bay Latrunculid sponges and to determine if the sponge-associated bacterial communities were conserved and species-specific. While the sponge specimens investigated in the study were found growing in close proximity to one another, their associated bacterial communities were distinct. Initial DGGE profiling of the sponge specimens indicated that each sponge species contained a bacterial population so conserved it could be used to distinguish between species. Subsequent 16S rRNA gene sequence analysis implied that the diversity and relative abundance of bacterial phyla within *Tsitsikamma favus* and *Tsitsikamma scurra* was similar, which was not entirely unexpected given that they belong to the same genus and share very similar chemical profiles. Both *T. favus* and *T. scurra* were dominated by OTU003 (representing approximately 14% and 5% of the total 454 sequence reads respectively) which was classified within the phylum Spirochaeta together with the *T. favus* clone library isolates Sp02-3 and Sp02-15. Previous studies on *T. favus* using 16S rRNA gene clone libraries (see section 3.3.3) suggested that the betaproteobacterial clone Sp02-1 was the overwhelmingly dominant species associated within *T. favus*, representing approximately 70% of the clone reads. Classification of the sequence reads to the phylum and class level also suggested that the dominant class within *T. favus* was the Betaproteobacteria. The discrepancy between the 454 sequences and clone library analysis may be due to primer bias since the templates used for sequencing were amplified using different primer pairs (see Fig. 3.1).Despite the consistent dominance of the class Betaproteobacteria within the *Tsitsikamma* sponges, the most abundant OTU for both *T. favus* and *T. scurra* is represented by a Spirochaete. The large number of sequence reads assigned to the class Betaproteobacteria by the RDP classifier could be because this class is represented by a high diversity of betaproteobacterial species and not one abundant betaproteobacterium.

In a study by Kamke *et al.* (2010) the activity profiles of sponge-associated bacteria were studied using rRNA transcription as an indicator of metabolic activity, with the Spirochaetes showing high metabolic activity. The high

abundance of Spirochaetes within the mesohyl of host sponges together with their elevated metabolic activity (Kamke *et al*., 2010; Neulinger *et al*., 2010) are indicators that these sponge symbionts are an active part of the sponge-associated bacterial community. With regards to *T. favus* and *T. scurra,* the high abundance and conservation of a particular Spirochaete species would suggest that this bacterium plays some role in the health of the sponge, perhaps by assisting in the production of secondary metabolites.

Despite some similarities, the *T. favus* and *T. scurra* specimens did display differences in the relative abundance of the dominant OTUs. *T. favus* shared a betaproteobacterium in common with the *Latrunculia sp.* whereas *T. scurra* had a higher relative abundance of a gammaproteobaterium. *T. scurra* was covered with a yellow encrusting sponge which had to be peeled off like a skin. All four *T. scurra* specimens collected were covered in this encrusting sponge and did not seem harmed or damaged in any way indicating a commensal or possibly even symbiotic relationship. The bacterial communities associated with these two sponges were, however, different both in terms of their DGGE profile (Fig. 4.1) and the 16S rRNA analysis, with Betaproteobacteri*a* and Unclassified bacteria dominating in *T. scurra* and unclassified Proteobacteria dominating in the yellow encrusting sponge. Lastly, the *Latrunculia sp*. which belongs to the family *Latrunculiidae* (as do *T. favus* and *T. scurra)* showed a distinct DGGE profile as well as differences in its associated bacterial community in the 16S rRNA gene sequence analysis. Also present at relatively high levels within the *T. scurra* sponges were a species of Planctomycetacia and Gammaproteobacteria, both of which are common sponge symbionts. A betaproteobacterium (OTU002) dominates in the bacterial population associated with the *Latrunculia sp.* and this betaproteobacterium is also present, albeit at a lower abundance, within *T. favus*.

Previous sponge diversity studies have highlighted three consistently dominant phyla, namely the Chloroflexi, the Acidobacteria and the Proteobacteria (Erpenbeck *et al.,* 2002; Holmes & Blanch, 2007; Lee *et al*., 2009; Montalvo & Hill, 2011; Taylor *et al.,* 2007; Trindade-Silva *et al.,* 2012; Webster *et al*., 2010; White *et al.,* 2012). However, the dominance of the class Betaproteobacteria is unique (see Chapter Three). The above OTU analysis provided insight into the dominant

species present within the Latrunculid sponges. OTU analysis can also provide insight into the degree of similarity between specimens as well as an estimate of the species richness associated with a specimen, and this is visually represented by means of a heatmap (Fig. 4.6) and Venn diagram (Fig. 4.7) respectively.

The microbial diversity associated with Algoa Bay Latrunculid sponges appears to be conserved and species-specific (based on results obtained using both DGGE and 454 pyrosequencing). There was also similarity observed between the bacterial populations associated with *T. favus* and *T. scurra*, which share common secondary metabolite profiles. This raises the question as to whether the observed similarity in bacterial populations is due to the similarity in chemical profiles which are allowing for the growth of certain microorganisms while inhibiting the growth of others; or whether the similarity in chemical profiles is due to the fact that species within the genus *Tsitsikamma* share similar bacterial communities which are responsible for the production of the 'sponge-associated' secondary metabolites. Isolation of culturable bacteria associated with *T. favus* will be discussed in Chapter Five.

4.5 References

Chao, A. (2005) Species richness estimation IN, Balakrishnan, N., Read, C.B., & Vidakovic, B. (Eds.). Encyclopedia of Statistical Sciences. New York, Wiley.

Erpenbeck, D., Breeuwer, J.A.J, van der Velde, H.C., van Soest, R.W.M. (2002) Unravelling host and symbiont phylogenies of halichondrid sponges (Demospongiae, Porifera) using a mitochondrial marker. *Marine Biology*, 141: 377–386.

Erwin, P. M., Olson, J.B., Thacker, R.W. (2011). Phylogenetic diversity, host specificity and community profiling of sponge-associated bacteria in the northern gulf of Mexico. *PLoS ONE,* 6: 1-16

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* 39: 783-791.

Hentschel, U., Hopke, J., Horn, M., Friedrich, A.B., Wagner, M. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied Environmental Microbiology,* 68: 4431–4440.

Hill, M., Hill, A., Lopez, N., Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). *Marine Biology*, 148: 1221–1230.

Holmes, B., Blanch, H. (2007). Genus-specific associations of marine sponges with group I crenarchaeotes. *Marine Biology*, 150: 759–772.

Hooper, G.J., Davies-Coleman, M.T., Kelly-Borges, M. & Coetzee, P.S. (1996). New alkaloids from a South African Latrunculid sponge. *Tetrahedron Letters,* 37: 7135-7138

Kamke, J., Taylor, M.W. & Schmitt, S. (2010). Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *ISME J,* 4: 498-508.

Kelly, M. & Samaai, T. (2002). Family Latrunculiidae Topsent, 1922. IN Hooper, J.N.A. & Van Soest, R.W.M. (Eds.) *System Porifera: A guide to the Classification of Sponges.* New York: Kluwer/Plenum Academic.

Lee, O.O., Wong, Y.H., Qian, P.Y. (2009). Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. *Applied and Environmental Microbiology*, 75: 3513–3521.

Li, Z., Hu, Y., Liu, Y., Huang, Y., He, L. (2007) 16S rDNA clone library-based bacterial phylogenetic diversity associated with three South China Sea sponges. *World Journal of Microbiology Biotechnology*, 23: 1265–1272.

Mohamed, N.M., Enticknap, J.J., Lohr, J.E., Mcintosh, S.M. & Hill, R.T. (2008). Changes in Bacterial Communities of the Marine Sponge *Mycale laxissima* on Transfer into Aquaculture. *Applied and Environmental Microbiology,* 74: 1209 - 1222.

Montalvo, N.F., Mohamed, N.M., Enticknap, J.J. & Hill, R.T. (2005). Novel actinobacteria from marine sponges. *Antonie van Leeuwenhoek,* 87: 29-36.

Muscholl-Silberhorn, A., Thiel, V. & Imhoff, J. (2008). Abundance and Bioactivity of Cultured Sponge-Associated Bacteria from the Mediterranean Sea. *Microbial Ecology,* 55: 94-106.

Neulinger, S.C., Stohr, R., Thiel, V., Schmaljohann, R., Imhoff, J.F. (2010). New phylogenetic lineages of the Spirochaetes phylum associated with *Clathrina* species (Porifera). *The Journal of Microbiology*, 48: 411-418.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* 4: 406-425.

Schloss, P. D., *et al*. (2009). Introducing MOTHUR: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, 75: 7537–7541.

Simpson, E. H. (1949). Measurement of diversity. *Nature*, 163: 688.

Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA),* 101: 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. . *Molecular Biology and Evolution,* In Press.

Taylor, M.W., Schupp, P.J., Dahllof, I., Kjelleberg, S., Steinberg, P.D. (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology,* 6: 121–130.

Taylor, M.W., Schupp, P.J., de Nys, R., Kjelleberg, S., Steinberg, P.D. (2005). Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica*. *Environmental Microbiology,* 7: 419–433.

Taylor, M.W., Radax, R., Steger, D. & Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiology and Molecular Biology Reviews,* 71: 295-347.

Trindade-Silva, A.E., Rua, C., Silva, G.G.Z., Dutilh, B.E., Moreira, A.P.B. (2012). Taxonomic and Functional Microbial Signatures of the Endemic Marine Sponge Arenosclera brasiliensis. *PLoS ONE*, 7(7): e39905.

Turque, A.S., Cardoso, A.M., Silveira, C.B., Vieira, R.P., Freitas, F.A.D. (2008). Bacterial communities of the marine songes Hymeniacidon heliophila and Polymastia janeirensis and their environment in Rio de Janeiro, Brazil. *Marine Biology*, 155: 135–146.

Webster, N.S. (2007). Sponge disease: a global threat? *Environmental Microbiology*, 9: 1363–1375.

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology,* 12: 2070-2082.

White, J.R., Patel, J., Ottesen, A., Arce, G., Blackwelder, P. (2012) Pyrosequencing of Bacterial Symbionts within Axinella corrugata Sponges: Diversity and Seasonal Variability. *PLoS ONE,* 7(6): e38204.

Wooley, J.C., Godzik, A., Friedberg, I. (2010). A Primer on Metagenomics. *PLoS Computational Biology*, 6(2):e1000667.

Yue, J.C., and Clayton, M.K. (2005). A similarity measure based on species proportions. *Communications in Statistics – Theory and Methods,* 34:2123-2131.

Chapter Five

ISOLATION AND CULTURE OF SPONGE-ASSOCIATED BACTERIA

5.1 Introduction

The sponge family *Latrunculiidae* are known for their production of a number of cytotoxic pyrroloiminoquinone compounds, including the discorhabdins and tsitsikammamines (Antunes *et al.,* 2004, Antunes *et al.,* 2005). The tsitsikammamines have, to date, only been isolated from the sponge species *Tsitsikamma favus* whereas the discorhabdins are common throughout the *Latrunculiidae* sponges. LCMS analysis of *Tsitsikamma scurra* specimens (section 2.3.3) showed the presence of the discorhabdins 2,4-debromo-3-dihydrodiscorhabdin C, 7,8-dehydro-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V which have also been isolated from both *T. favus* and *T. pedunculata* (Antunes *et al.,* 2003; Antunes *et al.,* 2004; Antunes *et al.,* 2005). A study of the diversity of bacteria associated with *T. favus* revealed the dominance of Betaproteobacteria within the *T. favus* associated microbial community (Chapter Three). Further 454 pyrosequencing analyses of T*. favus*, *T. scurra* and *Latrunculia sp.* specimens (Chapter Four) confirmed that, although the Betaproteobacteria were not necessarily the most dominant OTU, they did represent the most dominant class within the three Latrunculid sponge species. The relative rareness of the Betaproteobacteria as the most dominant class within a sponge-associated bacterial community made them an interesting target for isolation and culture studies. Marine sponges contain highly specific and conserved microbial communities, making them an attractive source for the discovery of novel sponge-associated bacteria which are assumed to be conserved by the sponges for a reason; and there is growing evidence to prove this hypothesis with the discovery of sponge-symbionts (as discussed in section 1.4).

Despite their relatively low numerical abundance within the Latrunculid sponges the actinomycetes are the most economically important prokaryotes (Tiwari & Gupta, 2012) because of their production of bioactive secondary metabolites (Berdy, 2005). The rate of discovery of new compounds from terrestrial

actinomycetes has decreased, making the pursuit of new groups of actinomycetes from different habitats of great significance in the search for new drugs (Tiwari & Gupta, 2012). The initial impression that the diversity of actinomycetes in the oceans was small and restricted has been dispelled by 16S rRNA phylogenetic studies (Webster *et al.,* 2001; Montalvo *et al*., 2005; Pathom-aree *et al.,* 2006; Gandhimathi *et al*., 2008). Instead, as research and development in marine microbiology increases, there has been an associated increase in the discovery of a number of novel actinomycete metabolites. The oceans; including the seawater, marine sediment, and vertebrate and invertebrate organisms living within it; are considered the greatest source of biodiversity on the planet, making them an obvious target of exploration for the discovery of new compounds for drug development (Hentschel *et al.,* 2003; Lam, 2006; Berdy, 2005; Tiwari & Gupta, 2012).

Cultivation of the conserved sponge bacterial community and the sponge-associated actinomycetes would provide a method of bioprospecting for novel bioactive compounds, as well as potentially providing a microbial source for supposed ‘sponge’ secondary metabolites (Kennedy *et al*., 2007; Haygood *et al*., 1999). Mincer *et al.* (2005) emphasized that if time is spent in developing media and growth conditions specific to the marine environment from which the samples were collected, it is possible to culture significant numbers of previously unidentified bacteria and optimize fermentation conditions for the production of novel secondary metabolites (Mincer *et al*., 2005; Newman & Hill, 2006).

The research described in this chapter was primarily aimed at the isolation of dominant sponge-associated bacteria from T. *favus*, but also included the isolation of actinomycetes from *T. favus*, T. *scurra* (and its associated yellow encrusting sponge) and *Latrunculia sp.* specimens.

5.2 Methods and Materials

5.2.1 Microbial strains and general culture conditions

All freshly collected sponge material was processed as described in section 2.2.1. Additionally, for the isolation of culturable *T. favus* associated bacteria and actinomycetes, approximately 1 cm^3 of fresh sponge material was pulverised in 1 ml ASW (refer to section 2.2.1) in a mortar and pestle. A 100 µl volume of the mashed sponge was spread onto isolation media and incubated at 28 °C. The isolation media used (see Appendix A1 for media recipes) and the incubation time are further discussed under the relevant headings. Once the relevant culture was obtained, the cells were rinsed off the plate with 10 ml ASW and 1.5 ml aliquots were pelleted by centrifugation at 16,000 x g for 5 min and the pellet stored at -80 °C until later being used for DNA extractions. Glycerol stocks were prepared by adding 750 µl cell/ASW mix to 750 µl 2 x MA2216 broth (Difco) containing 50% glycerol and stored at -80 °C.

The sponge isolates were streaked to single colonies and then re-streaked for the purposes of creating glycerol stocks and frozen pellets for DNA isolation purposes (as described above). DNA was isolated using the guanidinium thiocyanate protocol (as described in section 2.2.3). The 16S rRNA sequences were obtained using the PCR amplification protocol described in section 3.2.4. DGGE analysis was performed as described in section 3.2.3.

For antimicrobial testing, the test bacteria were streaked to single colonies on Luria agar plates and then patched from a single colony onto the respective plates. The plates were then incubated overnight at optimal growth temperatures of 28 °C for *Pseudomonas aeruginosa* (Gram-negative), *Bacillus subtilus* (Gram-positive) and a *Pseudomonas putida* strain RUKM3s (Gram-negative); and 37 °C for *Eschericia coli* (Gram-negative), *Shigella sonnei* (Gram-negative), *Staphylococcus aureus* (Gram-positive), *Klebsiella pneumonia* (Gram-negative), *Erwinia* (Gram-negative), and *Serratia marcescens* (Gram-negative). Two additional actinomycete isolates, UCT-05-011 #3 and UCT-05-011 3d, were provided by Dr Marelise Le Roes-Hill, (Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology) and were obtained

from sponges other than the genus *Tsitsikamma*. These actinomycete test strains were streaked to single colonies on ISP2 media and then patched from a single colony onto the respective plates before being incubated at 28 °C.

5.2.2 Isolation of dominant T. favus-associated bacterial strains

A 100 µl volume of a ten-fold serial dilution (10^0 to 10^{-6}) of the mashed *T. favus* specimen Sp26 was plated onto MA2216 and ISP2 and incubated at 28 °C for 24 hrs. A 100 µl volume was also inoculated into 10 ml of MA2216 broth and incubated (200 rpm, 28 °C, 7 days). Plates were checked for growth. If the plates had extensive colony growth, the plates were removed and processed. If there was little to no growth, the plates were left to incubate for up to a week. Individual colonies were streaked to single colonies and the remaining cell mass was rinsed off the solid media using sterile MA2216 broth and glycerol stocks and frozen pellets were stored at -80 °C. The guanidium thiocyanate DNA isolation method was used to extract total genomic DNA and the bacterial diversity was visualised using DGGE (as previously described in section 3.2.3).

A *Pseudovibrio sp.* was the most dominant colony seen growing on the solid media. A pure culture was obtained and identified using 16S rRNA PCR and sequencing (see section 3.2.4). Glycerol stocks of the *Pseudovibrio* isolate were spread plated onto ten MA2216 solid media plates (28 °C, 10 days) and inoculated into 2 x 500 ml MA2216 broth (200 rpm, 28 °C, 10 days). The cells were rinsed off the solid media with methanol, filtered through cotton wool and the methanol extract was cyclic loaded onto a PSDVB (HP20, 50 ml) column (see Chapter 2). The liquid cultures were filtered through cotton wool and loaded directly onto a second PSDVB (HP20, 100 ml) column (cyclic loading wasn't necessary because any excreted compounds were already in an aqueous medium). Both columns were rinsed with a 10 x column volume of ddH20 to remove all salts and other polar compounds. The columns were eluted with 3 x column volumes of 25%, 40%, 60%, 80% and 100% acetone (in de-ionised water). The fractions were back-loaded onto a PSDVB (HP20, 30 ml) column. The 25% and 40% fractions were eluted with 100 ml methanol while the 60%, 80% and 100% fractions were eluted with 100 ml acetone. All fractions were concentrated *in*

vacuo and analysed using 600 MHz proton NMR (as described in section 2.2.6) and ESI LCMS (as described in section 2.2.8).

For the isolation of the *T. favus* associated betaproteobacterium, 100 µl of each of five 10-fold serial dilutions (10^0 – 10^{-4}) of the MA2216 solid medium glycerol stock (chosen because it contained a band corresponding to ‘Sp02-1’ in the DGGE analysis) was plated onto 1/100 MA2216, MA2216* (1/100 MA2216 with 1.9% NaCl), 1/100 Nutrient Agar (NA) and Nutrient Agar* (1/100 NA with 1.9% NaCl). The plates were incubated at 28 °C for 2 weeks. Individual colonies were picked into 150 µl of liquid media (corresponding to the solid media they were isolated from) in 96-well microtitre plates as follows: each of the four types of media used were assigned three columns in the microtitre plate i.e. 24 colonies were picked from each type of medium used. The microtitre plates were incubated at 28 °C for 5 days with gentle shaking (20 rpm), 100 µl from 8 wells was combined (down the column), the total genomic DNA was extracted using the guanidium thiocyanate protocol, and the bacterial diversity was visualised using DGGE (as described in section 3.2.3). 50 µl MA2216 glycerol stock (50% glycerol:50% 2 x MA2216) was added to the remaining 50 µl in the microtitre plate and was stored at -80 °C.

5.2.3 Actinomycete isolation media and growth conditions

Five different actinomycete isolation media, namely ISP2, ISP5, R2A, M1 Agar and SCN media provided by Dr Marelise Le Roes-Hill (Biocatalysis and Technical Biology Research Group, Cape Peninsula University of Technology) and Professor Russell Hill (Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science), as well as MA2216 (a non-selective medium) were initially used (see Appendix A1 for media recipes). After sterilization (autoclave), the media was allowed to cool to about 40 °C and 1 ml of each of nalidixic acid (10 mg/ml in 0.2 M NaOH) and cycloheximide (10 mg/ml in triple distilled water) were added. The antibiotic nalidixic acid prevents the growth of Gram-negative bacteria, while cycloheximide prevents fungal growth. A 100 ul volume of the mashed sponge material was plated onto each of the above media and the plates were incubated at 28 °C for 2 – 12 weeks.

5.2.4 Screening of a crude T. favus extract for antimicrobial activity

Crude sponge extract (1.25 g dry mass, extracted using methanol as described in section 2.2.6) was added to a 0.1 M sodium acetate buffer (50 ml. pH 5.5) and mixed by vortexing for 1 min. The insoluble components were then separated by centrifugation (13000 rpm, 2 min) and 1 ml, 2 ml, 3.5 ml, and 5 ml respectively of the supernatant was added to 100 ml sterilized MA2216 broth and ISP2 broth (supplemented with 16 g bacto agar) at approximately 40 °C, before being poured as solid media plates. The positive control contained 5 ml of the sodium acetate buffer to negate any negative effect on the bacterial growth due to the buffer. The test bacteria and actinomycete isolates (both those supplied by Dr le Roes-Hill and those isolated from the Algoa Bay sponges) were first streaked to single colonies on MA2216 and ISP2 respectively and then streaked from a single colony onto the relevant sponge extract plates. Those that had diminished growth were considered sensitive to the sponge extract, whereas those that had normal growth (compared to the positive control) were considered resistant to the sponge extract.

5.2.5 Screening of actinomycete isolates for antimicrobial activity

Each individual colony found growing on the actinomycete isolation media was streaked onto a fresh ISP2 plate (without the antibiotics) to form a uniform zone of growth. All the sponge associated actinobacterial isolates were sent to Dr Paul Meyers at the Department of Molecular and Cell Biology, University of Cape Town for antimycobacterial screening. For screening purposes, each isolate was purified and maintained by repeated plate subculturing and grown in liquid culture with shaking as per the method of Everest *et al.* (2011). Incubation of isolates was always at 30 °C. Gram stains and streak plates were used to check purity of cultures.

Antimycobacterial activity was determined by carrying out standard bacterial overlays. Actinobacterial stab inoculations were grown on a solid agar plate for 9 days at 30 °C and overlaid with 6 ml of sloppy LB agar (0.7% w/v agar) containing *M. aurum* A+ from an overnight culture. For purposes of reproducibility and comparability between isolates, the volume of *M. aurum* A+ added to each overlay

was standardised using the equation: OD_{600} × volume to be added (in µl) = 160. In an attempt to induce the production of silent genes for antibiotic biosynthesis, two variations of five different media were used (standard recipes and versions where distilled water was replaced with ASW (annotated with a *)) (Kemp & Coyne 2011). The media used were yeast extract-malt extract (YEME), Czapek Solution agar (CZ) at pH 7.3 (Atlas, 2004), Modified Czapek solution agar (MC) at pH 7 (Nonomura & Ohara, 1971), Difco Middlebrook 7H9 agar prepared according to the manufacturer's specifications and supplemented with 10 mM glucose (albumin and catalase free) and R2A (Atlas, 2004). Plates were inspected and the zone of inhibition around the tested actinobacterial isolate was measured.

5.3 Results

5.3.1 Antimicrobial activity of the crude sponge extract

The pyrroloiminoquinones isolated from *T. favus* are well known for their cytotoxic properties (Antunes *et al*., 2004; Antunes *et al*., 2005) but little is known about their general antimicrobial properties. In previous studies, the secondary metabolites extracted from the sponge *T. favus* showed antimicrobial activity against the Gram-positive bacterium *Bacillus subtilis* (Antunes, 2003).

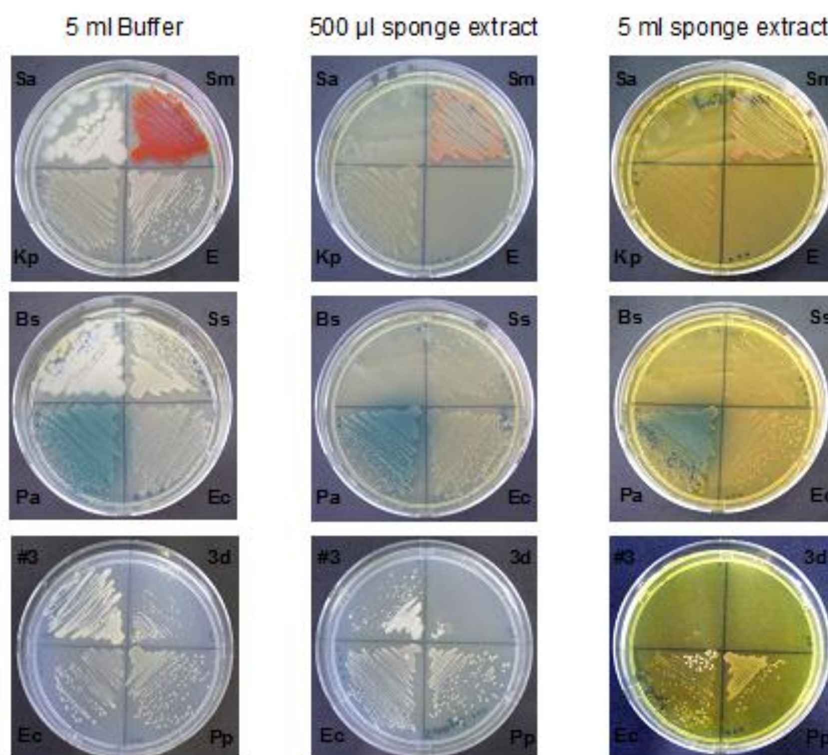


Figure 5.1 The effect of a crude methanol extract of *T. favus* on the growth of a range of Gram-positive and Gram-negative bacteria. Row 1 (clockwise starting from top left quadrant): *S. aureus* (Sa), *S. marcescens* (Sm), *Erwinia* (E), *K. pneumonia* (Kp); Row 2 (clockwise starting from top left quadrant): *B. subtilis* (Bs), *S. sonnei* (Ss), *E. coli* (Ec), *P. aeruginosa* (Pa). Row 3 (clockwise starting from top left quadrant): UCT-05-011 #3 (#3), UCT-05-011 3d (3d), *P. putida* strain (RUKM3s) (Pp), *E. coli* (Ec).

The sponge extract isolated from a May 2009 collection of *T. favus*, was tested for its general antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. All the Gram-positive bacteria tested (*S. aureus*, *B. subtilis*,

UCT-05-011 #3 and UCT-05-011 3d) were susceptible to the sponge extract, while the Gram-negative bacteria (*Pseudomonas aeruginosa, Pseudomonas putida* strain RUKM3s, *Eschericia coli*, *Shigella sonnei*, *Klebsiella pneumonia, Serratia marcescens*, with the exception of *Erwinia sp.*), were resistant (Figure 5.1). Thus the antimicrobial activity of the sponge extract appeared to inhibit the growth of Gram-positive bacteria, including two actinomycete strains. Thus it is not surprising that the Gram-negative Betaproteobacteria were numerically the most dominant class associated with *T. favus*.

5.3.2 Isolation and culture of T. favus associated bacteria

Previously, the clone library analyses of the 16S rRNA sequences of *T. favus*-associated bacteria (section 3.3.3) showed that a betaproteobacterium species (Sp02-1) was the dominant bacterial species in *T. favus*. Other conserved and relatively dominant *T. favus* bacteria belonged to the Oceanospirillales (Gammaproteobacteria, Sp02-2) Spirochaeta (Sp02-3), Pseudovibrio (Alphaproteobacteria, Sp02sw-5) and Unclassified Gammproteobacteria (Sp02-9). The unique chemistry associated with *T. favus*, the conservation of species amongst *T. favus* specimens, and the knowledge that sponges harbour bacterial symbionts for multiple reasons including the production of secondary metabolites for chemical defence, motivated attempts at culturing and isolation of *T. favus* associated bacteria.

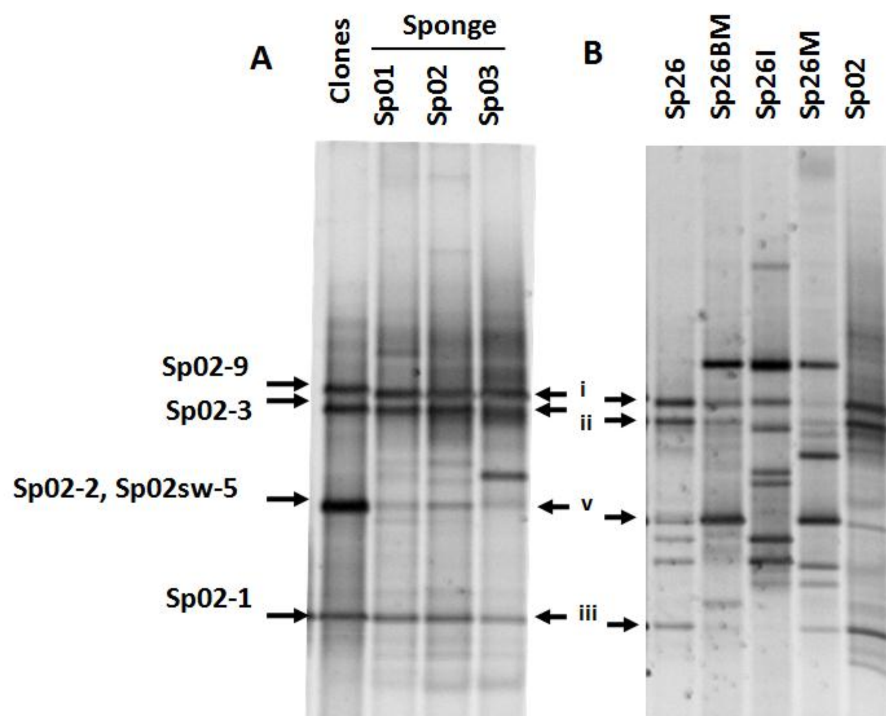


Figure 5.2 DGGE analysis of culturable *T. favus* associated bacteria. **(A)** Comparison of DGGE profiles of dominant species in clone libraries (Sp02-9, Sp02-3, Sp02-2, Sp02sw-5 and Sp02-1) with microbial diversity present in *T. favus* specimens Sp01 (1), Sp02 (2) and Sp03 (3). The relative positions of conserved bands i, ii, iii and v are indicated on the right. **(B)** DGGE profile of 'culturable' bacteria in *T. favus* on different solid and liquid media (Sp26BM – MA2216 broth, Sp26I – ISP2 solid medium and Sp26M – MA2216 solid medium) with dominant *T. favus* associated bands indicated on the left. Sp26 refers to *T. favus* specimen Sp26.

A number of different media were used (see section 5.2.2) to isolate the bacteria associated with *T. favus* specimen Sp26. To investigate whether any of the culturable organisms seen growing in/on the respective media were the dominant clones, genomic DNA was isolated (as described in section 5.2.2) and then used to PCR amplify 16S rRNA gene fragments that were analysed using DGGE. PCR products derived from recombinant plasmids containing the full-length 16S rRNA gene fragments of dominant OTUs associated with *T. favus* (Chapter 3) were used as markers for DGGE analysis. The positions of these markers corresponded to the dominant DGGE bands obtained from PCR amplification of gDNA from the *T. favus* sponge specimens (Fig. 5.2A). This 'marker' was then used to assess the 'culturable' bacteria from *T. favus* (Fig. 5.2B) and it appeared, based on the dominant DGGE bands, that at least 3 of the 5 dominant *T. favus* associated

bacteria were culturable, including the betaproteobacterium. The band corresponding to the betaproteobacterial clone Sp02-1 (band iii) is only clearly visible in the DGGE profile of colonies obtained from Sp26M (pulverised sponge material from *T. favus* specimen Sp26 spread plated onto MA2216). Further investigations into isolating the *T. favus* associated betaproteobacterium were therefore focused on isolates obtained from MA2216 solid medium. Despite the dominance of the betaproteobacterium in the 16S clone library, the corresponding DGGE band (iii) was quite faint in comparison to those corresponding to the other dominant *T. favus* associated bacteria.

There are several potential reasons for this result. First, the DGGE primers may not bind as well to the Sp02-1 sequence as to the other rRNA gene sequences under the PCR conditions used to produce the DGGE templates. Alternatively the bacterium represented by Sp02-1 may be present in lower numbers in the sponge than indicated by the clone library data. This is borne out by the data obtained from 454 pyrosequencing (see Chapter Four). With respect to the DGGE analysis of cultured bacteria, the lower intensity of the Sp02-1 band relative to the other dominant bands may also be due to the possibility that the Sp02-1 isolate grew slower compared to the other sponge-associated bacteria. However, the uniqueness of the Betaproteobacteria as the dominant class within the Latrunculid sponges warranted further efforts to culture the Sp02-1strain focussing on MA2216 medium agar plates.

The majority of the bacterial colonies observed growing on the MA2216 plates were dark brown in colour (Fig. 5.3A). The dark brown/green colour of the colonies, which seemed to be secreted into the surrounding medium, was similar to that of the pyrroloiminoquinone compounds characteristic of *Tsitsikamma* sponges. Colonies were picked and spread to single colonies on MA2216 agar to obtain pure cultures (confirmed by Gram-staining and light microscopy). gDNA was prepared from this isolate and amplified and the sequence of the 16S rRNA gene fragment was determined. A BLAST analysis showed a 100% match with *Pseudovibrio ascidiaceicola* which belongs to the class Alphaproteobacteria.

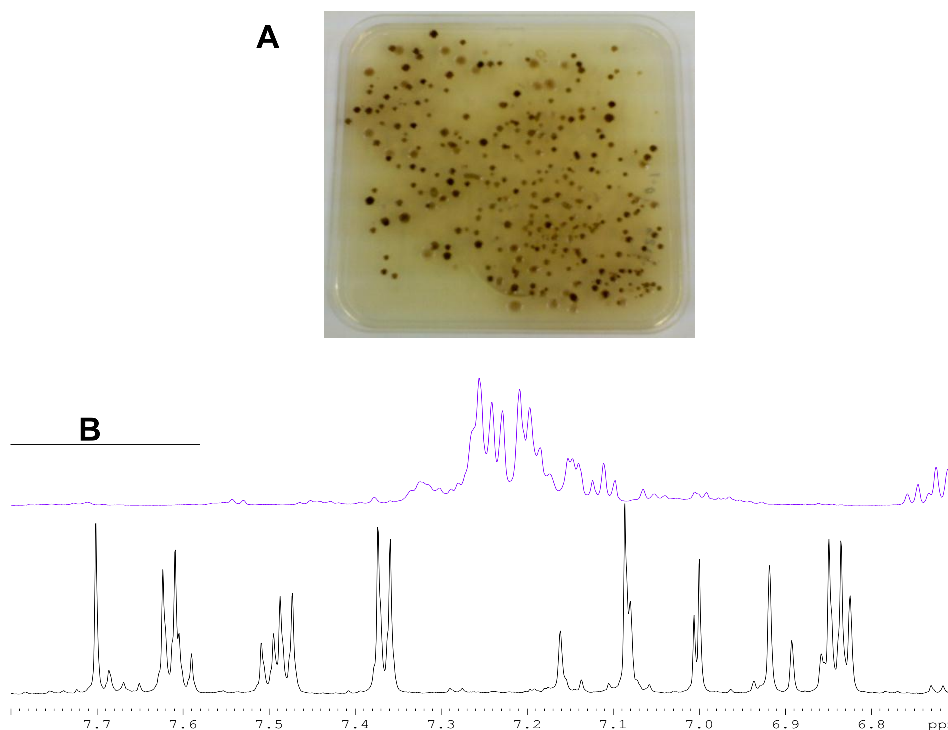


Figure 5.3 **(A)** Colonies of *P. ascidiaceicola* growing on MA2216 plates. **(B)** ^{1}H NMR of 25% acetone fraction of *P. ascidaiceicola* (purple) and *T. favus* (black)

The compounds produced by *P. ascidiaceicola*, in both solid and liquid cultures, were analysed using proton NMR (Fig. 5.3B) and ESI LCMS to look for the presence of pyrroloiminoquinones. No pyrroloiminoquinone-like compounds were detected, so it was concluded that the brown pigment secreted by *P. ascidiaceicola* was not a sponge-associated pyrroloiminoquinone. However, small amounts of toluenediamene (TDA), a mild antimicrobial, were detected using ESI LCMS.

Further studies were conducted to isolate and culture *T. favus* associated bacterial species. Two approaches were used. The first took into account the assumed numerical dominance of the betaproteobacterium Sp02-1 in *T. favus* (as indicated by the clone library analyses, Chapter Three) and so ten-fold dilutions were spread plated to isolate the numerically dominant culturable bacterial strains. The second approach was to use minimal media to select for the betaproteobacterial isolate. According to Janssen *et al.* (2002) the use of a dilute medium (1/100 dilution) allows for the isolation of slower growing bacterial isolates, including Proteobacteria (Janssen *et al.*, 2002). As mentioned previously, DGGE analysis of

colonies growing on MA2216 solid medium contained a band corresponding to the *T. favus* associated betaproteobacterial clone Sp02-1 (Fig. 5.2). Glycerol stocks of the colonies growing on the MA2216 solid medium where serially diluted (10^0 – 10^{-4}) and spread onto a number of different media, namely 1/100 MA2216, MA2216* (1/100 MA2216 with 1.9% NaCl), 1/100 Nutrient Agar and Nutrient Agar* (1/100 NA with 1.9% NaCl). The plates were incubated at 28 °C for 2 weeks and individual colonies were picked, inoculated into liquid media, pooled and gDNA extracted as described in section 5.2.2.

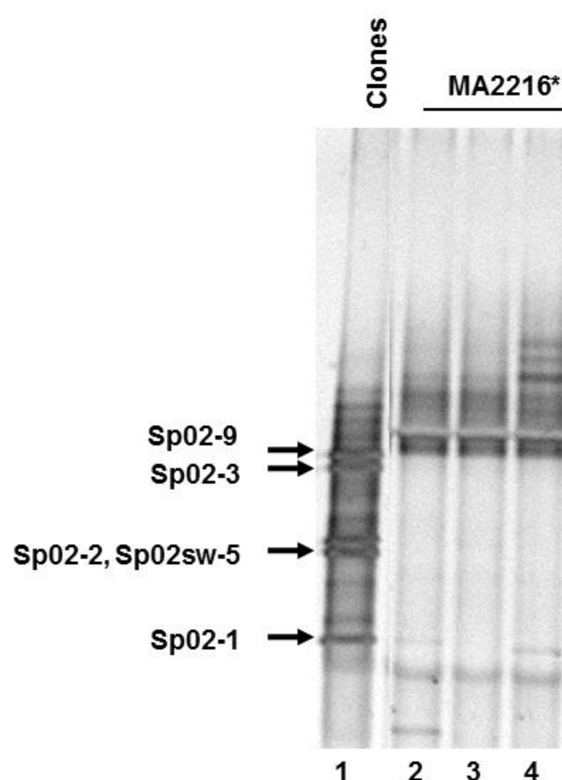


Figure 5.4 DGGE profile of colonies picked from MA2216*. Each DGGE well represents 8 individual colonies all picked from the same plate (as described in section 5.2.2). Only the lanes that were loaded with colonies from MA2216* are indicated because they alone contained a band corresponding to Sp02-1.

DGGE analysis of the pooled colonies indicated that the minimal medium MA2216* gave rise to colonies that contained a band corresponding to the *T. favus* associated betaproteobacterium Sp02-1 (Fig. 5.4, lanes 2 and 4). Glycerol stocks of each of the16 individual colonies that were used to generate the DGGE profile displayed in lanes 2 and 4 (Fig. 5.4) were spread to single colonies on

MA2216*, incubated and monitored over 10 days using light microscopy. At 4 days and 10 days of growth the colonies were assessed by Gram-stain and the results are represented in Fig. 5.5 A and B respectively.

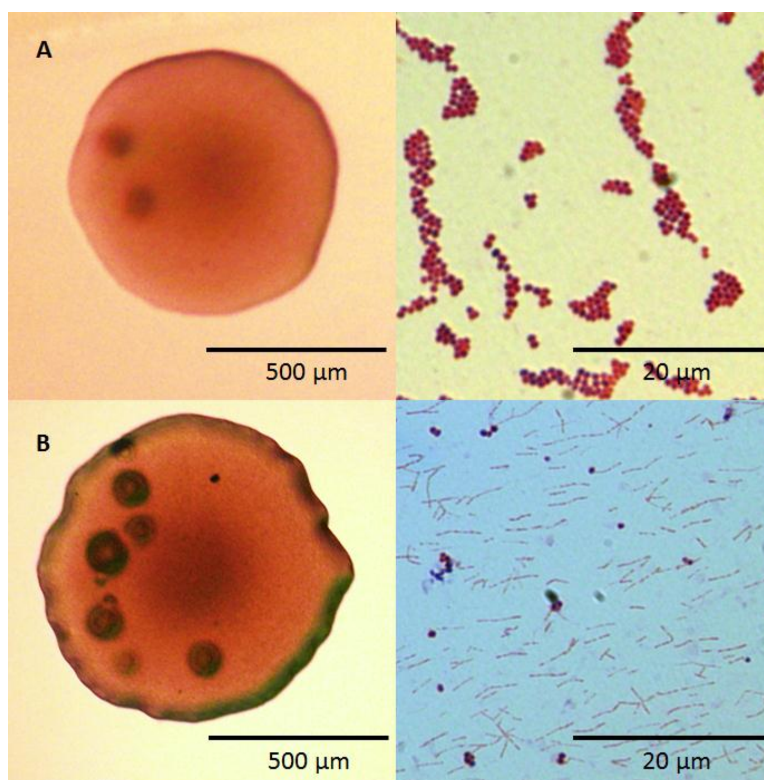


Figure 5.5 An 'Sp02-1' colony after 4 days **(A)** and 10 days **(B)** of growth on MA2216* at 28 °C. The image on the right is of the colony itself and the image on the left is a Gram-stain of the colony.

At first the cells grew as small, cream, opaque colonies with smooth edges and a smooth surface. After 4 days of growth (Fig. 5.5A) raised 'spots' started forming on the surface of the colony, which gradually increased in size with time (Fig. 5.5B, left panel). At 4 days the 'colonies' contained mostly Gram-positive cocci (Fig. 5.5A right panel). After 10 days of growth, the 'colonies' were almost completely dominated by Gram-negative, thin, rod-shaped cells (Fig. 5.5B, right panel). Betaproteobacteria are Gram-negative, and many of them are rod-shaped (Garrity, 2005); so it was possible that the rod-shaped bacteria observed in the colonies could have been the betaproteobacterium Sp02-1. Six individual colonies (at different stages of growth and all displaying the characteristic raised 'spots') were picked and genomic DNA was isolated for DGGE (Fig. 5.6) and 16S rRNA sequence analysis, which revealed that the coccus shaped cells were closely

related (98% identity) to the salt tolerant *Staphylococcus sp.* C1FRI H-TSB-6-HA (JF799910.1) isolated from the east coast of India. Unfortunately, regardless of the dominance of the rod shaped Gram-negative bacterium (assessed by Gram-stain), only the *Staphylococcus* species was identified using 16S rRNA techniques and the identity of the rod-shaped bacterium could not be confirmed. The faster growing *Staphylococcus* species appeared to form a protective layer that then allowed the rod-shaped bacteria to grow underneath it and it is this sub-surface growth that appears to push the gram positive cocci upwards to form the protrusions that were visible as raised 'spots' when viewed using a dissecting light microscope.

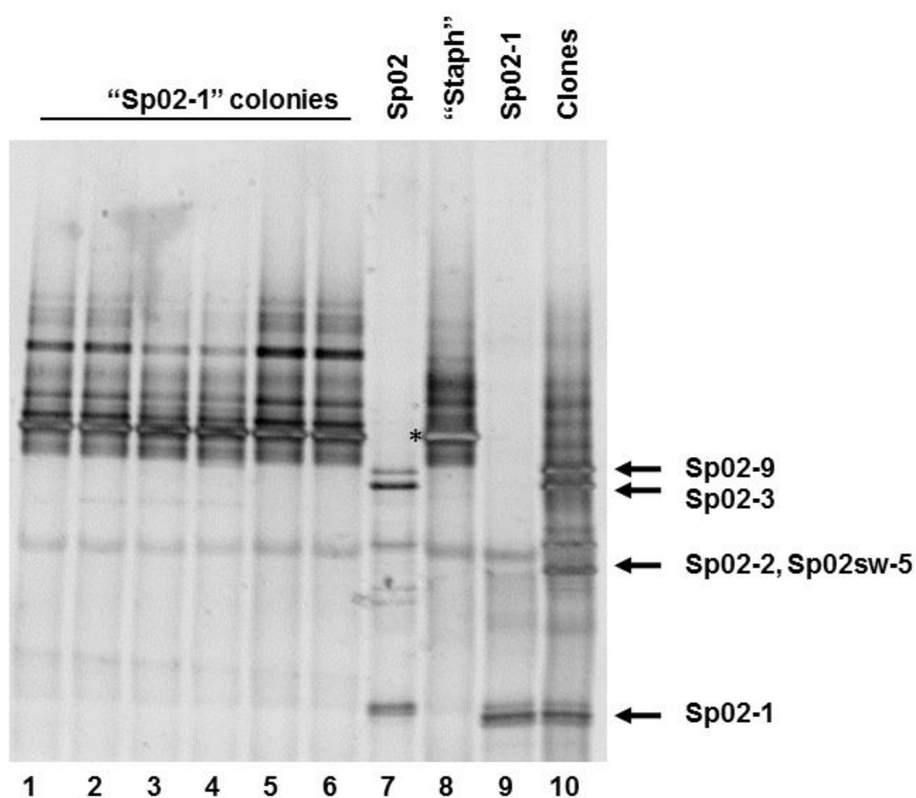


Figure 5.6 DGGE profiles of 6 individual colonies (lanes 1 to 6) that were picked after re-streaking and monitoring of the colonies containing a band corresponding to 'Sp02-1' in the DGGE profile displayed in Fig. 5.4. Lane 7 – DGGE profile of bacteria associated with *T. favus* specimen Sp02 (Sp02), lane 8 – DGGE profile of the *Staphylococcus sp.* identified ('Staph") with a '*' next to the most dominant band, lane 9 – DGGE profile of *T. favus* clone Sp02-1 (Sp02-1) and lane 10 – DGGE profile of the dominant *T. favus* associated clones (Clones).

DGGE analysis of 6 colonies picked according to the results described above indicated that the 6 colonies analysed did not contain a band corresponding to the betaproteobacterium Sp02-1 despite the presence of the rod-shaped bacterium. However, a band corresponding to the most dominant band in the *Staphylococcus sp.* DGGE profile (indicated with a '*') is present in each of the 6 colonies analysed. The *Staphylococcus* species appears to be present in each of the colonies picked but the presence of the betaproteobacterium could not be confirmed.

5.3.3 Isolation and culture of sponge-associated actinomycetes

Five different media were used to isolate actinomycetes from sponge tissue. No colonies were observed on the ISP5, SCN or R2A media after 2 weeks of incubation. The M1 Agar plates had a few colonies while the ISP2 plates produced the most colonies with the greatest morphological diversity. Morphologically distinct colonies growing on ISP2 agar plates that stained Gram-positive were selected for molecular identification using the sequence of the 16S rRNA gene. Twenty four actinomycetes isolates were identified based on BLAST analysis of their 16S rRNA gene sequences (Table 5.1).

Table 5.1: Diversity of Actinobacteria isolated from Algoa Bay sponges

Isolate	Top BLAST hit	Accession no.	% Similarity
RuSp01-3a	*Kocuria kristinae* DSM 20032	X80749	99
RuSp01-3b	*Kocuria kristinae* DSM 20032	X80749	99
RuSp02-2a	*Kocuria kristinae* DSM 20032	X80749	99
RuSp02-2b	*Kocuria kristinae* DSM 20032	X80749	99
RuSp02-3a	*Kocuria kristinae* DSM 20032	X80749	99
RuSp02-3b	*Kocuria kristinae* DSM 20032	X80749	99
RuSp02-4	*Streptomyces matensis* NBRC 12889	AB184221	100
RuSp02-6	*Streptomyces matensis* NBRC 12889	AB184221	100
RuSp03-3	*Rothia amarae* JCM 11375	AY043359	99
RuSp04-2	*Streptomyces albidoflavus* DSM 40455	Z76676	99
RuSp06-2	*Streptomyces viridochromogenes* NBRC 3113	AB184728	99
RU 1B-1	*Tsukamurella pulmonis* DSM 44142	X92981	99
RU 1B-2	*Streptomyces albidoflavus* DSM 40455	Z76676	99
RU 2B-1	*Streptomyces viridochromogenes* NBRC 3113	AB184728	99
RU 3B-1	*Gordonia soli* CC-AB07	AY995560	97
RU 3B-2	*Micrococcus yunnanensis* YIM 65004	FJ214355	100
RU 4/5	*Kocuria kristinae* DSM 20032	X80749	99
RU 6B-1b	*Gordonia bronchialis* DSM 43247	CP001802	98
RU-3S-3	*Streptomyces glomeratus* LMG 19903	AJ781754	98
RU-3S-7	*Amycolatopsis pretoriensis* NRRL B-24133	AY183356	99
Sp26 (2)	*Streptomyces violaceoruber* NBRC 12826	AB184174	99
Sp28 (1)	*Streptomyces albidoflavus* DSM 40455	Z76676	99
Sp28 (4)	*Rhodococcus sp.* VITSNK6	FJ973466.1	91
Sp29 (1)	*Streptomyces lilacinus* NBRC 3944	AB184819	100

The genus *Streptomyces* was the most well represented genus amongst the actinobacterial isolates. *Streptomyces* are aerobic, Gram-positive and filamentous and are morphologically diverse. They are currently the largest genus of

Actinobacteria with over 500 described species (Euzeby, 2008) and are most commonly found in soil and decaying vegetation but a number of species have been isolated from marine environments (Dharmaraj, 2010). They are considered to be the most pharmaceutically significant genus, producing over two thirds of the clinically useful antibiotics of natural origin (Kieser *et al.*, 2000). Several species of *Streptomyces* were isolated from the Algoa Bay sponge specimens, including *S. albidoflavus* (RUSp04-2, Sp28 (1), RU1B-2), *S. matensis* (RUSp02-4, RUSp02-6), *S. viridochromogenes* (RU2B-1), *S. glomeratus* (RU-3S-3), *S. violaceoruber* (Sp26 (2)) and *S. lilacinus* (Sp29 1)).

The genus *Kocuria* was the second most well represented genus. All the *Kocuria* isolates were yellow in colour, except for RUSp02-2a and b which were pink-yellow in colour and RUSp01-3a and b which were a very light yellow (almost white). The isolates all grew as individual convex powdery colonies with a rough surface and an uneven perimeter. According to the literature, members of the *Kocuria* genus are Gram-positive, aerobic, coccoid, non-encapsulated, nonhalophilic and non-endospore-forming (Kloos *et al.*, 1974, Kocur, 1986, Kovacs *et al.*, 1999). Their normal habitats include mammalian skin, soil, the rhizoplane and freshwater, although their occurrence in the marine environment is becoming more apparent. Seven species currently comprise the genus, namely *Kocuria kristinae, Kocuria palustris, Kocuria polaris, Kocuria rhizophila, Kocuria rosea, Kocuria varians* (Stackebrandt *et al.,* 1995, Kovacs *et al.*, 1999, Reddy *et al.*, 2003) and the more recently discovered *Kocuria marina* (Kim *et al.*, 2004).

5.3.4 Antimicrobial activity of sponge-associated actinomycetes

All of the actinomycete isolates were screened by Tarisayi Matongo in Dr Paul Meyer's laboratory (Molecular and Cell Biology Department, University of Cape Town) for growth inhibition of the Gram-negative *Mycobacterium aurum* A+, a non-pathogenic *Mycobacterium* strain considered to have a similar antibiotic susceptibility profile to *Mycobacterium tuberculosis* (Chung *et al.*, 1995).

Table 5.2: Antibiotic activity of Algoa Bay actinomycetes against *M. aurum*. The numbers indicate the zone of inhibition in millimetres on different actinomycete growth media as described in section 5.2.5 (taken from Matongo & Meyers, 2011).

Isolate	Species	YEME	YEME*	CZ	CZ*	MC	MC*	7H9	7H9*	R2A	R2A*
Sp26 (2)	*Streptomyces violaceoruber*	553	547	298		459	612				88
Sp28 (1)	*Streptomyces albidoflavus*					707	289				
Sp29 (1)	*Streptomyces lilacinus*	2617	761	1298		1367	452	1046			283
RUSp02-4	*Streptomyces matensis*	2359	851			276	415			1282	
RUSp02-6	*Streptomyces matensis*		330			327	603			1708	
RUSp04-2	*Streptomyces albidoflavus*		1869			251	179	459			
RUSp06-2	*Streptomyces matensis*	1923				339	398				
RU1B-2	*Streptomyces albidoflavus*	4365	990			262		459	1103		731
RU2B-1	*Streptomyces viridochromogenes*	5873	4423			594					
RU3S-3	*Streptomyces glomeratus*										
RUSp01-3a	*Kocuria kristinae*	3197	1445								
RUSp01-3b	*Kocuria kristinae*	368	2919								
RUSp02-2a	*Kocuria kristinae*										
RUSp02-2b	*Kocuria kristinae*										
RUSp02-3a	*Kocuria kristinae*	1649	>7834								
RUSp02-3b	*Kocuria kristinae*		>7834								
RUSp04/5	*Kocuria kristinae*	2438	901								
RU3B-1	*Gordonia soli*										
RU6B-1b	*Gordonia bronchialis*								38		
RUSp03-3	*Rothia amarae*										
RU1B-1	*Tsukamurella pulmonis*	2280									
RU3B-2	*Micrococcus yunnanensis*										
RU3S-7	*Amycolatopsis pretoriensis*		26				128		51		51
Sp28 (4)	*Rhodococcus sp.*					7	7			2626	

Two of the *Kocuria kristinae* isolates (RUSp02-3a and RUSp02-3b) had significant activity against *M. aurum* with a zone of inhibition that cleared the plate (>7834 mm). It is also important to note that the other *Kocuria* isolates were not as active, and some showed no activity at all (Table 5.2), suggesting that while these isolates were taxonomically identical with reference to their 16S rRNA gene sequences, they potentially represented different strains of the same species. All of the *Streptomyces* isolates (except for *S. glomeratus*) showed promising activity against *M. aurum* especially *Streptomyces albidoflavus* isolate RU1B-2. The other Actinobacterial isolates displayed limited activity against *M. aurum*.

5.4 Discussion

The culturing of *T. favus* associated bacteria was approached from two directions. Firstly, attempts were made to culture the dominant *T. favus* associated bacteria, with particular interest in the dominant betaproteobacterium Sp02-1 (as indicated by the clone library analyses, Chapter Three). Several dark brown colonies were observed and were identified as *Pseudovibrio ascidiaceicola*, an alphaproteobacterium. Initial interest in this isolate was due to its ability to produce a dark brown pigment which was released into the surrounding medium. The pyroloiminiquinones associated with *Tsitsikamma* sponges are also dark brown in colour but ESI LCMS analysis suggested that no pyrroloiminoquinones were produced by *P. ascidiaceicola*. Instead small amounts of TDA were detected. TDA was recently isolated from *Pseudovibrio sp.* D323 and related strains and is proposed to have mild antimicrobial activity (Penesyan *et al*., 2011). Further attempts to culture the dominant *T. favus* associated betaproteobacterium (Sp02-1) resulted in the isolation of a salt tolerant *Staphylococcus* species that appeared to be consistently associated with a rod shaped bacterium that was speculated to be Sp02-1. Unfortunately this could not be confirmed as subsequent DGGE analysis did not indicate the presence of the betaproteobacterial band. It is estimated that only a small percentage of the sponge-associated microorganisms are culturable so it was not unexpected that the betaproteobacterium would be difficult to culture. The *Staphylococcus* species belongs to the phylum Firmicutes, which were detected within the clonal library and within the 454 pyrosequencing analysis of *T. favus* associated bacteria (see Chapter Three).

The second approach involved the culturing of Actinobacteria associated with the Algoa Bay Latrunculid sponges. A small number of actinomycete isolates were cultured and of particular interest were two of the *Kocuria kristinae* strains (RUSp02-3a and RUSp02-3b) as well as the *Streptomyces albidoflavus* strain (RU1B-2) which showed significant antimicrobial activity against *M. aurum*. The discovery of a *Kocuria* species with antimicrobial activity is unusual because *Kocuria* species aren't generally renowned for their production of antimicrobial compounds. Conversely, *S. albidoflavus* is a well characterised species consisting of numerous sub-species and strains (Anderson & Wellington, 2001), and certain strains of *S. albidoflavus* are known to produce several bioactive compounds (Augustine *et al.,* 2005; Roy *et al.*, 2006). Further research into the secondary metabolite production of the *K. kristinae* and *S. albidoflavus* strains is currently underway.

5.5 References

Anderson, A.S. & Wellington, E.M.H. (2001). The taxonomy of *Streptomyces* and related genera. *International Journal of Systematic and Evolutionary Microbiology*, 51:797-814.

Antunes, E.M. (2003). Pyrroloiminoquinone metabolites from South African Latrunculid sponges. *Chemistry.* Grahamstown: Rhodes University.

Antunes, E.M., Beukes, D.R., Kelly, M., Samaai, T., Barrows, L.R., Marshall, K.M., Sincich, C. & Davies-Coleman, M.T. (2004). Cytotoxic pyrroloiminoquinones from four new species of South African Latrunculid sponges. *Journal of Natural Products,* 67: 1268-1276.

Antunes, E.M., Copp, B.R., Davies-Coleman, M.T. & Samaai, T. (2005). Pyrroloiminoquinones and related metabolites from marine sponges. *Natural Products Reports,* 22: 62-72

Atlas, R. M. (2004). *Handbook of Microbiological Media (3rd edition)*. Boca Raton, Fl: CRC Press.

Augustine, S.K., Bhavsar, S.P., Kapadnis, B.P. (2005). A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23. *Journal of Biosciences*, 30:201-211.

Berdy, J. (2005). Bioactive Microbial Metabolites. *The Journal of Antibiotics,* 58: 1-26.

Burja, A.M. & Hill, R.T. (2001). Microbial symbionts of the Australian Great Barrier Reef sponge, *Candidaspongia flabellata*. *Hydrobiologia,* 461: 41-47.

Chung, G. A., Aktar, Z., Jackson, S., Duncan, K. (1995). High-throughput screen for detecting antimycobacterial agents. *Antimicrobial agents and chemotherapy*, 39: 2235-2238.

Dharmaraj, S. (2010). Marine *Streptomyces* as a novel source of bioactive substances. *World Journal of Microbial Biotechnology*. 26:2123-2139.

Enticknap, J.J., Kelly, M., Peraud, O. & Hill, R.T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied Environmental Microbiology,* 72: 3724-3732.

Everest, G.J. & Meyers, P.R. (2008). *Kribbella hippodromi* sp . nov ., isolated from soil from a racecourse in South Africa. *International Journal of Systematic and Evolutionary Microbiology*, 58: 443-446

Euzéby, J.P. (2008). "Genus Streptomyces". *List of Prokaryotic names with Standing in Nomenclature*. http://www.bacterio.cict.fr/s/streptomycesa.html.

Everest, G.J. & Meyers, P.R. (2011). Evaluation of the antibiotic biosynthetic potential of the genus *Amycolatopsis* and description of *Amycolatopsis circi* sp . nov ., *Amycolatopsis equina* sp . nov . and *Amycolatopsis hippodromi* sp. nov. *Journal of Applied Microbiology*, 111: 300-311.

Fukunaga, Y., Kurahashi, M., Tanaka, K., Yanagi, K., Yokota, A. & Harayama, S. (2006). *Pseudovibrio ascidiaceicola* sp. nov., isolated from ascidians (sea

squirts). *International Journal of Systematic and Evolutionary Microbiology,* 56: 343-347.

Gandhimathi, R., Arunkumar, M., Selvin, J., Thangavelu, T., Sivaramakrishnan, S., Kiran, G.S., Shanmughapriya, S., Natarajaseenivasan, K. (2008). Antimicrobial potential of sponge associated marine actinomycetes. *Journal of Medical Mycology,* 18: 16-22.

Garrity, G. (2005). Bergey's Manual of Systematic Bacteriology, Volume II: The Proteobacteria IN Staley, J.T., Brenner, D.J., Krieg, N.R. (Eds.) New York: Springer Science.

Haygood, M.G., Schmidt, E.M., Davidson, S.K. & Faulkner, D.J. (1999). Microbial symbionts of marine invertebrates: Opportunities for microbial biotechnology. *Jouranl of Molecular Microbioliology and Biotechnology,* 1: 33-43.

Hentschel, U., Fieseler, L., Wherl, A., Gernert, C., Steinert, M., Hacker, J. & Horn, M. (2003). Microbial diversity of marine sponges. IN Weg, M. (Ed.) *Sponges (Porifera).* New York: Springer Berlin Heidelberg.

Hentschel, U., Hopke, J., Horn, M., Friedrich, A.B., Wagner, M., Hacker, J. & Moore, B.S. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied Environmental Microbiology,* 68: 4431-4440.

Hughes, C.C., MacMillan, J.B., Gaudêncio, S.P., Jensen, P.R. & Fenical, W. (2009). The Ammosamides: Structures of cell cycle modulators from a marine-derived *Streptomyces* species. *Angewandte Chemie International Edition*, 48:725-727.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. & Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Applied Environmental Microbiology,* 68: 2391-2396.

Kamke, J., Taylor, M.W. & Schmitt, S. (2010). Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *ISME J,* 4: 498-508.

Kemp I. K. & Coyne V. E. (2011). Identification and characterisation of the Mpeg1 homologue in the South African abalone *Haliotis midae*. *Fish & Shellfish Immunology,* 31: 754-764.

Kennedy, J., Marchesi, J.R. & Dobson, A.D. (2007). Metagenomic approaches to exploit the biotechnological potential of the microbial consortia of marine sponges. *Applied Microbiology and Biotechnology,* 75: 11 - 20.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000). *Practical Streptomyces Genetics* (2nd ed.). Norwich, England: John Innes Foundation

Kim, S.B., Nedashkovskaya, O.I., Mikhailov, V.V., Han, S.K., Kim, K.-O., Rhee, M.-S. & Bae, K.S. (2004). *Kocuria marina* sp. nov., a novel actinobacterium isolated from marine sediment. *International Journal of Systematic Evolutionary Microbiology,* 54: 1617-1620.

Kloos, W.E., Tornabene, T.G. & Schleifer, K.H. (1974). Isolation and characterization of micrococci from human skin, including two new species: Micrococcus lylae and Micrococcus kristinae. *International Journal of Systematic Bacteriology,* 24: 79 - 101.

Kocur, M. (1986). Genus Micrococcus Cohn 1872. IN Sneath, P.H.A., Mair, N.S., Sharpe, M.E. & Holt, J.G. (Eds.) *Bergey's Manual of Systematic Bacteriology.* Baltimore: Williams & Wilkins.

Kovacs, G., Burghardt, J., Pradella, S., Schumann, P., Stackebrandt, E. & Marialigeti, K. (1999). Kocuria palustris sp. nov. and Kocuria rhizophila sp. nov., isolated from the rhizoplane of the narrow-leaved cattail (Typha angustifolia). *International Journal of Systematic Bacteriology,* 49: 167-173.

Lam, K.S. (2006). Discovery of novel metabolites from marine actinomycetes. *Current Opinion in Microbiology,* 9: 245-251.

Matongo, T. & Meyers, P. (2011) Honours Thesis: Identification and primary antibiotic screening of Actinobacterial isolates from the South African sea sponge *Tsitsikamma favus*. Department of Molecular and Cell Biology, University of Cape Town.

Mincer, T.J., Fenical, W. & Jensen, P.R. (2005). Culture-Dependent and Culture-Independent Diversity within the Obligate Marine Actinomycete Genus Salinispora. *Applied and Environmental Microbiology,* 71: 7019-7028.

Miyanaga, A., Janso, J.E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustaì•Quio, A.S., Fielding, E.N., Carter, G.T., Jensen, P.R., Feng, X., Leighton, M., Koehn, F.E. & Moore, B.S. (2011). Discovery and Assembly-Line Biosynthesis of the Lymphostin Pyrroloquinoline Alkaloid Family of mTOR Inhibitors in Salinispora Bacteria. *Journal of the American Chemical Society,* 133: 13311-13313.

Montalvo, N.F., Mohamed, N.M., Enticknap, J.J. & Hill, R.T. (2005). Novel actinobacteria from marine sponges. *Antonie van Leeuwenhoek,* 87: 29-36.

Muscholl-Silberhorn, A., Thiel, V. & Imhoff, J. (2008). Abundance and Bioactivity of Cultured Sponge-Associated Bacteria from the Mediterranean Sea. *Microbial Ecology,* 55: 94-106.

Nagata, H., Ochiai, K., Aotani, Y., Ando, K., Yoshida, M., Takahashi, I., Tamaoki, T. J. (1997). Lymphostin (LK6-A), a novel immunosuppressant from *Streptomyces* sp. KY11783: Taxonomy of the producing organism, fermentation, isolation and biological activities. *The Journal of Antibiotics*, 50: 537–542.

Newman, D.J. & Hill, R.T. (2006). New drugs from marine microbes: the tide is turning. *J Ind Microbiol Biotechnol,* 33: 539 - 544.

Nonomura, H. & Ohara, Y. (1971). Distribution of actinomycetes in soil. VIII. Green spore group of *Microtetraspora*, its preferential isolation and taxonomic characteristics. *Journal of Fermentation Technology,* 49: 1-7

O' Halloran, J.A., Barbosa, T.M., Morrissey, J.P., Kennedy, J., O' Gara, F. & Dobson, A.D.W. (2011). Diversity and antimicrobial activity of Pseudovibrio

spp. from Irish marine sponges. *Journal of Applied Microbiology,* 110: 1495-1508.

Pathom-Aree, W., Stach, J., Ward, A., Horikoshi, K., Bull, A. & Goodfellow, M. (2006). Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. *Extremophiles,* 10: 181-189.

Penesyan, A., Tebben, J., Lee, M., Thomas, T., Kjelleberg, S., Harder, T. & Egan, S. (2011). Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio sp.* D323 and related sponge-associated bacteria. *Marine Drugs,* 9: 1391-1402.

Radwan, M., Hanora, A., Zan, J., Mohamed, N., Abo-Elmatty, D., Abou-El-Ela, S. & Hill, R. (2010). Bacterial community analyses of two Red Sea sponges. *Marine Biotechnology,* 12: 350-360.

Reddy, G.S.N., Prakash, J.S.S., Prabahar, V., Matsumoto, G.I., Stackebrandt, E. & Shivaji, S. (2003). *Kocuria polaris* sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. *International Journal of Systematic and Evolutionary Microbiology,* 53: 183-187.

Roy, R.N., Laskar, S., Sen, S.K. (2006). Dibutyl phthalate, the bioactive compound produced by *Streptomyces albidoflavus* 321.2. *Microbial Research*, 161:121-126.

Schläppy, M.-L., Schöttner, S., Lavik, G., Kuypers, M., De Beer, D. & Hoffmann, F. (2010). Evidence of nitrification and denitrification in high and low microbial abundance sponges. *Marine Biology,* 157: 593-602.

Stackebrandt, E. (2006). The Genus *Stomatococcus*: *Rothia mucilaginosa*, basonym *Stomatococcus mucilaginosus. The Prokaryotes.*

Tiwari, K., Gupta, R.K. (2012). Rare actinomycetes: a potential storehouse for novel antibiotics. *Critical Reviews in Biotechnology,* 32:108-132.

Taylor, M.W., Schupp, P.J., Dahllöf, I., Kjelleberg, S. & Steinberg, P.D. (2004). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental Microbiology,* 6: 121-130.

Taylor, M.W., Radax, R., Steger, D. & Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiology and Molecular Biology Reviews,* 71: 295-347

Wang, G. (2006). Diversity and biotechnological potential of the sponge-associated microbial consortia. *Journal of Industrial Microbiology and Biotechnology,* 33: 545-551.

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology,* 12: 2070-2082.

Webster, N.S., Wilson, K.J., Blackall, L.L. & Hill, R.T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology,* 67: 434-444.

Chapter Six

GENERAL DISCUSSION AND FUTURE PROSPECTS

6.1 General Discussion

The pyrroloiminoquinone class of secondary metabolites have been found exclusively in sponges and microorganisms, with the exception of wakayin which was isolated from an Ascidian. Antunes *et al.* (2005) proposed a putative biosynthetic pathway for the pyrroloiminoquinones (Fig. 1.16) that starts with tryptophan as a precursor. Miyanaga *et al.* (2011) elucidated the biosynthetic pathway for the production of the pyrroloiminoquinone lymphostin in the marine actinobacteria *Salinispora tropica* and *Salinispora arenicola*, also using tryptophan as a precursor. Genome sequencing of *S. tropica* and *S. arenicola* revealed a unique hybrid NRPS-PKS locus that has been assigned to the production of the pyrrolo[4,3,2-*de*]quinoline core characteristic of pyrroloiminoquinone metabolites. *T. favus*, *T. scurra* and *T. pedunculata* have the discorhabdins 7,8-dehydro-3-dihydrodiscorhabdin C, 14-bromo-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V in common. According to the biosynthetic pathway, proposed by Antunes *et al.* (2005) all of the secondary metabolites isolated from the *Tsitsikamma* sponges have makaluvamines, which have been shown to have a microbial source (Ishibashi *et al.,* 2001), as an intermediate. This lead to the hypothesis that the pyrroloiminoquinone core and/or many of the intermediates and/or the final secondary metabolites associated with sponges within the *Tsitsikamma* genus are produced by one or a group of closely related microbial symbionts.

This study focused on *Tsitsikamma favus,* which is unique amongst members of the genus *Tsitsikamma* due to its production of tsitsikammamine A and B, as well as *Tsitsikamma scurra* which does not produce tsitsikammamine A and B. The principle aim was to determine whether these two sponge species were associated with conserved microbial species that might be related to their production of secondary metabolites.

Tsitsikamma favus and *Tsitsikamma scurra* (closely associated with a yellow encrusting sponge) were collected from Evan's Peak Algoa Bay, together with a neighbouring and newly described *Latrunculia sp.* The sponge specimens were identified using traditional taxonomic methods coupled with molecular and chemotaxonomic based techniques. The bacterial community associated with each of the sponge specimens was then characterised using several molecular techniques in order to compare the bacterial populations associated with each of the Latrunculid sponges. Culturable sponge-associated bacteria were then isolated and screened for the production of bioactive compounds.

Traditional taxonomic methods identified *Tsitsikamma favus*, *Tsitsikamma scurra* (surrounded by a yellow encrusting sponge) and a new species of *Latrunculia*. Phylogenetic analysis, using both the *COX1* and 28S rRNA gene fragments, confirmed the taxonomic identification and identified the yellow encrusting sponge as a member of the family *Mycaliidae*. However, the *COX1* fragment was unable to differentiate between *T. favus* and *T. scurra* even when the extended fragment was used whereas the 28S rRNA gene fragment proved to be more successful. This highlights the need for the use of a reliable barcoding fragment (or multiple barcoding fragments if one is not sufficient) that are consistently used by sponge taxonomists. To date, there is limited data available on molecular classification of sponges, and what is available is spread across multiple barcoding fragments. This makes the characterisation of sponges using phylogenetic techniques unfeasible and traditional taxonomy is still the most reliable characterisation method. Chemotaxonomic analysis of *T. scurra* revealed that it contained the pyrroloiminoquinones 2,4-debromo-3-dihydrodiscorhabdin C, 7,8-dehydro-3-dihydrodiscorhabdin C and 14-bromo-1-hydroxy-discorhabdin V in common with both *Tsitsikamma favus* and *Tsitsikamma pedunculata* (Antunes *et al*., 2003, Antunes *et al*., 2004; Antunes *et al*., 2005). However, *T. scurra* did not contain tsitsikammamine B thus reconfirming the notion that the tsitsikammamines appear to be characteristic of *T. favus* specifically.

The uniqueness of the tsitsikammamines to *T. favus* motivated an investigation into the link between the unique chemistry associated with *T. favus* and its associated bacterial community. The *T. favus* associated bacterial community;

investigated using DGGE, clonal libraries of full length 16S rRNA genes, as well as 454 pyrosequencing; was shown to be highly conserved, stable over time and dominated by the class Betaproteobacteria. *T. favus* was found to contain a relatively unique distribution of bacteria compared to previous sponge-associated bacterial diversity studies (Li & Liu, 2006; Turque *et al.*, 2008; Lee *et al.*, 2009; Radwan *et al.*, 2010; Hardoim *et al.*, 2009; Wang *et al.*, 2009; Sipkema & Blanch, 2010; Webster *et al.*, 2010). Interestingly, a recent and comprehensive phylogenetic analysis by Simister *et al.* (2012) on the 7 546 publicly available sponge derived 16S rRNA sequences revealed a number of sponge specific sequence clusters which were most well represented within the Chloroflexi, Cyanobacteria, Poribacteria, Betaproteobacteria and Acidobacteria (Simister *et al.,* 2012). These phyla may not be the most dominant bacteria within previously investigated sponges, but they do appear to be the most conserved within sponge communities in general.

There was a general consensus that sponges harboured a select community of "sponge-associated" bacteria and that this core community, although conserved within the phylum Porifera, was universal to sponges in general (Hentschel *et al.,* 2002; Hill *et al.,* 2006; Olson & McCarthy, 2005). However, a number of studies indicate that this sponge-associated bacterial community is not as universal as previously thought, and that contrary to this, an increasing number of sponges have been shown to have highly conserved, species-specific bacterial communities (Erwin *et al.,* 2011; Holmes & Blanch, 2007; Lee *et al*., 2009; Li *et al.,* 2007; Taylor *et al*., 2007). A theory that is gaining momentum due to advances in pyrosequencing capabilities is that the sponge microbiome can be classified into three main groups, namely species-specific, variable and core. Using currently available data it has been estimated that the species-specific taxa occupy approximately 72% of the sponge-associated microbial population whereas the core community is thought to occupy as little as 2% (Trindade-Silva *et al.,* 2012). However, our current knowledge on sponge-symbiont interactions is derived from a relatively small number of host sponges given that there are at least 15 000 sponge species worldwide (Trindade-Silva *et al.,* 2012). Research into the sponge-associated microbial communities associated with unique sponge species from varying geographical locations is needed in order to understand the true

distribution and importance of these sponge symbionts from both an ecological as well as an application based perspective.

With the development of more accessible pyrosequencing technologies comes the ability to analyse microbial communities associated with higher organisms far more holistically. As mentioned in Chapter 3, traditional Sanger sequencing methods are still of importance due to the longer sequencing reads obtained and are therefore still necessary when closely comparing species. However, in terms of large-scale diversity studies, the use of high throughput pyrosequencing provides a more accurate assessment of relative abundances of bacterial phylotypes and allows us to study and compare even rare microbial symbionts which may still be of significance. Pyrosequencing analyses of sponge-associated bacterial communities, to date, has been done on the Red Sea sponges *Hyrtios erectus, Stylissa carteri* and *Xestospongia testudinaria* (Lee *et al.*, 2010), the Great Barrier Reef sponges *Ianthella basta, Ircinia ramose* and *Rhopaloeides odorabile* (Webster *et al.*, 2010), the Caribbean reef sponge *Axinella corrugata* (White *et al.,* 2012) and the Brazilian sponge *Arenosclera brasiliensis* (Trindade-Silva *et al.,* 2012). However, to our knowledge, this is the first study that uses NGS techniques to investigate and compare the microbial diversity associated with closely related marine sponges found growing in close proximity to one another

The microbial community associated with *T. favus* appeared to be highly conserved, but was it species specific? Also, did the two sponges within the genus *Tsitsikamma* share similarities in their microbial communities, and if so how similar were they? The hypothesis was that *T. favus* and *T. scurra* would have similar microbial communities because they shared similarities in their secondary metabolite profiles. However, the exclusivity of the tsitsikammamines to *T. favus* would imply that there would be differences in the microbial distribution if the associated microbes were in some way linked to the secondary metabolite production. Additionally, Latrunculid sponges (including *T. scurra*, *T. favus* and the *Latrunculia sp.*) are known to produce pyrroloiminoquinones including discorhabdins. The microbial community of the *Latrunculia sp.* was therefore hypothesised to have some similarities to the *Tsitsikamma* sponges, whereas the

yellow encrusting sponge (family *Mycaliidae*) would have a very diiferent associated microbial population.

Investigations into Latrunculid sponges collected in Algoa Bay, including *T. scurra* (and its associated yellow encrusting sponge) and *Latrunculia sp*. that grew in close proximity to *T. favus*, revealed that the bacterial communities associated with these sponge species were not only highly conserved but also species specific. Initial DGGE analysis revealed that each of the sponge species investigated had a distinct and conserved DGGE 'fingerprint' and this became the most reliable molecular technique for distinguishing between sponge species. Further investigation into *T. favus* and *T. scurra* using NGS revealed that the *Tsitsikamma* sponges shared a dominant 'Spirochaete' species (OTU003) in common, as well as some other dominant OTUs, albeit as different relative percentages of the associated bacterial population. The *Latrunculia sp.* was dominated by a single betaproteobacterial species (OTU002) that represented over 19% of the sponge associated bacterial community and was also present in *T. favus* (at approximately 1.8% abundance). The yellow encrusting sponge so closely associated with *T. scurra* had a different bacterial community to that of *T. scurra* (except for *T. scurra* specimen Sp07 and yellow encrusting sponge Sp08 where cross contamintation between the two sponges was assumed to have occurred due to the difficulty involved in separating them). The Algoa Bay sponges investigated appeared to have conserved and species specific bacterial communities, with the two species within the genus *Tsitsikamma* sharing similar, yet distinct, profiles.

It can be concluded that despite their close proximity, the sponge species investigated harboured distinct and species specific bacterial communities that were stable over time. This would imply that their associated bacterial community is not a product of their environment, but that it has been selected and conserved. The results for the DGGE and OTU analysis appeared to be the most congruent. The consistently faint DGGE band associated with the betaproteobacterial clone Sp02-1 would suggest that its abundance within the *T. favus* associated bacterial population is not as dominant as suggested by the clone library. However, the results obtained from the clone library analyses cannot be discredited as the

betaproteobacterial clone Sp02-1 may still play a significant role in the *T. favus* associated microbial community.

The phylum Porifera is known to produce many biologically active compounds that have been isolated and studied since the 1950's (Bergmann & Feeney, 1950), with 287 new compounds isolated from sponges in 2009 alone (Blunt *et al.,* 2011). Marine microorganisms are also proving to be an invaluable source for the isolation of novel pharmaceutically relevant compounds (273 new compounds were reported in 2009, Blunt *et al.,* 2011). There is increasing evidence that sponge 'symbionts' may be responsible for the biologically active compounds previously attributed to the sponge itself (Bewley *et al.,* 1996; Flowers *et al.,* 1988; Stierle *et al*., 1988; Thomas *et al*., 2010). Attempts were made to culture the *T. favus* associated bacterial community, with a focus on the betaproteobacterium Sp02-1. A *Pseudomonas ascidiaceicola* strain was isolated due to its dominance on the MA2216 plates and its ability to produce a dark brown pigment which resembled the dark brown colour of the pyrroloiminoquinone compounds isolated from Latrunculid sponges. However, fermentation studies did not indicate the presence of pyrroloiminoquinones and instead trace amounts of toluenediamine, a mild antimicrobial recently isolated from *Pseudovibrio sp.* D323, were detected. A salt tolerant *Staphylococcus* species was also isolated, growing in conjunction with what appeared to be the betaproteobacterial isolate Sp02-1, but this could not be confirmed and it was assumed that attempts to culture the *T. favus* associated betaproteobacterium were unsuccessful.

An alternate approach involved the isolation of actinomycetes from the Latrunculid sponges despite their low abundance within the Algoa Bay sponges, as indicated by both the clone library and 454 pyrosequencing. Actinomycetes are well known for their production of bioactive secondary metabolites and a study by Miyanaga *et al.* (2011) revealed a unique hybrid NRPS-PKS locus that was assigned to the production of the pyrrolo[4,3,2-*de*]quinoline core characteristic of pyrroloiminoquinone metabolites in the marine actinomycetes *S. tropica* and *S. arenicola.* The actinomycete isolates were screened for antimycobacterial activity, with the *Kocuria* isolates (RUSp02-3a and RUSp02-3b) and *Streptomyces* isolate (RU1B-2) showing the most promise. A number of *Kocuria* strains were isolated,

but with varying activity against *M. aurum.* This reinforces the theory that it is very important to check the antibiotic producing profile of all isolates, even those belonging to the same species, as failure to do so could mean missing out on a strain with new and interesting antibiotic activity.

The bacterium responsible for the production of the pyrroloiminoquinones and/or their precursors was not conclusively isolated. Perhaps a yet uninvestigated isolate is producing the compounds of interest and/or a number of bacteria are producing the compounds in unison. Alternately, maybe the conservation of the bacterial communitites within the Latrunculid sponges is not because they are responsible for the production of the pyrroloiminoquinones but rather because the presence of different pyrroloiminoquinone compounds within the sponge species is shaping their associated bacterial communities.

6.2 Future Prospects

The microbial communities associated with the Algoa Bay Latrunculid sponges appeared to be conserved and species specific. The 16S rDNA gene is present in all bacterial species and provided an indication of the total bacterial diversity present in the sponge. OTU analysis of the 16S rDNA gene fragment (to the species level) revealed which bacterial species were the most abundant, but most abundant does not necessarily imply that it is the most metabolically active. Cellular concentrations of 16S rRNA are generally correlated with growth rate and activity (DeLong *et al.*, 1989; Poulsen *et al.*, 1993) hence the rRNA itself can yield useful information about which community members are active. The generation of RNA libraries would provide insight as to which members of the complex bacterial community are most active, and assist in differentiating between symbiotic and transient bacteria with the assumption that the symbiotic bacteria would form a more active part of the sponge community than the transient bacteria which are passing through and/or being digested.

Attempts at culturing the dominant *T. favus* associated bacteria, in an attempt to isolate a bacterium responsible for the production of the associated pyrroloiminoquinones and/or their precursors, were unsuccessful. The isolation of

the actinomycetes proved to be more successful from a bioprospecting point of view, and several of the relatively small number of actinomycetes isolated during this study were shown to have antimycobacterial activity. Further studies into the fermentation of these isolates and the extraction and elucidation of the secondary metabolites are currently underway.

Many bacteria associated with sponges have not been cultured despite considerable effort. Alternative strategies, such as genomic approaches aimed at isolating biosynthetic genes and expressing them in surrogate hosts, are therefore necessary. This involves the construction and screening of metagenomic libraries, in which large pieces of DNA isolated from mixed populations without prior cultivation are cloned and screened for targeted genes. A metagenomics approach by Miyanaga *et al.* (2011) revealed a unique hybrid NRPS-PKS locus that has been assigned to the production of the pyrrolo[4,3,2-*de*]quinoline core characteristic of pyrroloiminoquinone metabolites in *S. tropica* and *S. Salinispora*. A similar study into the genes responsible for pyrroloiminoquinone production in Latrunculid sponges could prove to be very insightful.

The South African coastline, like the country itself, is rich in species diversity and is also relatively underexplored. Other marine invertebrates, including Ascidians (phylum Chordata) and Nudibranchs (phylum Mollusca) are also prolific along this coastline and are of interest due to their production of biologically active secondary metabolites. Research into the secondary metabolite production and associated bacterial communities of the Porifera, Chordata and Mollusca discovered along the South African coastline would provide invaluable insight into the potential of these invertebrates, and their associated bacterial communities, as sources of novel compounds.

6.3 References

Antunes, E.M. (2003). Pyrroloiminoquinone metabolites from South African Latrunculid sponges. *Chemistry.* Grahamstown: Rhodes University.

Antunes, E.M., Beukes, D.R., Kelly, M., Samaai, T., Barrows, L.R., Marshall, K.M., Sincich, C. & Davies-Coleman, M.T. (2004). Cytotoxic Pyrroloiminoquinones from Four New Species of South African Latrunculid Sponges. *Journal of Natural Products,* 67: 1268-1276.

Antunes, E.M., Copp, B.R., Davies-Coleman, M.T. & Samaai, T. (2005). Pyrroloiminoquinones and related metabolites from marine sponges. *Natural Products Reports,* 22: 62-72.

Bergmann, W. & Feeney, R.J. (1950). The isolation of a new thymine pentoside from sponges. *Journal of the American Chemical Society,* 72: 2809-2810.

Bewley, C.A., Holland, N.D. & Faulkner, D.J. (1996). Two classes of metabolites from<i>Theonella swinhoei</i> are localized in distinct populations of bacterial symbionts. *Cellular and Molecular Life Sciences,* 52**:** 716-722.

Blunt, J.W., Copp, B.R., Munro, M.H.G., Northcote, P.T. & Prinsep, M.R. (2011). Marine natural products. *Natural Product Reports,* 28**:** 196-268.

Erwin, P. M., Olson, J.B., Thacker, R.W. (2011). Phylogenetic diversity, host specificity and community profiling of sponge-associated bacteria in the northern gulf of Mexico. *PLoS ONE,* 6: 1-16

Flowers, A.E., Garson, M.J., Webb, R.I., Dumdei, E.J. & Charan, R.D. (1998). Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). *Cell and Tissue Research,* 292**:** 597-607.

Hardoim, C.C.P., Costa, R., Araujo, F.V., Hajdu, E., Peixoto, R., Lins, U., Rosado, A.S. & Van Elsas, J.D. (2009). Diversity of Bacteria in the Marine Sponge *Aplysina fulva* in Brazilian Coastal Waters. *Applied Environmental Microbiology,* 75: 3331-3343.

Hentschel, U., Hopke, J., Horn, M., Friedrich, A.B., Wagner, M. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied Environmental Microbiology,* 68: 4431–4440.

Hill, M., Hill, A., Lopez, N., Harriott, O. (2006). Sponge-specific bacterial symbionts in the Caribbean sponge, *Chondrilla nucula* (Demospongiae, Chondrosida). *Marine Biology*, 148: 1221–1230.

Holmes, B., Blanch, H. (2007). Genus-specific associations of marine sponges with group I crenarchaeotes. *Marine Biology*, 150: 759–772.

Ishibashi, M., Iwasaki, T., Imai, S., Sakamoto, S., Yamaguchi, K. & Ito, A. (2001). Laboratory culture of the myxomycetes: Formation of fruiting bodies of *Didymium bahiense* and its plasmodial production of makaluvamine A. *Journal of Natural Products,* 64: 108-110.

Lee, O.O., Wong, Y.H., Qian, P.Y. (2009). Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. *Applied and Environmental Microbiology*, 75: 3513–3521.

Lee, O.O., Wang, Y., Yang, J., Lafi, F.F., Al-Suwailem, A. & Qian, P.-Y. (2010). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. ISME J 5: 650-664.

Li, Z.Y. & Liu, Y. (2006). Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis. *Letters in Applied Microbiology* 43: 410-416.

Li, Z., Hu, Y., Liu, Y., Huang, Y., He, L. (2007) 16S rDNA clone library-based bacterial phylogenetic diversity associated with three South China Sea sponges. *World Journal of Microbiology Biotechnology*, 23: 1265–1272.

Miyanaga, A., Janso, J.E., Mcdonald, L., He, M., Liu, H., Barbieri, L., Eustaì•Quio, A.S., Fielding, E.N., Carter, G.T., Jensen, P.R., Feng, X., Leighton, M., Koehn, F.E. & Moore, B.S. (2011). Discovery and assembly-line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in *Salinispora* bacteria. *Journal of the American Chemical Society,* 133: 13311-13313.

Olson, J.B., McCarthy, P.J. (2005). Associated bacterial communities of two deepwater sponges. *Aquatic Microbiology and Ecology,* 39: 47–55.

Radwan, M., Hanora, A., Zan, J., Mohamed, N., Abo-Elmatty, D., Abou-El-Ela, S. & Hill, R. (2010). Bacterial Community Analyses of Two Red Sea Sponges. *Marine Biotechnology,* 12**:** 350-360.

Simister, R.L., Deines, P., Botte, E.S., Webster, N.S. & Taylor, M.W. (2012). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environmental Microbiology*, 14:517-524.

Sipkema, D. & Blanch, H.W. (2010). Spatial distribution of bacteria associated with the marine sponge *Tethya californiana*. *Marine Biology,* 157: 627-638.

Stierle, A.C., Cardellina, J.H. & Singleton, F.L. (1988). A marine *Micrococcus* produces metabolites ascribed to the sponge *Tedania ignis*. *Experientia,* 44**:** 1021.

Taylor, M.W., Radax, R., Steger, D. & Wagner, M. (2007). Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiology and Molecular Biology Reviews,* 71: 295-347.

Thomas, T.R.A., Kavlekar, D.P. & Lokabharathi, P.A. (2010). Marine Drugs from Sponge-Microbe Associations - A Review. *Marine Drugs,* 8**:** 1417-1468.

Trindade-Silva, A.E., Rua, C., Silva, G.G.Z., Dutilh, B.E., Moreira, A.P.B. (2012). Taxonomic and Functional Microbial Signatures of the Endemic Marine Sponge Arenosclera brasiliensis. *PLoS ONE*, 7(7): e39905

Turque, A.S., Cardoso, A.M., Silveira, C.B., Vieira, R.P., Freitas, F.A.D. (2008). Bacterial communities of the marine songes Hymeniacidon heliophila and Polymastia janeirensis and their environment in Rio de Janeiro, Brazil. *Marine Biology*, 155: 135–146.

Wang, Y. & Qian, P. (2009) Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. *PLoS One* 4(10): e7401.

Webster, N.S., Taylor, M.W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M. & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology,* 12: 2070-2082.

White, J.R., Patel, J., Ottesen, A., Arce, G., Blackwelder, P. (2012). Pyrosequencing of Bacterial Symbionts within Axinella corrugata Sponges: Diversity and Seasonal Variability. *PLoS ONE,* 7(6): e38204.

APPENDICES

Appendix A1 – Media recipes

ISP2 (International Streptomyces Project isolation media 2): Bacto yeast extract (4 g), bacto malt extract (10 g), bacto dextrose (4 g), sodium chloride (20 g) and bacto agar (16 g).

ISP5 (International Streptomyces Project isolation media 5**):** L-asparagine monohydrate (1 g), glycerol (10 g), potassium hydrogen phosphate (1 g), trace salts solution (1 ml), sodium chloride (15 g) and bacto agar (16 g). The trace salts solution contained iron sulphate heptahydrate (0.1 g), manganese chloride tetrahydrate (0.1 g), zinc sulphate heptahydrate (0.1 g), double distilled water (100 ml).

R2A: Bacto yeast extract (1 g), peptone (1 g), casamino acids (1 g), glucose (1 g), starch (1 g), sodium tartrate (0.6 g), potassium hydrogen phosphate (0.6 g), magnesium sulphate heptahydrate (0.1 g), sodium chloride (15 g) and bacto agar (16 g).

M1 Agar: Starch (10 g), bacto yeast extract (4 g), bacto peptone (2 g), sodium chloride (15 g) and bacto agar (16 g).

SCN (Starch Casein Nitrate agar): Starch (10 g), Casein (0.3 g), potassium nitrate (2 g), magnesium sulphate heptahydrate (0.05 g), calcium carbonate (0.3 g), iron sulphate heptahydrate (0.01 g), sodium chloride (15 g) and bacto agar (16 g).

MA2216 solid media: MA2216 Broth (34 g, Difco), and bacto agar (16 g).

Appendix A2

Permission letters from the Editor of *Marine Biotechnology* to use figures and excerpts from Walmsley *et al.* (2012): Diversity of bacterial communities associated with the Indian Ocean sponge *Tsitsikamma favus* that contains the bioactive pyrroloiminoquinones, tsitsikammamine A and B. *Marine Biotechnology,* 14:681-691.

SPRINGER LICENSE
TERMS AND CONDITIONS

Jun 06, 2012

This is a License Agreement between Tara Walmsley ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2923010371530
License date	Jun 06, 2012
Licensed content publisher	Springer
Licensed content publication	Marine Biotechnology
Licensed content title	Diversity of Bacterial Communities Associated with the Indian Ocean Sponge *Tsitsikamma favus* That Contains the Bioactive Pyrroloiminoquinones, Tsitsikammamine A and B
Licensed content author	Tara A. Walmsley
Licensed content date	Jan 1, 2012
Type of Use	Thesis/Dissertation
Portion	Excerpts
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	None
Title of your thesis / dissertation	Investigating the link between microbial diversity and secondary metabolite production in endemic South African marine sponges
Expected completion date	Sep 2012
Estimated size(pages)	300
Total	0.00 USD

Terms and Conditions

Introduction
The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business

Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other

person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the 'Netherlands Arbitrage Instituut' (Netherlands Institute of Arbitration).***OR:***

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other terms and conditions:

v1.3

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK500793593.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support:
customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

SPRINGER LICENSE
TERMS AND CONDITIONS

Jun 06, 2012

This is a License Agreement between Tara Walmsley ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2923000981272
License date	Jun 06, 2012
Licensed content publisher	Springer
Licensed content publication	Marine Biotechnology
Licensed content title	Diversity of Bacterial Communities Associated with the Indian Ocean Sponge *Tsitsikamma favus* That Contains the Bioactive Pyrroloiminoquinones, Tsitsikammamine A and B
Licensed content author	Tara A. Walmsley
Licensed content date	Jan 1, 2012
Type of Use	Thesis/Dissertation
Portion	Figures
Author of this Springer article	Yes and you are the sole author of the new work
Order reference number	
Title of your thesis / dissertation	Investigating the link between microbial diversity and secondary metabolite production in endemic South African marine sponges
Expected completion date	Sep 2012
Estimated size(pages)	300
Total	0.00 USD
Terms and Conditions	

Introduction
The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use

indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with

respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the 'Netherlands Arbitrage Instituut' (Netherlands Institute of Arbitration).***OR:***

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other terms and conditions:

v1.3

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK500793589.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.