# Isolation, Diversity and Antimicrobial Activity of Sponge-Associated Bacteria from South Australian Marine Environments

by

Yitayal Shiferaw Anteneh

*Thesis*

*Submitted to Flinders University*

*for the degree of*

**Doctor of Philosophy**

**College of Medicine and Public Health**

**May,2020**

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

# Table of contents

# List of Tables

# List of Figures

## Acknowledgments

Firstly, I would like to thank my supervisors Prof. Christopher Franco and Prof. Melissa Brown who have supported me during my research project. They provided me all possible guidance and advice for my PhD project. I greatly appreciate their patience and suggestions for my writing.

Secondly, I would like to thank Australian Government for sponsoring my PhD study through the Australian Government Research Training Program International Fee Offset Scolarship.

Special thanks to sponge collection team Mr. John Naumann and Mr. Matt (Flinders University Marine Archaeology Department) and Mr Peng Su for teaching me most of the equipment in the lab. I also would like to thank Dr. Hanna Krysinska and Kushari Burns for their excellent assistance. Thanks to Onuma Kaewkla, Evita Chrisnayanti, Qi Young, Elmo Thomas, Tivie for helping me, sharing ideas and having a happy time in the laboratory.

Thanks for Dr Mike Perkins and Mr Jesson (Flinders University) for NMR and mass analysis of the extract. I also thank Dr Adarsha Gupta helping me for GC lipid analysis and Dr, Qi Yang for sponge samples identification and sponge microbiome analysis (Both Flinders University)

Thanks to my family, my wife, Tigist , and my daughter , Soliana, who have made me to forget all of the difficult time during this long journey.

# Abbreviations

| | |
|---|---|
| AFB | Acid.Fast Bacillus |
| AFLP | Amplified Fragment Length Polymorphism |
| AMR | Antimicrobial resistance |
| ARDRA | Amplified Ribosomal DNA Restriction Analysis |
| ATCC | American Type Culture Collection |
| BCRC | Bioresource Collection and Research Centre |
| BLASTN | Basic Local Alignment Search Tool Nucleotide |
| BP | Base pair |
| b.p. | Boiling point |
| °C | Degree centigrade |
| CFU | Colony forming units |
| DAP | Diaminopimelic acid |
| DNA | Deoxyribonucleic acid |
| DSM | German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) |
| F | Forward |
| FAME | Fatty Acid Methyl Ester |
| FISH | Fluorescence in situ hybridization |
| GB | Glenelg Block |

| | |
|---|---|
| GC | Gas Chromatography |
| G+C | Guanine + Cytosine |
| HPLC | High performance liquid chromatography |
| Hrs | Hours |
| IJSEM | International Journal of Systematic and Evolutionary Microbiology |
| ITS | Internal transcribed spacer |
| ISP2 | International Streptomyces Project 2 |
| JCM | Japan Collection of Microorganisms |
| KCTC | Korean.Collection.for Type.Cultures |
| LC-MS | Liquid Chromatography - Mass Spectrometry |
| M | Molar |
| MHz | MegaHertZ |
| MBC | Minimum Bactericidal Concentration |
| Min | Minute |
| MIC | Minimum Inhibitory Concentration |
| ml | Millilitre |
| mM | Milli molar |
| N | Normal |
| NCBI | National Cntre for Biotechnology Information |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| PEG | Polyethylene Glycol |
| PFGE | Pulse Field Gel Electrophoresis |
| R | Reverse |
| RAPD | Randomly Amplified Polymorphic DNA |
| RB | Rapid Bay |
| Rep-PCR | Repetitive Extragenic Palindromic Polymerase Chain Reaction |
| RF | Retention Factor |
| RFLP | Restriction Fragment Length Polymorphism |
| RO | Reverse Osmosis |
| Rpm | Revolution Per Minute |
| SA | South Australia |
| sp. | Species (singular) |
| spp. | Species (plural) |
| TBE | Tris Borate EDTA |
| TLC | Thin Layer Chromatography |
| TSI | Triple Sugar Iron |
| ΔTm | Change in Temperature |
| µl | Microliter |
| V | Volt |

# Summary

The world is in the search of novel antibiotics from various sources as current epidemics due to multidrug-resistant microbes threaten the existence of life. Since the terrestrial environments have been exploited for many years for the search of compounds, the probability of getting new active compounds from these sources is low. Therefore, considering less explored sources is essential and marine sponges have received some attention as novel biologically active compounds are increasingly reported from them. Most studies claimed that many of the current discovered active metabolites from marine sponges originated from associated symbionts rather than the host sponges. As a result, it is important to study the diversity of microorganisms from sponges from various sources. This study was designed to isolate bacteria from marine sponges of South Australia to investigate their diversity, factors affecting diversity and antimicrobial activities of the isolates against human microbial pathogens. Previous molecular-based studies in this area indicated more than 90% of the microbial community in the sponges are unculturable. This study attempted to culture this bacterial population by employing multiple cultivation strategies.

Twelve sponge samples were collected from Rapid Bay and Glenelg beaches of South Australia at a depth of 10-15 m. The bacterial population was isolated using seven primary isolation agar media which were incubated at different temperatures and oxygen levels over 3 months. Bacterial isolates were identified and categorized with the help of microscopic and macroscopic features, and restriction fragment length polymorphism (RFLP) analysis of their 16S rRNA gene PCR products. Subsequently, selection of bacterial isolates were identified using their 16S rRNA gene sequence. The bacterial isolates were tested for antimicrobial activities against human pathogenic bacteria and fungi. For selected active strain, antimicrobial produced in large scale, purified and identified.

A total of 1234 bacterial colonies were isolated, and these were categorised into 383 types based on micro and macroscopic features. Enzymatic digestion of 16S rRNA gene PCR products produced 38 RFLP patterns. 16S rRNA gene sequencing of representatives from each pattern identified four phyla; Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes, containing 21 genera. Actinobacteria was the most dominant phylum isolated, while *Streptomyces* was the most frequent genus isolated. Three novel bacterial isolates and one novel fungus were identified using polyphasic taxonomy  Comparison of culture-dependent and independent approaches revealed discrepancies, where genera observed by isolation not seen by next generation sequencing.

A significant variation of bacterial diversity was observed depending on the growth media, sponge collection sites, sponge types, time of incubation and incubation conditions. Furthermore, the bacterial isolates markedly varied depending on their temperature tolerance and NaCl preference for growth.

A total of 169 bacteria were tested for antimicrobial activities against human pathogenic bacteria and fungi. Approximately 41% of the tested bacterial strains displayed antimicrobial activities. Specifically, 37% of the bacterial isolates showed activity against multidrug-resistant *Staphylococcus aureus*, and 21% of them produced an antifungal activity at least against one of the tested fungi. Antimicrobial production was highly influenced by media type and incubation period. A novel water soluble antibiotic effective against multidrug-resistant *Staphylococcus aureus* was discovered.

The findings of the study indicated that the marine sponges of South Australia are the sources of novel and highly diverse bacterial strains. The isolation approaches helped to report 5 genera which were not reported in previous related studies. Furthermore, the approaches resulted in the isolation of many uncommon genera at a time compared to similar studies. The success of isolation of highly diverse and novel bacterial isolates depends on various factors and considering them during future studies helps to reflect the true picture of the culturable bacterial population from sponges.

Antimicrobial screening and further analysis indicated that the bacterial isolates from these sites could be alternative sources for the search of new antimicrobials effective against multidrug-resistant microorganisms. Other colleagues in the research group also reported herbicidal and anticancer activities from these bacterial strains. They also produced biologically active enzymes such as alginate lyase. These diverse biological activities may stimulate many scholars to exploit the area for the search of different biological applications of sponge-associated bacteria.

# Chapter one: Introduction and literature review

## 1.1 Marine sponges

### 1.1.1 Sponge distribution, biology, and physiology

Marine sponges which comprise the phylum Porifera, are one of the earliest living forms that exist in the world (~630 million years) ( Gutleben *et al.*, 2020; Maloof *et al.*, 2010). Factors such as the ability to adapt to various environmental changes and the effect of competition with other organisms, allowed sponges to maintain their continual existence in the marine environment for many eons (W. Müller & I. Müller, 2003a). Sponges are obligate marine organisms that attach themselves to various substrates and filter out large amounts of water to obtain the required food particles. They are sedentary filter-feeders, able to pump thousands of litres of water each day (Bell, 2008), and some claim the ability of sponges to absorb dissolved organic matter (Goeij *et al.*, 2008). Marine sponges have a worldwide distribution (Figure 1.1) and are vital members of underwater communities. They are higher in number and diversity compared to other marine organisms, and even the sponge species diversity outnumbers the combined species diversity of other organisms in the community (Van Soest *et al.*, 2012). According to the world Porifera databases (available: www.marinespecies.org/porifera), there are more than 8,370 species of sponges spread among 680 genera and four different classes including Calcarea, Demospongiae, Hexactinellida, and Homoscleromorpha (Gazave *et al.*, 2010). The class Demospongiae alone represents about 83% of the total valid species. Except for Spongillina, the suborder of Demospongiae, which resides in freshwater, the majority of sponges reside in seawater (Van Soest *et al.*, 2012).

### 1.1.2 Sponges cellular organization

As indicated in Figure 1.2, sponges have a simple cellular organization having specialized cells with various functions, which themselves are not assembled into either tissues or organs. All sponges are equipped with pinacocytes, T-shaped or flattened cells, which cover the exterior of the sponge, internal system of canals, and microscopic chambers. These chambers are lined by choanocytes, flagellated cells which help to generate water currents important for the filtering function of the sponges. Mesohyl is a collagenous matrix which fills the space between canals and chambers, and is

composed of supporting fibres and inorganic structures of the skeleton; they also contain a large number of symbiont microbes (Van Soest *et al.*, 2012).

Removed due to copyright restriction

Figure 1.1 Worldwide distribution of marine sponges, taken from Van Soest *et al.*, 2012.

### 1.1.3 Sponge skeletons

Marine sponges appear in various sizes and morphological forms depending on the nature of internal minerals and organic skeletons produced by specialized cells. If present, sponge skeletons are mainly composed of spicules (discrete siliceous or calcareous elements) (Müller *et al.*, 2007), sponging (organic collagenous fibres), and in a few occasions aspicular (massive limestone constructions) as presented in Figure 1.3 (Van Soest *et al.*, 2012). The skeletal organisation varies depending on the class of the sponge. Calcareous spicules seen in the class Calcarea, siliceous spicules are observed in the class Hexactinellida and the class Demospongia and Homoscleromorpha contain either the combination of siliceous spicules and spongin or aspiculate which contain spongin skeletons.


Figure 1.2 Ultrastructure of marine sponge.

(http://www.okc.cc.ok.us/biologylabs/documents/Porifera_Cnidaria/Porifera.htm)

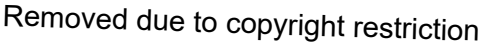



Figure 1.3 Sponge skeletal components. **a** calcareous spicules; **b** siliceous spicules; **c** spongin.

(http://www.okc.cc.ok.us/biologylabs/documents/Porifera_Cnidaria/Porifera.htm)

The consistency of sponges varies depending on the type and density of the building blocks. They may appear as soft, compressible, fragile or hard. They also exist in different forms such as flat and cup-shaped and their sizes range from millimetres to meters. Sponges have several macroscopic and microscopic openings and their shape varies among different species and genera and even within the same species depending on the external factors (Van Soest *et al.*, 2012).

### 1.1.4 Sponge physiology

Unlike other multicellular organisms, marine sponges lack organized systems and organs but rather have an aquiferous system which serves as circulatory, digestive and excretory systems comparable to the one observed in higher organisms. Most of the mature sponges are immobilized filter feeders that can filter different materials including micro-eukaryotes, bacteria, and particulate matter from seawater and pump them via the canal systems onto their bodies. Diffusion is a means by which oxygen enters the body of sponge and food particles engulfed by phagocytic cells in mesohyl and waste are removed in the constant water current throughout the body. It has been indicated on a daily basis that sponges pump about 24,000 L of water per kg of their mass (Taylor *et al.*, 2007). On the contrary, some deep-sea sponge species (~120 species) are unable to filter feed and are carnivores where they capture their food particles with hook-like structures (Van Soest *et al.*, 2012).

Sponges do not have adaptive immunity, though they present an innate immunity including immunoglobulin and interferon-like systems (W. Müller & I. Müller, 2003b; Bibi *et al.*, 2017), and compounds with anti-microbial and anti-inflammatory activities have been identified from sponge tissues. It is not clear from where the sponge-associated metabolites originate though marked similarities have been observed to compounds produced from microbes (Bibi *et al.*, 2017).

## 1.2 Sponge microbiome

The mesohyl part of most sponges is heavily populated by microbial symbionts which are consist of bacteria, fungi, viruses, and archaea (Taylor *et al*. 2007; Webster and Taylor 2012; Webster *et al.*, 2013). Depending on the amount of microbial community the sponges have, they are broadly classified as high and low microbial abundance sponges. In the former group, the microbial mass accounts for about $10^8$-$10^{10}$ bacteria per gram of sponge wet weight (Hentschel *et al.*, 2006), which surpasses by many folds the amount that exists in the surrounding seawater (Friedrich *et al.*, 2001), and in most cases, the microbial symbionts represent about 40-60% of the sponge biomass (Hill *et al*., 2006). In the latter category, low amounts of microorganisms ($10^5$-$10^6$ bacteria per gram of sponge wet weight) exist, which is comparable to the amount found in natural seawater (Hentschel *et al.*, 2006).

In most cases, the symbiont microbial community in sponges are preserved as they are vertically inherited from parent sponges during reproduction (Ereskovsky *et al.*, 2005). These microbial communities in sponges are stable, sponge-specific and vary greatly from transit bacteria. Frequently, genetically related sponge from different geographical areas presented similar symbiont microbiota, which proposed the existence of a specific association between microbial symbionts and host sponges (Thoms *et al*., 2003; Reveillaud *et al*., 2014).

The symbionts assist the sponges in metabolic waste processing, and protection from various chemicals and infectious agents (Taylor *et al*., 2007; Bayer *et al.*, 2008; Hoffmann *et al*., 2009; Thomas *et al*., 2010; Webster & Taylor, 2012; Bibi *et al.*, 2017; Gogineni & Hamann, 2018). The host, in exchange, provides vital nutrients including the source of protein and carbohydrates. As far as the bacteria resist killing by sponges, the mesohyl is by far the most nutrient-rich environment for them compared to the surrounding water body. The occurrence of a large and highly diverse microbial community in the mesohyl encourages various forms of interaction among microorganisms as well as the host sponges (Taylor *et al.*, 2004b). Synthesis of defence chemicals allows bacteria to survive more in sponges' environment. It is indicated that the majority of defence chemicals isolated from sponges mainly originated from microbial symbionts (Proksch *et al.*, 2010; Freeman *et al.*, 2012).

The presence of a large diversity of microorganisms in sponges is documented in several studies including observation under electron microscopy (Wilkinson, 1978), and microbial cultivation in artificial media (Webster & Hill, 2001a). In addition to these, more advance methods like 16S rRNA gene amplification and fluorescence *in situ* hybridization (FISH) exposed the presence of large and uncommon microbial community in sponges which have escaped detection by routine culture systems (Hentschel *et al.*, 2002; Olson & McCarthy, 2005). Though most of the microbial community in sponges were not cultured, different sequence-based studies revealed sponges associated with more than 26 phyla (Webster *et al*., 2010; Lee *et al*., 2011). Even recent massive DNA sequencing studies reported the existence of more than 47 bacterial phyla (Reveillaud *et al*., 2014).

The application of universal primers for the 16S rRNA gene allowed for several years for the discovery of numerous phylogenetically complex microbial communities from marine sponges of various locations (Hentschel *et al.*, 2002; Hentschel *et al.*, 2006). These diverse groups of microorganisms

are mostly affiliated with the following phyla: Proteobacteria (α and ϒ), Actinobacteria, Acidobacteria, Firmicutes, Bacteroidetes, and Chloroflexi (Taylor *et al*., 2007a; Webster *et al*., 2010; Hentschel *et al.*, 2012; Schmitt *et al*.,2012). Furthermore, the application of 16S rRNA gene analysis helped in the identification of a new phylum of Poribacteria from sponges (Fieseler *et al.*, 2004). Apart from genetic studies, various phyla of bacteria including Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes, and Verrucomicrobia were cultured from marine sponges (Burja & Hill, 2001; Zhang *et al.*, 2006; Abdelmohsen *et al.*, 2010). Unlike marine sponges, very low diversity of bacteria was reported from freshwater sponges with only some representatives of α and β Proteobacteria, Chloroflexi, and Actinobacteria (Gernert *et al.*, 2005).

Among the many bacteria isolated from marine sponges, bacteria from the phylum Actinobacteria are important as this bacterial group is well-known for the production of pharmaceutically-active metabolites (Blunt *et al.*, 2018). Different studies with the application of both culture-dependent and culture-independent approaches identified various bacterial genera of Actinobacteria from marine sponges (Yang *et al*.,2015; Liu *et al.*, 2019). The most common reported genera of these groups include *Streptomyces, Micromonospora, Gordonia*, *Pseudonocardia, Micrococcus, Actinoalloteichus, Rhodococcus, Nocardia, and Nocardiopsis* (Baig *et al.*, 2020). Ellis *et al* (2017) isolated several Actinobacteria including *Streptomyces, Nocardiopsis, Micromonospora,* and *Verrucosispora* from marine sponges (Ellis et al., 2017). Remarkably, some Actinobacteria like *Salinispora* previously only reported from marine sediment have now been isolated from sponges (Bose *et al.*, 2017). *Salinispora* is the first Actinobacterium isolated specifically from sponges in the presence of seawater (Jensen *et al*., 2007; Bose *et al.*, 2017). One species of *Salinispora, Salinispora tropica* devoted the majority of its genome to secondary metabolites synthesis, which is higher than the previously documented Actinobacteria (Udwary *et al.*, 2007; Doroghazi & Metcalf, 2013). Marine sponges are not only the source of common Actinobacteria genera but also the source of rare and new Actinobacteria genera including *Verrucosispora, Pseudonocardia, Actinokineospora*, *Amycolatopsis*, *Saccharopolyspora, Nonomuraea*, *Actinomadura, Saccharomonospora*, and *Knoellia.* We recommend a review by Abdelmohsen *et al*. (2014) for these and more diverse Actinobacteria from marine sponges (Abdelmohsen *et al.*, 2014). Table 1.1 presents some of the recent novel bacteria isolated from marine sponges.

Table 1.1 Some examples of recent novel bacteria from marine sponges.

| Sponges type | Geographic area | Name of the isolates | Phylum | References |
|---|---|---|---|---|
| *Spongin* | South Korea | *Aquimarina spongiicola* sp. nov. | Bacteroidetes | (Choi *et al.*, 2018) |
| *Arenosclera brasiliensis* | Rio de Janeiro Brazil | *Pseudovibrio brasiliensis* sp. nov. | Proteobacteria | (Fróes *et al.*, 2018) |
| *Verongula gigantea* | West coast of San Salvador, The Bahamas | *Sansalvadorimonas verongulae* gen. Nov., sp. nov. | Proteobacteria | (Goldberg *et al.*, 2018) |
| *Haliclona* | Justus Liebig University Giessen, Germany | *Litorimonas haliclonae* sp. nov. | Proteobacteria | (Schellenberg *et al.*, 2018) |
| Unidentified | Yangpu Bay, Hainan, China | *Zhouia spongiae* sp. nov. | Bacteroidetes | (Zhuang *et al.*, 2018) |
| *Amphimedon viridis* | Praia Guaeca' (Sao Paulo, Brazil) | *Williamsia spongiae* sp. nov. | Actinobacteria | (Afonso de Menezes *et al.*, 2017) |
| *Antho dichotoma* | Trondheim fjord in Norway | *Actinoalloteichus fjordicus sp. nov*. | Actinobacteria | (Nouioui *et al.*, 2017) |
| *Haliclona* | Justus Liebig University of Giessen, Germany | *Winogradskyella haliclonae* sp. nov. | Bacteroidetes | (Schellenberg *et al.*, 2017) |
| *Aplysina fulva* | Archipelago of Saint Peter and Saint Paul (Equatorial Atlantic Ocean) | *Streptomyces atlanticus* sp. nov. | Actinobacteria | (Silva *et al.*, 2016) |
| *Scopalina ruetzleri* | Saint Peter and Saint Paul Archipelago, in Brazil, | *Saccharopolyspora spongiae* sp. nov. | Actinobacteria | (Souza *et al.*, 2017) |

Table 1.1 (Continued)

| Sponges type | Geographic area | Name of the isolates | Phylum | References |
|---|---|---|---|---|
| *Xestospongia sp.* | Andaman Sea, Phuket Province, Thailand | *Nocardia xestospongiae* sp. nov. | Actinobacteria | (Thawai *et al.*, 2017) |
| Unidentified | The coast of Sanya City, PR China | *Streptomyces spongiicola* sp. nov. | Actinobacteria | (Huang *et al.*, 2016) |
| *Rhabdastrella* sp. | Sado Island, Japan. | *Spongiiferula fulva* gen. nov., sp. nov. | Bacteroidetes | (Yoon *et al.*, 2016a) |
| *Unidentified* | Sado Island, Niigata, Japan | *Spongiimicrobium salis gen. nov., sp. nov.* | Bacteroidetes | (Yoon *et al.*, 2016b) |
| *Glodia corticostylifera* | Sa˜o Paulo, Brasil. | *Marmoricola aquaticus* sp. nov. | Actinobacteria | (de Menezes *et al.*, 2015) |
| Unidentified | Chuuk State, Federated States of Micronesia | *Amphritea spongicola* sp. nov. | Proteobacteria | (Jang *et al.*, 2015) |
| *Leucosolenia* sp. | Lough Hyne, Co. Cork, Ireland | *Maribacter spongiicola* sp. nov. | Bacteroidetes | (Jackson *et al.*, 2015a) |
| *Suberites carnosus* | Lough Hyne, Co. Cork, Ireland | *Maribacter vaceletii* sp. nov. | Bacteroidetes | (Jackson *et al.*, 2015b) |
| *Spheciospongia vagabunda* | Red Sea, Ras Mohamed, Sinai, Egypt | *Actinokineospora spheciospongiae sp. nov.* | Actinobacteria | (Kämpfer *et al.*, 2015) |
| unidentified | Uljin County in the coastal area of the East Sea, Korea | *Kiloniella spongiae* sp. nov. | Proteobacteria | (Yang *et al.*, 2015) |
| *Aplysina aerophoba* | Rovinj, Croatia | *Rubrobacter aplysinae* sp. nov. | Actinobacteria | (Kämpfer *et al.*, 2014) |

Table 1.1 (Continued)

| Sponges type | Geographic area | Name of the isolates | Phylum | References |
|---|---|---|---|---|
| *Amphilectus fucorum* | Lough Hyne, County Cork, Ireland | *Aquimarina amphilecti* sp. nov. | Bacteroidetes | (Kennedy *et al.*, 2014) |
| *Tethya* sp. | Kagoshima, Japan | *Spongiivirga citrea* gen. nov. sp. nov. | Bacteroidetes | (Yoon *et al.*, 2014) |
| *Axinella verrucosa* | Israeli coast near Sdot Yam | *Aureivirga marina* gen. nov., sp. nov. | Bacteroidetes | (Haber *et al.*, 2013a) |
| *Axinella polypoides* | Sdot Yam, Israel | *Luteivirga sdotyamensis* gen. nov., sp. nov. | Bacteroidetes | (Haber *et al.*, 2013b) |
| Unidentified | Weno Island, Chuuk State, Federated State of Micronesia | *Microbulbifer pacificus* sp. nov. | Proteobacteria | (Jeong *et al.*, 2013) |
| *Xestospongia* sp. | Andaman Sea, Thailand | *Verrucosispora andamanensis* sp. nov. | Actinobacteria | (Supong *et al.*, 2013a) |
| *Haplosclerida* | Coast of Japan at Numazu | *Endozoicomonas numazuensis sp. nov.* | Proteobacteria | (Nishijima *et al.*, 2013) |
| *Axinella dissimilis* | Ireland | *Pseudovibrio axinellae* sp. nov. | Proteobacteria | (O'Halloran *et al.*, 2013) |
| Unidentified | Gulf of Thailand | *Micromonospora spongicola* sp. nov. | Actinobacteria | (Supong *et al.*, 2013b) |
| Unidentified | Omura Bay, Nagasaki, Japan | *Spongiimonas flava* gen. nov., sp. nov. | Bacteroidetes | (Yoon *et al.*, 2013) |
| *Scleritoderma cyanea* | West coast of Curacao | *Vibrio caribbeanicus* sp. nov. | Proteobacteria | (Hoffmann *et al.*, 2012) |

Table 1.1 (Continued)

| Sponges type | Geographic area | Name of the isolates | Phylum | References |
|---|---|---|---|---|
| *Plakortis simplex* | Bay of Bengal, Gopalpur, Orissa, India | *Planococcus plakortidis* sp. nov. | Actinobacteria | (Kaur *et al.*, 2012) |
| *Xestospongia testudinaria* | Jeddah, Saudi Arabia | *Marinobacter xestospongiae* sp. nov. | Proteobacteria | (Lee *et al.*, 2012a) |
| Unidentified | Numazu City in Japan | *Spongitalea numazuensis* gen. nov., sp. nov. | Bacteroidetes | (Mitra *et al.*, 2012) |
| *Halichondria oshoro* | Jeju Island, Republic of Korea | *Spongiibacterium flavum* gen. nov., sp. nov. | Bacteroidetes | (Yoon & Oh, 2012) |
| Unidentified sponge | Dachan reef, China | *Micromonospora yangpuensis* sp. nov. | Actinobacteria | (Zhang *et al.*, 2012) |
| *Chondrilla nucula* | Florida Keys, USA | *Kangiella spongicola* sp. nov. | Proteobacteria | (Ahn *et al.*, 2011) |
| *Hymeniacidon flavia* | Jeju Island, Republic of Korea | *Sphingomonas jejuensis* sp. nov. | Proteobacteria | (Park *et al.*, 2011) |
| *Hymeniacidon flavia* | Coast of Jeju Island, South Korea. | *Formosa spongicola* sp. nov. | Bacteroidetes | (Yoon & Oh, 2011a) |
| Unidentified | Oshima, Natsudomari Peninsula, Aomori Prefecture, Japan | *Porifericola rhodea* gen. nov., sp. nov. | Bacteroidetes | (Yoon *et al.*, 2011b) |
| *Haliclonae* sp. | Tateyama City, Japan | *Streptomyces tateyamensis* sp. nov., *Streptomyces marinus* sp. nov. and *Streptomyces haliclonae* sp. nov. | Actinobacteria | (Khan *et al.*, 2010) |

Table 1.1 (Continued)

| Sponges type | Geographic area | Name of the isolates | Phylum | References |
|---|---|---|---|---|
| *Axinella polypoides* | Banyuls-sur- Mer, France | *Streptomyces axinellae* sp. nov. | Actinobacteria | (Pimentel-Elardo *et al.*, 2009) |
| *Haliclona sp.* | Cebu, Philippines | *Saccharopolyspora cebuensis* sp. nov. | Actinobacteria | (Pimentel-Elardo *et al.*, 2008) |
| Unidentified sponge | Curaçao, Netherlands Antilles | *Tsukamurella spongiae* sp. Nov. | Actinobacteria | (Olson *et al.*, 2007) |
| *Hymeniacidon perleve* | Dachan reef, China | *Actinoalloteichus hymeniacidonis* sp. nov. | Actinobacteria | (Kwon *et al.*, 2006) |

## 1.3 Overview of active compounds from marine sponges

The marine environment is an abundant source of active natural compounds with biological and pharmaceutical properties. About 9% of the total number of natural compounds listed in the 2006 Chapman and Hall Dictionary of Natural Products have originated from marine environments (Singh & Pelaez, 2008). Each year, the amount of metabolically-active compounds from the marine environment increases and sponges contribute a large share with about 200 new active compounds every year (Mehbub *et al.*, 2014). Mehbub *et al*., (2014) reviewed in-depth the active natural compounds produced from marine sponges and they indicated variation depending on the sponge species. Sponges are considered the largest reservoir of novel metabolites (Blunt *et al.*, 2007; Taylor *et al.*, 2007b; Gogineni & Hamann, 2018). Due to the existing variation of marine environments with the terrestrial counterparts, marine sponges may be exposed to different challenges which stimulate them to produce various biologically-active molecules as defence mechanisms, some of which may be novel (Gogineni & Hamann, 2018). These active metabolites from marine sponges could be nucleoside derivatives, terpenoids, polyethers, alkaloids, macrolides, or peptides with various biological activities including anti-inflammation, anti-cancer and antimicrobial properties (Newman,

2008; Gogineni & Hamann, 2018). Table 1.2 below presents some of the active metabolites from marine sponges.

Table 1.2 Overview of active secondary metabolites from marine sponges.

| **Sponge sources** | **Active compounds** | **Biological activities** | **References** |
|---|---|---|---|
| *Iricinia fusca* | Imidazole | Antibacterial | (Meesala *et al.*, 2018) |
| *Monanchora clathrata* | Monanchoramides | Anticancer | (Raslan *et al.*, 2018) |
| *Topsentia* sp. | Tulongicin | Antibacterial | (Liu *et al.*, 2017) |
| *Tedania anhelans* | Pyrrolo | Antimicrobial | (Visamsetti *et al.*, 2016) |
| *Ircinia* sp. | Indole | Anti-mycobacterial | (Abdjul *et al.*, 2015) |
| *Xestospongia* sp. | Polyacetylene | Antimicrobial | (Ayyad *et al.*, 2015) |
| *Dactylospongia* sp. | Puupehenol | Antioxidant<br>antimicrobial | (Hagiwara *et al.*, 2015) |
| *Dysidea granulosa* | Polybrominated diphenyl ethers | Antimicrobial | (Sun *et al.*, 2015a) |
| *Biemna ehrenbergi* | Ehrenasterol | Antimicrobial | (Youssef *et al.*, 2015) |
| *Echinoclathria gibbosa* | β-sitosterol<br>Pentacosenoate | Antimicrobial<br>Antiinflammatory<br>Anticancer | (Mohamed *et al.*, 2014) |

Table 1.2 (Continued)

| Sponge sources | Active compounds | Biological activities | References |
|---|---|---|---|
| *Dendrilla nigra* | Denigrins A–C | Antitubercular | (Murali Krishna Kumar *et al.*, 2014) |
| *Haliclona crassiloba* | Halicrasterols A–D | Antimicrobial | (Cheng *et al.*, 2013) |
| *Corticium* sp. | Plakinamine M | Anti-Mycobacterium tuberculosis | (Lu *et al.*, 2013) |
| *Agelas* sp. | Nagelamides X-Z | Antimicrobial | (Tanaka *et al.*, 2013) |
| *Petrosid* Ng5 Sp5 | 22(S)-hydroxyingamine A, dihydroingenamine D | Antiplasmodial | (Ilias *et al.*, 2012) |
| *Iotrochota purpurea* | Purpuroines A–J | Antibiotic | (Shen *et al.*, 2012) |
| *Neopetrosia proxima* | Neopetrosiamine A | Antimycobacterial, Anti-malarial | (Wei *et al.*, 2010) |
| *Melophlus sarassinorum* | Sarasinosides | Antimicrobial | (Dai *et al.*, 2005) |
| *Psammaplysilla purpurea* | Purpurealidin | Antimicrobial | (Tilvi *et al.*, 2004) |
| *Arenosclera brasiliensis* | Arenosclerins | Antimicrobial | (Torres *et al.*, 2000) |
| *Xestospongia* sp. | Polyacetylene | Antimicrobial | (Ayyad *et al.*, 2015) |
| *Topsentia* sp. | Tulongicin | Antibacterial | (Liu *et al.*, 2017) |
| *Tedania anhelans* | Pyrrolo | Antimicrobial | (Visamsetti *et al.*, 2016) |

## 1.4 Active metabolites from sponge-associated bacteria

Antimicrobial resistance (AMR) is an emerging global threat, reducing the chance of prevention and curing of infectious diseases due to microbial pathogens (Indraningrat *et al.*, 2016). A recent report from WHO indicated that communicable diseases due to AMR microbes are responsible for vast amount of morbidity and mortality, which could responsible for a significant amount of economic loss, with an estimated to reach about 10 trillion USD in 2050 (WHO, Geneva, Switzerland, 2014) (Indraningrat *et al.*, 2016).

The emergence of multi-drug resistance in common infectious bacteria such as *Mycobacterium tuberculosis* (Projan, 2003), *Pseudomonas aeruginosa* (Moellering, 2011) and *Staphylococcus aureus* (Aminov, 2010) have been detected, which makes it difficult to cure diseases due to these organisms with existing antibiotics. These life-threatening scenarios demand urgent discovery of bioactive metabolites with high clinical significance (Aminov, 2010). The chance of rediscovering the known antimicrobials from the widely searched environments is high (Taylor & Wright, 2008) though few new compounds such as turbomycin A and B (Gillespie *et al.*, 2002) and teixobactin (Ling *et al.*, 2015) were still isolated from soil.

Unlike the terrestrial environment, less attention has been given to the marine environment for the discovery of antibiotics due to accessibility issues. Yet, the marine environment contains a wide variety of novel antimicrobial compounds (Hughes & Fenical, 2010). Recent review indicates the rate of discovery of novel natural compounds from marine bacteria has increased (Blunt *et al.*, 2018) compared to the previous reviews from this group (Blunt *et al.*, 2007; Blunt *et al.*, 2009; Blunt *et al.*, 2013), where the genus *Streptomyces* remains to be the main force behind the discovery of novel and interesting chemistry. Among the many organisms in this environment, marine sponges are associated with a variety of active secondary metabolites that contribute to the improvement of human health (Taylor *et al.*, 2007; Laport *et al.*, 2009; Thomas *et al.*, 2010; Fuerst, 2014).

Due to the requirement of large amounts of biomass for the production of antibiotics and mass cultivation of marine sponges being difficult, the utilization of sponges as a source of antibiotics is hindered (Indraningrat *et al.*, 2016). The good thing is that various studies have indicated that most of the biologically active compounds reported from marine sponges originated from associated bacteria. This is because of the structural similarity of the compounds from marine sponges with those compounds identified from terrestrial microorganisms (Taylor *et al.*, 2007; Laport *et al.*, 2009; Thomas *et al.*, 2010; Fuerst, 2014). Moreover, numerous studies have identified a wide range of antimicrobial activities from sponge-associated microbes, which makes these microbial populations an important source for novel antimicrobials (Santos-Gandelman *et al.*, 2014; Graça *et al.*, 2015; Hoppers *et al.*, 2015).

The phylum Actinobacteria contributes to the major share of active metabolites from the sponges (Tawfike *et al.*, 2019). A comprehensive study from Germany revealed the presence of various Actinobacteria with a range of antimicrobial activities (Schneemann *et al.*, 2010a). Bibi *et al*. (2017), Indraningrat *et al.* (2016), and Fuerst (2014) also reviewed metabolites with different biological activities from sponge-associated culturable and unculturable microbial population. Clinically significant bioactive compounds from marine sponge-associated microbes have been reported from different geographical areas including the Great Barrier Reef of Australia, South China Sea, Mediterranean Sea, Indonesia, Papua New Guinea, Indo-Pacific region, etc. (Kennedy *et al.*, 2009; Bibi *et al.*, 2017). Table 1.3 presents some of the novel antimicrobials from marine sponge-associated bacteria and Figure 1.4 highlights a few examples of the chemical structure of these compounds.

Table 1.3 Overview of active secondary metabolites from sponge-associated bacteria.

| **Microorganism** | **Sponge source** | **Metabolite** | **Biological activity** | **References** |
|---|---|---|---|---|
| *Bacillus tequilensis* | *Callyspongia diffusa* | pyrrolo | Antibacterial | (Kiran *et al.*, 2018) |
| *Rhodococcus sp. UA13* | *Callyspongia aff. Implexa.* | rhodozepinone | Antitrypanosomal | (Elsayed *et al.*, 2017) |

Table 1.3 (Continued)

| Microorganism | Sponge source | Metabolite | Biological activity | References |
|---|---|---|---|---|
| *Streptomyces* sp. RV15 | *Dysidea tupha* | naphthacene glycoside | Antibacterial | (Reimer *et al.*, 2015) |
| *Kocuria*, *Micrococcus* | Unidentified | kocurin | Anti-MRSA | (Palomo *et al.*, 2013) |
| *Bacillus* sp. | Unidentified | apteniol A | Antimicrobial | (Devi *et al.*, 2010) |
| *Streptomyces* sp. Hedaya 48 | *Aplysina fistularis* | saadamycin | Antifungal | (El-Gendy & El-Bondkly, 2010) |
| *Streptomyces* sp | *Halichondria* | mayamycin 53 | Anticancer, Antimicrobial | (Schneemann, Kajahn, *et al.*, 2010) |
| *Nocardiopsis* sp. | *Halichondria panicea* | nocapyrones | Anticancer, Antimicrobial | (Schneemann, Ohlendorf, *et al.*, 2010c) |
| *Streptomyces* sp. HB202 | *Haliclona simulans* | mayamycin | Anti-MRSA | (Schneemann, Nagel, *et al.*, 2010b) |
| *Salinispora* | *Pseudoceratina clavata* | rifamycin B, rifamycin SV | Anti-bacterial | (Hewavitharana *et al.*, 2009) |
| *Pseudoalteromonas maricaloris* KMM 636T | *Fascaplysinopsis reticulata* | bromoalterochromide A | Antimicrobial | (Speitling *et al.*, 2007) |
| Unknown bacterium | *Dysidea avara* | 2-methylthio-1,4-naphthoquinone | Antimicrobial and Anti-angiogenic activities | (Thakur *et al.*, 2005) |
| *Pseudoalteromonas piscicida* | *Hymeniacidon perleve* | norharman (β-carboline alkaloid) | Antimicrobial | (Zheng *et al.*, 2005) |

| | | | | |
|---|---|---|---|---|
| *Pseudomonas sp.F92S91* | Unidentified | α-pyrones | Antibacterial | (Singh *et al.*, 2003) |
| *Bacillus cereus* | *Halichondria japonica* | pyridine and thiazole | Anti-bacterial | (Suzumura *et al.*, 2003) |
| *Pseudomonas* sp. | *Suberea creba* | quinolones | Antibacterial | (Debitus *et al.*, 1998) |
| *Pseudomonas* sp. 1531-E7 | *Homophymia* sp. | 2-undecyl-4-quinolone | Antiviral | (Gamini *et al.*, 1996) |

Removed due to copyright restriction

Figure 1.4 Structures of some antimicrobials from marine associated bacteria.

## 1.5 Antimicrobial resistance

### 1.5.1 Brief history

A well-known actinomycetes related researcher, Selman Waksman, described in the early twentieth century the term antibiotic to reflect a compound produced from microorganisms that inhibit or kill another microorganism (Hopwood, 2007; Davies & Davies, 2010). Many of the current antibiotics were produced from the phylum Actinobacteria particularly from the soil associated genus *Streptomyces* (Barka *et al.*, 2016). Prior to antibiotics discovery, chemotherapy depends on synthetic

drugs such as sulfa drugs (Aminov, 2010). Though Alexander Fleming observed the effect of Penicillin on bacteria in 1928, it only appeared in use after the late 1930s where Howard Florey and Ernst Chain turned penicillin from a laboratory curiosity into a life-saving drug (Hopwood, 2007). Penicillin exerts its inhibitory effects by inhibiting bacterial cell wall synthesis and discovered effective against Gram-positive bacteria but not to Gram-negative bacteria as they have extra lipid bilayer (Peterson & Kaur, 2018).

Following the discovery of Penicillin, a massive search was on for antibacterial agents from different microorganisms in the soil and it was found that most antibacterials were associated with actinomycete cultures compared to other bacteria or fungi. Many of the discovered antibacterial agents were not used as they were associated with toxicity until the breakthrough in 1943 where streptomycin produced by *Streptomyces griseus* (Waksman *et al.*, 1946). This antibiotic binds the 30S subunit of the prokaryotic ribosome and inhibit protein synthesis, and they are found to be effective both against Gram-negative bacteria and tubercle bacillus (Hopwood, 2007). From this point forward the golden era for antibiotics (1940–1990) started which involved different pharmaceutical industries in different parts of the world who. managed to discover antibiotics that inhibit almost all vital process in bacterial cells. Now, seven general types of antibiotics have been discovered based on their mode of action and structure, including β-lactams and glycopeptides (cell wall synthesis inhibitor); aminoglycosides, macrolides, and tetracyclines (protein synthesis inhibitor); daptomycin (inhibit cell membrane function); and platensimycin (inhibit fatty acid biosynthesis) (Peterson & Kaur, 2018).

### 1.5.2 Antimicrobial resistant in human pathogens

Though the discovery of antibiotics for curing infectious disease is a major success, extensive and indiscriminate utilization of them over the last 7 decades lead to the appearance of resistant strains for almost every antibiotic in the pipeline. Antibiotic resistant was documented for the very early drugs such as sulphonamide in the late 1930s, soon after discovery (Davies & Davies, 2010). The discovery of penicillinase in *Staphylococcus aureus* and *Streptococcus pneumoniae* before the wide use of penicillin indicated the presence of resistant mechanisms in nature (Davies & Davies, 2010; Ogawara, 2016). Likewise, soon after the availability of methicillin, *S. aureus* developed resistance

which is widely known as Methicillin resistant *Staphylococcus aureus* (MRSA).(Davies & Davies, 2010). These findings indicated sooner or later the use of every antibiotic resulted in the development of resistant strains. In the USA alone, antimicrobial-resistant is associated with 23,000 deaths every year (Centres for Disease Control and Prevention report). The recent appearance of MDR and XDR (extremely drug resistant) bacterial strains such as *S. aureus*, *Mycobacterium tuberculosis*, and *Acinetobacter baumannii* impose serious public health issue where only a few options remain to treat disease caused by these bacteria. The term 'superbugs,' is given to represent human commensal flora which acquired resistant and become highly virulent like MRSA and vancomycin resistant enterococci (VRE), or those environmental bacteria like *Pseudomonas aeruginosa* and *A. baumannii* which are intrinsically resistant and become opportunistic pathogens (Wright, 2007; Miller *et al.*, 2014).

### 1.5.3 Mechanisms of resistance

Bacteria depends on two general genetic mechanisms for the development of antibiotics resistance. The first is a mutation in genes frequently linked to mechanisms of action of the antibiotics and the second is the acquisition of external DNA coding for resistance determinants via horizontal gene transfer (Munita & Arias, 2016). The most common mutational resistance mechanisms associated with antimicrobial target modification, drug uptake reduction, efflux pump activation (fast removal of the compound from the inner cell), and universal metabolic pathways modification through regulatory networks variation. Bacteria apart direct mutation, they acquire resistant gene from the environment through the process of transformation, transduction, and conjugation (Munita & Arias, 2016).

Bacterial antibiotic resistant can be intrinsic or acquired. The most common types of intrinsic resistance are non-specific efflux pump, enzymes inactivation and antimicrobial impermeability which all are chromosomally encoded (Fajardo *et al.*, 2008; Cox & Wright, 2013). The AcrAB/TolC efflux pump in *Escherichia coli* is one example of intrinsic resistance mechanisms which export out large varieties of inhibitor molecules such as dyes, detergents, disinfectant and different classes of antibiotics (Nikaido & Takatsuka, 2009). Permeability barrier due to outer membrane which is associated with vancomycin resistance in Gram-negative bacteria is also an example of intrinsic

resistance (Arthur & Courvalin, 1993). The acquired resistance in most cases resulted from horizontal transfer of resistant gene which included plasmid encoded efflux pumps and enzymes that change drugs or its active sites (Van Hoek *et al.*, 2011). Unlike intrinsic resistant, acquired resistance poses serious public health issues as resistant highly disseminated with the help of plasmid (Dantas & Sommer, 2012; Martínez *et al.*, 2015).

#### 1.5.3.1 Antibiotics modification

Resistant to aminoglycosides by most pathogenic Gram positive and negative bacteria are associated with its modification with the help of various types of aminoglycoside modifying enzymes (AMEs) (Schwarz *et al.*, 2004; Ramirez & Tolmasky, 2010). In most cases the genes for AMEs located in mobile genetic elements, but in the environmental bacterial strains such as *Providencia* and *Acinetobacter* species, chromosomal determinants of AMEs are observed (Yoon *et al.*, 2014). Apart from AMEs, chloramphenicol acetyl transferases (CATs) are also the most common resistance mechanisms observed both in Gram-positive and negative pathogenic bacteria (Schwarz *et al.*, 2004). Another enzyme responsible antibiotic modification in pathogenic bacteria is β-lactamase, which can degrade the β-lactam ring of penicillin (Foster, 2017). The gene responsible for this enzyme is mostly located in mobile genetic elements and this contributes to the easy spread of resistance in the community. Due to massive mutation associated this gene, their number has increased and one of this type of mutation occurred on TEM-3 which lead to the degradation of 3rd generation cephalosporins, placing it into the category of Extended Spectrum β-lactamases (ESBLs) (Paterson and Bonomo, 2005).

#### 1.5.3.2 Antibiotic efflux

Reduction of antibiotic permeability and/or their efflux are also the common mechanisms by which pathogen develop resistance. Decrease permeability is the common intrinsic observation among Gram negative bacteria due to the nature of their cell membrane, which inhibits hydrophilic antibiotics such as vancomycin (Nikaido, 2003). Further hydrophilic antibiotics resistant to Gram negative bacteria observed following mutation in porin genes or their expression (Li *et al.*, 2012). In addition to these, several active pumps have been identified in pathogenic bacteria (Gram positive

and negative). They are generally categorized into five families: ATP Binding Cassette (ABC), Major Facilitator Superfamily (MFS), Resistance-Nodulation-Division (RND), Multidrug and Toxin Extrusion (MATE), and Small Multidrug Resistance (SMR) (Sun *et al.*, 2014; Schindler & Kaatz, 2016). ATP dependent transport work only for ABC and for the rest, substrate transport is coupled with the ion gradients. Though most transport proteins carry only specific transport, multidrug/polyspecific exporters are observed in each family (Schindler & Kaatz, 2016).

Bacteria may have intrinsic or acquired genes which code for antibiotic efflux pumps. The most common inherent genes are acrAB/tolC (*E. coli*), norA (*S. aureus*), and lmrA (*Lactococcus lactis*). Among these three the tripartite RND pump AcrAB/TolC system is the most understood. This is a unique system which exports antibiotics in a single step by linking the inner and outer membranes via fusion (Martínez, 2019). In most pathogenic bacteria, the acquired antibiotic efflux genes located on mobile genetic elements, and they are characterized by various types of tet genes situated on plasmids in both Gram-negative and Gram-positive bacteria (Roberts, 2005). Remarkably, synergy observed between simple Tet pump proteins (MFS family) and RND pumps which enhanced in the minimum inhibitory concentration for tetracycline (Nikaido & Takatsuka, 2009). This showed how the acquired resistant augmented by the intrinsic system.

#### 1.5.3.3 Target modification and protection

Pathogenic bacteria display several target modifications to resist the antibiotic. The famous example is the modification observed in MRSA where the bacteria acquired external Penicillin binding protein 2 a (PBP2a) where the transpeptidase part is insensitive to the action of various β-lactams. The mecA gene, which is found on an MGE called Staphylococcal chromosomal cassette (SCCmec), is coded PBP2a in *S. aureus* (Fishovitz *et al.*, 2014; Vickers, 2017). Vancomycin resistant by *Enterococci* is another example associated with target modification, which is a result due to the acquisition of the van gene cluster (Miller *et al.*, 2014).

In addition to target modification, enzymatic change of the target conferred resistance to a macrolide. This resistant resulted in a large group of erythromycin ribosomal methylation (erm) genes which methylate a specific adenine in the 23S rRNA (Weisblum, 1995). These genes are located

in mobile gene elements and they occur widely both in Gram positive and negative bacteria (Palmieri *et al.*, 2011). Resistance due to target protection is observed in clinical strains of *S. aureus*. Genes which are located in MGEs region of *S. aureus* coded for Tet(M) and Tet(O) proteins, and their attachment to the ribosome aids elimination of tetracycline (Trieber *et al.*, 1998).

## 1.6 Approach for isolation of bacteria from marine sponges

Most of the bacterial community in the environment is not yet cultured and they remain the undetected majority in the environment, with an expected total number estimated to range between $4 \times 10^{30}$ and $6 \times 10^{30}$ (Whitman *et al.*, 1998). They are also described as uncultivable as only a small percentage are readily cultured irrespective of their origin including soil, marine environment or human gut (Davis *et al.*, 2005; Eckburg *et al.*, 2005). This also applies to sponge-associated bacteria where researchers only managed to cultivate 0.1 to 11% from various sponge samples (Hacker *et al.*, 2001; Olson & McCarthy, 2005). Recently, isolation of sponge-associated bacteria received the attention of researchers as the majority of active compounds identified from host sponges were claimed to originate from symbionts (Lee *et al.*, 2001; Flatt *et al.*, 2005; Mehbub *et al.*, 2014).

For many years, the cultivation of microorganisms has been the basis for most microbiology studies. Though the recently developed molecular techniques such as metagenomics allow for genetic and functional descriptions of uncultured bacteria, cultivation of bacteria is still a cornerstone for the understanding of their physiology and the contribution of these bacteria in the ecology, host health, and natural compound productions (Sipkema *et al.*, 2011; Stewart, 2012). However, existing knowledge for creating the necessary natural conditions for all microorganisms is limited and most of the common artificial culture ingredients are unable to mimic all the required conditions for microbial growth (Alain & Querellou, 2009).

Significant differences were observed between microenvironments to which bacteria are exposed and from their apparent natural environment. The difference is marked for strains living inside the mesohyl of marine sponges, where the microenvironment has low similarity to the surrounding macro-environment, which is generally used in most cultivation experiments. Unlike the surrounding environment, anaerobic conditions frequently occur in the sponge mesohyl whenever the sponge

does not pump water (Hoffmann *et al.*, 2005; Hoffmann *et al.*, 2008). Besides, the presence of siderophores in the mesohyl contributes to a higher concentration of iron (Onuki & Kamino, 2000). Incorporation of sponge-specific lectins in a medium may also support the cultivation of sponge specific bacteria (Müller *et al.*, 1981).

Several innovative strategies have been employed to bring these microorganisms into the culture. Modifying the common culture media and culture conditions is considered one of the strategies to grow the previously uncultured microbial communities. Microorganisms vary significantly from each other and their growth requirements can include special nutrients, temperature optima, oxygen levels, specific pH and incubation times (Reichelt & Baumann, 1974; Tripp *et al.*, 2008). Various studies managed to cultivate different types of bacteria by manipulating these isolation conditions (Köpke *et al.*, 2005) and the incorporation of environmental supplements to the basal media (Hamaki *et al.*, 2005). Some of the employed strategies include aerobic and microaerophilic conditions, combinations of different low and high nutrient media with or without antibiotics (Abdelmohsen *et al.*, 2010; Schwartz *et al.*, 2014). Some were successful in cultivating new microorganisms by only increasing the incubation time ( Stevenson *et al.*, 2004; Davis *et al.*, 2005; Stott *et al.*, 2008; Song *et al.*, 2009) and reducing inoculum size (Davis *et al.*, 2005).

The co-culturing of two or more microorganisms has allowed for the growth of previously uncultured microorganisms. This co-cultivation or community culturing creates a favourable condition for microorganisms to support each other through the exchange of metabolites and/or signalling molecules, as mostly observed in biofilm production in bacteria (Nadell *et al.*, 2008). Successful cultivation of previously uncultured bacteria was indicated when bacteria grew together with helper strains, which released growth stimulation factors in the environment (Ohno *et al.*, 2000; Nichols *et al.*, 2008). Various studies incorporated cell-free extracts, culture supernatant, known signalling molecules such as cyclic AMP and growth factors into the basal media for isolation of previously uncultured bacteria (Bruns *et al.*, 2002; Tanaka *et al.*, 2004; Bae *et al.*, 2005; Nichols *et al.*, 2008; D'Onofrio *et al.*, 2010).

Dilution of samples to extinction and culturing them in microtiter plates allowed researchers to grow bacteria in their pure form (Rappé *et al.*, 2002; Giovannoni & Stingl, 2007). Methods like high-

throughput cultivation use dilution extinction concepts and culturing them in a nutrient-poor medium in microwells, which reduce the inoculum size to support the culturing of pure bacteria (Button *et al.*, 1993; Simu & Hagström, 2004; Stingl *et al.*, 2008; Song *et al.*, 2009).

In addition to the above-mentioned strategies, many innovative approaches have been reported for growth of uncultured bacteria including growing of microorganisms using microcapsules (Zengler *et al.*, 2002), diffusion-chambers (Kaeberlein *et al.*, 2002; Bollmann *et al.*, 2007; Gavrish *et al.*, 2008; Bollmann *et al.*, 2010; Steinert *et al.*, 2014), soil substrate membrane systems (Ferrari *et al.*, 2005; Ferrari *et al.*, 2008; Ferrari & Gillings, 2009), and micro-petri dish (Ingham *et al.*, 2007).

## 1.7 Polyphasic strategy for bacterial taxonomy

Taxonomic data plays a significant role in a clear understanding of the biodiversity and interactions of living organisms from various environmental sources (Gevers *et al.*, 2005). In particular, for prokaryotes, taxonomic information is an essential component for the full identification of microbial strains from different sources (Moore *et al.*, 2010). The first attempt at introducing bacteria taxonomy was initiated in the late 19$^{th}$ century, where phenotypic characteristics such as morphology, growth requirements or pathogenicity were the basis for bacteria classification (Lehmann & Neumann, 1896). Then, bacteria classification was achieved using physiological and biochemical properties (Ramasamy *et al.*, 2014). In the 1960s and 1980s, bacterial identifications were achieved with the help of chemotaxonomy (Minnikin *et al.*, 1975), numerical taxonomy and DNA-DNA hybridization techniques (Brenner *et al.*, 1969; Stackebrandt & Goodfellow, 1991). The introduction of DNA amplification and sequencing techniques, especially of the 16S rRNA gene, in the 1980s, facilitated faster and more accurate bacterial classification (Coenye & Vandamme, 2004; Konstantinidis & Tiedje, 2007). From the beginning of the mid-1990s, the classification of bacteria was further supported by whole-genome sequencing (Kunin *et al.*, 2008).

Despite the remarkable improvement and development of various genome-based methods such as multilocus sequence analysis and average nucleotide identity, whole-genome analysis (Goris *et al.*, 2007; Konstantinidis & Tiedje, 2007) is not still accepted as a source of taxonomic information. As a result, the current taxonomy of prokaryotes relies on polyphasic combinations of phenotypic,

chemotaxonomic and genotypic characteristics. This is achieved using a side-by-side comparison of the above characteristics with those of type strains of established species. In the absence of type strains, the novelty of an unusual taxon is established by fulfilling a set of minimum standards (Ramasamy *et al.*, 2014).

### 1.7.1 Genotypic classification

DNA-DNA hybridization, DNA G+C content and 16S rRNA gene sequence analysis are the most frequently investigated genetic criteria for bacterial taxonomy (Stackebrandt & Ebers, 2006; Tindall *et al.*, 2010). With the help of these criteria, the definition of a bacteria species has been refined. DNA-DNA similarity of 70% or more with 5°C or less ΔTm value among members of the group can be a definition of the same species, provided that other polyphasic data agrees with it (Prakash *et al.*, 2007).

Due to its inherent characteristics such as its ubiquitous existence, stability, high conservation and a poor chance of horizontal transfer, the 16S rRNA gene is considered an essential genetic marker for taxonomy. 16S rRNA gene sequence differences detected in archaea and bacteria were the basis of prokaryote classification (Woese *et al*., 1990; Fox *et al*., 1992; Olsen & Woese 1993; Brenner *et al*., 2001; Stackebrandt *et al*., 2002). Due to this, 16S rRNA gene sequencing and phylogenetic analysis have increased the right way for the categorization of bacteria at certain taxonomic levels. The cut of values of $\leq$98.7% and $\leq$95% similarity has been indicated for placing of isolates into novel species and genus, respectively as long as the isolates compared with valid type strains (Stackebrandt *et al.*, 2002; Stackebrandt & Ebers, 2006). Such values usually correlated with DNA-DNA hybridization results (Rosselló-Mora, 2006). Stackebrandt and colleagues suggested that 16S rRNA gene sequence similarity might even replace DNA-DNA hybridization (Stackebrandt *et al.*, 2002; Stackebrandt & Ebers, 2006).

Gross classification of prokaryotes can be frequently possible with their DNA G+C content. For example, the phylum Actinobacteria classify as high-G+C-content Gram-positive bacteria compared to the Fermicutes as they labelled as the low-G+C-content Gram-positive bacteria. For some bacteria, a variation of > 5 and > 10% within strains has been used to categorize the strain as a

different species or genera, respectively (Goodfellow *et al.*, 1997; Ramasamy *et al.*, 2014). With the advancement of DNA-based molecular techniques, systems of bacteria identification and taxonomy have been transformed. These methods comprise of Restriction Fragment Length Polymorphism (RFLP), plasmid profiling, Ribotyping, Amplified Ribosomal DNA Restriction Analysis (ARDRA), Pulsed Field Gel Electrophoresis (PFGE) and Randomly Amplified Polymorphic DNA (RAPD) (Prakash *et al.*, 2007). The modern classification of bacteria depends on a combination of different markers and approaches as indicated in Figure 1.5 below.



Figure 1.5 Illustrative representation of techniques and markers considered in the modern polyphasic approach for resolving the bacterial hierarchy, taken from Prakash *et al.*, 2007.

### 1.7.2 Chemotaxonomic classification

The chemotaxonomic classification depends on the analysis of microbial chemical composition or chemotaxonomic markers to place them at specific taxon. These chemotaxonomic markers unevenly distribute, and the selection of specific types depends on the bacteria. The most common markers include the chemical composition of the cell wall amino acids, lipids, proteins, menaquinones, muramic acid types, and sugars, all of which are used frequently for chemotaxonomic classification. Analysis of the cell wall composition, in particular for peptidoglycan, is very important for the taxonomy of bacteria as its nature varies markedly within suborders of bacteria ( Berd, 1973; Barka

*et al.*, 2016;). Peptidoglycan characteristics such as the sugar contents, the position of amino acid in the tetrapeptide side chain and the isomers of 2, 6-diaminopimelic acid (DAP) are used for classification of bacteria into various genera (Barka *et al.*, 2016). Apart from cell wall peptidoglycan, properties such as the pattern of cellular fatty acids, type of menaquinone, phospholipids, and composition of sugars are essential components for the classification of bacteria at specific taxonomic levels (Barka *et al.*, 2016).

### 1.7.3 Phenotypic classification

Phenotypic methods of classification assess the observable genotypic expression of the bacteria which includes morphological, physiological and biochemical properties of the organism. Before the era of genotypic techniques, comparative studies on the phenotypic properties of the bacteria were the only methods for bacteria taxonomy. The most common phenotypic tests in the classic microbiology laboratory for bacteria taxonomy includes characteristics of the organism on different growth substrates, growth characteristics at different ranges of pH, salt and temperature and as well as susceptibility against antimicrobial agents. Since the phenotypic data can be affected by various environmental factors, each test should be performed side-by-side with the known type strains (Prakash *et al.*, 2007; Ramasamy *et al.*, 2014). Identification of isolate as a novel requires several steps as displayed in Figure 1.6.

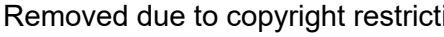



Figure 1.6 Flow diagram depicting the step-by-step process for the taxonomical description of newly isolated strains followed by its deposition in culture collection centres and publication, taken from Prakash *et al*., 2007.

## 1.8 Research Plan

### 1.8.1 Rationale of the study

Currently, due to various factors such as a high rate of drug resistance and the appearance of new infectious diseases, the searches for new active antimicrobial compounds are increasing (Strobel *et al.*, 2004). Bacteria, particularly Actinobacteria from marine environments, contribute more than half of the antibiotics recently discovered (Lazzarini *et al.*, 2000). For the past several years due to

the overutilization of terrestrial bacteria, the chance of getting new bacteria as well as new compounds is drastically reduced with a success rate of less than 5% (Marcus *et al.*, 1999; Taylor & Wright, 2008). Therefore, it is necessary to increase the search area to new and less explored environments. Marine sponges are one of the areas recently got the attention as most of the newly discovered metabolites were originated either the sponge itself or associated microorganisms (Webster & Hill, 2001a; Lee *et al.*, 2001; Blunt *et al.*, 2007; Taylor *et al.*, 2007; Blunt *et al.*, 2018; Gogineni & Hamann, 2018).

Australia completely separated from Antarctica before 40 million years ago to form an isolated continent. Due to its isolation from the rest of the world for many years, Australia is the home for a huge amount of endemic and diverse organisms compared to other areas. In addition, the country contains a large span of latitude with different climate zones, which contribute to the greater biodiversity of flora and fauna on land and sea (Biodiversity unit,1994). Like other organisms, Australia, particularly the Southern region, is a source of huge diversity and endemic sponge species (Van Soest *et al.*, 2012). Sorokin *et al.*,(2007) and Sorokin & Currie (2008) observed a significant variation in the chemical composition among sponges of South Australia, and this is the base to hypothesised the associated symbionts and/or the metabolites from them are different. Therefore, this study was designed to isolate these sponges-associated bacteria using different culturing approaches and investigate factors that affect their diversity. The study also focused on the production of active compounds from sponge-associated bacteria and testing their inhibitory activities against human pathogenic bacteria and fungi.

### 1.8.2 Research questions

This project was a search for answers to the following questions. How does one isolate novel bacteria that have been observed in sponges using molecular techniques? Do these bacteria produce novel secondary metabolites?

### 1.8.3 Hypothesis

The hypotheses of this project are:

1) Unique and diverse strains of bacteria from marine sponges of South Australia are cultivable.
2) These sponge-associated bacteria are a potential source of biologically active secondary metabolites.

### 1.8.4 Aims of the project

The main aims of this project were to discover novel and more diverse bacteria from sponges of the South Australian marine environment and new antimicrobials from them. Specifically, this project has the following objectives:

- ❖ To develop new techniques for the isolation of sponge-associated bacteria
- ❖ To fully characterize the isolated bacteria
- ❖ To screen and characterize secondary metabolites from the sponge-associated bacteria with antimicrobial activity against a range of human pathogenic microorganisms.

### 1.8.5 Approach followed for bacterial isolation in this project

For isolation of bacteria from sponges, one should mimic the growth conditions to the sea environment. As a result, this project implemented different approaches. One of the major components of seawater is sodium chloride. We believed that the incorporation of sodium chloride in artificial media affects the isolation of bacteria from sea materials. Therefore, all primary isolation media in this project made of sterilized seawater to obtain the required NaCl.

Temperature is one of the many factors which affects the isolation of bacteria. While the commonly used incubation temperature for isolation of bacteria is 30-37$^{O}$C, the average temperature of the sea environment is lower. Sponges used in this study have been obtained from waters with a temperature of 15-20$^{O}$C. Hence, the media in this project were incubated at different temperatures to increase the chance of bacterial isolation.

The oxygen tension in the sea is different from the laboratory atmosphere. As a result, in addition to incubating at the usual oxygen level of room temperature, this project used methods to reduce the oxygen tension comparable to the sea environment.

We used different pH ranges for the isolation of bacteria. This is because the pH preference of bacteria from sponges might not similar to bacteria from various sources such as soil and plants.

The mesohyl part of the sponge is a good source of bacteria. In this project, isolation media were prepared in such a way to match the nutrient composition of mesohyl. To achieve this, different concentrations of amino acids, sugars and sponge extracts were added in the isolation media.

As the distributions of bacteria are normally not homogenous in a sample, successful isolation of bacteria is determined by the number of specimens plated onto the isolation medium. In this project, a large volume of sponge specimen was plated in multiple plates on multiple isolation media.

The presence of bacteria in a plated samples are only visualised when the bacteria grow to form a colony. Colony formation depends on several factors including the vegetative growth stage of the bacterium, its intrinsic growth rate, its ability to germinate. Therefore, it is necessary to prolong the incubation time to allow for colony formation. Here, we incubated each culture medium for at least 16 weeks. This time allows for the emergence of slow-growing and possibly more diverse and unique isolates.

Bacteria with short doubling time will affect the growth of those having a slow growth rate when they grow in the same medium. To avoid this situation, we removed early appearing colonies and subcultured onto a new medium. This allows a favourable condition for the growth of all bacteria possibly present in the specimens. Figure 1.7 below presents a diagrammatic overview of the study.

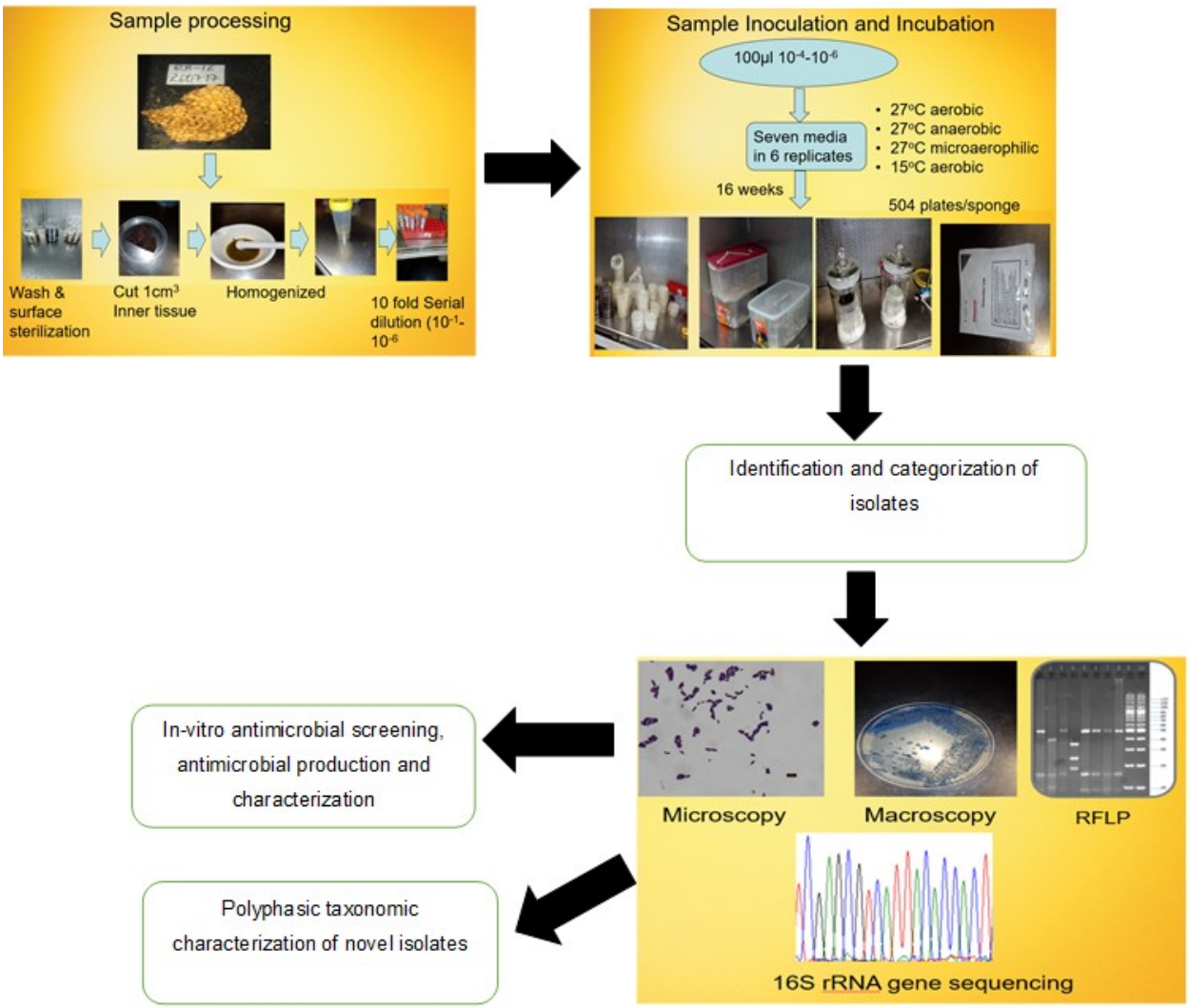



Figure 1.7 Flow diagram of study outline.

# Chapter Two: General Methods and Materials

## 2.1 Maintenance of bacterial cultures

This chapter describes the common methods and materials which were established during this study and often cited in subsequent chapters. These methods are categorized as microbiological, chemotaxonomical and molecular.

### 2.1.1 Culture conditions

Bacterial strains isolated from sponge samples were subcultured onto starch yeast peptone agar (SYP), nutrient agar (NA), tryptone soy agar (TSA) and ISP2 media at 27$^{o}$C and 30$^{o}$C for a period of 3 (for most bacteria) to 14 (Actinobacteria) days until good growth was observed for the subsequent experiments.

### 2.1.2 Sub-culturing and storage of bacteria strains

The pure strains of bacteria were maintained on agar slants of SYP, TSA, NA and ISP2 media until they were transferred to glycerol for long-term storage. The cultures were placed overnight into sterile 50% glycerol at -20$^{o}$C before being transferred into -80$^{o}$C in cryostat tubes. When needed, bacteria were subcultured from the frozen state onto the surface of SYP, TSA, NA and ISP2 media.

### 2.1.3 Morphological characterization

Macroscopic morphological characteristics of the bacteria strains were assessed by subculturing them onto ISP2, SYP, TSA, NA, mannitol soybean agar (MS) and half strength potato dextrose agar (HPDA). Colony colour, consistency, spore formation, diffusible pigment production and degree of general growth were the properties that were considered to place strains in the specific groups.

### 2.1.4 Microscopic observation of strains

Preliminary categorization of the bacterial strains was achieved by observing them under light microscopy. Direct wet mount, Gram stains, Ziehl-Neelsen (AFB) stains, and lactophenol cotton blue stains were used to observe bacteria under the microscopy (procedures for each staining technique are in Appendix 2). Slide culture methods were also applied for microscopic examination of sporulated bacteria such as *Streptomyces*. Here, the bacteria strains were subcultured onto ISP2

medium and a sterile microscopic coverslip was placed at a 45$^{o}$ angle to the inoculum. After a few days of incubation, the cover slipe was removed, stained with lactophenol cotton blue and the isolates analyzed in terms of the presence or absence of substrate and aerial mycelium, spore chain morphology and spore structures.

### 2.1.5 Biomass preparation for DNA extraction

Bacterial strains were subcultured onto SYP, ISP2 and TSA agar for periods of 3-14 days at 27$^{o}$C until good growth was observed. Two to three loopful of bacteria and bacterial spores were transferred to 2 ml tubes for further DNA extraction protocols.

### 2.1.6 Biomass preparation for fatty acid methyl ester (FAME) analysis of bacterial cell

Bacterial strains were grown onto TSA plates at 27$^{o}$C and 30$^{o}$C until good growth was achieved. Two loops of bacteria cells were transferred into 250 ml Erlenmeyer flasks containing 50 ml TSB. The inoculated flasks were incubated for 10 days at 27$^{o}$C and 30$^{o}$C on a shaker at 150 rpm for mass production of bacteria. The biomass was harvested by centrifugation at 3000 x g for 10 min and washed and recentrifuged three times with sterilized RO water. About forty milligrams of wet cells were placed in a clean 13 x 100 cm glass test tube for subsequent FAME analysis using methods in section 2.3.6.

### 2.1.7 Biomass production for phospholipids, menaquinone and mycolic acid analysis

Biomass production and its clean-up for phospholipids, menaquinone and mycolic acid analysis were similar to the protocol stated in section 2.1.6. The bacterial biomass was freeze-dried using VirTis freeze-drying equipment and the lyophilized bacterial biomass were ready for immediate analysis or they were stored at -20$^{o}$C.

### 2.1.8 Biomass production for sugar cell wall and DAP analysis

Bacterial strains were subcultured to TSA agar at 27$^{o}$C and 30$^{o}$C until good growth was observed. Two to three loopful of bacterial cells were transferred into a 10 cm Pyrex tube for further acid hydrolysis and analysis.

## 2.2 Methods for the isolation of bacteria from sponges of South Australia marine environment

### 2.2.1 Sponges sample collection, processing, and classification

Twelve sponge samples were collected from South Australian marine environments by scuba diving. Specifically, 4 samples which coded as GB 1, GB 08, GB 21 and GB 23 were collected from Glenelg blocks (34° 58' 406" S, 138° 30' 494" E) and 8 sponge samples which coded as RB 1, RB 2, RB 3, RB 11, RB 12, RB 16, RB 17, and RB 18 were collected from Rapid Bay Jetty (35.5229° S, 138.1854° E), at a depth of 10-15 m, water temperature of 15°C and salinity of 36.5-37 PSU. Glenelg blocks is a city beach where human and domestic animal contacts are high and there is runoff from a local river. In contrast, Rapid Bay jetty has a much lower frequency of human contact.

The samples were collected in Ziplock plastic bags containing fresh seawater and transported to the laboratory in an icebox. In the laboratory, the survival of the sponge samples was maintained using an aquarium system. The sponge samples were processed as described previously in similar studies (Hentschel *et al.*, 2001; Lafi *et al.*, 2005; Zhang *et al.*, 2006; Gandhimathi *et al.*, 2008; Kennedy *et al.*, 2009; Selvin *et al.*, 2009; Abdelmohsen *et al.*, 2010; Santos *et al.*, 2010; Li *et al.*, 2011; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Xi *et al.*, 2012; Ekiz *et al.*, 2014; Graça *et al.*, 2015; Sun *et al.*, 2015; Ellis *et al.*, 2017; Matobole *et al.*, 2017; Bibi *et al.*, 2018;.Kuo *et al.*, 2019). Briefly, the sponges were thoroughly rinsed with 2 L of autoclaved natural seawater and the surface of the sample disinfected with 70% ethanol. This was followed by drying of the samples in a sterile laminar flow chamber, and a section removed and processed immediately for microbial isolation, while some parts kept in a jar with 70% ethanol for sponge morphological identification and the rest was frozen (-80°C) in sterile Ziplock bags for future analysis.

Seven sponge samples were classified by morphological characterization as indicated in the previous study (Hooper & van Soest, 2002) and methods (Hooper, 2000). Histological sections and spicule Preparation have followed the methods in 'sponguide' (Hooper, 2000). All classifications were in line with the revised Demosponge classification (Morrow & Cardenas, 2015). Sponge samples coded as GB 1 classified as *Geodia* sp. And the other three sponge samples from Glenelg Blocks (GB 08, GB 21,

and Gb 23) found to be *Chondrosida* spp. The three sponge samples from Rapid Bay (RB 1, RB 2, and RB 3) were morphologically identified as *Ircinia* sp., *Poecilosclerida* sp., *Crella* sp., respectively.

Five sponge samples collected from Rapid Bay (RB 11, Rb 12, Rb 16, Rb 17, and RB 18) were identified by the molecular locus of 28S rRNA gene sequencing. Sponges DNA from frozen samples were extracted by conventional hexadecyltrimethylammonium bromide (CTAB)-based protocol (Taylor *et al.*, 2004b). In short, the frozen sponge samples were crashed by liquid nitrogen and their tissue was lysed with CTAB extraction buffer. The mix then combined with polyvinylpyrrolidone (PVP) and β -mercaptoethanol that help to remove tannins and phenolic compounds. The addition of phenol: chloroform: isoamyl allowed the separation of nucleic acids from proteins and polysaccharides, and finally the DNA was precipitated with iced isopropanol and reconstitute with 50 µl injection water and stored at -20°C. The quality and the quantity of the DNA assessed with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and those with high quality was considered for PCR reactions.

The DNA samples were amplified, sequenced, analysed following previous methods (Yang *et al.*, 2017; Yang *et al.*, 2019a; Yang *et al.*, 2019b). Briefly, Sponges DNA was amplified for D3- D5 regions of 28S rRNA gene by the primer set of NL4F (5'-GAC CCG AAA GAT GGT GAA CTA-3,) and NL4R (5' -ACC TTG GAG ACC TGA TGC G-3') (Nichols, 2005). Thermocycler conditions were as follows: a 10-min initial denaturation at 95 °C; 35 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min; and a final extension step at 72 °C for 7 min. The PCR products were cleaned and sent for sequencing to Macrogen, South Korea. NCBI database was used for BLASTn of the sequence and the five sponge samples were identified as *Sarcotragus* sp. (EF646841) (RB 11), *Carteriospongia foliascens* (KC869574) (RB 12), *Aplysilla sulfurea* (EF646837) (RB 16), *Dendrilla* sp. (KU533858) (RB 17), and *Tedania tubulifera* (KJ620377) (RB 18). The map of collection sites and photos of sampled sponges present in Figure 2.1 and Figure 2.2 below. Sponge samples were identified by Dr Qi Yang.

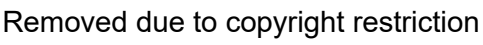



Figure 2.1 Map for sponge sample collection sites. **a** Rapid Bay; **b** Glenelg Block; **c** actual collection site where the sponge attached with the substrate.

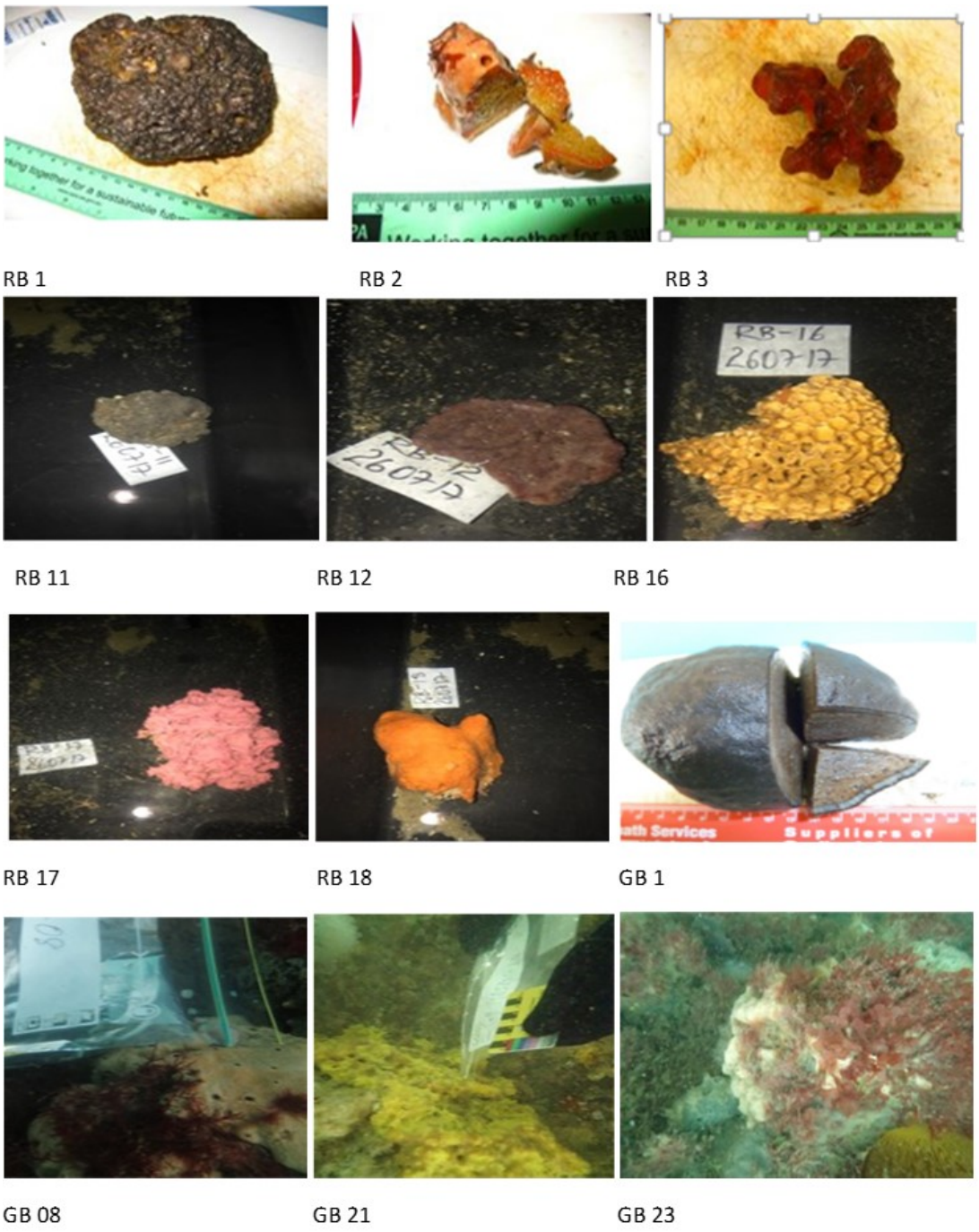



Figure 2.2 Twelve sponge samples collected from two sites. RB 1, RB 2, RB 3, RB 11, RB 12, RB 16, RB 17, and RB 18 were collected from Rapid Bay; GB 1, GB 08, GB 21, and GB 23 were collected from Glenelg blocks.

### 2.2.2 Isolation of sponge-associated bacteria

Approximately 1 $cm^3$ of dried sponge pieces were cut out using a sterile scalpel from the internal mesohyl area and homogenized using a clean, sterile pestle and mortar. To facilitate homogenization, 3 ml of autoclaved seawater was added. From the homogenate, a 10-fold dilution series ($10^{-1}$ to $10^{-6}$) was prepared and 100 µl of the three highest dilutions were inoculated in six replicates using a sterile L-shaped spreader onto seven isolation media. The media were selected from previous studies (Kennedy *et al.*, 2009; Lafi *et al.*, 2005; Li *et al.*, 2011; Ellis *et al.*, 2017; Hentschel *et al.*, 2001; Graça *et al.*, 2015; Abdelmohsen *et al.*, 2010; Bibi *et al.*, 2018; Flemer *et al.*, 2012; Kuo *et al.*, 2019; Margassery *et al.*, 2012; Matobole *et al.*, 2017; Santos *et al.*, 2010; Selvin *et al.*, 2009; Zhang *et al.*, 2006; Mincer, Fenical *et al*., 2005; Hayakawa and Nonomura 1987), which can support isolation of possible representative bacteria from sponges. The recipes of the seven-isolation media which are listed below and other which are used in this project are indicated in Table 2.1. Each isolation medium was supplemented with filter-sterilized (0.2 µm) 100 µg/ml cycloheximide to inhibit fungal growth.

a) SYP
b) Asparagine peptone agar (ASP)
c) Natural seawater agar (SWA)
d) Humic acid vitamin agar (HV)
e) ½ strength NA
f) Marine agar (MA)
g) ½ strength TSA

Table 2.1 Complete recipe of media used in the study

| Media | Ingredients (g/l) | Media | Ingredients (g/l) |
|---|---|---|---|
| Starch Yeast peptone agar (SYP) | Soluble starch (10)<br>Yeast extract (4)<br>Peptone (2)<br>Agar (18) | Asparagine pepton agar (ASP) | L-asparagine (0.1)<br>$K_2HPO_4$ (0.5)<br>$FeSO_4$ (0.001)<br>$MgSO_4$ (0.1)<br>Peptone (2)<br>Sodium propionate (4)<br>NaCl (20)<br>Agar (18)<br>RO water (1 litre) |
| Natural seawater agar (Seaagar medium) | Agar (18)<br>Seawater (1 litre) | Tryptic Soy Agar (TSA) | Tryptic Soy Broth (Oxoid) (30)<br>Agar (15) |
| Tryptone Soy broth (TSB) and Trypton Soya agar (TSA) | (Oxiod# CM0129 Media prepared following manufacturer's instruction | Nutrient Agar (NA) | Peptone (5)<br>NaCl (5)<br>Yeast extract (2)<br>Beef extract (1)<br>Agar (15) |
| Half Strength potato dextrose agar (HPDA) | PDA (Oxoid # CM0139) (19.5), Agar.(7.5) | Antibiotics medium No.1 (Seed agar, AAM) | (Oxoid # CM0327) The medium prepared following the provider's direction |

Table 2.1 (Continued)

| Media | Ingredients (g/l) | Media | Ingredients (g/l) |
|---|---|---|---|
| ISP 2 (Yeast extract-malt extract agar) | Malt extract (10)<br>Yeast extract (4)<br>Glucose (4)<br>Agar (20) | Potato Dextrose agar (PDA) | (Oxoid # CM0139) the medium was prepared following the manufacturer's instruction |
| Marine agar medium (MA) | NaCl (24)<br>$MgCl_2.6H_2O$ (11)<br>$Na_2SO_4$ (4)<br>$CaCl_2.6H_2O$ (2)<br>KCl (0.7)<br>KBr (0.1)<br>$H_3BO_3$ (0.03)<br>$NaSiO_3.9H_2O$ (0.005)<br>$SrCl_2.6H_2O$ (0.04)<br>NaF (0.003)<br>$NH_4NO_3$ (0.002)<br>$Fe_3PO_4.4H_2O$ (0.001)<br>Bacto peptone (5)<br>Agar (15)<br>RO water (1 litre) | Humic acid vitamin agar (HVA) | Humic acid (1)<br>$Na_2HPO_4$ (0.25)<br>KCl (0.85)<br>$MgSO_4.7H_2O$ (0.025)<br>$FeSO_4.7H_2O$ (0.05)<br>$CaCO_3$ (0.01)<br>Agar (18)<br>Vitamin B 100x (added after media autoclaved) (1ml)<br>Prepare NaOH 0.2N then 1g of humic acid was dissolved in 10ml of NaOH 0.2N. |

Table 2.1 (Continued)

| Media | Ingredients (g/l) | Media | Ingredients (g/l) |
|---|---|---|---|
| Vitamin B (100x) per 100 ml RO water | Thiamine-hydrochloride (5 mg)<br>Riboflavin (5 mg)<br>Niacin (5 mg)<br>Pyridoxine-hydrochloride (5 mg)<br>Inositol (5 mg)<br>Ca-panthotenate (5 mg)<br>p-aminobenzoic acid (25 mg)<br>Biotin (25 mg)<br>Adjust pH to 4.5 and filter sterilised | Nutrient Broth (NB) and Nutrient agar (NA) | (Oxoid # CM01) The media prepared following the manufacturer's instruction and 1.5 % (w/v) agar was added for NA |
| Sabouraud Dextrose Agar (SDA) | Dextrose (Glucose) (40)<br>Agar (15)<br>Distilled Water (1 litre)<br>pH 5.6 | Malt Extract agar (MEA) | Malt extract (30)<br>Agar (15) |
| CMA Cornmeal Agar (CMA) | Cornmeal agar premix (17)<br>R.O water (1 litre) | MS (Mannitol Soya agar (MS) | Mannitol (20), Soya flour (20)<br>Agar (20), R.O water (1 litre) |

Table 2.1 (Continued)

| Media | Ingredients (g/l) | Media | Ingredients (g/l) |
|---|---|---|---|
| Trace elements solution HO-LE (filter sterilised) | $H_3BO_3$ (2.85)<br>$MnCl_2.4H_2O$ (1.8)<br>Sodium tartrate (1.77)<br>$FeSO_4.7H_2O$ (1.36)<br>$CoCl_2.6H_2O$ (0.04)<br>$CuCl_2.2H_2O$ (0.027)<br>$Na_2MoO_4.2H_2O$ (0.025)<br>$ZnCl_2$ (0.02)<br>R.O. water (1 litre) | | |

* All ingredients are per litre of half strength sterilised natural seawater unless otherwise indicated. All media were adjusted to pH 7.2 before autoclaving unless otherwise indicated.

The plates were sealed with parafilm and kept in plastic lunch boxes lined with wet paper towels to prevent drying of the media. The plates were incubated for up to 16 weeks under different conditions: one set each of plates was kept at 27$^oC$ under aerobic, anaerobic and microaerophilic environments and the fourth set incubated aerobically at 15$^oC$. The anaerobic environment was generated in a sealed jar using an anaerobic generation kit (AnaeroGen$^{TM}$, Sigma-Aldrich) and its adequate establishment was checked using a methylene blue indicator, which turns colourless in the absence of oxygen. Microaerophilic conditions were established by lighting a candle in a candle jar with the lid closed to deplete the oxygen and established some degree of $CO_2$. The appearance of colonies was observed on all the plates, they were counted, and colonies removed completely and recorded every week. Subculturing and subsequent identification of anaerobic isolates were processed in anaerobic chambers, and in all times the anaerobic environment was maintained within

the sealed jar. Similarly, the microaerophilic environment was always maintained during bacterial identification from this incubation condition. Each sponge sample was tested on seven media, with six replicates and three dilutions. Each set of 126 plates was incubated at 27$^{o}$C aerobic, 27$^{o}$C anaerobic, 27$^{o}$C microaerophilic and 15$^{o}$C aerobic conditions, bringing the total to 504 plates per sponge sample.

### 2.2.3 Purification of isolates

Colonies were picked from primary isolation medium once a week, for 16 weeks, as previously described by Kaewkla and Franco (Kaewkla & Franco, 2013) and sub-cultured successively onto SYP, TSA, ISP2, and NA plates until pure cultures were attained as estimated by colony similarity and Gram stain cell morphology criteria (Li *et al.*, 2011). Pure cultures were stored on plates and agar slants for short periods, and colonies were also placed in 30% glycerol and stored at -80$^{o}$C for future use (Laich *et al.*, 2013).

### 2.2.4 Preliminary identification and categorization of pure isolates

All pure isolates were sub-cultured in a grid system onto ISP2, SYP, TSA, NA, MS and HPDA media and incubated until good growth was achieved. The colony morphology, texture, colour, consistency, nature of the spores, and growth pattern on the media were the features employed, if present, for grouping the isolates. Microscopic observation of hyphae in a wet and stained smear and Gram stain characteristics of the isolates were also considered to place the isolates into representative clusters. Procedures for microscopic tests indicated in Appendix 1.

### 2.2.5 Bacterial genomic DNA extraction

Bacterial DNA was extracted by a cetyltrimethylammonium bromide (CTAB) method as described previously (Kurtzman and Robnett 1998). Two to three loopful of purified bacterial colonies were removed from one of the purification media and resuspend into modified CTAB (containing an equal volume of 10% CTAB in 0.7 M NaCl and 240 mM potassium phosphate buffer). After the addition of zirconium beads and phenol: chloroform: isoamyl alcohol (25:24:1), the samples were shaken for 5 min in Mini-Beadbeater-16 (BioSpec products), a high-energy cell disrupter with shaking speed of

3450 oscillations/min with the capacity of 16 samples at a time. Overheating can denature DNA, and this was avoided by pausing the instrument every minute for 30 seconds. Then the samples were incubated for 1 hr at 65°C and the supernatant was collected in a clean Eppendorf tube after centrifugation at 16000 x g for 5 min at 4°C. The DNA-containing supernatant was extracted using chloroform: isoamyl alcohol (24:1) and the supernatant was again collected following centrifugation at 16000 x g for 5 min at room temperature. Two-fold volumes of PEG/NaCl precipitating solution (1.6 M NaCl, 30% (w/v) polyethylene glycol 6000) was added to the supernatant and after 2 hrs of incubation at 4°C, the DNA pellet was obtained following centrifugation at 18000 x g for 10 min at 4°C. Finally, the DNA pellet was washed twice with iced 70% ethanol and resuspended with 50 µl of water for injection and stored at -20°C until analysis. The concentration of each DNA extract was measured using Nanodrop 1000. The purity of DNA was examined by the absorbance ratio of 260/230, 260/280 and agarose gel electrophoresis. Pure DNA has 1.8 and 2-2.2 absorbance ration for 260/280 and 260/230, respectively. Good quality DNA result discrete band in agarose gel electrophoresis with minimum DNA shearing as indicated in Figure 2.3. Refer Section 2.2.6.2 for agarose gel electrophoresis. Depending on the concentration, either the DNA pellet was diluted or more volume was used for further downstream analysis.

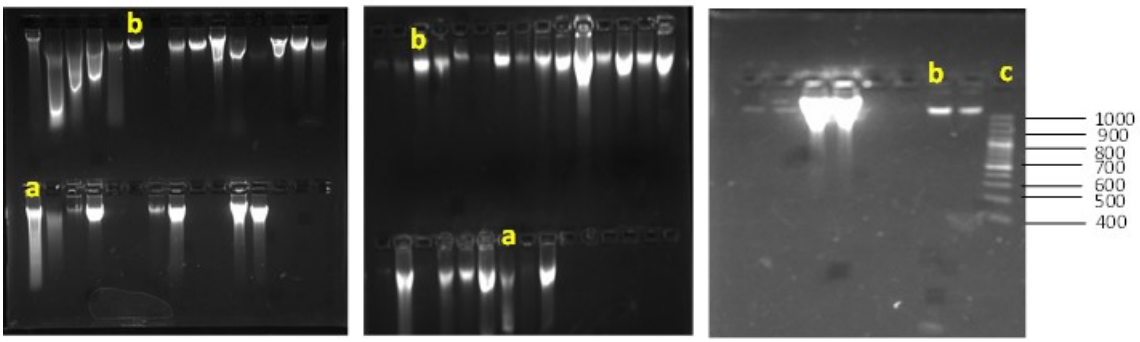



Figure 2.3 Agarose gel electrophoresis results of DNA extracts from bacterial strains. **a** sheared DNA; **b** discrete band good quality DNA; **c** 100 bp DNA ladder (BioLabs INC, NEW ENGLAND).

## 2 2.6 16S rRNA gene amplification

The primers used for the PCR reactions were universal 16S rRNA primers. The 16S rRNA gene was amplified separately in two segments using the primers pairs 27F (5'GAGAGTTTGATCCTGGCTCAG3') (Lane, 1991) and 765R (5'CTGTTTGCTCCCCACGCTTTC3') (Damiani *et al.*, 1997), which yield about 738 bp and the pair 704F (5'GTAGCGGTGAAATGCGTAGA3) (Damiani *et al.*, 1997) and 1492R (5'CACGGATCCTACGGGTACCTTGTTACGACTT3') (Weisburg *et al.*, 1991), which yield about 790 bp PCR products.

### 2.2.6.1 PCR conditions

The 16S rRNA gene was amplified using the primers set: 27F and 765R for all strains, and 704F and 1492R for those strains selected for full-length sequencing. The PCR reactions were done in a 50 µl reaction mixture with the following reagents: 2 µl of 5 µmol 27F, 704F, 765R, and 1492R primers, 24 µl of readymade Taq 2x master mix (NEW ENGLAND BioLabs®$_{Inc.}$), which contain DNA polymerase and nucleotides, 1 µl $MgCl_2$, 19 µl of injection water and 2 µl of DNA extracts. All amplification reactions were carried out in a Swift Thermal Cycler (Esco GB Ltd), with a reaction cycle of 95$^{o}$C for 10 min, 35 cycles of 94$^{o}$C for 1 min, 52$^{o}$C for 1 min and 72$^{o}$C for 2 min, followed by a cycle of 72$^{o}$C for 10 min and 12$^{o}$C cooling.

### 2.2.6.2 Agarose gel electrophoresis

DNA extracts, as well as their PCR products, were analysed using agarose gel electrophoresis for quality and successful amplification. Agarose concentrations of 0.8%, 1%, and 2% were used for observation of DNA extracts, PCR products, and RFLP fragments, respectively. The measured concentration of respective agarose was dissolved in 0.5x TBE buffer, which was prepared from 5x TBE stock (54 g Tris base, 27.5 g boric acid, 20 millilitres of 0.5 M EDTA (pH 8.0) in 1 litre of deionized water), and completely melted in a microwave. A proportional volume of Gelred™, Fisher Biotec Australia, was added in melted agarose (1 µl Gelred for every 10 ml gel volume). After cooling at 50$^{o}$C, the gel was poured into a casting tray with a sample comb and allowed to solidify at room temperature. Once the gel was set, the comb was removed. The DNA sample was mixed with Gel Loading Dye Blue (6x) (New England Biolabs®$_{inc}$) and then loaded into the sample wells with the gel

submerged in 0.5x TBE. The sample DNA was run through the gel for various durations depending on the gel concentration, voltage, and size of the tray. At the same time, the 100 bp DNA ladder (New England Biolabs®$_{inc}$) was used as a standard comparison of the DNA length.

### 2.2.6.3 Preparation of PCR products for sequencing

All PCR products were cleaned by an UltraClean™ PCR Clean-Up, DNA purification Kit (Catalog # 12500-100 MoBio Laboratories, Inc.). For the specified volume of PCR product, five times the volume of the spin bind solution was mixed with it, and the resulting mixture was added into a spin filter column in Eppendorf tubes. After centrifugation of the tubes at 13,000 x g for 30 seconds, the spin filter unit was maintained and the liquid flow-through from the tube was discarded. The spin filter was treated with 300 µl SpinClean buffer and followed by centrifugation at 13,000 x g for 30 seconds, the liquid flow-through was discarded. The spin filter unit was further centrifuged for 2 minutes at 13,000 x g and 50 µl Injection water was added into the spin filters and incubated at room temperature for 5 minutes followed by centrifugation for 2 minutes at 13,000 x g. The clean PCR products were collected in the collection tubes and stored at -20$^{o}$C. One microliter of cleaned PCR product was run in 1% agarose gel to check the quality. Representative PCR products from each RFLP pattern were sent for sequencing to Macrogen, South Korea, and the results were subjected to a BLASTN search of the NCBI database.

### 2.2.7 Restriction endonuclease digestion of PCR products

RFLP of the amplified product was used to group the isolates according to the method of Cook and Meyers (2003). The amplified products of 27F and 765R were digested first with *Hha*I and in some cases with *Pst*I restriction enzymes for RFLP-based categorization of the isolates. This part of the gene is more variable compared to the other half which was amplified by 704F and 1492R primers.

### 2.2.7.1 RFLP working conditions

The first segments of 16S rRNA gene PCR products were cleaved using restriction endonuclease *Hha*I and *Pst*I (Promega, USA). The following reaction conditions were used for the final 20 µl volume: 4 µl PCR products, 1 µl restriction enzyme, 2 µl 10x buffer, 0.2 µl bovine serum albumin (all Promega)

and 12.2 µl of injection water. This mixture was incubated at 37$^{o}$C for 18 hrs to achieve complete digestion of the product and finally, the product was placed in 65$^{o}$C for 15 min to inactivate the enzyme.

#### 2.2.7.2 Visualization of RFLP digestion products

The patterns of the RFLP digestion products were observed by 2% agarose gel electrophoresis. The preparation of the gel documented in Section 2.2.6.2. 100 bp DNA ladder (New England Biolabs®$_{inc}$) was loaded and run together with the digestion product at 100 v for 1.30 hrs. The band size of each RFLP digestion product was compared with the DNA ladder and every isolated bacterium was grouped based on similarity on the pattern of bands in the gel.

## 2.3 Chemotaxonomy studies of bacterial cell wall contents

### 2.3.1 DAP analysis

The DAP analysis was performed as described previously (Kaewkla *et al*, 2016; Bousfield, 1985 ). Two loopful of bacterial cells, prepared using a method explained in Section 2.1.8, were transferred to 10 ml Pyrex tubes containing 1 ml of 6 N HCl for hydrolysis. After thorough mixing, the tubes were placed at 121$^{o}$C for 15 min. Two millilitres of sterilised Reverse osmosis (RO) water was added to the cooled mixture, mixed thoroughly and filtered using Whatman paper No 1. The filtrate was evaporated to dryness using a boiler followed by the addition of 1 ml of sterilised RO water. This process was repeated twice and 40 µl of sterilised RO water was added for the final dried residue. The final hydrolysate products were stored at -20$^{o}$C till further analysis with TLC.

Analyses of DAP with TLC depend on the use of TLC cellulose plates (Merck 5552 cellulose coated aluminium sheets), which were pretreated by heating at a temperature of 110$^{o}$C for 1 hr. The buffer system consisting of pyridine: 6 M HCl: RO $H_20$: MeOH (10:4:26:80) was equilibrated for at least 1 hr in a tank. The hydrolysate was diluted 1 in 10 with sterile RO water, and 2.5-20 µl was spotted in a 1 cm lane and dried with a hairdryer held 40 cm above the plate. A standard mix (Sigma) containing both-*meso* and -*LL* isomers of DAP (0.1 M DAP in 0.2 M NaOH) was spotted and run together with the test until the solvent system reaches approximately 1.5 cm from the top of the TLC plate. Once

air-dried, the TLC plate was sprayed with a 0.2% ninhydrin in acetone. The sprayed TLC plate was allowed to dry and placed in a dry heat oven of 100$^{o}$C for 5-10 min to enhance colour development. The plate remained covered overnight to enhance the development of yellow DAP spots. The DAP spots appeared olive green fading to yellow, where the LL-isomer travels more than the *meso*-isomer. The type of DAP for the sample is determined by comparing the distance it travelled with the standard DAP.

### 2.3.2 Sugar cell wall analysis

Bacterial cell wall sugars analysis was performed as described previously (Hasegawa, 1983). Two loopful of bacterial biomass (Section 2.1.8) were transferred to 10 ml Pyrex tube containing 200 µl of 0.25 N HCl followed by heating at 121$^{o}$C for 15 min. Once cooled, the hydrolysate was centrifuged at 12000 x g for 5 min, and the debris was removed by filtering through Whatman paper no 1 and the filtrates either stored at -20$^{o}$C or analysed immediately with TLC. Two microliters of the test and reference bacterial hydrolysate were spotted onto the cellulose TLC sheet (Merck 5552 cellulose coated aluminium plates). Also, 1 µl of 1% solution of arabinose, ribose, galactose, glucose, mannose, rhamnose and xylose was spotted as standard. The mobile phase consisting of n-butanol: RO $H_2O$: pyridine: toluene (10:6:6:1) was equilibrated in the TLC tank for 1 hr and then air-dried TLC plate was run for the period until the solvent reached approximately 1.5 cm from the end. The dried TLC plate was sprayed with aniline phthalic acid (aniline 2 ml; phthalic acid 3.3 g; water-saturated n-butanol 100 ml) for colour development. For further colour enhancement, the dried plate was heated in a dry heat oven at 100$^{o}$C for 10 min. The sugar pattern of the test sample was obtained by comparing the distance it travelled with specific standard sugars.

### 2.3.3 Menaquinone analysis

Menaquinone analysis was performed as described previously (Alderson, 1985). The bacterial biomass was prepared using the protocol in Section 2.1.7. Approximately 50 mg of lyophilized bacterial biomass was placed in 8.5 ml of polytetrafluoroethene capped tubes followed by the addition of 2 ml of each of the following solutions: methanol-0.3% NaCl solution (100:10) and petroleum ether (b.p. 60-80$^{0}$C). The mixture was mixed end to end in a shaker for 15 min and the

upper layer was transferred to a small vial. Furthermore, 1 ml of petroleum ether was added and mixed in the same way. The collected upper layers were pulled together and evaporate with $N_2$. The resulted menaquinone extract was purified by using TLC. The extract was spotted about 4 cm on 10 x 10 cm pre-coated kieselgel 60 $F_{254}$ plastic-backed plates (Merck # 5735) and vitamin K (Sigma) was used as a standard. The buffer system consisting of hexane: diethyl ether (85:15) was equilibrated in a TLC tank for 1 hr and the plate was run until the solvent reached 1.5 cm from the end. Once dried, the plate was observed by using UV (254 nm) and the menaquinone has appeared as a dark brown spot with an Rf value of about 0.8, the same as vitamin K. The menaquinone band was scraped and placed into 1 ml of acetone. The mixture was vortexed followed by shaking for 30 min and centrifugation at 8000 x g for 5 min. The resulting supernatant was dried with a vacuum evaporator and resuspended in 50 µl of acetone. Analyses of menaquinone were done by reverse-phase LC-MS employing UV detection and electrospray mass spectrometry (ESI). This method used a ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm (Agilent Part No. 993967-902) column, and with the solvent system of isopropanol: methanol (1:1) at a flow rate of 1 ml/min.

### 2.3.4 Mycolic acid analysis

Bacterial biomass for this analysis was prepared as described in Section 2.1.7 and the analysis was performed as described previously (Minnikin, 1980; Minnikin, 1975). Approximately 5 mg of lyophilized biomass was placed in a 10 ml Pyrex tube which contains 40 µl of concentrated sulphuric acid and 1 ml each of methanol and toluene. The mixture was incubated for 18 hrs at 75$^{o}$C and once cooled, 400 µl of hexane was added and the upper part which contains mycolic acid was collected following thoroughly mixing the resultant solution. Ten microlitres of mycolic acid extracts from test bacteria, a known mycolic acid producing bacteria, and extracts from a non-mycolic bacteria, were spotted on silica gel 60 $F_{254}$ TLC plates ( Merck 5554 aluminium sheets), and run on buffer system of petroleum ether: diethyl ether (85:15) for 30 min. After drying, the TLC plate was sprayed with a 10% ethanolic solution of molybdophosphoric acid (10 g of molybdophosphoric acid dissolved in 100 ml of absolute ethanol). The bluish-green spot of mycolic acid developed well after the plate heated at 120$^{o}$C for 15 min. Methyl ester of mycolic acid was presented with Rf value between 0.1-0.5, while methyl esters of non-hydroxylated fatty acids resulted in higher Rf values, such as 0.8 to 1.

### 2.3.5 Peptidoglycan analysis

The analysis was performed as described previously (Schumann, 2011). Two loopful of bacterial cells, prepared as described in Section 2.1.7, were transferred to a 10 ml Pyrex tube containing 1 ml of 6 N HCl for hydrolysis. The bacterial biomass was subjected to acid hydrolysis at 120$^{o}$C for 16 hrs. The peptidoglycan was analyzed using two-dimensional TLC. Five microliters of the hydrolyzate were spotted onto silica gel 60 $F_{254}$ TLC plates (Merck 5554 aluminium sheets) and run in the first direction with a solvent system of isopropanol-acetic acid-water (75:10:15 v/v/v) till the end. This solvent system was equilibrated in a TLC tank overnight. The TLC plate was dried overnight in a fume hood and the plate was run in the same direction with the same solvent. Once completely dry, the plate was subjected to the second direction run with the solvent system of α-picoline-25% $NH_4OH$-water (70: 2:28 v/v/v). Once air-dried, the TLC plate was sprayed with a 0.2% ninhydrin in acetone. The sprayed TLC plate was allowed to dry and placed in a dry heat oven at 100$^{o}$C for 5-10 min to enhance colour development. The TLC pattern of the test bacterium was compared with the pattern of known bacterial species.

### 2.3.6 Fatty acid methyl ester (FAME) analysis

Bacterial biomass for FAME analysis was prepared following methods described in Section 2.1.6 and was extracted following the standard MIDI protocols (Sasser, 1990). About 40 mg of wet cells were placed in a clean 13 x 100 cm glass test tube and FAME was extracted using the following 4 steps. The first step was saponification. One millilitre of 3.75 M NaOH in methanol was added to each tube containing cells and after the tubes were closed with Teflon-lined caps and subjected to quick vortex followed by heating in a water bath at 95$^{o}$C for 5 min. Following these steps, the tubes were vigorously vortexed for 10 seconds then incubated at 95$^{o}$C for 30 min.

Two millilitres of 3.2 N HCl in methanol was added to each tube and briefly vortexed. The tubes were then placed in an 80 $\pm$ 1$^{o}$C water bath for 10 $\pm$ 1 min. The liquid-liquid extraction step removed fatty acid methyl esters from the acidic aqueous phase and transferred them to the organic phase. One and a quarter millilitre of hexane: methyl tert-butyl ether (1:1) was added to each cooled tube followed by recapping and gently mixed by tumbling end over end in a laboratory shaker for about

10 min. The tubes were uncapped and the aqueous (lower) phase was pipetted out and discarded. The final step was base wash which reduces contamination of the injection port liner, the column, and the detector. Three ml of 0.3 M NaOH was added to each tube. The tubes were recapped and then fixed to a laboratory shaker for 5 min. About two-thirds of the organic phase was pipetted into a GC vial which was capped and put in the fridge. Then, the sodium sulphate was added to the sample to eliminate water and pipette samples to a small GC vial for analysis or stored at -20$^{o}$C. The Sherlock ACTIN6 software version 6.2B was used for analysis side by side with the nearest type strains.

### 2.3.7 Phospholipid analysis

The bacterial biomass was prepared using the protocol described in Section 2.1.7 and the phospholipid pattern determined as described previously (Minnikin, 1984; Komagata, 1988) using 5% ethanolic molybdophosphoric acid, α-naphthol, ninhydrin, molybdenum blue reagent, and periodate-Schiff spray. Approximately 50 mg of lyophilized bacterial biomass was placed in 8.5 ml of polytetrafluoroethene capped tubes followed by the addition of 2 ml of each of the following solutions: methanol-0.3% sodium chloride solution (100:10) and petroleum ether (b.p. 60-80$^{0}$C). The mixture was mixed end to end in a shaker for 15 min and the upper layer was removed. One millilitre of petroleum ether was further added and following mixed end to end the upper layer was removed and the resulting lower layer was heated in a boiling water bath for 5 min and cooled in a water bath at 37$^{0}$C for 5 min. Chloroform-methanol-water (90:100:30) ( 2.3 ml) was added to this mixture, mixed end to end in a shaker for 60 min, then centrifuged at 30,000 x g for 1 min, and the supernatant transferred to an 8.5 ml tube. In the remaining sediment, 0.75 ml of chloroform-methanol-water (50:100:40) was added, mixed end to end for 30 min and following centrifugation at 30,000 x g for 1 min, the supernatant was collected and pooled with the previous. On the combined supernatants, 1.3 ml each of chloroform and aqueous sodium chloride was added and the mixture was thoroughly mixed, centrifuged and the upper layer was discarded. Finally, the lower layer which contained the phospholipid was evaporated to dryness with a rotary evaporator.

During analysis, the dried polar lipid was dissolved with 60 µl of chloroform-methanol (2:1, v/v) and 10 µl of it was applied to the corner of 6.6 X 6.6 cm pieces of TLC sheet (Merck 5554 aluminium-backed silica gel sheets). These plates were run into two-dimensional TLC with solvent systems of

methanol-water (65:25:4, by vol) in the first direction, followed by acetic acid-methanol-water (40: 7.5: 6: 2, by vol.) in the second direction. Each plate was sprayed with specific chemicals (Appendix 2) to detect the type of phospholipid present.

# Chapter Three: Isolation of bacteria from marine sponges of South Australia and characterization by morphology, RFLP analysis, and 16S rRNA gene sequencing

## 3.1 Introduction

Studies on biologically active metabolites are increasingly important as they can be linked to various biotechnological applications, including the search for new antibiotics effective against multidrug-resistant pathogens (Payne *et al.*, 2007). Multidrug-resistance has become a global challenge as increases in infections due to multidrug-resistant bacteria such as *Escherichia coli*, *Salmonella* spp., and methicillin-resistant *Staphylococcus aureus* (MRSA) are now prevalent both in clinical settings as well as in the food industry (Chambers & Deleo, 2009; Flemer *et al.*, 2012). This problem requires proper stewardship of the current antibiotics as well as the continuous search for effective novel antibiotics. Natural environments are still an important source for the discovery of novel antibiotics and significant efforts are in place for chemical synthesis of antimicrobials (Bull & Stach, 2007; Claverias *et al.*, 2015). Microbes from terrestrial environments have been investigated for many years and the chance of finding novel products from these sources is minimal. Therefore, it is crucial to concentrate the search for microbes from less explored areas such as marine environments (Claverias *et al.*, 2015; Goodfellow & Fiedler, 2010).

Marine sponges are host to a large variety of bacteria, with about 26 bacterial phyla reported in a recent study (Lee *et al.*, 2011) or more if using a molecular technique (Yang *et al.*, 2019a). These sponge-associated bacteria, via the production of biologically active secondary metabolites, protect the sponges against the harmful effects of pathogens, competitors, fouling organisms and predation (Taylor *et al.*, 2007). It has been also established that sponges produce active secondary metabolites that can have antimicrobial and anti-inflammatory properties (Brady *et al.*, 2009; Piel, 2009). Undeniably, sponges are the most productive marine producers of unique metabolites, with more than 3500 novel compounds having been described from sponges between 1985 and 2008 (Hu *et al.*, 2011; Mehbub *et al.*, 2014). The observation of structural similarities among compounds from sponges and sponge-associated microbiota are the basis to hypothesise that metabolites from sponges have originated from the associated microbes (Wang, 2006). Furthermore, several studies managed to isolate the same compounds from bacteria as those reported from the sponge hosts (Stierle *et al.*, 1988; Bewley *et al.*, 1996; König *et al.*, 2006). Therefore, the observation and discovery

indeed indicated that marine sponges can be an ideal source for the search of microbes having the potential to produce new antimicrobial agents (Flemer *et al.*, 2012).

Previous studies have reported the isolation of bacteria from marine sponges which displayed various biological activities (Abdelmohsen *et al.*, 2010; Santos *et al.*, 2010; Hu *et al.*, 2011; Li *et al.*, 2011; Radjasa *et al.*, 2011; O'Halloran *et al.*, 2011; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Graça *et al.*, 2015; Ellis *et al.*, 2017; Matobole *et al.*, 2017; Bibi *et al.*, 2018; Kuo *et al.*, 2019). South Australian marine environments are a source of a huge diversity of sponge species with approximately 60% of them being endemic. Variations in the chemical composition among sponges in South Australia have been reported (Sorokin *et al.*, 2007; Sorokin & Currie, 2008) and it is expected that the associated bacteria could also be different either in terms of diversity or in the type of metabolites they produce. Therefore, this section of the project was focused on the isolation of bacteria from marine sponges of South Australia and identify them based on Macro and Microscopic morphology and RFLP patterns. Furthermore, this section also investigated the underlying factors which affect bacterial isolation and their relative diversity.

## 3.2 Methods and materials

### 3.2.1 Sponges sample collection, processing, isolation, and purification

Methods of sponge sample collection, processing, isolation, and purification were described in general method sections 2.2.1, 2.2.2, and 2.2.3. The colony numbers and isolation dates were recorded every week for 16 weeks.

### 3.2.2 Identification of pure isolates

#### 3.2.2.1 Macroscopic morphological characterization

Macroscopic morphological characterizations of the bacterial strains by subculturing them onto ISP2, SYP, TSA, NA, MS, and HPDA were described in 2.1.3.

#### 3.2.2.2 Microscopic morphological characterization

Microscopic characterizations of bacterial strains using different stained and unstained preparations were described in section 2.1.4.

### 3.2.3 Molecular characterizations of the isolates

#### 3.2.3.1 Genomic DNA extraction

Bacterial DNA for all isolates was extracted using the protocol described in section 2.2.5.

#### 3.2.3.2 16S r RNA gene amplification

The protocols for PCR, primer selection, electrophoresis detection of PCR products and preparation of PCR products for sequencing were described in sections 2.2.6.1, 2.2.6 and 2.2.6.3

### 3.2.4. Restriction endonuclease digestion of PCR products

Methods for RFLP digestion and visualization of the patterns were described in sections, 2.2.7.1 and 2.2.7.2.

### 3.2.5. Sponge metagenomic DNA isolation, sequencing, and data processing

The sponge species with the highest culturable bacterial diversity was selected to conduct amplicon-based metagenomic sequencing on Next Generation Sequencing platform Illumina MiSeq. The DNA extraction method was described in the previous study (Yang *et al*., 2015). Purity and quantity of DNA were determined with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The qualified DNA samples (A260/280: 1.8–2.0; Conc. > 100 ng/µl) extracted from different sponge individuals for each species were selected and kept at −20 °C for subsequent sequencing.

Illumina MiSeq amplicon library establishment was following the protocol developed in the previous study (Yang *et al*., 2019b). Briefly, The three primers for the selected 16S rRNA gene region V1V3 are 28F-519R (28F: 5’-GAG TTT GAT CNT GGC TCA G-3’; 519R: 5’-GTN TTA CNG CGG CKG CTG-3’) (Crou´e

*et al*., 2013), 518F-926R for the V4V5 region (518F: 5'-CCA GCA GCY GCG GTAAN-3'; 926R: 5'-CCG TCA ATT CNT TTR AGT-3') (Nelson *et al*., 2014) and 803F-1392R for the V5V8 region (803F: 5'-TTA GAN ACC CNN GTA GTC-3'; 1392R: 5'-ACG GGC GGT GWG TRC-3') (Engelbrektson *et al*., 2010). PCR was performed based on the protocol presented in the previous study (Caporaso *et al*.,2011).

Sequencing was run multiple times (n>3) for each amplicon. The demultiplexing and quality filter (at Phred > = Q20) for the Illumina MiSeq dataset was processed by script split libraries.py in QIIME pipeline (version 1.9.1) (Caporaso *et al*., 2010). The multiplexed reads were assigned to samples based on their nucleotide barcode (demultiplexing). Quality filtering was performed based on the characteristics of each sequence, removing any low quality or ambiguous reads. Closed-reference picking was selected in our study (see OTU picking strategies in QIIME) (Caporaso *et al*., 2010). By default, QIIME applied the uclust (Edgar 2010) consensus taxonomy classifier to attempt to assign taxonomy to each representative sequence. The OTU representative sequences were aligned using PyNAST tool (Caporaso *et al*., 2010) and the filtered alignment file was then used to build a phylogenetic tree using a tree-building program (FastTree (Price *et al*., 2010)). Finally, an OTU table (otu table.biom) was summarized to show the OTU abundances with taxonomic identifiers for each OTU based on Greengenes taxonomy. The raw sequencing reads were deposited in the GenBank at the National Center for Biotechnology Information (BioProject ID: PRJNA490791).

### 3.2.6 Growth at different oxygen levels

In this study, isolates were primarily obtained from aerobic, microaerophilic and anaerobic conditions. To assess whether isolates are maintained under the different oxygen levels, all bacterial isolates were re-cultured onto ASP and SYP agar and incubated at 27$^{o}$C under aerobic and strict anaerobic conditions. Growth at the specific oxygen level was assessed by observing colony growth. All tests were carried out in duplicate on both media and the cultures were kept for 2 weeks before discarding of plates with no visible colony growth.

### 3.2.7 Temperature and NaCl tolerance tests

For temperature tolerance tests, all bacterial isolates were inoculated on ASP and SYP agars in a grid of four and the plates were incubated at 3, 15, 27, 37 and 45$^{o}$C for 2 weeks. In the same manner, the

bacterial isolates were inoculated onto ASP and SYP media containing NaCl concentrations (w/v) of 0, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 16% and incubated at 15$^{o}$C and 27$^{o}$C for 2 weeks. Both tolerance tests were carried out in duplicate on both media. The agar plates for NaCl tolerance were prepared using distilled water to remove the effect of any unintended salt. Results were recorded as positive or negative for all treatments by observing the presence or absence of colony growth at specified incubation periods.

## 3.3 Results

### 3.3.1 Isolation and macro and microscopic identification of bacteria from marine sponges

A total of 1234 colony-forming bacteria were isolated from 12 sponge samples collected from Glenelg block (310 CFU) and Rapid Bay Jetty (924 CFU), beaches in South Australia. The bacterial isolates with their coded as prefix RB and GB to reflect sponge collection sites, its sponge sources, colony characteristics, and isolation conditions were indicated in Appendix 3. As indicated in Figures 3.1-3.3, the bacterial isolates appeared in different macro and microscopic features in primary and identification media.

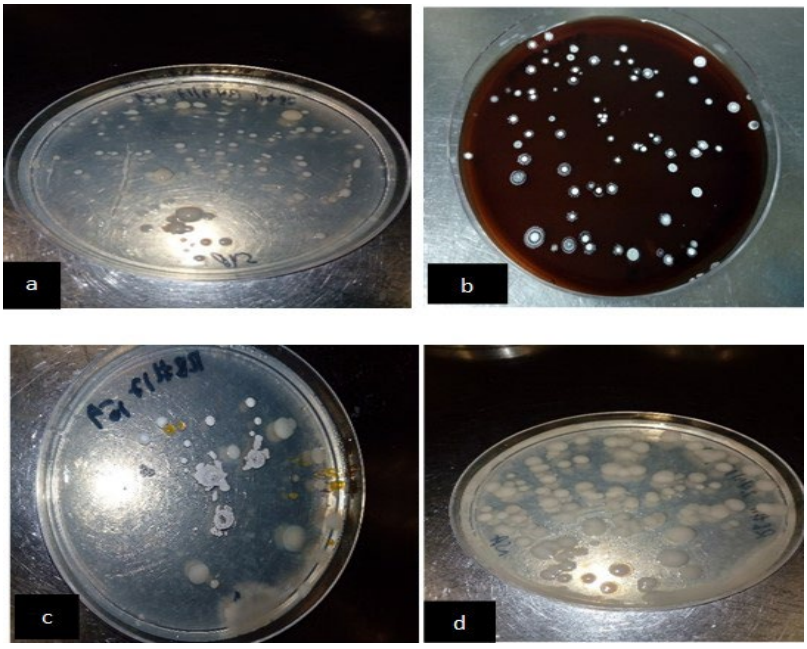



Figure 3.1 Bacterial isolates at different primary isolation media. **a** bacterial isolates in SYP; **b** bacterial isolates in HV; **c** bacterial isolates in ASP; **d** bacterial isolates in MA.

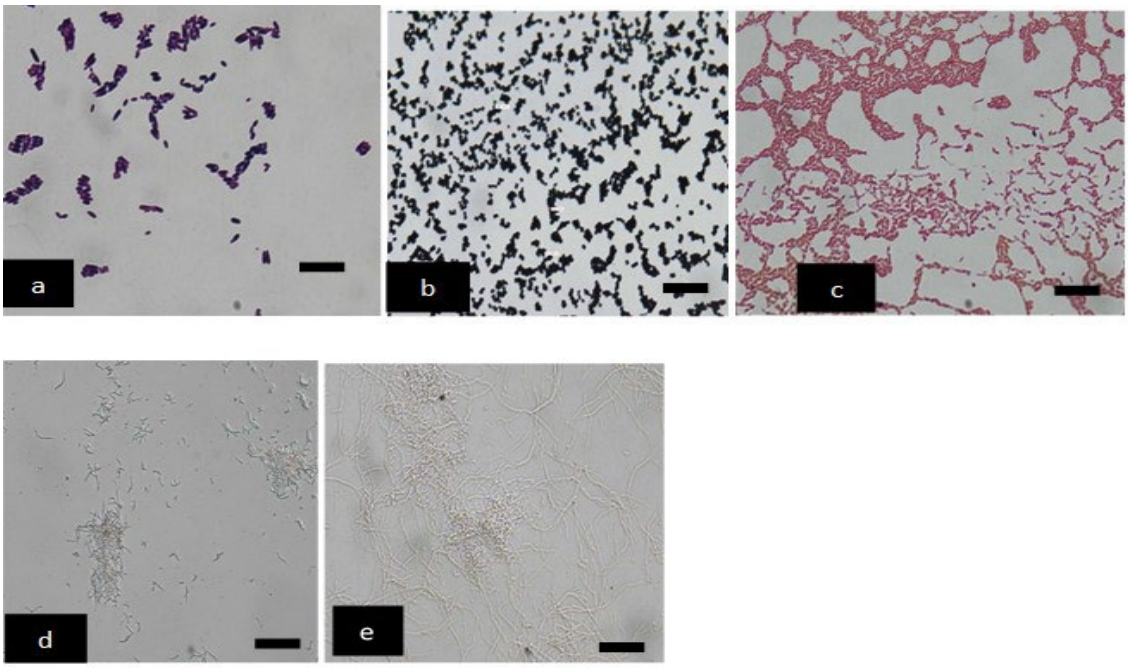



Figure 3.2 Gram stain and wet mount microscopic features of some bacterial isolates with 40x magnification and scale of 10 µm. **a**, **b**, **c** Gram-positive and negative bacteria; **d**, **e** wet mount preparation revealed filamentous bacteria.

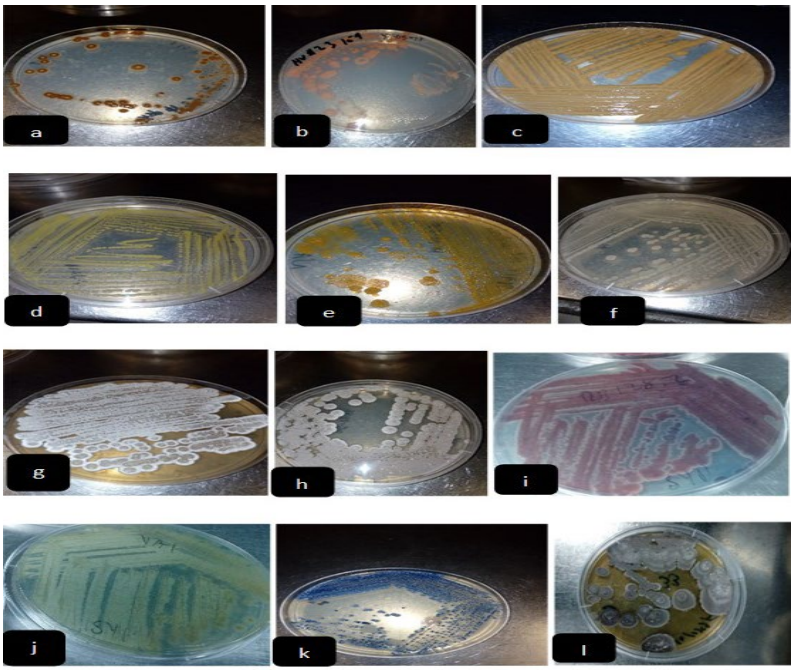



Figure 3.3 Pure bacterial isolates in ISP2 and NA media. **a** to **f** wet, non-spore forming bacterial strains with various morphological forms; **g** to **l** spore-forming bacterial strains with various spore appearances and pigmentation.

The majority (637) of the isolates (Figure 3.4a) were Gram-positive bacteria, followed by filamentous (375) and Gram-negative (222) bacteria. These bacterial isolates were further categorized into 383 bacterial types depending on the morphological variations in different media and microscopic characteristics. One hundred and ninety-five Gram-positive bacteria with common shapes of bacilli, coccobacilli and cocci were clustered for further identification. Similarly, 118 bacteria with aerial and substrate filaments and 70 Gram-negative bacilli and coccobacilli were selected for further identification. Morphologically, non-filamentous bacteria displayed various types of colony consistency (wet, dry, mucoid) and colours (creamy white, yellow, orange, pink, brown, and black) (Figure 3.4b). The filamentous bacteria also appeared in different morphological forms both in the presence and absence of aerial mycelium. Grey (light and dark), yellow, green, blue, brown, purple and pink were the most common spore colours and pigments of the filamentous bacteria (Figure 3.4c). The filamentous bacteria produced two general types of spores; the first type is the one that was easily removed from the medium and the other type firmly attached with it.

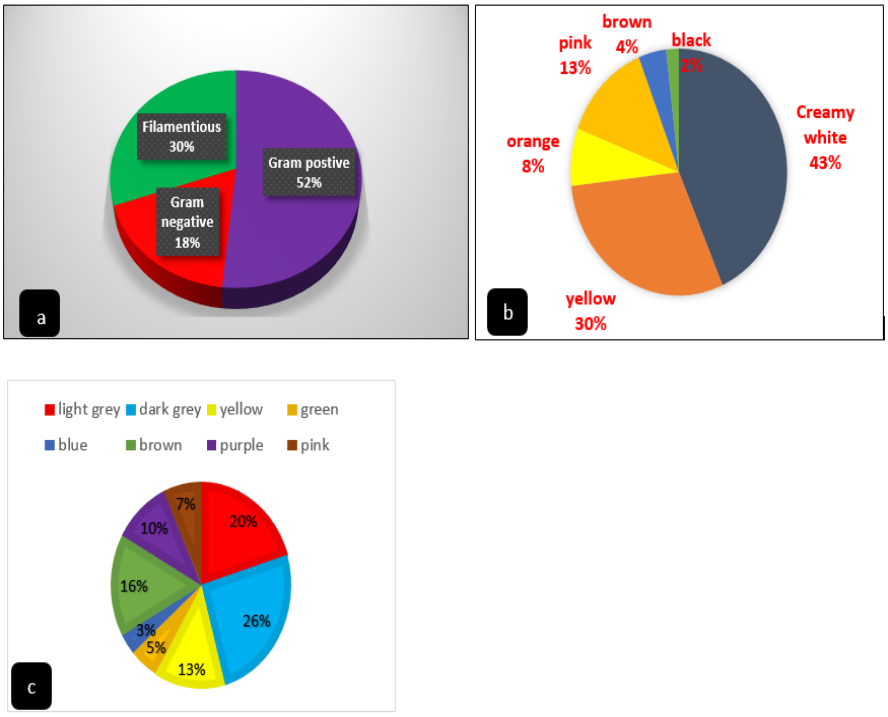



Figure 3.4 Pie charts presenting the different morphological and colony colours of the bacterial isolates. **a** microscopic morphology of the total CFU; **b** the colony appearances of the non-filamentous bacteria (Gram-positive and negative); **c** the colony colours of filamentous bacteria.

### 3.3.2 Identification of the genera in PCR-RFLP patterns with *HhaI* and *PstI* restriction enzymes digestion and 16S rRNA gene sequence

Partial 16S rRNA gene amplicons (obtained using Section 2.2.7) of the 383 bacteria were subjected to restriction enzyme digestion first with *HhaI.* The *HhaI* digestion was able to categorize the 383 bacteria into 32 patterns and their similarities were cross-checked by observing the macro and microscopic features of strains within each pattern group. Pattern groups 7, 21 and 31, which varied morphologically, were further digested with *Pst*I resulting in six more pattern groups of 33-38 (Table 3.1). An example of a digest is indicated in Figure 3.5.

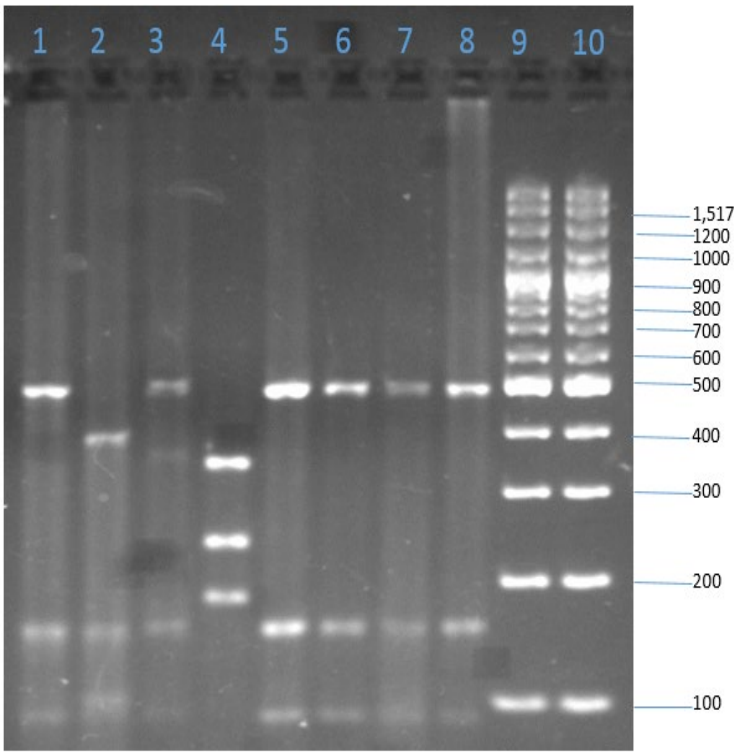



Figure 3.5 Visualization of the 16S rRNA PCR-RFLP patterns with *Hha*I enzyme digest on 1.8% agarose gel electrophoresis. lane 1 RB56 (pattern 31); lane 2 RB71(pattern 33); lane 3 YA2 (pattern 31); lane 4 RB166 (pattern 29); lane 5 YA19 (pattern 31); lane 6 YA22 (pattern 31); lane 7 RB12 (pattern 31); lane 8 RB151(pattern 31); lane 9 and 10 100 bp DNA ladder (BioLabs INC, NEW ENGLAND).

Table 3.1 Identification of the genera via PCR-RFLP patterns with *Hha*I and *Pst*I restriction enzyme digestion and 16S rRNA gene sequence

| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 15 | GB17*, RB180, RB188, RB194, RB203, YAL, YA45 | 600,500,200 | *Gordonia* | Actinobacteria |
| 19 | L3*, L40*, RB202, YAJ, F12* | 400,380,350,250,180 | *Isoptericola* | Actinobacteria |
| 1 | GB23* | Uncut | *Janibacter* | Actinobacteria |
| 21[a] | RB16, RB41, RB42, RB61, RB96, RB62, RB8, RB9, RB10, RB11, RB39, RB40*, RB44, RB63, RB64, RB133, RB143, RB149, RB173, | 400,160,100 | *Kocuria* | Actinobacteria |
| 33[b] | RB71*, RB123, RB79, RB100, RB17, RB50 | 400,160,110,100 | *Kocuria* | Actinobacteria |
| 34[b] | RB43, RB104, RB107*, RB94, RB171, RB108 | 400,300,150,100 | *Kocuria* | Actinobacteria |
| 20 | RBA4*, GB56, GB60, RB2, RB4, RB59, RB187, RB197, RB213, RB204, RB207, RB196, RB206, RB159, GBYAF, GBYAG, RBYA44 | 600,350,250,200,180 | *Microbacterium* | Actinobacteria |

Table 3.1 (Continued)

| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 23 | RB3, RB5*, RB164 | 400,350,300 | *Micrococcus* | Actinobacteria |
| 24 | RBLC12*, RBYA5, RBYA38, GBYAK, RBYAR | 500,250,240 | *Micrococcus* | Actinobacteria |
| 26 | RBYA34*, RBYA35, RBYA39, RB132 | 350,160,150,120 | *Micrococcus* | Actinobacteria |
| 29 | GB9, GB55, RB166* | 300,250,180 | *Micrococcus* | Actinobacteria |
| 17 | LRBC1*, RB214, RB191 | 400,220,200,100 | *Mycolicibacterium* | Actinobacteria |
| 30 | RBYA11*, RB78, RB212, RB200, RBYA9, RB83, RB80 | 400,320 | *Pseudonocardia* | Actinobacteria |
| 12 | GB24, GB63, RBLC21*, RB75, RB82, RB163, RBYA12, RBYAN | 350,310 | *Rhodococcus* | Actinobacteria |
| 38[b] | RBH8*, RB47, RB177 | 500,450,350,210,100 | *Streptomyces* | Actinobacteria |

Table 3.1 (Continued)

| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 37[b] | RB179, RBLC14* | 350,310,250,200 | *Streptomyces* | Actinobacteria |
| 31[a] | RB12*, RB18, RB19, RB29, RB31, RB45, RB46, RB48, RB49, RB51, RB53, RB56*, RB57, RB60, RB65, RB66, RB67, RB68, RB69, RB70, RB74, RB215, RB76, RB77, RB210, RB89, RB110, RB112, RB113, RB114, RB115, RB116, RB117, RB119, RB120, RB121, RB124, RB126, RB127, RB128, RB129, RB130, RB134, RB135, RB136, RB139, RB140, RB142, RB145, RB146, RB147, RB150, RB151*, RB152, RB153, RB154*, RB155, RB158, RB186, RB190, GBYA1, RBYA2*, RBYA3, RBYA7, RBYA13, RBYA18, RBYA19*, RBYA20*, RBYA21, RBYA22*, RBYA23, GBYA27*, RBYA28, RBYA32, RBYA33 , RBYA8, RBYAE, RBYAQ | 500,160,100 | *Streptomyces* | Actinobacteria |
| 32 | RB15, RB20, RB21, RB22, RB23, RB24, Rb25, RB26, RB27*, RB28, RB30, RB32, RB33, RB34, RB35, RB36, RB54, RB111, RB118, RB131, RB38*, RB144, RB181, RB182, RB183 | 450,160,80,100 | *Streptomyces* | Actinobacteria |

Table 3.1 (Continued)

| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 3 | GB37*, GB77, YA4 | 400,210 | *Muricauda* | Bacteroidetes |
| 1 | GB21*, RBYAI* | Uncut | *Bacillus* | Firmicutes |
| 5 | GB10, GB66, GB67, RB199, RB208, RB209, RBYAD, RBL30* | 450,200 | *Bacillus* | Firmicutes |
| 7[a] | GB2, GB25, GB48*, GB50, GB51, GB52, GB54, RB1, RB13, RB52 , RB37*, RB98, RB88, RB90, RB91, RB92, RB93, RB95, RB99*, RB137, RB138*, RB141, RB148*, RB160,RB162, RB165, RB167, RB178, RB192, RB193, GBYA14, GBYA15, GBYA16, GBYA24, RBYA25, GBYA10, GBYA30, RBYA51, GBYA49, GBYA50, RBYA41, RBYA43, RBYA48, RBYA6*, RBYAM, RBYAH, RBYAO, RBYAS, RBLC2*,RBYAU | 350,200,180 | *Bacillus* | Firmicutes |
| 8 | RBD5*, RB219, RB169, RB175, RB216 | 350,200,100 | *Bacillus* | Firmicutes |
| 13 | GB14, GB53, Rb 14*, RB97, RB103, RB109, RB157, RB217, RB170, RB174, RB201, RBYA17, RBYA26, RBYA31, RBYA36*, RBYA40 | 350,250,200,100 | *Bacillus* | Firmicutes |

Table 3.1 (Continued)

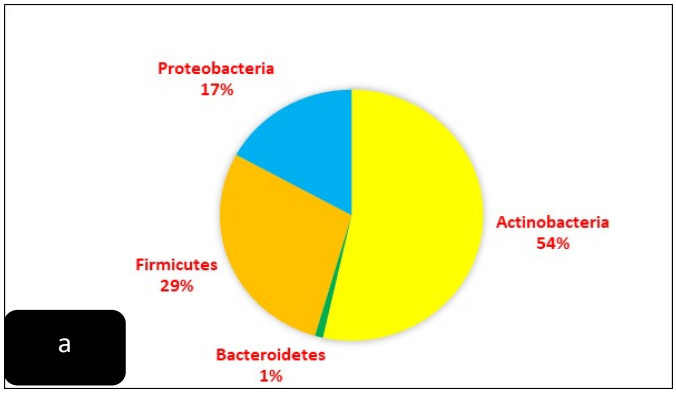
| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 35[b] | RBL35*, RB156, RBYA37 | 400,300,220,200 | *Bacillus* | Firmicutes |
| 36[b] | GB1, GB3* | 500,350,250,220,200,180 | *Bacillus* | Firmicutes |
| 22 | GB19, GB27, GB31, RB62* | 400,300,100 | *Bacillus* | Firmicutes |
| 9 | GB41, GB72, GB73, RBLC7*, RB189, RBYAA, RBYAC | 350,220,180,150,100 | *Fictibacillus* | Firmicutes |
| 10 | GB74, GB75, RBLC15*, RBLC16*, RB184, GBYA47 | 300,200,150 | *Flasibacillus* | Firmicutes |
| 18 | RBL11*, RB218, RB72, RB172, RB176 | 600,500,220,100 | *Staphylococcus* | Firmicutes |
| 16 | RB85, RB86*, RB211, RB168, RB205 | 700,120 | *Leisingera* | Proteobacteria |
| 4 | GB11*, GB16, GB18, GB71, GB80 | 450,400 | *Limimaricola* | Proteobacteria |
| 6 | GB32, GB33*, GB36, GB45, GB46*, GB65, GB69, RB6, RB101, RB102, RB105, RB106 | 350,200,180 | *Limimaricola* | Proteobacteria |

Table 3.1 (Continued)

| Pattern No | Isolate No | Fragment sizes | Genus | Phylum |
|---|---|---|---|---|
| 25 | RB122* | 420,400 | *Pseudoalteromonas* | Proteobacteria |
| 14 | GB4, RBLC17*, RB161, RB185, RB198, RBYA46 | 600,180,100 | *Pseudomonas* | Proteobacteria |
| 11 | GB7, GB81, GB15*, GB76, GB79 | 350,180 | *Rhodovulum* | Proteobacteria |
| 1 | GB12* | Uncut | *Sulfitobacter* | Proteobacteria |
| 2 | GB5, GB29, GB28, GB38, GB40, GB42, GB44*, GB49, GB59, GB64, GB61, GB70, GB78, RB55, RB58*, RB81, RB84, RB87 | 400,350 | *Sulfitobacter* | Proteobacteria |
| 27 | GB22, GB26, GB39*, GB47, GB57, GB58, RB7, RB73* | 400,350,300,220,200,100 | *Sulfitobacter* | Proteobacteria |
| 28 | GB6*, GB20, GB34, GB35, RB195 | 600,200 | *Sulfitobacter* | Proteobacteria |

Three hundred and eighty-three bacterial isolates were categorized into 38 RFLP patterns with two enzymes (*Hha*I and *Pst*I ) and a representative from each pattern sequenced for partial 16S rRNA gene. **a** groups further digested with *PstI;* **b** groups obtained after *PstI* digestion; ***** bacterial isolates selected for 16S rRNA gene sequencing.

As indicated in Table 3.1 above, 63 bacteria were selected randomly as representatives of each RFLP pattern and subjected to partial 16S rRNA gene sequencing. The result placed the 383 bacteria into 21 genera under the four phyla of Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes. The percentage distribution of each phylum and genus was indicated in Figure 3.6a and b, and of the 383 bacteria isolated, the most abundant (54%) belonged to the phylum Actinobacteria, followed by Firmicutes (28%), Proteobacteria (17%) and Bacteroidetes (1%). Among the 21 genera identified, the genus *Streptomyces* was the most frequently isolated (30%) followed by *Bacillus* (23.8%) with the least abundant being *Janibacter* and *Pseudoalteromonas,* each with 0.3%. Nearly half of the isolated genera belong to the phylum Actinobacteria.



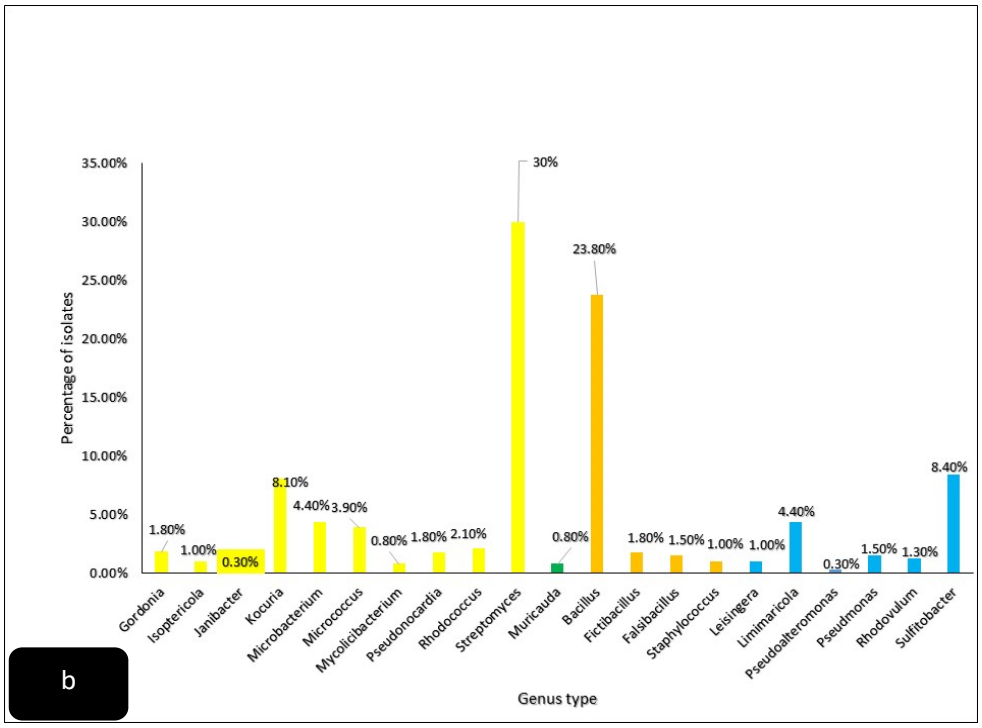




Figure 3.6 Percentage distribution of bacterial isolates in terms of phyla and genera. **a** the percentage distribution of the four phyla; **b** the percentage distribution of the 21 genera.

The RFLP patterns (38) of the isolates with respect to incubation conditions and temperatures are presented in Table 3.2. The majority (89.5%) of the RFLP patterns isolated at 27$^{o}$C aerobic incubation followed by 71% at 15$^{o}$C aerobic, 50% at 27$^{o}$C microaerophilic, and 47.4% at 27$^{o}$C anaerobic. Twenty-one percent of the patterns were common among all incubation conditions, 29% were common among three incubation conditions, and 32% were common among two incubation conditions. More than half (53%) of patterns which are isolated from 27$^{o}$C aerobic incubation consist of genera belongs to phylum Actinobacteria, 42% from 27$^{o}$C microaerophilic consist of genera from phylum Actinobacteria, 41% of the patterns form 15$^{o}$C aerobic incubation represented by genera from phylum Actinobacteria, and only 11% of those patterns isolated from 27$^{o}$C anaerobic incubation comprised of genera from phylum Actinobacteria. Of interest, almost all (105 of 111) *Streptomyces* spp. were isolated aerobically at 27$^{o}$C with only 2 Actinobacterial genera were isolated anaerobically. In contrast, all except two of the non-Actinobacterial genera could be isolated under anaerobic conditions. The complete list of bacterial RFLP patterns with respect to isolation conditions indicated in Appendix 4.

The percentage similarity of partial and full 16S rRNA gene sequence of the 63 selected bacterial strains with type cultures is shown in Table 3.3 and it was found that 18 bacterial strains (29% of those sequenced) showed a sequence similarity of less than 99%. Ten out of 29 sequenced bacterial strains from phylum Actinobacteria, 3 out of 21 bacterial strains from phylum Firmicutes, 4 out of 12 bacterial isolates from phylum Proteobacteria, and one out of one bacterial strain from phylum Bacteroidetes displayed sequence similarities of less than 99%. And theses bacterial strains could be potential candidates for full sequencing in the search for putative novel isolates. Just to confirm this novelty, three of the less common genera were selected among the possible candidates (due to limited scholarship duration) for full 16S rRNA gene sequencing and the complete polyphasic taxonomy. The three bacterial strains were confirmed novel and the name *Isoptericola rapidicum* L4O (MK367392), *Muricauda gelenilca* GB37 (MK367393) and *Mycolicibacterium rapidicum* LC1 (MK358953) were assigned. Their full description of these novel isolates will be presented in a subsequent chapter. The 16S rRNA gene sequences of the bacterial strains are indicated in Appendix 5.

Table 3.2 Distribution of RFLP patterns, genus, and phylum in terms of isolation temperatures and oxygen levels

| Genus | Pattern No | 27°C aerobic | 27°C anaerobic | 27°C Microaerophilic | 15°C aerobic | Genus | Pattern No | 27°C aerobic | 27°C anaerobic | 27°C Microaerophilic | 15°C aerobic |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Gordonia* | 15 | 4[a] | - | 1 | 2 | *Micrococcus* | 29 | 1[a] | - | - | 2 |
| *Isoptericola* | 19 | 2[a] | - | 2[a] | 1[a] | *Mycolicibacterium* | 17 | 2[a] | - | 1 | - |
| *Janibacter* | 1 | 1[a] | - | - | - | *Pseudonocardia* | 30 | 7[a] | - | - | - |
| *Kocuria* | 21 | 7 | - | 3 | 9[a] | *Rhodococcus* | 12 | 1 | 3[a] | 2 | 2 |
| *Kocuria* | 33 | 5[a] | - | - | 1 | *Streptomyces* | 38 | 3[a] | - | - | - |
| *Kocuria* | 34 | 5[a] | - | - | 1 | *Streptomyces* | 37 | 2[a] | - | - | - |
| *Microbacterium* | 20 | 11 | 4[a] | 2 | - | *Streptomyces* | 31 | 76[a9] | - | 3 | 1 |
| *Micrococcus* | 23 | 2 | - | - | 1[a] | *Streptomyces* | 32 | 24[a2] | - | 2 | - |
| *Micrococcus* | 24 | 2[a] | - | - | 3 | *Muricauda* | 3 | 3[a] | - | - | - |
| *Micrococcus* | 26 | 3[a] | - | - | 1 | *Bacillus* | 1 | - | 1[a] | - | 1[a] |

Table 3.2 (Continued)

| Genus | Pattern No | 27°C aerobic | 27°C anaerobic | 27°C Microaerophilic | 15°C aerobic | Genus | Pattern No | 27°C aerobic | 27°C anaerobic | 27°C Microaerophilic | 15°C aerobic |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bacillus* | 5 | 2 | 2 | 2[a] | 2 | *Staphylococcus* | 18 | 5[a] | - | - | - |
| *Bacillus* | 7 | 9[a2] | 15[a] | 8[a2] | 17[a2] | *Leisingera* | 16 | 1 | 2 | - | 2[a] |
| *Bacillus* | 8 | 2 | 1[a] | | 2 | *Limimaricola* | 4 | 1[a] | 2 | - | 2 |
| *Bacillus* | 13 | 5[a] | 4 | 3[a] | 4 | *Limimaricola* | 6 | 3[a] | 1 | 2 | 6[a] |
| *Bacillus* | 35 | 2[a] | - | - | 1 | *Pseudoalteromonas* | 25 | - | 1[a] | - | - |
| *Bacillus* | 36 | - | - | - | 2[a] | *Pseudomonas* | 14 | 1[a] | 2 | 1 | 2 |
| *Bacillus* | 22 | 2 | | 1[a] | 1 | *Rhodovulum* | 11 | - | 3[a] | - | 2 |
| *Fictibacillus* | 9 | 2 | 3 | 2[a] | - | *Sulfitobacter* | 1 | 1[a] | - | - | - |
| *Falsibacillus* | 10 | - | 4[a] | 2[a] | - | *Sulfitobacter* | 2 | 2 | 7 | 3[a] | 6[a] |
| *Sulfitobacter* | 27 | 1 | 3[a] | 1 | 3[a] | *Sulfitobacter* | 28 | - | 1 | 1[a] | 3 |

[a] Isolates selected for 16S rRNA gene sequencing. The number of isolates sequenced is indicated next to the letter

Table 3.3 Percentage similarity of 16S rRNA partial gene sequencing with type strains

| Isolates | Highest match | % | RFLP groups | Genbank accession number | Isolates | Highest match | % | RFLP group | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|
| LC2 | *Bacillus flexus* NBRC 15715$^T$ | 98.97 | 7 | MN310326 | RB154 | *Streptomyces sundarbansensis* MS1/7$^T$ | 98.30 | 31 | MN310351 |
| LC35 | *Bacillus pumilus* ATCC 7061$^T$ | 98.44 | 35 | MN310333 | GB39 | *Sulfitobacter indolifex* HEL-45$^T$ | 98.87 | 27 | MN310322 |
| GB21 | *Bacillus qingshengii* G19$^T$ | 98.25 | 1 | MN310332 | RB37 | *Bacillus algicola* KMM 3737$^T$ | 99.46 | 7 | MN310361 |
| GB17 | *Gordonia otitidis* NBRC 100426$^T$ | 99.1 | 15 | MN310343 | YA36 | *Bacillus haynesii* NRRL B-41327$^T$ | 99.47 | 13 | MN310336 |
| GB17[a] | *Gordonia otitidis* NBRC 100426$^T$ | 98.89 | 15 | MN310343 | GB48 | *Bacillus hwajinpoensis* SW-72$^T$ | 100 | 7 | MN315519 |
| L40 | *Isoptericola rhizophila* BKS 3-46$^T$ | 97.87 | 19 | MN310363 | GB3 | *Bacillus hwajinpoensis* SW-72$^T$ | 100 | 36 | MN315520 |
| L40[a] | *Isoptericola rhizophila* BKS 3-46$^T$ | 98.3 | 19 | MK367392 | YAI | *Bacillus hwajinpoensis* SW-72$^T$ | 99.89 | 1 | MN310359 |
| L3 | *Isoptericola rhizophila* BKS 3-46$^T$ | 97.87 | 19 | MN315523 | LC16 | *Bacillus hwajinpoensis* SW-72$^T$ | 99.68 | 10 | MN310328 |

Table 3.3 (Continued)

| Isolates | Highest match | % | RFLP groups | Genbank accession number | Isolates | Highest match | % | RFLP group | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|
| F12 | *Isoptericola rhizophila* BKS 3-46$^T$ | 97.87 | 19 | MN315524 | RB73 | *Bacillus hwajinpoensis* SW-72$^T$ | 99.46 | 5 | MN310357 |
| GB46 | *Limimaricola hongkongensis* DSM 17492$^T$ | 98.95 | 6 | MN310319 | RB138 | *Bacillus infantis* NRRL B-14911 | 99.46 | 7 | MN310331 |
| GB33 | *Limimaricola hongkongensis* DSM 17492$^T$ | 98.56 | 6 | MN315531 | D5 | *Bacillus licheniformis* ATCC 14580$^T$ | 99.15 | 8 | MN310334 |
| GB11 | *Limimaricola hongkongensis* DSM 17492$^T$ | 98.77 | 4 | MN315530 | RB62 | *Bacillus siamensis* KCTC 13613$^T$ | 99.14 | 22 | MN310330 |
| YA34 | *Microbacterium aquimaris* JS54-2$^T$ | 98.74 | 26 | MN310337 | RB99 | *Bacillus simplex* NBRC 15720$^T$ | 99.87 | 7 | MN310354 |
| A4 | *Microbacterium aquimaris* JS54-2$^T$ | 99.35 | 20 | MN315525 | LC30 | *Bacillus simplex* NBRC 15720$^T$ | 99.48 | 5 | MN310327 |
| GB37 | *Muricauda aquimarina* SW-63$^T$ | 97.67 | 3 | MN310365 | RB148 | *Bacillus simplex* NBRC 15720$^T$ | 99.3 | 7 | MN310352 |

Table 3.3 (Continued)

| Isolates | Highest match | % | RFLP groups | Genbank accession number | Isolates | Highest match | % | RFLP group | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|
| GB37[a] | *Muricauda aquimarina* SW-63$^T$ | 98.13 | 3 | MK367393 | RB14 | *Bacillus sonorensis* NBRC 101234$^T$ | 99.36 | 13 | MN310358 |
| LC1 | *Mycolicibacterium iranicum* DSM 45541$^T$ | 98.68 | 17 | MN310364 | YA6 | *Bacillus algicola KMM 3737$^T$* | 99.68 | 7 | MN310362 |
| LC1[a] | *Mycolicibacterium iranicum* DSM 45541$^T$ | 98 | 17 | MK358953 | LC15 | *Falsibacillus pallidus* CW 7$^T$ | 99.12 | 10 | MN310329 |
| GB15 | *Rhodovulum iodosum* N1$^T$ | 98.09 | 11 | MN310321 | LC7 | *Fictibacillus rigui* WPCB074$^T$ | 99.48 | 9 | MN310335 |
| RB38 | *Streptomyces ambofaciens* ATCC 23877$^T$ | 98.60 | 32 | MN310342 | GB23 | *Janibacter indicus* CGMCC 1.12511$^T$ | 99.79 | 1 | MN310348 |
| YA19 | *Streptomyces anthocyanicus* NBRC 14892$^T$ | 98.55 | 31 | MN310340 | RB107 | *Kocuria kristinae* NBRC 15354$^T$ | 99.45 | 34 | MN310353 |
| YA20 | *Streptomyces bacillaris* NBRC 13487$^T$ | 98.87 | 31 | MN315528 | RB40 | *Kocuria kristinae* NBRC 15354$^T$ | 99.45 | 21 | MN310355 |

Table 3.3 (Continued)

| Isolates | Highest match | % | RFLP groups | Genbank accession number | Isolates | Highest match | % | RFLP group | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|
| RB86 | *Leisingera methylohalidivorans* DSM 14336$^T$ | 99.10 | 16 | MN310320 | RB27 | *Streptomyces lienomycini* LMG 20091$^T$ | 99.83 | 32 | MN315546 |
| LC12 | *Micrococcus aloeverae* AE-6$^T$ | 99.60 | 24 | MN310338 | RB27[a] | *Streptomyces lienomycini* LMG 20091$^T$ | 99.92 | 32 | MN315546 |
| RB166 | *Micrococcus aloeverae* AE-6$^T$ | 99.43 | 29 | MN315526 | YA22 | *Streptomyces prasinosporus* NRRL B-12431$^T$ | 98.40 | 31 | MN310366 |
| RB5 | *Micrococcus aloeverae* AE-6$^T$ | 99.60 | 23 | MN310360 | YA22[a] | *Streptomyces prasinosporus* NRRL B-12431$^T$ | 98.94 | 31 | MN315547 |
| RB122 | *Pseudoalteromonas marina* Mano4$^T$ | 99.28 | 25 | MN310325 | LC14 | *Streptomyces setonii* NRRL ISP-5322$^T$ | 99.85 | 37 | MN310347 |
| LC17 | *Pseudomonas oleovorans subsp. oleovorans* DSM 1045$^T$ | 100 | 14 | MN315527 | YA2 | *Streptomyces tendae* ATCC 19812$^T$ | 99.82 | 31 | MN310341 |

Table 3.3 (Continued)

| Isolates | Highest Match | % | RFLP groups | Genbank accession number | Isolates | Highest Match | % | RFLP group | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|
| YA11 | *Pseudonocardia carboxydivorans* Y8$^T$ | 99.74 | 30 | MN310349 | RB56 | *Streptomyces violaceorubidus* LMG 20319$^T$ | 99.65 | 31 | MN310346 |
| LC21 | *Rhodococcus corynebacterioides* DSM 20151$^T$ | 99.06 | 12 | MN310339 | RB58 | *Sulfitobacter delicates* DSM 16477$^T$ | 99.89 | 2 | MN310356 |
| RB71 | *Kocuria kristinae* NBRC 15354$^T$ | 99.86 | 33 | MN310345 | GB12 | *Sulfitobacter faviae* S5-53$^T$ | 99.30 | 1 | MN315529 |
| L11 | *Staphylococcus hominis subsp. novobiosepticus* GTC 1228$^T$ | 100 | 18 | MN315522 | GB44 | *Sulfitobacter faviae* S5-53$^T$ | 99.27 | 2 | MN310324 |
| H8 | *Streptomyces antimycoticus* NBRC 12839$^T$ | 100 | 38 | MN310344 | GB6 | *Sulfitobacter indolifex* HEL-45$^T$ | 99.20 | 28 | MN310323 |
| YA27 | *Streptomyces badius* NRRL B-2567$^T$ | 99.84 | 31 | MN315521 | | | | | |
| RB128 | *Streptomyces hyderabadensis* OU-40$^T$ | 99.44 | 31 | MN310350 | | | | | |

[a] full 16S rRNA gene sequence was obtained

### 3.3.3 Diversity of bacteria isolated from different parameters

The effect of media for bacterial isolates abundance and diversity was examined and the results were presented in Figure 3.7 and Table 3.4. The highest number of CFU and morphological forms were observed on the ASP medium followed by HV and with the lowest on the SWA medium (Figure 3.7). Among the 21 isolated genera, 76.2% were recovered using ASP medium followed by 66.7% from TSA and 61.9% from NA with the lowest (42.9%) from SWA medium (Table 3.4). Specifically, *Streptomyces*, *Micrococcus*, *Bacillus*, *Isoptericola*, and *Kocuria*.were isolated from all media. The genus *Gordonia* was isolated from all media except SWA; five media allowed for isolation of *Microbacterium* (except SWA & HV) and *Rhodococcus* (except SWA and MA); four media allowed for isolation of *Leisingera* (except SYP, NA, and TSA)*, Limimaricola* (except ASP, SWA, and TSA)*, Muricauda* (except ASP, SWA, and HV)*,* and *Pseudonocardia* (except TSA, NA, and SYP); three media allowed for isolation of *Falsibacillus* (ASP, NA, and TSA), *Fictibacillus* (NA, TSA, and MA), *Staphylococcus* (ASP, SWA, and TSA)*, Sulfitobacteria* (ASP, SWA, and HV)*,* and *Mycolicibacterium* (SYP, NA, TSA); two media allowed for *Pseudomonas* (ASP, TSA) and only single medium allowed for isolation of *Rhodovulum* (ASP)*, Pseudoalteromonas* (SYP)*,* and *Janibacter* (ASP). ASP medium was the most effective both in the number of bacteria isolated as well as genera diversity.

To determine which ingredients were essential compared to others in medium composition, a small scale experiment was conducted by culturing bacteria in different ASP media by removing one ingredient at a time. The isolation rate of specific ingredient deficient media compared with the complete medium and the results indicated, the growth of the bacteria reduced by half in each experiment setups and no significant effect observed in leaving of one component over the other. The availability of all components was equally important for the maximum isolation of bacteria.

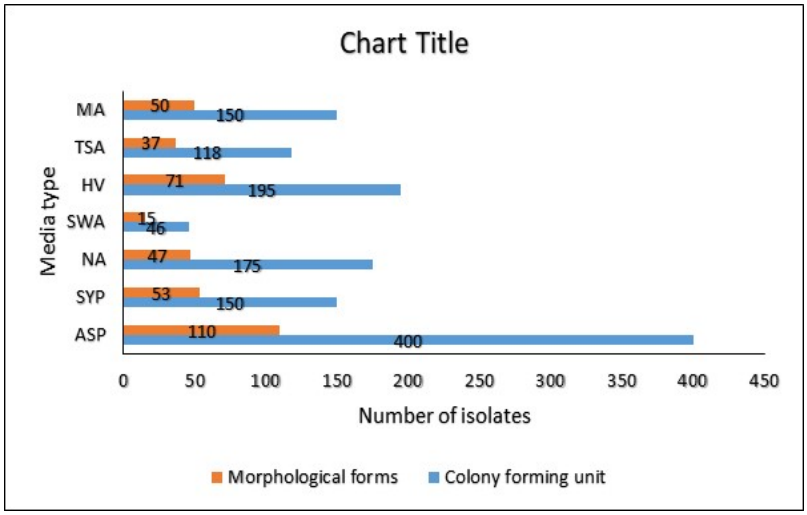



Figure 3.7 The distribution of CFU (1234) and morphological types (383) of bacterial isolates among seven isolation media.

Table 3.4. The diversity of bacterial genera within seven isolation media.

| Genus name | Media type | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | ASP | SYP | NA | SWA | HV | TSA | MA | |
| *Gordonia* | 10 | 4 | 2 | 0 | 1 | 2 | 3 | 22 |
| *Isoptericola* | 6 | 9 | 4 | 3 | 5 | 2 | 3 | 32 |
| *Janibacter* | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| *Kocuria* | 30 | 19 | 11 | 5 | 8 | 15 | 10 | 98 |
| *Microbacterium* | 11 | 8 | 12 | 0 | 0 | 14 | 9 | 54 |
| *Micrococcus* | 15 | 9 | 5 | 2 | 3 | 6 | 8 | 48 |
| *Mycolicibacterium* | 0 | 5 | 2 | 0 | 0 | 3 | 0 | 10 |
| *Pseudonocardia* | 8 | 0 | 0 | 2 | 8 | 0 | 4 | 22 |
| *Rhodococcus* | 8 | 6 | 3 | 0 | 4 | 4 | 0 | 25 |
| *Streptomyces* | 100 | 44 | 43 | 13 | 90 | 17 | 45 | 352 |
| *Muricauda* | 0 | 4 | 1 | 0 | 0 | 3 | 2 | 10 |
| *Bacillus* | 85 | 26 | 56 | 5 | 42 | 32 | 43 | 289 |
| *Fictibacillus* | 0 | 0 | 12 | 0 | 0 | 6 | 4 | 22 |
| *Falsibacillus* | 6 | 0 | 9 | 0 | 0 | 4 | 0 | 19 |
| *Staphylococcus* | 5 | 0 | 0 | 3 | 0 | 8 | 0 | 16 |
| *Leisingera* | 6 | 0 | 0 | 4 | 2 | 0 | 4 | 16 |
| *Limimaricola* | 0 | 13 | 15 | 0 | 11 | 0 | 15 | 54 |
| *Pseudoalteromonas* | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 3 |
| *Pseudomonas* | 17 | 0 | 0 | 0 | 0 | 2 | 0 | 19 |
| *Rhodovulum* | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| *Sulfitobacter* | 74 | 0 | 0 | 9 | 21 | 0 | 0 | 104 |
| Total | 400 | 150 | 175 | 46 | 195 | 118 | 150 | 1234 |

The number and diversity of bacterial isolates were assessed in terms of sponge collection sites and types of sponge species. (Figure 3.8). The total bacteria isolates, as well as morphological types, were considerably varied among sponge types from two collection sites. Among sponge samples collected from Rapid Bay, sponge number RB 16 (*Aplysilla sulfurea*) displayed the highest bacterial population (35.5%) and morphological types (41%), followed by sponge number Rb 18 (*Tedania tubulifera*)-26% (bacterial population) and 20% (morphological types), and RB 17 (*Dendrilla* sp.)-14% (bacterial population) and 10.5% (morphological types). Quite a few numbers of bacterial population compared to the above three sponges were observed in sponges number RB 1 (*Ircinia* sp.), RB 2 (*Poecilosclerida* sp.), RB 3 (*Crella* sp.), RB 11 (*Sarcotragus* sp.) and RB 12 (*Carteriospongia foliascens*) where the least number observed from RB 12.

Similarly, among sponge samples collected from Glenelg blocks, sponge number GB 08 (*Chondrosida* sp.) resulted the highest bacterial population (39%) and 37.5% (morphological types), followed by GB 23 (*Chondrosida* sp.)-28% (bacterial population) and 30% (morphological types), GB 21 (*Chondrosida* sp.)-19% and 16% bacterial population and morphological types, respectively with the lowest number isolated from GB 1(*Geodia* sp.).

The genera distribution also varied within sponge types (Figure 3.8b) and among the sites of sponge collection (Table 3.5). Fourteen types of genera (*Gordonia*, *Kocuria*, *Microbacterium*, *Pseudonocardia*, *Bacillus*, *Fictibacillus*, *Falsibacillus*, *Leisingera*, *Limimaricola*, *Pseudomonas*, *Rhodovulum*, *Sulfitobacter*, *Staphylococcus*, and *Micrococcus*) were isolated from the four sponge samples which are collected from Glenelg blocks. Among them, except *Staphylococcus* and *Micrococcus,* all were isolated from sponge GB 08. Eight genera were isolated from sample GB 23, 6 genera were isolated from each of sponge sample GB 1 and GB 21. Four genera (*Kocuria*, *Bacillus*, *Limimaricola*, and *Sulfitobacter*) were common among GB 1, GB 21, and GB 23; genus Microbacterium observed both in GB 21 and GB 23 while genus *Fictibacillus* was isolated both from GB 1 and GB 23. *Falsibacillus, Staphylococcus* and *Leisingera*, and *Micrococcus* were detected only in Gb 21, GB 23 and GB 1, respectively.

Except for the genus *Fictibacillus*, all of the isolated genera were observed within at least one of the 8 sponge samples collected from Rapid Bay. Sponge number RB 16 presented 15 genera (*Gordonia*, *Kocuria*, *Isoptericola*, *Janibacter*, *Micrococcus*, *Mycolicibacterium*, *Pseudonocardia*, *Rhodococcus*, *Streptomyces*, *Muricauda*, *Bacillus*, *Staphylococcus*, *Pseudoalteromonas*,

*Pseudomonas*, and *Sulfitobacter*), 10 genera from RB 18 (*Kocuria*, *Isoptericola*, *Microbacterium*, *Micrococcus*, *Rhodococcus*, *Streptomyces*, *Bacillus*, *Leisingera*, *Pseudomonas*, and *Sulfitobacter*), 7 genera from RB 12 (*Kocuria*, *Microbacterium*, *Streptomyces*, *Bacillus*, *Falsibacillus*, *Limimaricola,* and *Sulfitobacter*), 6 genera from RB 17 (*Isoptericola*, *Microbacterium*, *Micrococcus*, *Rhodococcus*, *Streptomyces*, and *Bacillus*), 5 genera from RB 2. (*Micrococcus*, *Pseudonocardia*, *Streptomyces*, *Bacillus*, and *Sulfitobacter*), RB 1. (*Micrococcus*, *Streptomyces*, *Bacillus*, *Falsibacillus*, and *Sulfitobacter*) and RB 3 (*Micrococcus*, *Streptomyces*, *Bacillus*, *Limimaricola*, and *Rhodovulum*), and 4 genera from RB 11 (*Kocuria*, *Streptomyces*, *Bacillus*, and *Limimaricola*). Except for *Streptomyces* and *Bacillus*, no single genera shared by all sponges in this site.

Among 14 genera which were isolated from Glenelg Block, 35.7% belongs to phylum Actinobacteria (*Kocuria*, *Gordonia*, *Microbacterium*, *Micrococcus* and *Pseudonocardia*), while 50% of the genera isolated from Rapid bay belongs to the phylum Actinobacteria which included the five genera from Glenelg and *Streptomyces*, *Isoptericola*, *Janibacter*, *Mycolicibacterium* and *Rhodococcus*. Among non-actinobacterial genera all except *Fictibacillus*, *Pseudoalteromonas* and *Muricauda* were isolated from the two sites, where the first only isolated from Glenelg and the last two only from Rapid Bay.

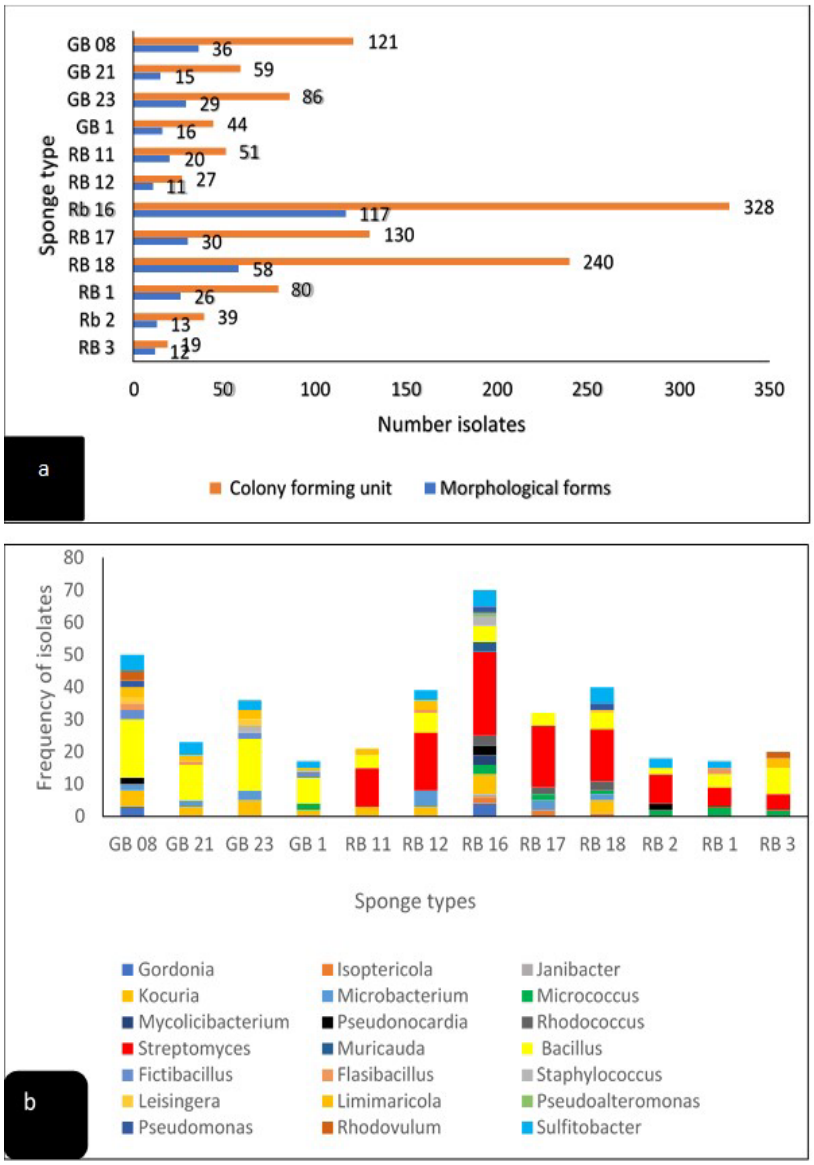



Figure 3.8 The frequency of genus diversity among 12 sponge samples collected from Glenelg and Rapid Bay. **a** the total CFU and morphological forms in each sponge sample; **b** the diversity of bacterial isolates in each sponge sample at the genus level. RB 1 (*Ircinia* sp.), RB 2 (*Poecilosclerida* sp.), RB 3 (*Crella* sp.), RB 11 (*Sarcotragus* sp.), RB 12 (*Carteriospongia foliascens*), RB 16 (*Aplysilla sulfurea*), RB 17 (*Dendrilla* sp.), Rb 18 (*Tedania tubulifera*), Gb 1 (*Geodia* sp.),.GB 08 (*Chondrosida* sp.), GB 21 (*Chondrosida* sp.), GB 23 (*Chondrosida* sp.).

Table 3.5 The diversity of genera among sites of sponge collection.

| Genera | Sponge collection sites | | Genera | Sponge collection sites | |
|---|---|---|---|---|---|
| | Glenelg Block | Rapid Bay | | Glenelg Block | Rapid Bay |
| *Gordonia* | 3 | 4 | *Bacillus* | 53 | 38 |
| *Isoptericola* | 0 | 5 | *Fictibacillus* | 7 | 0 |
| *Janibacter* | 0 | 1 | *Falsibacillus* | 2 | 4 |
| *Kocuria* | 15 | 16 | *Staphylococcus* | 2 | 3 |
| *Microbacterium* | 7 | 10 | *Leisingera* | 4 | 1 |
| *Micrococcus* | 2 | 13 | *Limimaricola* | 9 | 8 |
| *Mycolicibacterium* | 0 | 3 | *Pseudoalteromonas* | 0 | 1 |
| *Pseudonocardia* | 2 | 5 | *Pseudomonas* | 2 | 4 |
| *Rhodococcus* | 0 | 8 | *Rhodovulum* | 3 | 2 |
| *Streptomyces* | 0 | 111 | *Sulfitobacter* | 13 | 19 |
| *Muricauda* | 0 | 3 | | | |

The abundance and diversity of bacteria at different incubation periods were assessed (Figure 3.9 and Table 3.6). The cumulative bacterial number sharply increased in the first three weeks of incubation and the rate of change remained constant until 6 weeks followed by a serge until 9 weeks (Figure 3.9). In terms of diversity at the genus level, in the first three weeks of incubation, the isolates were dominated by genera other than from phylum Actinobacteria. At this incubation periods, all genera under phylum Firmicutes (*Bacillus*, *Fictibacillus*, *Falsibacillus,* and *Staphylococcus*) were isolated but only one genus (*Streptomyces*) from phylum Actinobacteria was observed. By week five, half of the Actinobacterial genera and all genera in the other three phyla were isolated. As the incubation period approached to 9 weeks, all the genera were detected, and no new genera were detected until 16 weeks of incubation (Table 3.6).

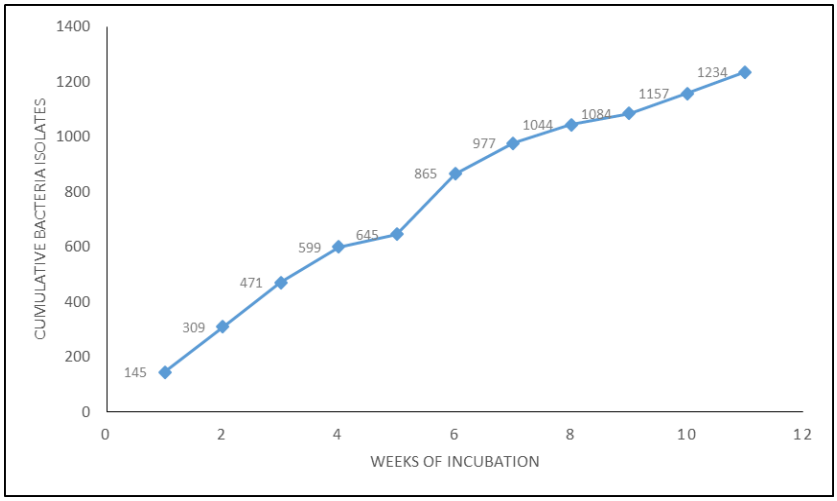



Figure 3.9 Cumulative bacteria abundance over the 16 weeks of incubation.

Table 3.6 Bacterial genus diversity isolated with increasing lengths of incubation time.

| Incubation periods | Genera identified | | |
|---|---|---|---|
| ≤ 3weeks | *Bacillus*<br>*Fictibacillus*<br>*Falsibacillus*<br>*Leisingera* | *Limimaricola*<br>*Muricauda*<br>*Pseudomonas*<br>*Rhodovulum* | *Staphylococcus*<br>*Streptomyces* |
| 4-5 weeks | *Gordonia*<br>*Isoptericola*<br>*Kocuria*<br>*Leisingera* | *Micrococcus*<br>*Microbacetrium*<br>*Pseudoalteromonas*<br>*Sulfitobacter*<br>*Streptomyces* | |
| 6-9 weeks | *Gordonia*<br>*Isoptericola*<br>*Janibacter*<br>*Kocuria*<br>*Limimaricola* | *Microbacterium*<br>*Micrococcus*<br>*Muricauda*<br>*Mycolicibacterium*<br>*Pseudonocardia* | *Rhodococcus*<br>*Streptomyces*<br>*Sulfitobacter*<br>*Rhodovulum* |
| ≥ 10 weeks | *Streptomyces*<br>*Kocuria*<br>*Microbacterium* | *Limimaricola*<br>*Sulfitobacter*<br>*Pseudonocardia* | |

The diversity of the isolated bacteria was assessed at different incubation conditions as indicated in Figure 3.10. Forty percent of the bacteria were isolated from $27^{O}$C aerobic incubation, followed by $27^{0}$C anaerobic, $15^{o}$C aerobic and $27^{o}$C microaerophilic incubations. About half of the morphological forms (52.2%) were isolated under $27^{o}$C aerobic incubation, followed by $15^{o}$C aerobic (20.9%), $27^{o}$C anaerobic (15.7%) and $27^{o}$C microaerophilic (11.2%) incubations (Figure 3.10a). In terms of genera diversity, 19 genera apart from *Falsibacillus* and *Pseudoalteromonas* were isolated under aerobic conditions, 13 from microaerophilic and 11 genera from anaerobic incubation (Figure 3.10b). Seven genera were detected from all oxygen levels; these were *Bacillus*, *Fictibacillus*, *Lamimaricola*, *Rhodococcus*, *Microbacterium*, *Pseudomonas* and *Sulfitobacter*, while *Pseudonocardia*, *Janibacter, Micrococcus, Muricauda, Staphylococcus* were isolated from only aerobic and *Pseudoalteromonas* isolated only from anaerobic conditions. The isolates were tested for their strict oxygen requirement(s) and the results indicated all of the 60 strains which were isolated from anaerobic incubation grew in aerobic setups, indicative of facultative anaerobic properties, and 50 out of 280 bacteria isolated under aerobic conditions did not grow under strictly anaerobic conditions. All genera observed in $27^{o}$C incubation versus 12 genera (6 of them were from the phylum Actinobacteria) at $15^{o}$C (Table 3.7).

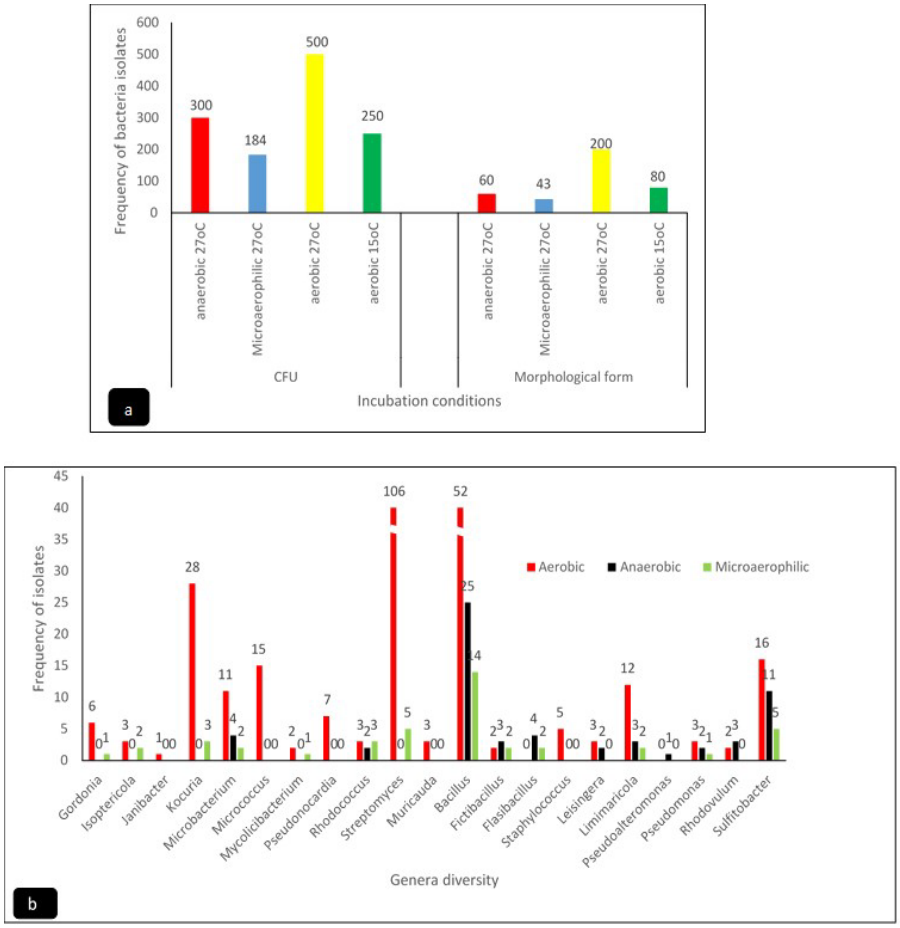



Figure 3.10 Abundance and diversity of bacteria isolated under different incubation conditions. **a** total CFU and morphological forms at four incubation conditions; **b** the genera diversity of bacteria isolated from the three oxygen levels.

Table 3.7 Genus diversity of 383 bacterial isolates with respect to isolation temperatures.

| Genera | Isolation temperatures | | Genera | Isolation temperatures | |
|---|---|---|---|---|---|
| | 15°C | 27°C | | 15°C | 27°C |
| *Gordonia* | 2 | 5 | *Bacillus* | 30 | 61 |
| *Isoptericola* | 1 | 4 | *Fictibacillus* | 0 | 7 |
| *Janibacter* | 0 | 1 | *Falsibacillus* | 0 | 6 |
| *Kocuria* | 11 | 20 | *Staphylococcus* | 0 | 5 |
| *Microbacterium* | 0 | 17 | *Leisingera* | 2 | 3 |
| *Micrococcus* | 7 | 8 | *Limimaricola* | 8 | 9 |
| *Mycolicibacterium* | 0 | 3 | *Pseudoalteromonas* | 0 | 1 |
| *Pseudonocardia* | 0 | 7 | *Pseudomonas* | 4 | 2 |
| *Rhodococcus* | 2 | 6 | *Rhodovulum* | 2 | 3 |
| *Streptomyces* | 25 | 86 | *Sulfitobacter* | 13 | 19 |
| *Muricauda* | 0 | 3 | | | |

### 3.3.4 Tolerance tests

All 383 bacterial isolates were tested for their salt and temperature tolerances. Five bacterial isolates were observed to grow at temperatures ranging from 3-37°C, with four of them belonging to the genus *Bacillus* and which were initially isolated at 27°C and one from the genus *Pseudomonas,* which was primarily isolated from 15°C incubation (Figure 3.11). Twenty-one percent of the isolates had broader tolerances of either 3-27°C or 15-37°C, while the majority of the isolates grew within a narrower temperature range. Bacterial isolates from phylum Firmicutes tolerate more the above-mentioned wider temperatures (29%) compared to bacterial isolates from phylum Actinobacteria (18%), Proteobacteria (13%) and none for Bacteroidetes. The maximum number of bacterial grew at temperatures of 15-27°C (270) followed by 151 bacteria at a range of 27-37°C and 41 bacteria at a range of 3-15°C. The temperature ranges of 15-27°C tolerated by 90% of bacterial isolates from phylum Proteobacteria, 74% from Firmicutes and 61% from Actinobacteria. The complete data for temperature tolerances indicated in Appendix 6.

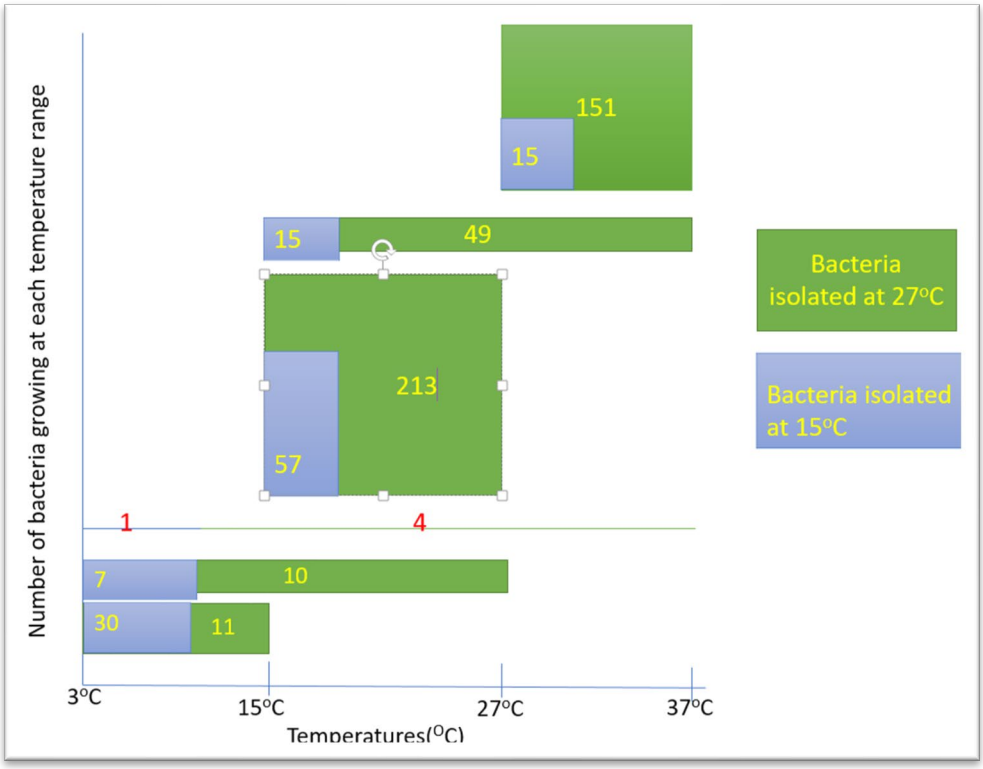



Figure 3.11 Growth capability of 383 bacterial isolates at different ranges of temperature.

The NaCl tolerance tests indicated some bacteria were able to grow in concentrations of 1-14 % NaCl (w/v), but the maximum number of bacterial isolates was observed at 4% NaCl. The tolerance declined from 6% and none grew at 16% NaCl. The raw data is presented in Appendix 7. Though a high percentage of isolates (58.7%) did not show a mandatory requirement for any NaCl in the growth medium, a considerable number of bacteria (41.3%) depend on the certain concentration of NaCl for their growth (Figure 3.12). Specifically, all bacterial isolates from phylum Bacteroidetes, 57.6% from Proteobacteria, 54% from Firmicutes and 29.8% from Actinobacteria required NaCl for their growth.

Furthermore, 65.2 % of these NaCl-requiring bacteria showed growth at NaCl concentrations of >10%, while only 7% of those that could grow in the absence of NaCl, demonstrating an ability to grow at salt concentrations of ≥10% and more. It was also observed that the bacteria grew better in media prepared with seawater compared to media containing NaCl and the sporulation of some bacteria enhanced in the presence of a certain concentration of NaCl.

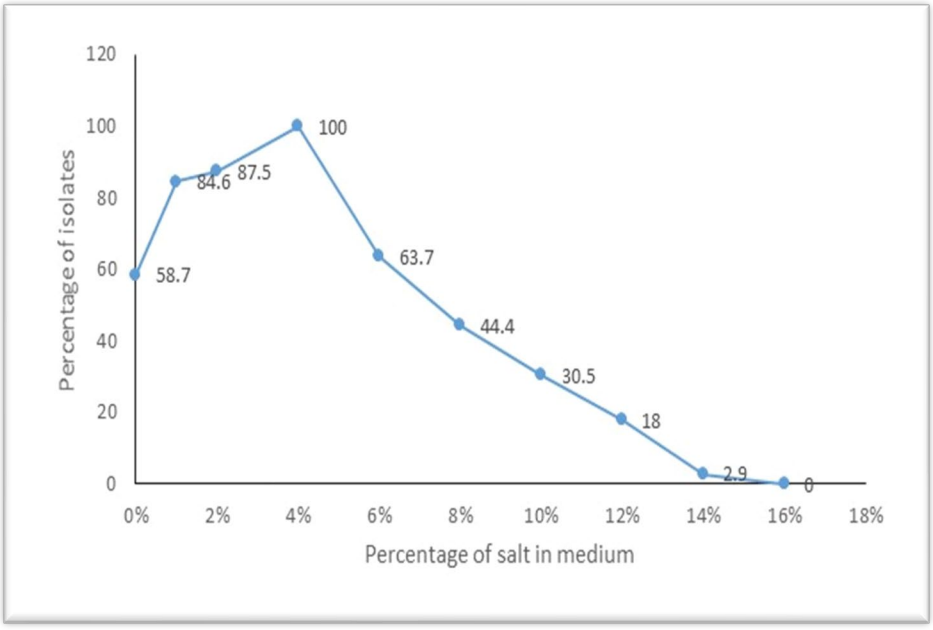



Figure 3.12 Bacterial growth at different NaCl concentrations presented as a percentage of total numbers.

### 3.3.5 Sponge *Aplysilla sulfurea* (RB 16) microbiome revealed by NGS

The identification of sponge samples and the NGS study was carried out by Dr Qi Yang for this study. Next-generation sequencing was done to the sponge that showed the highest genera diversity. Amplicon-based metagenomic sequencing generated an average of 93225, 100626, and 114703 reads by three primer sets for 16S rRNA gene regions V1V3, V4V5, and V5V8, respectively In a total of 12 microbial phyla (Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria, Planctomycetes, Proteobacteria, Chlamydiae, Verrucomicrobia, Chloroflexi, Crenarchaeota, and Spirochaetes) were uncovered. Among the combined dataset (regarded as 100%), 149 genera are identified (Table 3.8) and the complete list of the genera present in Table 3.9. The primer set for V1V3 revealed 8 microbial phyla, including 48 known genera and 3 candidate genera. The primer set for V4V5 revealed 11 microbial phyla, including 80 known genera and 5 candidate genera. The primer set for V5V8 revealed 9 microbial phyla, including 86 known genera and 8 candidate genera. Comparing the microbial profiles revealed by different primers, seven core phyla and 18 shared genera were identified and the complete data presented in Appendix 9.

Table 3.8 Microbial phyla and genera revealed by different primers for sponge *Aplysilla sulfurea* (RB 16*)*

| | V1V3 | V4V5 | V5V8 | Total | Shared |
|---|---|---|---|---|---|
| Phylum | 8 | 11 | 9 | 12 | 7 |
| Known genera | 48 | 80 | 86 | 137 | 17 |
| Candidate genera | 3 | 5 | 8 | 12 | 1 |

Table 3.9 Known and candidate genera identified by next generation sequencing

| **137 known genera identified by NGS and also via isolation** | | | |
|---|---|---|---|
| g__Acidaminobacter | g__Desulfovibrio | g__Parachlamydia | g__Thermoanaerobacter |
| g__Acinetobacter | g__Dialister | g__Paracoccus | g__Treponema |
| g__Actinobacillus | g__Dinoroseobacter | g__Persicirhabdus | g__Turicibacter |

Table 3.9 (Continued)

| 137 known genera identified by NGS and also via isolation | | | |
|---|---|---|---|
| g__Actinomyces | g__Dolichospermum | g__Persicobacter | g__Ulvibacter |
| g__Aggregatibacter | g__Elizabethkingia | g__Phaeobacter | g__Veillonella |
| g__Agrobacterium | g__Erwinia | g__Photobacterium | g__Verrucomicrobium |
| g__Alkaliphilus | g__Erythrobacter | g__Planctomyces | g__Vibrio |
| g__Alteromonas | g__Ferrimonas | g__Pleomorphomonas | g__Waddlia |
| g__Amaricoccus | g__Flavobacterium | g__Plesiomonas | g__Winogradskyella |
| g__Aminobacter | g__Fluviicola | g__Polaribacter | g__Wolbachia |
| g__Amycolatopsis | g__Fusibacter | g__Porphyromonas | g__Zoogloea |
| g__Antarctobacter | g__Fusobacterium | g__Prevotella | g__Candidatus Xiphinematobacter |
| g__Aquicella | g__Gemella | g__Prochlorococcus | g__Capnocytophaga |
| g__Aquimarina | g__Glaciecola | g__Propionibacterium | g__Chryseobacterium |
| g__Arcobacter | g__Gramella | g__Pseudaminobacter | g__Clostridium |
| g__Atopobium | g__Granulicatella | g__Pseudidiomarina | g__Cobetia |
| g__Bacteroides | g__Haemophilus | g__Pseudoalteromonas | g__Cohaesibacter |
| g__Bdellovibrio | g__Inquilinus | g__Pseudomonas | g__Collinsella |
| g__Bifidobacterium | g__Jannaschia | g__Pseudonocardia | g__Congregibacter |
| g__Burkholderia | g__Kaistia | g__Psychrilyobacter | g__Coprococcus |
| g__Campylobacter | g__Kingella | g__Reinekea | g__Coraliomargarita |
| g__Candidatus Arthromitus | g__Klebsiella | g__Rhodobacter | g__Corynebacterium |

Table 3.9 (Continued)

| 137 known genera identified by NGS and also via isolation | | | |
|---|---|---|---|
| g__Candidatus Portiera | g__Lactobacillus | g__Robiginitalea | g__Coxiella |
| g__Candidatus Protochlamydia | g__Leptotrichia | g__Roseivirga | g__Crenothrix |
| g__Candidatus Rhabdochlamydia | g__Leuconostoc | g__Roseobacter | g__Desulfobacter |
| g__Desulfocapsa | g__Loktanella | g__Roseovarius | g__Maribacter |
| g__Desulfosarcina | g__Luteolibacter | g__Rothia | g__Ruegeria |
| g__Desulfosporosinus | g__Lutimonas | g__Rubritalea | g__Megasphaera |
| g__Marivita | g__Selenomonas | g__Microbulbifer | g__Shewanella |
| g__Micrococcus | g__Staphylococcus | g__Streptococcus | g__Streptomyces |
| g__Moraxella | g__Stenotrophomonas | g__Moritella | g__Nautella |
| g__Sutterella | g__Neptunomonas | g__Synechococcus | g__Nitrosopumilus |
| g__Syntrophomonas | g__Octadecabacter | g__Tenacibaculum | g__Oleibacter |
| g__Tepidibacter | g__Oribacterium | g__Thalassobius | g__Parabacteroides |
| g__Thalassomonas | | | |
| **12 candidate genera identified by NGS** | | | |
| g__A17 | g__HB2-32-21 | g__[Prevotella] | g__WH1-8 |
| g__BD2-13 | g__HTCC | g__T78 | g__ZD0117 |
| g__Blvii28 | g__MSBL3 | g__Ucs1325 | g__5-7N15 |

## 3.4 Discussion

The marine environments of South Australia contain huge masses of sponge species and most of them are endemic (Sorokin *et al.*, 2007; Sorokin & Currie, 2008). However, few studies have reported the isolation and diversity of bacteria from marine sponges from these environments.

In this study, the bacterial communities of twelve sponge samples were investigated by culturing to obtain a total of 1234 CFU comprised of Gram-positive (52%), Gram-negative (18%), and filamentous bacteria (30%). With more than 0.77 µg of the original sample added from each sponge extract which leads to the isolation of a larger number and diversity of bacteria. Application of different macro and microscopic properties allowed to group these (1234 CFU) into 383 morphological types. Many bacterial isolates found to be similar in macroscopic features and further analysis using different stains and microscopy placed them into different categories. This highlights the importance of a combination of methods to place a large mass of bacterial isolates into manageable clusters. Further genetic analysis revealed bacteria with similar morphologies not always appeared in the same phylum and genera. Similarly, some bacteria with different morphological forms appeared to be the same genera and phyla. These findings further strengthen the importance of multiple approaches for the identification of bacteria from marine sponges as well as related microbial community studies.

In comparison, similar studies from Brazil with a comparable number of sponge samples yielded a lower number of 158 CFU (Santos *et al.*, 2010). Several studies reported a reasonable amount of bacteria from marine sponges with different numbers and morphological forms (Lafi *et al.*, 2005; Margassery *et al.*, 2012; Bibi *et al.*, 2018) and our study further supports the finding that marine sponges are a font of bacteria with various microscopic and morphological forms.

When attempting to characterise large numbers of isolates, restriction enzyme digestion of the 16S rRNA gene amplicons followed by sequencing of selected strains was found to be an efficient strategy for their rapid identification (Gernert *et al.*, 2005; Zhang *et al.*, 2006). A combination of two enzymes, *Hha*I and *Pst*I*,* allowed the categorization of the 383 bacteria isolates into 38 manageable groups, which confirmed the high discriminatory power of the two enzymes. As shown by Cook and Meyers (2003), it was demonstrated that all the genera of Actinobacteria could be identified with the use of four enzymes (Cook & Meyers, 2003). The microscopic and

morphological forms of each pattern were checked for agreement and if all bacteria within the same pattern displayed similar properties. RFLP is a relatively cheap method that can be applied to characterise the diversity of microbial communities.

Partial 16S rRNA gene sequencing of 63 randomly selected bacteria from each RFLP pattern placed the isolates into 21 genera belonging to four phyla, Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes. In terms of phyla diversity, the same types and number of phyla were reported with various isolation studies (Lafi *et al.*, 2005;.Kennedy *et al.*, 2009; Margassery *et al.*, 2012; Flemer *et al.*, 2012; Kuo *et al.*, 2019). Some also reported three of the above phyla with the absence of Actinobacteria and/or Bacteroidetes (Hentschel *et al.*, 2001; Santos *et al.*, 2010; Graça *et al.*, 2013; Graça *et al.*, 2015). Some researchers such as Abdelmohsen *et al* (2010) and Zhang *et al.* (2006) were focused only on specific phylum (Actinobacteria) and reported a significant number of bacterial isolates and genera diversity.

The relative abundance of each phylum varies substantially where phylum Actinobacteria was the most abundant followed by Firmicutes, Proteobacteria and an insignificant amount of Bacteroidetes. In agreement with our report, a high abundance of phylum Actinobacteria was reported in similar studies (Webster *et al.*, 2001; Kennedy *et al.*, 2009; Liu *et al.*, 2019; Baig *et al.*, 2020). However, some studies also reported a low level of the phylum Actinobacteria compared to Proteobacteria (Flemer *et al.*, 2012; Margassery *et al.*, 2012; Graça *et al.*, 2015; Kuo *et al.*, 2019). Indeed, in our studies, the four sponges from Glenelg had relatively low numbers of this phylum. Like our study, consistently very low levels of Bacteroidetes were reported from similar studies (Lafi *et al.*, 2005; Kennedy *et al.*, 2009).

This study presented 21 genera and 10 of them belong to the phylum Actinobacteria, 6 of them belong to Proteobacteria, 4 Firmicutes, and 1 Bacteroidetes. Of the 10 identified Actinobacterial genera, the genus *Streptomyces* was dominant. *Streptomyces* have a proven ability to produce natural antibiotics (Zhang *et al.*, 2006; Kennedy *et al.*, 2009; Bibi *et al.*, 2018) and this higher isolation from South Australian marine sponges encourages the further search for novel *Streptomyces* with antimicrobial properties. The observed morphological variations (more than 6 types) within the genus *Streptomyces* could be a good indicator that this genus has different genetic makeup and that in turn attribute to difference in metabolites they produced.

As far as we know*, Fictibacillus, Falsibacillus, Isoptericola, Limimaricola, Muricuda,* and *Mycolicibacterium* were not reported in similar studies. Furthermore*, Janibacter, Microbacterium, Leisingera, Rhodovulum, Sulfitobacter, Pseudonocardia* were reported rarely in related studies but not all at the same time in any single study (Lafi *et al.*, 2005; Kennedy *et al.*, 2009; Abdelmohsen *et al.*, 2010; Li *et al.*, 2011; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*, 2015; Ellis *et al.*, 2017; Indraningrat *et al.*, 2019; Kuo *et al.*, 2019). Unlike most studies, this study plate more volume of sponge, and incubated them at different incubation conditions for longer periods. These may help to isolate a higher number of less common bacterial genera compared to other studies. In contrary, several types of genera which were not reported in this study such as *Micromonospora*, *Pseudovibrio*, and *Vibrio* were isolated from marine sponges (Zhang *et al.*, 2006; Selvin *et al.*, 2009; Abdelmohsen *et al.*, 2010; Li *et al.*, 2011; Flemer *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*, 2015; Matobole *et al.*, 2017; Bibi *et al.*, 2018). These observations reflect some degree of area dependent variation on phylum and genera distributions. Furthermore, the methodology this study used might be still not enough to reflect the true pictures of the bacterial community in sponges.

Our findings predict that marine sponges will continue to be a good source of diverse bacterial isolates with novelty. This is especially the case when similarity analysis of these bacterial isolates with type strains showed about 29% could a potential candidate for novelty, as were found similar in other studies (Abdelmohsen *et al.*, 2010; Ahn *et al.*, 2011; Afonso de Menezes *et al.*, 2017). The observed variation in quality and quantity of bacterial isolates from different geographical sites and a high chance of discovering novel bacteria encourages the continued screening of bacteria from marine sponges to widen the existing ecological information as well as the chance of isolating new genera and phyla which could be a source of useful metabolites.

Next-generation sequencing of microbiota from *Aplysilla sulfurea* (RB 16), sponge sample that showed the highest genera diversity, revealed the presence of 12 phyla and about 149 genera, with the dominancy of Proteobacteria. Less than 5% of the genera identified by NGS were isolated Forty percent of those genera isolated by culture methods (*Pseudoalteromonas*, *Pseudomonas, Pseudonocardia, Pseudoalteromonas*, *Staphylococcus*, and *Streptomyces)* were shared by NGS. However, ten genera out of 16 were not observed by NGS. Like our study, several studies reported few shared genera or species among culture-dependent and culture-independent approaches (Kisand & Wikner, 2003; Sun *et al.*, 2010; Yashiro *et al.*, 2011; Jackson

*et al.*, 2013; Stefani *et al.*, 2015). The findings highlighted the two approaches are complementary and it should be combined to reflect the true picture of the microbial community in the environment. Here, several factors could be indicated for these variations. One may be primers used for amplification do not work equally for all genera in the sponge community, which demands optimization of the NGS protocol (These supported by our findings where the variation of phyla and genera observed among the three primers). Another possible explanation is that the combination of the isolation methods followed in this study may support the isolation of uncommon genera which are present in low abundance and possibly not detected by NGS. It is also true that the protocol used in this study is not still enough to reflect the true culturable population of bacteria in sponges, as most of the named genera in NGS are culturable. This observation reinforces the need for more novel isolation protocols.

Several factors were observed to influence the diversity of bacterial isolates from our set of marine sponges. Quite remarkable variations in genera diversity were observed among sponge samples collected from the same site as well as across the two sites. Generally, higher diversities of bacterial genera were isolated from sponge samples collected from Rapid Bay compared to Glenelg blocks. Those isolates from Rapid Bay were mainly dominated by the phylum Actinobacteria and even the genus *Streptomyces* was only isolated from this site. However, bacteria other than Actinobacteria were dominant from the Glenelg samples. Variations in diversity not only observed among sites but also within the sponge types. For example, sponge *Aplysilla sulfurea* (RB 16) showed a higher genera diversity compared to other sponge species. Furthermore, abundance and diversity in genera within the same species were observed. GB 08 showed more genera diversity compared to GB 21 and GB 23, all are belonging to *Chondrosida* spp.

Similarly, other studies have reported spatial variation in abundance and diversity of bacteria among sample sites and sponge types (Webster *et al.*, 2001; Lafi *et al.*, 2005; Thiel *et al.*, 2007; Abdelmohsen *et al.*, 2010; Ellis *et al.*, 2017). Even some bacterial species were sponge specific and not found in other sponges (Erwin *et al.*, 2011; Schwartz *et al.*, 2014). Though it is difficult to tell about the cause of these variations, spatial variations on the nature of sponges in South Australia marine environments have been documented (Sorokin *et al.*, 2007; Sorokin & Currie, 2008) and this may affect the nature of the associated bacteria. Rapid Bay is farther from the human habitation compared to Glenelg and the possible variations in the chemical composition

of sponges, the nutrient availability, water flow, water temperature, and salinity, and distance from human and animal contact could be possible determinants for the richness and variability of bacteria within sponge communities (Galand *et al.*, 2009; Schmitt *et al.*, 2012).

The diversities of bacterial isolates were highly affected by the types of media used. Our findings indicated that the importance of media containing different nutrients for isolation of more diverse bacteria, as demonstrated by isolation of distinct genera on ASP and HV media compared to fewer genera isolated with nutrient-poor SWA medium. Other studies have also acknowledged the importance of media for the isolation of high abundant and more diverse bacteria (Sipkema *et al.*, 2011; Schwartz *et al.*, 2014). Furthermore, it showed that the specific advantage of one medium type over the other (Matobole *et al.*, 2017; Bibi *et al.*, 2018). Substantial CFU and morphological variations were observed depending on the composition of the medium as well as the presence or absence of enrichment and antibiotics (Selvin *et al.*, 2009; Sipkema *et al.*, 2011; Schwartz *et al.*, 2014). Abdelmohsen *et al.*, (2010) reported media-dependent variation in the number of bacterial isolates, as well as genera diversity and they also indicated the incorporation of sponge extracts on medium help for isolation of novel bacteria. The uneven distribution of genera with the isolation media strengthens the importance of multiple media to obtain more of the culturable bacterial community within sponges.

Incubation period dependent variations in bacterial diversity were an important facet in this study. In the first few weeks of incubation, the isolates were dominated mostly by the genus Bacillus and few other common non-actinobacteria genera. As the incubation period increased the genus diversity increased with a shift towards Actinobacteria. Incubation time-dependent variation on the abundance and the diversity of bacteria as indicated by Kaewkla and Franco (2013) is once again highlighted in this study, in which about 50% of the isolates were obtained after six weeks of incubation. Bibi *et al.*, (2018) reported 8 genera of bacteria and filed to report any from phylum Actinobacteria due to 5-7 days of incubation. This reinforces the importance of providing an adequate period of incubation to bring out the less common bacteria in the culture system.

Bacterial diversity was also influenced by oxygen levels and incubation temperature. In general, the more genera belonging to the non-actinobacteria genera were found under limited oxygen levels compared to the phylum Actinobacteria. These findings indicate the isolation of specific

bacteria depends on oxygen levels and this underpins the importance of multiple incubation conditions for isolation of more diverse bacteria from sponge samples. The substantial differences in the physicochemical nature of marine environments were indicated (Dang *et al.*, 2019), which may have a role in shaping the compositions of the bacteria in the system. Strict oxygen requirement analysis revealed all the strains isolated from anaerobic conditions grew in the presence of oxygen, which implied the dominance of facultative anaerobes. The presence of anaerobic bacteria in sponges has been seen before (Selvin *et al.*, 2009; Mohamed *et al.*, 2010; Sipkema *et al.*, 2011; Schwartz *et al.*, 2014). The occurrence of time-based anaerobic patches inside a sponge was indicated and this condition could arise from a change of water pumping rate or oxygen depletion in the night (Hoffmann *et al.*, 2005; Hoffmann *et al.*, 2008).

The composition of bacteria from the marine environment may be affected by a change in temperature (Hicks *et al.*, 2018) and the observed range of temperature tolerance (Figure 3.11) of the majority of isolates was rather narrow 12-13$^{o}$C range, with only 1% able to grow between 3 and 37$^{o}$C and 20% of all isolates able to withstand a temperature range of 22-24$^{o}$C. The narrow temperature tolerance of most of the isolates predicts changes in microbial diversity with changes in the ambient temperature of the seawater and a poor ability to adapt to higher temperatures, such as those associated with climate change.

The tolerance of bacterial isolates to NaCl (Figure 3.12) indicated that the majority (59%) did require for NaCl, which implies that the parts of the internal tissue sponge may be NaCl free. However, 41% of bacteria were dependent on the presence of a certain amount of NaCl for their growth. Therefore, all marine-derived bacteria do not have an absolute requirement of salt for growth of bacteria, though all grew in the presence of 4% (w/v) NaCl and some also preferred higher NaCl concentrations. Genera dependent preference of salt reported in a similar study, where all of the α-Proteobacteria, 81% of γ-Proteobacteria and 20% of Actinobacteria had an absolute salt requirement (Kennedy *et al.*, 2009).

## 3.5 Conclusions

The findings of this study allowed us to conclude that the marine sponges of South Australia can yield considerable cultivable diversity if a comprehensive isolation strategy is implemented. Yet factors such as temperature, oxygen levels and salt concentration at the time of isolation are not

necessarily the optimum conditions for the growth of the isolate as was noted in this study. For example, whereas all isolation media contained at least 3% (w/v) NaCl, more than half the isolates did not require any NaCl to grow.

This study identified factors such as medium containing specific essential nutrients, long incubation periods, sponges from a more pristine site, and sponge species type as determinants for the isolation of highly diverse and abundant bacterial population.

Though the methodology used in this study allowed for the isolation of more diverse bacteria compared to previous studies, even those not observed in NGS, date from NGS revealed that there are still many bacteria that have yet to be cultured and innovative techniques are needed to increase the rate of cultivability.

The observed uneven distribution of bacteria for different factors suggest the requirement of a combination of isolation and incubation conditions to reflect the true picture of bacteria in marine sponges.

The phylum Actinobacteria and the genus *Streptomyces* were the most dominant bacteria isolated. Since the phylum Actinobacteria, particularly the genus *Streptomyces*, are the known sources of natural active metabolites including antibiotics, the marine sponges of South Australia should be the primary target for those searching natural compounds. Furthermore, a considerable amount of other known natural compound producers such as Proteobacteria and Firmicutes were isolated and this strengthens the area is a potential target for the search of bacteria with antimicrobial potentials.

Cost-effective identification of large numbers of bacteria is possible using a combination of morphology, RFLP patterns of the partial 16S rRNA gene amplicon and full amplicon sequencing of some representatives.

Finally, this section proves the hypothesis that marine sponges of South Australia could be a potential source for diverse bacteria with a high chance of novelty. Furthermore, this section proves the possibility of culturing diverse bacterial strains from marine sponges if one design optimum isolation strategies.

# Chapter Four: Polyphasic Taxonomic Characterization of putative novel isolates

## 4.1 Introduction

Polyphasic taxonomy depends on different genotypic and phenotypic techniques to provide a comprehensive description of microorganisms. Though several molecular techniques currently in use for bacterial identification, polyphasic taxonomy is still the cornerstone for categorization of candidate bacteria isolates as novel from various sources (Kaewkla & Franco, 2016; Afonso de Menezes *et al.*, 2017; Kaewkla *et al.*, 2017; Choi *et al.*, 2018; Landwehr *et al.*, 2018; Lee *et al.*, 2018; Chaiya *et al.*, 2019).

While studying the diversity of bacteria from marine sponges of South Australia, which was described in Chapter 3, three putative novel bacteria and one novel yeast were identified. This chapter describes the complete polyphasic characterization of these strains in terms of their morphological, physiological, chemotaxonomic and genotypic features. The methods used are common to all three strains and will be presented first followed by separate sections on each of the new species together with their respective 'closest' type strains. The last section of this chapter is about new the fungal genus and it is presented separately as the methods used are different from the bacterial strains.

## 4.2 Materials and methods

### 4.2.1 Morphological studies

The morphological properties of potential novel bacteria isolates (referred hereafter as 'candidate') were compared side by side with their nearest type strains by culturing them on various media such as TSA, SYP, NA, MA, and ISP2. Each candidate was inoculated onto the media in duplicate and the culture was incubated at 30$^{o}$C for 7 days. The bacteria were compared for the nature of the growth and colour of the colonies.

### 4.2.2 Physiological and biochemical characteristics

The candidate and type strains were frequently cultured onto TSA, SYP and NA to obtain an inoculum for each test. An example of positive Biochemical reactions indicated in Appendix 8.

#### 4.2.2.1 Growth at various temperatures

Both the candidate and type strains were tested for the ability to grow at different temperatures following previous methods (Kurup, 1973). Each candidate was inoculated into TSA medium and the culture incubated at 10, 15, 27, 30, 37 and 45$^{o}$C in duplicate. The growth was assessed at 2, 4 and 6 days.

#### 4.2.2.2. Growth at various pH ranges

The potential of bacterial growth at different pH ranges was determined on TSA plates. After autoclaving of the medium, the pH was adjusted to 4, 5, 6, 7 and 8 by using sterile 1 N HCl or 1 N NaOH. The plates were inoculated and incubated at 30$^{o}$C in duplicate. The growth was recorded at 2, 4 and 6 days.

#### 4.2.2.3 Growth at various NaCl concentrations

The growth tolerance of bacteria at different concentrations of NaCl was tested on TSA medium. Plates with NaCl concentrations of 1%, 3%, 5%, 10%, 15%, 20% were prepared in duplicate following previous method (Kurup, 1973), and the bacteria were inoculated on them and incubated for 7 days. The results were recorded as positive or negative for bacterial growth.

#### 4.2.2.4 Growth under different organic substrates

The ability of candidate and type strains to utilize different organic substrates was assessed according to Yurkov, (1994). The utilization of organic substrates for growth was investigated under aerobic conditions in liquid medium containing (per litre RO water) 0.3 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.05 g $CaCl.2H_2O$, 0.3 g $NH_4Cl$, 0.3 g $K_2HPO_4$, 15.0 g NaCl, 0.005 g yeast extract, 20 pg vitamin B12, and 1 ml of trace elements solution. The organic substrates of D-glucose, glycerol, D- fructose, raffinose, cellobiose, sucrose, lactose, mannose, arginine, alanine, succinate, and malate were added at a concentration of 1 g/litre at pH 7.6 to 7.8. The results were recorded 4 days after inoculation.

#### 4.2.2.5 Carbon source utilization

The following sources of carbon were used: D-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, D-melezitose, Methyl $\alpha$-D-glucoside, L-rhamnose, D-sucrose,

D-trehalose, D-xylose, lactose, Myo-inositol, D-mannitol, D-sorbitol. Inorganic basal medium consisting $(NH_4)2HPO4$ (1 g/l), $MgSO_4.7H_2O$ (0.2 g/l) and agar (15 g/l) were prepared following previous method (Gordon, 1974). The pH was adjusted to 7 and 0.04% bromocresol purple solution was added as an indicator and the medium was autoclaved. Four millilitres of the medium were transferred to 15 ml tubes and a 10% solution of each carbohydrate was made and filter sterilised and added into the basal inorganic medium to get a final concentration of 1% carbohydrate. The tube was adjusted to form a slant and each bacterium was inoculated on the slant in duplicate. The tubes inoculated with bacteria and those without bacteria as control were incubated at $30^0$C for 6 days and observed every day for a change of purple colour to a yellow, positive indicator of acid production for carbohydrate.

#### 4.2.2.6 Starch hydrolysis

The previous method for starch hydrolysis was employed for this study (Gordon *et al.,* 1974). The basal medium consisting of Nutrient agar was prepared and its pH was adjusted to 7.1 following the addition of 1.5% of starch. The bacteria were streaked heavily perpendicular to the plate and the culture was incubated at $30^o$C for 6 days. Then the plates were covered with Lugol's Iodine (0.1% w/v iodine and 0.2% w/v KI) and kept at room temperature for 15 min. The appearance of a clearing zone beneath the bacterial inoculation area was an indicator of a positive reaction for starch hydrolysis.

#### 4.2.2.7 Decomposition of Urea

A 75 ml of urea broth consisting of 10 ml filter sterilised urea, 10 g/l $KH_2PO_4$, 9.5 g/l $Na_2HPO_4$, 1 g/l yeast extract and 20 ml of 0.04% phenol red solution were prepared in RO water and the pH was adjusted at 6.7, as indicated in previous study (Gordon *et al.,* 1974). Five millilitres of the broth was transferred to a McKinney vial and the bacteria were inoculated into it. After the culture was incubated for 6 days at $30^o$C, the appearance of a pink colour due to the presence of urease activities was recorded.

#### 4.2.2.8 Decomposition of Casein

The approach for casein decomposition was indicated by the previous method (Gordon *et al.,* 1974) using skim milk media which was made of 5 g of skim milk and 1 g of agar in 50 ml of RO

water. The skim milk powder and the agar were autoclaved separately, and the two components were mixed thoroughly when the temperature reached about 45$^{o}$C and poured into the plate. Each candidate was inoculated heavily across the agar plates and the plates were incubated at 30$^{o}$C for two weeks. The appearance of the clear zone under the inoculum was an indicator of positive for casein hydrolysis.

#### 4.2.2.9 Decomposition of adenine, xanthine, hypoxanthine, and L-tyrosine

Method for assimilation of adenine, xanthine, hypoxanthine, and L-tyrosine was described by Gordon *et al.* (1974). 100 ml of the basal medium, Nutrient agar, with a pH of 7 was prepared and autoclaved. Ten millilitres of each compound were suspended separately in RO water with the following concentration: 0.4 g xanthine and 0.5 g of hypoxanthine, adenine, and L-tyrosine and they were autoclaved. Each compound was mixed with the basal medium and poured to the plate. The bacterial isolates were streaked heavily across the plate and then incubated at 30$^{o}$C for 2 weeks. A zone of clearance underneath and around the culture indicated the decomposition of the compounds.

#### 4.2.2.10 Catalase production

The ability of bacteria for the production of the enzyme catalase was determined using the previous method (Kurup, 1973). A loop full of a one-week-old culture of the candidate in TSA was mixed with freshly prepared 5% $H_2O_2$ on clean slides. The appearance of foam indicated the presence of catalase.

#### 4.2.2.11 Use of organic acids

Organic acids utilization was assessed by using previous methods (Kurup, 1973). The basal medium consisted of 20 ml 0.04% phenol red 1 g/L NaCl, 0.2 g/L $MgSO_4.7H_2O$, 0.1 g/L $(NH_4)2HPO_4$, 0.5 g/L $KH_2PO_4$, and 18 g/L agar in 1 litre of RO water. In each litre of a medium, 2 g of each sodium salts of acetate, benzoate, citrate, malate, propionate, and tartrate was added. Tubes of slant were prepared for each sodium salts and the bacterial isolates were streaked on it. The tubes were incubated at 30$^{o}$C for 2 weeks and change in colour from yellow to purple was a positive indicator of sodium salts utilization.

#### 4.2.2.12 Oxidase test

This test was designed to detect the presence of a cytochrome oxidase system that catalyses the transfer of electrons into the redox dye. A loop full of bacteria was streaked on a moistened filter paper soaked with tetramethyl-p-phenylenediamine redox dye. After 10-30 seconds, the blue colour appeared in the streaking area, indicating the change of the redox dye due to the transfer of electron- a positive test for oxidase activity (Acharya, 2015).

#### 4.2.2.13 TSI test

This test was designed to assess the ability of bacteria to ferment three sugars - Lactose, Sucrose and Glucose and production of $H_2S$ (Stager *et al.*, 1983). TSI agar (Oxoid) was prepared and autoclaved. The slant of the medium was prepared, and the bacteria were inoculated both the butt and slant. The culture was incubated at 30$^{o}$C for 6 days and change in colour of the butt and slant were assessed and the results were interpreted accordingly. The fermentation of glucose was indicated by the presence of yellow colour in the butt and red in the slant. Yellow butt and slant indicated all the sugars were fermented and blackening in the media was an indication of $H_2S$ production.

#### 4.2.2.14 3-days Arylsulfatase test

This test was performed for the identification of *Mycobacterium* species. Depending on the species, various concentrations of arylsulfatase is produced (Bhalla *et al.*, 2018). The capacity to produce a noticeable degree of this enzyme is a biochemical characteristic applied for the distinction of some *Mycobacterium* species. The test was performed in broth containing (g/l) of $PO_4^{3-}$ (2.5), $KH_2PO_4$ (1), $(NH4)_2SO_4$ (0.5), $C_5H_8NO_4Na$ (0.5), $Na_3C_6H_5O_7$ (0.4), $MgSO_4$ (0.05), $C_6H_8FeNO_7$ (0.04), $C_8H_{11}NO_3$ (0.001), $ZnSO_4$ (0.001), $CuSO_4$ (0.001), biotin (0.0005), $CaCl_2$ (0.0005), ADC enrichment (100 ml), and $C_{20}H_{13}K_3O_{11}S_2$ (0.65). The final pH was adjusted at 6.6 at 25$^{o}$C. The candidate strain was inoculated at 30$^{o}$c for three days. If the bacterium produces arylsulfatase, it splits the phenolphthalein substrate, releasing free phenolphthalein, which turns pink to red when alkali is added to the medium.

ADC enrichment per litre of deionized water: bovine albumin fraction v (50 g), dextrose (20 g), sodium chloride (8.5 g) and catalase (0.03 g).

#### 4.2.2.15 Pyrazinamidase test

This test was designed for the differentiation of some *Mycobacterium* species for their ability to produce Pyrazinamidase which converts pyrazinamide to pyrazinoic acid (Bloom, 1994). Pyrazinamidase agar medium with the following ingredients per litre of deionized water was prepared for the test: $Na_2HPO_4$ (2.5 g), $C_4H_8N_2O_3$ (2 g), $C_3H_3NaO_3$ (2 g), $KH_2PO_4$ (1 g), casein peptone (0.5 g), $C_6H_8FeNO_7$ (0.5 g), tween ® 80 (0.2 g), pyrazinamide (0.1 g), $MgSO_4$ (10 mg), $CaCl_2$ (0.5 mg), $ZnSO_4$ (0.1 mg), $CuSO_4$ (0.1 mg) and agar (15 g).

#### 4.2.2.16 Nitrate reduction test

This test differentiates bacteria based on their ability to produce nitrate reductase enzyme which reduces nitrate to nitrite (Van Netten *et al.*, 1987). The test was performed in nitrate broth made of Peptone 5 g/l, Meat extract 3 g/l and $KNO_3$ 1 g/l in RO water. The broth was inoculated with bacterial suspension and incubated overnight at 30 or 37°C. After that, 6-8 drops of nitrite reagent A and nitrite reagent B were added. A positive reaction was indicated with the presence of a cherry red colouration within a few minutes.

#### 4.2.2.17 Growth on MacConkey agar without crystal violet

This test was employed to differentiate some species of mycobacterium for their ability to grow in MacConkey agar without crystal violet (Shojaei *et al.*, 2013). The medium was composed of g/l of RO water: Enzymatic digest of casein (18.5), Enzymatic digest of animal tissue (1.5), Lactose (10), Bile salts mixture (5), Neutral red (0.04) and agar (12). The pH was adjusted at 7.4 ± 0.2 at 25°C. The candidate was inoculated onto the medium and after 3 days of incubation, the result was reported as presence or absence of visible colony growth.

#### 4.2.2.18 Antimicrobial susceptibility testing

Disc diffusion antimicrobial susceptibility testing was performed to assess the antibiotic susceptibility of the candidate strains. Antibiotic assay medium (AAM: Oxoid) was used for the antibacterial activity assay. Candidates were grown in tryptone soy broth (TSB: Oxoid) at 37°C by shaking at 150 rpm overnight. The growth was assessed by measuring optical density (OD) at 600nm and OD of 0.25 of the test organisms were added to AAM at the ratio of 1 ml per 25 ml of the medium which was then poured into a 9 cm Petri plate. After drying, Oxoid™ Cefaclor

Antimicrobial Susceptibility Disks of Mupirocin (5 µg), Chloramphenicol (30 µl), Ciprofloxacin (5 µg), Gentamicin (10 µg), Colistin sulphate (10 µg), Doxycycline (10 µg), Cephalexin (30 µg), Rifampicin (5 µg), Vancomycin (10 µg) and Clindamycin (2 µg) were added and the plates were incubated up to 5 days before reporting on zones of inhibition.

### 4.2.3 Chemotaxonomy studies

#### 4.2.3.1 DAP cell wall analysis

The biomass for L40, GB37, LC1, and their corresponding type strains of *Isoptericola rhizophila* JCM 19252, *Muricauda aquimarina* JCM 11811 and *Mycolicibacterium iranicum* JCM 17461, respectively were prepared by culturing them onto TSA following the protocol described in section 2.1.8. DAP of each strain was extracted using high heat aided acid hydrolysis and few steps of purification as described in section 2.3.1. Finally, 2 µl of each DAP hydrolysate was placed in TLC plates alongside known DAP standards for analysis as indicated in section 2.3.1. and Figure 4.1 below presents the results of the DAP analysis of the bacterial isolates.

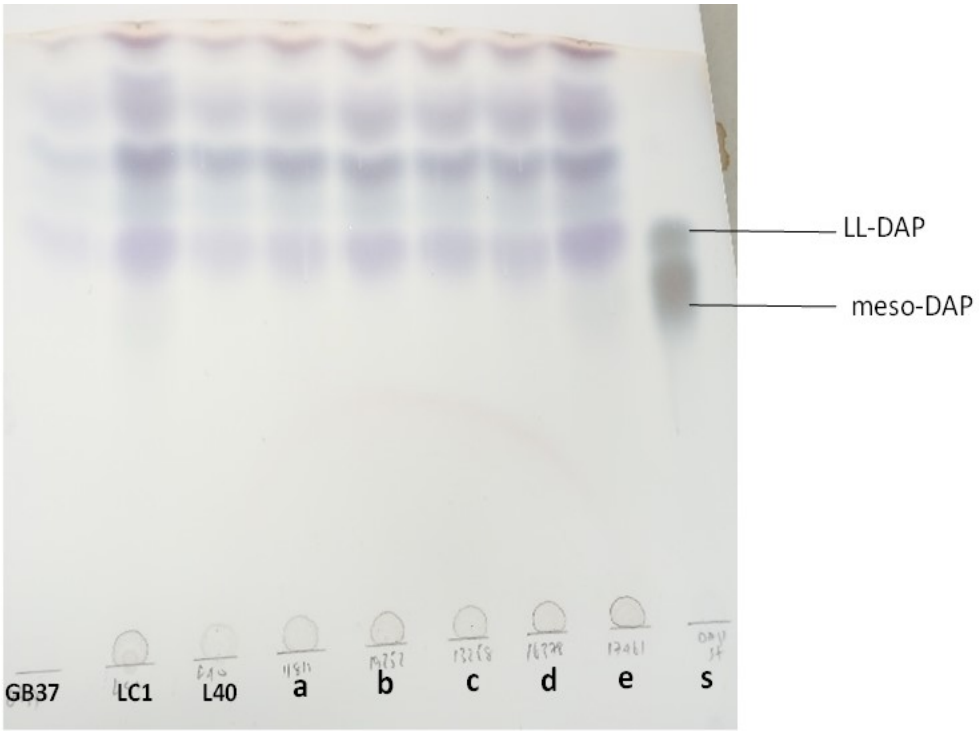



Figure 4.1 DAP analysis results of three putative novel bacterial isolates (GB37, LC1, and L40). **a** *Muricauda aquimarina* JCM 11811; **b** *Isoptericola rhizophila* JCM 19252; **c** *Muricauda ruestringensis* DSM 13258; **d** *Isoptericola halotolerans* DSM 16376; **e** *Mycolicibacterium iranicum* JCM 17461; **s** standard mix containing both-*meso* and -*LL* isomers of DAP.

4.2.3.2 Sugar cell wall analysis

The starting material for sugar cell wall analysis for three candidate isolates and type strains were prepared as described in section 2.1.8. The sugar cell wall of each bacterium was extracted using acid hydrolysis in the presence of a high degree of heat as indicated in section 2.3.2. Two microliters of each of the hydrolysate alongside sugar standards placed in TLC plates and sugar analysis proceeded using protocols indicated in section 2.3.2. Figure 4.2 below presents the results of the analysis.

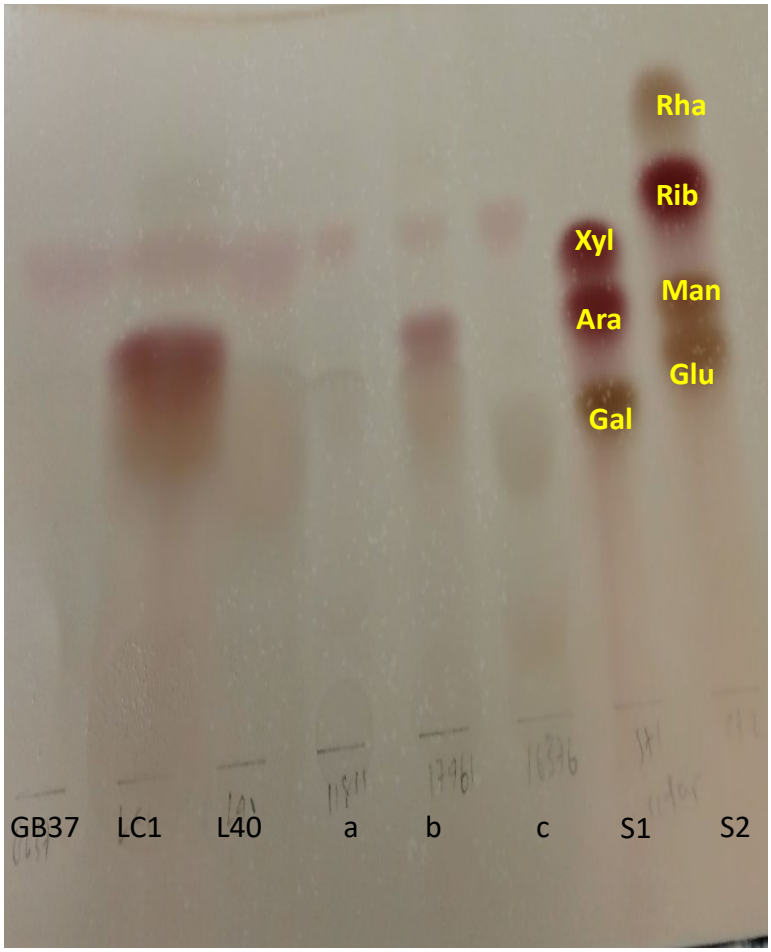



Figure 4.2 Cell wall sugar profile of the three putative novel bacterial isolates (GB37, LC1, and L40) compared to type strains. **a** *Muricauda aquimarina* JCM 11811; **b** *Mycolicibacterium iranicum* JCM 17461; **c** *Isoptericola rhizophila* JCM 19252; S1 sugar standards galactose, arabinose, xylose; S2 sugar standards glucose, mannose, ribose, and rhamnose.

4.2.3.3 Menaquinone analysis

The biomass for Menaquinone analysis for each bacterial strain was prepared in 50 ml liquid culture as indicated in section 2.1.8 and the menaquinone was extracted using chloroform: methanol (2:1) and their presence was confirmed and purified by using TLC as described in

section 2.3.3. The pure extracts of menaquinone were analysed using LC-MS employing UV detection and electrospray ionisation using ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm (Agilent part No. 993967-902) column and The TLC solvent system was isopropanol: methanol (1:1) at a flow rate of 1 ml/min.

### 4.2.3.4 Phospholipid analysis

The bacterial biomass for phospholipid analysis for the three putative novel bacterial isolates was prepared using the protocol described in Section 2.1.7 and the analysis was determined using different dyes as described in section 2.3.7. Some examples of positive reactions indicated in Figures 4.3-4.7.

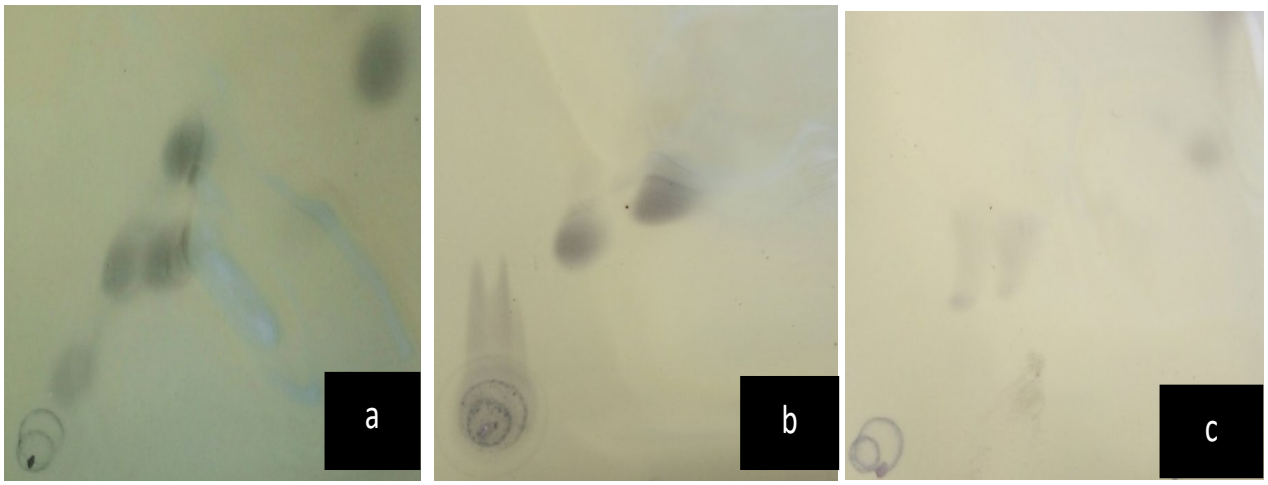



Figure 4.3 Total lipid of bacterial isolates spraying with 5% Ethanolic molybdophosphoric acid. **a** strain GB37; **b** strain LC1 and **c** strain L40.

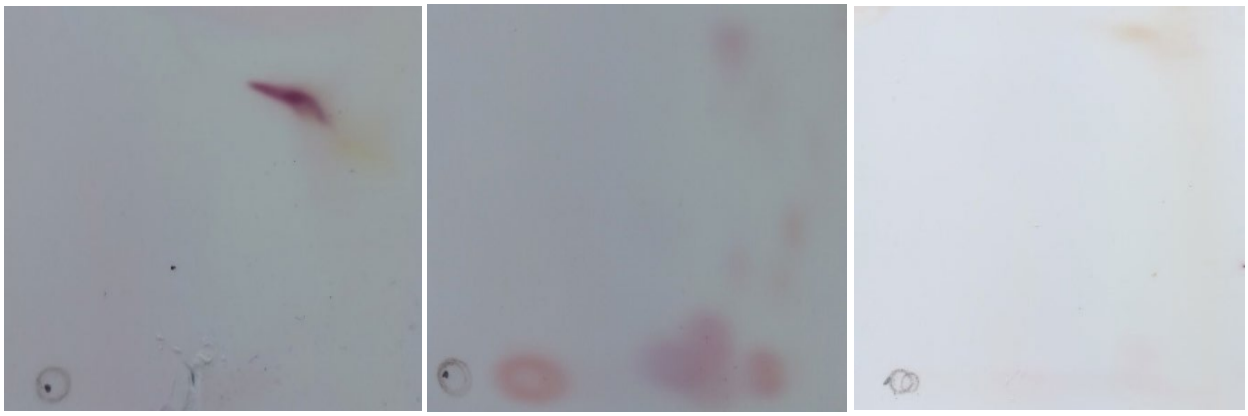


Figure 4.4 Ninhydrin spray positive for PE and PS as indicated by the pink spot.

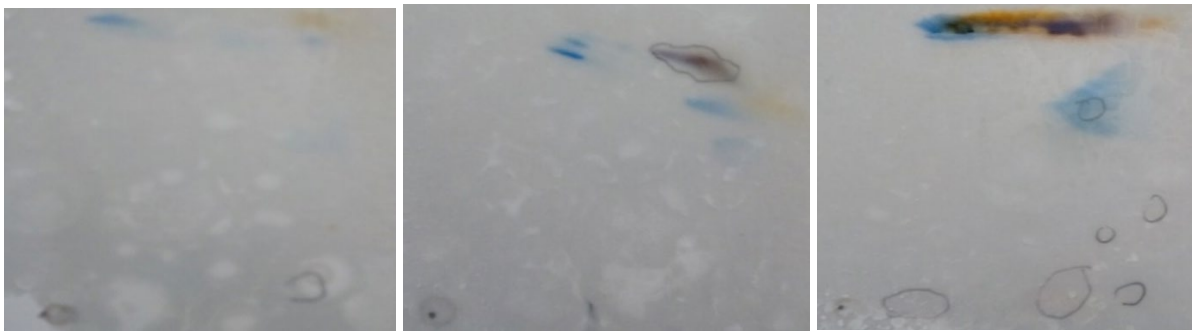


Figure 4.5 A spray of molybdenum blue reagent is used to detect lipids containing phosphate esters; PA, CL, SM, PE, PS as indicated by blue spots.

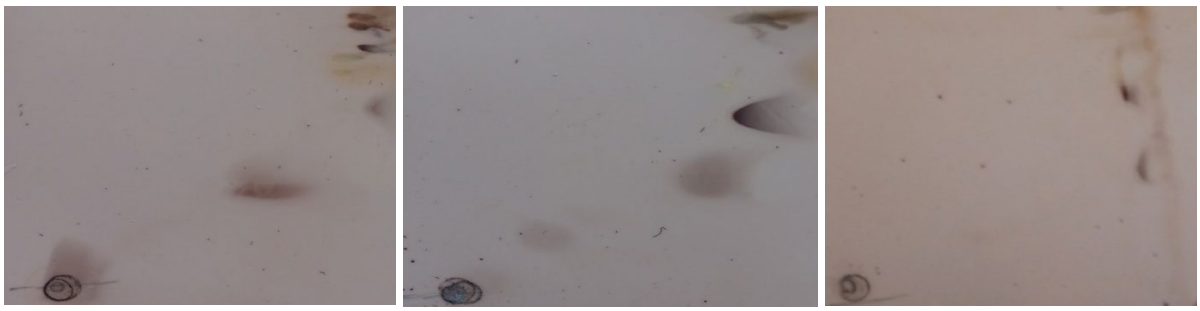


Figure 4.6 Alpha Naphthol sulphuric acid spray revealing the presence of glycolipids as brown Spots.

Figure 4.7 Spraying with the periodate-Schiff spray reagent for the detection of alpha-glycols. Glycolipid, including PIMs, present a slowly developing blue spot, while PI presents a yellow spot.

#### 4.2.3.5 Fatty acid methyl ester (FAME) analysis

The biomass from FAME analysis was prepared by the methods described in section 2.1.8. The FAME was extracted using four steps of saponification, methylation, extraction and base washing as indicated in section 2.3.6. The extract was analysed using methods described in section 2.3.6.

### 4.2.4 Genomic studies

#### 4.2.4.1 Phylogenetic and genomic studies

The genomic DNA of the novel bacteria isolates was extracted using the method described in section 2.2.5. The DNA was used for PCR amplification of the 16S rRNA gene using two primer sets 27F-765R and 704F-1492R. The PCR products were cleaned and sequenced for each half and their sequences were aligned to get the full sequence of 16S rRNA gene. The full 16S rRNA gene sequences of the bacteria were subjected to BLASTN on the NCBI database and http://www.ezbiocloud.net/eztaxon to find the closest type cultures. The phylogenetic trees of each bacterium were constructed by using MEGA 7.0 version.

#### 4.2.4.2 DNA-DNA hybridisation

DNA-DNA hybridizations between candidate and type strains were determined by using Spectrophotometric determination of DNA Hybridization from DNA renaturation rates following a previous method (Huss, 1983). In brief, about 20-30 ug/ml Genomic DNA of the bacteria was extracted by the method described in section 2.2.5 and the purified DNA was dialyzed with double strength standard saline citrate buffer (SSC; 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). The DNA concentration was adjusted spectrophotometrically at 260 nm. Sheared DNA was stored at-20$^{o}$C. T70 UV/VIS Spectrometer was used to measure the absorbance of renaturation. Four completely sealable cuvettes that contained the following DNA samples were prepared: A-DNA of candidate bacteria, B-DNA of the type strain, C- equal DNA volume and concentration of candidate and type strains and D-2 X SSC as a blank. The bacterial DNA was denatured in cuvettes at 100$^{o}$C for 10 min in a water bath and this was followed by quick cooling to start re-association reactions at appropriate renaturation temperature (TOR); which was obtained from TOR= 0.51 x % (G + C) + 47.0 (Gillis *et al*. , 1970). The decreased renaturation absorbance was measured and plotted at 260 nm over 30 min every 15 sec. The renaturation rates V' were determined as decrease in absorbance/ min (ΔA/t), and the degree of binding (%D) was calculated according to the formula given by De Ley *et al*. (1970): %D = 100 x (4V'M - V'A - V'B) / $2\sqrt{V'AxV'B}$, where M- is absorbance of cuvettes of equal volume of candidate and type strains, A- absorbance of candidate strains, B- absorbance of type strain.

#### 4.2.4.3 G+C content

The G+C content of the bacteria was determined with a slight modification of the HPLC method described in a previous study (Mesbah, 1989). The bacterial DNA was extracted with the method described in section 2.2.5 and the DNA was reconstituted with 0.01xSSC. Genomic DNA of test bacteria and two standard DNA from calf thymus (Sigma) and *E.coli* (Sigma) were denatured as follow: In 1.5 ml Eppendorf tubes, the genomic DNA of the three bacteria were dissolved in 0.01xSSC (25µl of 2-25 µg DNA) and the mixtures were placed in 100$^{o}$C boiling bath for 10 min and then immediately placed the mixture in ice. Fifty microliters of 30 mM sodium acetate (pH 5.3), 5 µl of 20 mM $ZnSO_4$ and 3 µl of P1 nuclease (340 units/ml) (Sigma # N8630) were added, mixed by hand and incubated at 37$^{o}$C for 2 hr. After that, 5 µl of 0.1 M glycine HCl buffer (pH 10.4) and 5 µl of Antarctic alkaline phosphatase (NEB) at 200 units/ml (prepared freshly) were added and incubated at 37$^{o}$C for 6 hr. The degraded genomic DNA was kept at -20$^{o}$C until the HPLC analysis could be performed. 0.01x SSC was used as a negative control. The following condition was applied for the gradient HPLC analysis of G+C content. The mobile phase was 12% methanol in 20 mM triethylamine phosphate, pH 5.1. The HPLC column was ZORBAX Eclipse XDB-C18, 4.6 x 150 mm, 5 µm (Agilent Part No. 993967-902). The flow rate was 1 min/ml and the temperature was controlled at 37$^{o}$C. The HPLC run for 10min and the peak areas corresponding to each of the four nucleotides (dC, dG, dA, and dT) of the strain and standard bacteria were used to calculate % G+C contents.

## 4.3 Results and Discussion

### 4.3.1 Discovery of three novel bacteria from marine sponges

Three novel bacterial strains have been discovered and their novelty confirmed by using a polyphasic study. The following sections present the polyphasic results for each of the three bacterial candidate strains.

### 4.3.2 *Isoptericola rapidicum* strain L40

#### 4.3.2.1 Morphological properties

The morphological characteristics of *Isoptericola rapidicum s*train L40 were assessed by comparing with its closest type strains of *Isoptericola halotolerans, Isoptericola rhizophila*, and

*Isoptericola chiayiensis.* The *Isoptericola rapidicum* strain L40 showed morphological characteristics belonging to the genus *Isoptericola* with the production of yellow substrate mycelium with the absence of aerial mycelium. Cultural characteristics of *Isoptericola rapidicum* strain L40 compared to type strains indicated in Table 4.1. The strain L40 grew well in media prepared in natural seawater compared to media without seawater. The colours of the colonies were compared with standard yellow colour charts (Yellow Colour Hue Range, Colour Name List of Yellow Colours, HEX, HSL: www.workwithcolor.com/yellow-color-hue-range).

Table 4.1 Morphological characteristics of *Isoptericola rapidicum* strain L40 in five media compared with three type strains.

| Media | *Isoptericola rapidicum strain* L40 | | *Isoptericola halotolerans* | | *Isoptericola rhizophila* | | *Isoptericola chiayiensis* | |
|---|---|---|---|---|---|---|---|---|
| | Growth | Colony colour | Growth | Colony colour | Growth | Colony colour | Growth | Colony colour |
| TSA | Poor | Pale Yellow | Good | Yellow | Good | Yellow | Poor | Yellow |
| SYP | Good | Golden Yellow | Poor | Pale Yellow | Poor | Pale Yellow | Poor | Cream Yellow |
| NA | Poor | Pale Yellow | Good | Lemon Yellow | Poor | Good | Poor | Beige Yellow |
| ISP2 | Good | Golden Yellow | Good | Yellow | Good | Pale Yellow | Poor | Ivory Yellow |
| MA | Good | Yellow | Good | Yellow | Good | Yellow | Good | Yellow |

### 4.3.2.2 Physiological and biochemical properties

Side by side comparison of *Isoptericola rapidicum* strain L40 with type strains was assessed for various physical and biochemical properties and the differential properties are indicated in Table 4.2. The candidate strain L40 produced acid from D-glucose, D-maltose, D-mannitol, myo-inositol, and sucrose but not from L-rhamnose, D-sorbitol. The candidate could not decompose adenine, xanthine, hypoxanthine, and L-tyrosine but degraded starch and demonstrated catalase and oxidase activities. The strain could assimilate sodium salts of benzoate, malate, propionate,

and tartrate but not acetate and citrate. The strain demonstrated the ability to grow at NaCl concentrations of 1-15%, pH of 7-8 and temperature of 10-30$^{o}$C.

All strains were positive for growth at a temperature range of 15-37$^{o}$C; pH of 7 and 8; NaCl concentrations of 1-15%; utilization of glycerol, D- fructose, arginine, alanine and malate; acid production from D-cellobiose, D-fructose, and D-mannitol; all three sugar fermented in TSI; degradation of starch; catalase production; susceptibility to rifampicin, vancomycin, clindamycin, doxycycline, and cephalexin. At the same time, all strains were negative for growth at 45$^{o}$C, pH of 4 and 5, NaCl concentration of 25%, acid production from D-melezitose, degradation of casein, assimilation of sodium benzoate, sodium malate, sodium propionate, and sodium tartrate, $H_2S$ production, and assimilation of adenine, L-tyrosine and xanthine.

Table 4.2 Phenotypic properties of *Isoptericola rapidicum s*train L40 that differentiates it from related type strains

| Characteristics | *Isoptericola rapidicum s*train L40 | *Isoptericola halotoleran*$^{T}$ | *Isoptericola rhizophila*$^{T}$ | *Isoptericola chiayiensis*$^{T}$ |
|---|---|---|---|---|
| Carbohydrate utilization | | | | |
| D-galactose | + | + | - | + |
| D-glucose | + | - | - | + |
| D-mannose | - | + | - | + |
| D-Xylose | - | + | + | - |
| Raffinose | + | - | - | - |
| Acid production from: | | | | |
| L-arabinose | + | + | - | - |
| D-galactose | + | + | - | - |
| D-glucose | + | - | - | + |
| D-maltose | + | + | - | - |

| | | | | |
|---|---|---|---|---|
| D-mannose | + | - | - | + |
| Raffinose | + | - | - | - |
| Methyl$\alpha$-D- glucoside | + | - | - | - |
| L-rhamnose | - | + | + | - |
| sucrose | + | + | - | - |
| D-xylose | + | - | + | + |
| lactose | - | + | + | - |
| myo-inositol | + | - | - | - |
| D-sorbitol | - | + | + | + |
| Growth at: 10$^{o}$C | + | + | - | - |
| Salt tolerance: 20% | - | + | + | - |
| Decomposition of urea | + | - | - | + |
| hypoxanthine | + | - | + | + |
| Indole production | - | + | - | + |
| Sodium acetate | - | + | + | - |
| Sodium citrate | - | - | + | + |
| Oxidase test | + | - | + | + |
| mupirocin (5 µg) | R | S | S | S |
| chloramphenicol (30 µg) | S | S | S | R |
| ciprofloxacin (5 µg) | R | S | R | S |
| gentamicin (10 µg) | R | S | S | S |

+ Positive for tested characteristics; - Negative for tested characteristics; R resistance; S susceptible

### 4.3.2.3 Chemical properties

The following cellular chemicals were identified from candidate strain. Galactose, mannose, and xylose (cell wall sugars), LLDAP (DAP type), phosphatidylglycerol, phatidylinositolmannosides, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid ( phospholipids types), A4α, variation L-Lys-D-Asp (peptidoglycan type). The major menaquinone was MK-9($H_4$). The major cellular fatty acids in comparison with the type strains were presented in Table 4.3. The fatty acid compositions of the reference strains analysed were qualitatively similar but quantitatively different from those of the novel strain.

Table 4.3 FAME patterns of *Isoptericola rapidicum s*train L40 in comparison with type strains

| Fatty acid | *Isoptericola rapidicum* L40 | *Isoptericola rhizophila*$^T$ JCM 19252 | *Isoptericola halotolerans*$^T$ DSM 16376 | *Isoptericola chiayiensis*$^T$ BCRC 16888 |
|---|---|---|---|---|
| Straight-chain fatty acids | | | | |
| $C_{12:0}$ | 0.07 | 0.5 | 0.5 | 0.11 |
| $C_{13:0}$ | 0.03 | 0.26 | 1.26 | 0.17 |
| $C_{14:0}$ | 0.5 | 2.37 | 3.17 | 0.76 |
| $C_{16:0}$ | 5.53 | 4.09 | 2.75 | 5.21 |
| $C_{17:0}$ | 0.53 | 0.29 | 0.57 | 0.52 |
| Branched fatty acid | | | | |
| iso-$C_{13:0}$ | 0.27 | 1.27 | 2.39 | 0.51 |
| iso-$C_{14:0}$ | 3.88 | 21.87 | 10.07 | 4.47 |
| iso-$C_{17:0}$ | 0.7 | 0.69 | 0.46 | 0.66 |
| iso-$C_{16:0}$ | 3.59 | 7.60 | 1.81 | 3.44 |
| anteiso-$C_{15:0}$ | 66.45 | 45.48 | 61.82 | 65.11 |
| anteiso A-$C_{15:0}$ | 1.02 | 0.53 | 0.23 | 0.92 |

| | | | | |
|---|---|---|---|---|
| anteiso-$C_{17:0}$<br><br>iso-$C_{15:0}$ | 10.26<br><br>6.6 | 3.76<br><br>8.65 | 3.77<br><br>8.96 | 9.29<br><br>6.82 |
| Hydroxy fatty acid<br><br>$C_{17:0}$ 3-OH | 0.13 | 1.07 | 0.76 | ND |

#### 4.3.2.4 Genotypic properties

##### 4.3.2.4.1 G+C content

*Isoptericola rapidicum s*train L40 has a G+C content of 70.4%

##### 4.3.2.4.2 16S rRNA gene sequence analysis

The 16S rRNA gene sequence was determined for 1,382 bases of strain L40$^T$ (MK367392) as indicated in Appendix 5. The strain L40 presents the highest 16S rRNA gene sequence similarity to *Isoptericola halotolerans* DSM 16376$^T$ (98.4%), *Isoptericola rhizophila* JCM 19252$^T$ (98.19%), and *Isoptericola chiayiensis* BCRC 16888$^T$ (97.61%).

##### 4.3.2.4.3 Phylogenetic analysis for *Isoptericola rapidicum s*train L40

The nearly complete sequence of strain L40 (1382 bp; GenBank accession no. MK367392) was examined for phylogenetic analysis. All published type strains of the genus *Isoptericola* were included in the construction of the phylogenetic dendrogram indicated in Figure 4.8. The phylogenetic tree displayed the *Isoptericola rapidicum s*train L40 is the member of the genus *Isoptericola* within the family *Promicromonosporaceae* which belong to the same clade with the closest neighbour strain*, Isoptericola rhizophila* JCM 19252$^T$. This affiliation was confirmed with both neighbour-joining and maximum-parsimony (Appendix 5) algorisms.

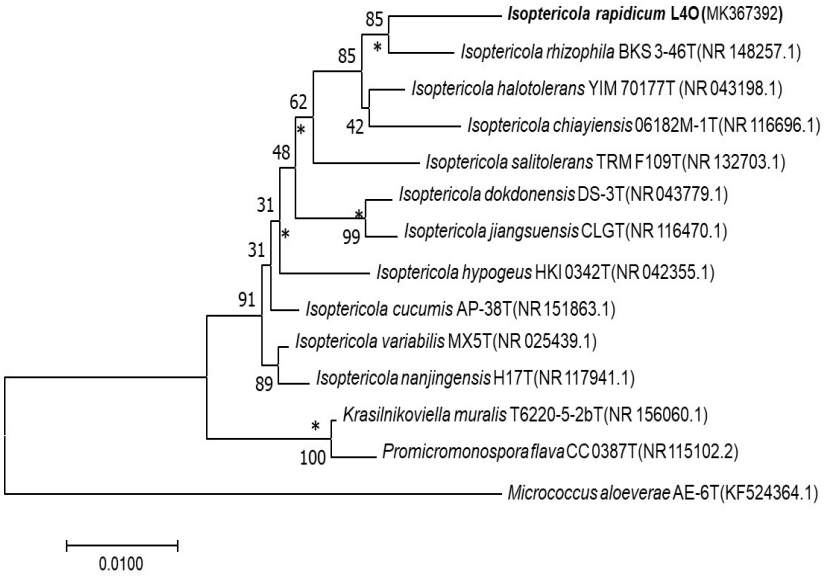



Figure 4.8 Phylogenetic tree analysis of strain L40. Neighbour-joining tree showing the relationship between strain L40 with related members of the genus *Isoptericola*. The asterisk indicates branches of the tree that were also identified by other algorithms. The numbers at the node indicated the levels of bootstrap support based on 1000 resampled datasets. The scale bar represents changes per nucleotide. *Micrococcus aloeverae* AE-6T as outgroup.

### 4.3.2.5 Differentiation of strain L40 with its closest strains

Phenotypic and genotypic studies displayed that *Isoptericola rapidicum s*train L40 is different compared to the validly published type strains of the genus *Isoptericola*. The physical and biological properties that differentiated the candidate strain with the closest species, *Isoptericola halotolerans* DSM 16376$^T$, and the closest phylogenetic neighbour, *Isoptericola rhizophila* JCM 19252$^T$ was indicated in Table 4.2. *Isoptericola rapidicum s*train L40 differed from *Isoptericola rhizophila* with the negative reaction of acid production from L-rhamnose, lactose and D-sorbitol; assimilation of sodium acetate and sodium citrate; growth at 20 % salt; and not respond to antimicrobials Mupirocin and Gentamicin. Unlike *Isoptericola rhizophila* the candidate strain showed a positive reaction to acid production from L-arabinose, D-galactose, D glucose, maltose, D-mannose, raffinose, methyl $\alpha$-D- glucoside, sucrose, and myo-inositol; growth at 10$^o$C and decomposition of urea. The candidate strain L40 also showed some different profiles for carbohydrates utilization and decomposition of certain substrates as presented in Table 4.2.

DNA–DNA relatedness of *Isoptericola rapidicum s*train L40 with the closest type strain of *Isoptericola rhizophila* and *Isoptericola halotolerans* was assessed by using the optical

renaturation method as mentioned in section 4.2.4.2. The result indicated the candidate strain L40 displayed DNA-DNA relatedness of 39.9% with *Isoptericola rhizophila* and 34.5% with *Isoptericola halotolerans*. (The experiments were conducted twice which resulted in 40.5 and 39.3% for *Isoptericola rhizophila* and 35 and 34% of DNA-DNA relatedness in the first and second run, respectively).

The polyphasic taxonomy indicates the strain L40 is a novel species of the genus *Isoptericola* and the name *Isoptericola rapidicum* sp.nov. was proposed.

### 4.3.2.6 Description of novel *Isoptericola rapidicum* strain L40$^T$

*Isoptericola rapidicum* (ra'.pid.icum. adj. rapidicum related to the source of the sponge sample, Rapid bay).

The bacteria are Gram-positive and they appeared as rod-shaped or coccoid, non-motile with the formation of primary mycelium but no aerial mycelium and spores. Their growth was good in SYP, MA and ISP2 but not on TSA, and NA. They produced about 1 mm diameter yellow colour, mainly golden yellow, circular colonies within 3 days of incubation at 27$^o$C. The strain grows well at a NaCl concentration of 1-5%, pH of 7-8 and temperature of 27$^o$C at an aerobic condition. The strain didn't decompose adenine, xanthine, L-tyrosine and casein but positive for urea and hypoxanthine decomposition. They are positive for catalase, oxidase, starch hydrolysis and acid production from L-arabinose, D-galactose, D glucose, maltose, D-mannose, raffinose, methyl $\alpha$-D- glucoside, sucrose, and myo-inositol. They are negative for the assimilation of sodium salts, indole production and acid production from L-rhamnose, lactose, and D-sorbitol.

Candidate strain *Isoptericola rapidicum* L40 contained LL-DAP, galactose, mannose, and xylose (cell wall sugars), phosphatidic acid, phosphatidic acid, phosphatidylglycerol, phosp-atidylethanolamine, and diphosphatidylglycerol (Phospholipids types), and A4$\alpha$, variation L-Lys-D-Asp, (peptidoglycan). The major menaquinone was MK-9($H_4$). The major cellular fatty acids are anteiso-$C_{15:0}$ (66.45%), anteiso-$C_{17:0}$ (10.26%), iso-$C_{15:0}$ (6.6%), $C_{16:0}$ (5.53%), iso-$C_{16:0}$ (3.59%), iso-$C_{14:0}$ (3.88%), and anteiso A-$C_{15:0}$(1.2%). The G+C content of the DNA of the type strain is 70.4 mol%.

The type strain, *Isoptericola rapidicum* L40$^T$ (=KCTC 49304$^T$, BCRC 81227$^T$), was isolated from a marine sponge *Aplysilla sulfurea* (RB 16) collected from Rapid Bay Jetty, South Australia.

### 4.3.2.7 Affiliation of novel Isoptericola rapidicum strain L40$^T$ with other members of Isoptericola

The genus *Isoptericola* first described by Stackebrandt and his colleagues following the reclassification of Cellulosimicrobium variabile Bakalidou et al. 2002 as *Isoptericola variabilis* gen. nov., comb. nov. (Stackebrandt *et al.*, 2004). The members of the genus *Isoptericola* are non-motile, facultative anaerobes, Gram-positive, bacillus or cocci, and that can produce primary mycelium. They produce acids from some carbohydrates. Actinobacteria Micrococcales/ Promicromonosporaceae/ Isoptericola. The genus characterized by peptidoglycan of A4α, L-Lys-D-Asp variation; menaquinones of MK-9 ($H_2$) and /or MK-9 ($H_4$); the major fatty acids of $C_{15:0}$ anteiso, $C_{15:0}$ iso, $C_{16:0}$, $C_{17:0}$ anteiso, $C_{14:0}$, and $C_{16:0}$ iso and phospholipids of phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylinositol, where unknown glycolipids and/or phospholipids may occur. The DNA G+C content ranges 70.0-74.1 mol % (Kaur *et al.*, 2014). The strain *Isoptericola rapidicum* L40 presented most of the above chemical propertes and its placement in genus *Isoptericola rapidicum* is supported (Zhang *et al.*, 2005; Tseng *et al.*, 2011; Kaur *et al.*, 2014).

During write up of this thesis, the genus *Isoptericola* consists of 10 species with validly published names and as indicated in Table 4.4, all are identified from various environmental sources. Comparison of 16S rRNA gene sequence analysis of L40 with other members of the genus *Isoptericola* showed that the strain has the highest percentage similarity with *Isoptericola halotolerans* (98.4%) and *Isoptericola rhizophila* (98.19%). They also share sequence similarity with the rest of the members with percentages ranging from 97.61 and 96.58%. The phylogenetic analysis indicated the strain L40 appeared in the same clade with *Isoptericola rhizophila* with a higher bootstrap value of 85. However, the strain L40 belonged to different clusters with the closely related strain of *Isoptericola halotolerans* which is in a cluster containing *Isoptericola chiayiensis,* which showed a similarity of 97.61% (Table 4.4).

Table 4.4 List of validly published species of genus *Isoptericola*

| Isolate name | Sources | % similarity | References |
|---|---|---|---|
| *Isoptericola halotolerans* sp. nov. | Saline soil | 98.41 | (Zhang *et al.*, 2005) |
| *Isoptericola rhizophila* sp. nov. | Soil sample<br><br>collected from the rhizosphere of Ficus benghalensis<br><br>(Banyan tree) | 98.19 | (Kaur *et al.*, 2014) |
| *Isoptericola chiayiensis* sp. nov. | Soil sample | 97.61 | (Tseng *et al.*, 2011) |
| *Isoptericola nanjingensis* sp. nov. | Soil sample | 97.50 | (Huang *et al.*, 2012) |
| *Isoptericola variabilis* gen. nov., comb. nov | Hindgut of the Australian termite | 97.17 | (Stackebrandt *et al.*, 2004) |
| *Isoptericola cucumis* sp. nov. | Root tissue of cucumber | 97.06 | (Kämpfer *et al.*, 2016) |
| *Isoptericola salitolerans sp. nov.* | Salt Lake | 96.87 | (Guan *et al.*, 2013) |
| *Isoptericola hypogeus* sp. nov. | Tufa | 96.66 | (Groth *et al.*, 2005) |
| *Isoptericola jiangsuensis* sp. nov. | Beach soil | 96.65 | (Wu *et al.*, 2010) |
| *Isoptericola dokdonensis* sp. nov., | Soil sample | 96.58 | (Yoon *et al.*, 2006) |

### 4.3.3 *Muricauda gelenilca* strain GB37

#### 4.3.3.1 Morphological properties

The morphological characteristics of *Muricauda gelenilca* strain GB37 were assessed by comparing with its closest type strains of *Muricauda aquimarina* JCM 11811$^T$, *Muricauda ruestringensis* DSM 13258$^T$ and *Muricauda lutimaris* KCTC 22173$^T$. The strain *Muricauda gelenilca* GB37 showed morphological belong to the genus *Muricauda* with the production of yellow, Gram-stain-negative, non-motile, and rod-shaped colonies. Cultural characteristics of strain *Muricauda gelenilca* GB37 compared to type strains were indicated in Table 4.5. The candidate strain grew well in medium prepared in natural seawater compared to others and the growth

was not prominent on medium without seawater. The colour of the colonies was compared with standard yellow colour charts (Yellow Colour Hue Range, Colour Name List of Yellow Colours, HEX, HSL: www.workwithcolor.com/yellow-color-hue-range).

Table 4.5 Morphological characteristics of *Muricauda gelenilca* strain GB37 in five media compared with three type strains.

| Media | *Muricauda gelenilca* strain GB37 | | *Muricauda aquimarina*$^T$ | | *Muricauda ruestringensis*$^T$ | | *Muricauda lutimaris*$^T$ | |
|---|---|---|---|---|---|---|---|---|
| | Growth | Colony colour | Growth | Colony colour | Growth | Colony colour | Growth | Colony colour |
| TSA | Poor | Pale Yellow | Good | Yellow | Good | Yellow | Poor | Yellow |
| SYP | Good | Golden Yellow | Poor | Pale Yellow | Poor | Pale Yellow | Poor | Pale Yellow |
| NA | Poor | Pale Yellow | Good | Lemon Yellow | Poor | Good | Poor | Yellow |
| MA | Good | Golden Yellow | Good | Golden Yellow | Good | Yellow | Good | Yellow |

### 4.3.3.2 Physiological and biochemical properties

Various physical and biochemical properties of the candidate strain *Muricauda gelenilca* GB37 were examined alongside with its closest type strains and those properties which showed difference are only presented in Table 4.6. The strain GB37 produced acid from D-fructose, D-galactose, D-maltose, and myo-inositol but not from L-arabinose, L-rhamnose, and D-glucose. The strain did not decompose adenine, hypoxanthine, and starch but degraded xanthine and L-tyrosine and showed catalase and oxidase activities. The candidate strain utilized sodium citrate but not other tested sodium salts. The candidate also grew at NaCl concentration of 1-15%, pH of 7-8 and temperatures of 15-30$^o$C.

The four tested bacterial strains were positive for growth at temperature ranges of 15-30$^o$C, pH of 6-8, NaCl concentrations of 1-10%; utilization of fructose, succinate, raffinose, cellobiose, sucrose, lactose and mannose, acid production from D-galactose, D-maltose, D-mannose, and D-trehalose; catalase production; susceptible to rifampicin, vancomycin, and clindamycin. Similarly,

all strains displayed negative results for growth at 45°C, pH 4 and 5; NaCl concentration of 20%; and acid production from D-melezitose, methyl α-D- glucoside; degradation of casein; assimilation of sodium salts of acetate, benzoate, malate, tartrate, and propionate; $H_2S$ production; and assimilation of adenine and hypoxanthine.

Table 4.6 Phenotypic properties of *Muricauda gelenilca* GB37 that differentiate it from related type strains

| Characteristics | *Muricauda gelenilca* GB37 | *Muricauda aquimarina*$^T$ | *Muricauda ruestringensis*$^T$ | *Muricauda lutimaris*$^T$ |
|---|---|---|---|---|
| Organic substrate utilization | | | | |
| glucose | + | - | + | + |
| malate | - | - | + | + |
| alanine | + | - | + | - |
| arginine | + | - | + | - |
| Acid from carbohydrate: | | | | |
| L-arabinose | - | + | + | + |
| D-cellobiose | - | + | + | - |
| D-fructose | + | + | + | - |
| D-glucose | - | + | + | + |
| D-melezitose | - | - | - | + |
| L-rhamnose | - | + | + | - |
| sucrose | - | + | + | - |
| D-xylose | - | + | + | + |
| *Lactose* | + | + | + | - |
| *myo*-inositol | + | - | - | - |

| | | | | |
|---|---|---|---|---|
| D-mannitol | - | + | + | - |
| D-sorbitol | - | + | + | - |
| Growth at: 10°C | + | - | + | - |
| Salt tolerance: 15% | + | + | - | - |
| Decomposition of urea | - | - | - | + |
| xanthine | + | - | - | - |
| L-tyrosine | + | - | - | - |
| Decomposition of starch | - | + | + | - |
| Sodium citrate | + | - | - | - |
| Oxidase test | + | - | - | - |
| mupirocin (5 µg) | S | R | R | R |
| chloramphenicol (30 µl) | S | S | R | R |
| ciprofloxacin (5 µg) | R | R | R | S |
| gentamicin (10 µg) | S | R | R | S |
| colistin sulphate (10 µg) | R | R | S | R |
| doxycycline (10 µg) | S | S | R | R |
| cephalexin (30 µg) | S | S | R | S |

+ Positive for tested characteristics; - Negative for tested characteristics; R resistance; S susceptible

#### 4.3.3.3 Chemical properties

The chemical properties of strain GB37 including cell wall sugars, types of DAP, phospholipids, menaquinone, peptidoglycan type, and FAME pattern were analysed. The DAP was LL DAP. The cell wall sugar was rhamnose and the phospholipids types were phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, and phosphatidylcholine. The major menaquinone was

MK-6. The major cellular fatty acids in comparison with the type strains were presented in Table 4.7. The fatty acid compositions of the reference strains were qualitatively similar to candidate strain but quantitatively different.

Table 4.7 FAME patterns of strain *Muricauda gelenilca* GB37 in comparison with type strains

| Fatty acid | *Muricauda gelenilca* GB37 | *Muricauda aquimarina*$^T$ JCM 11811 | *Muricauda ruestringensis*$^T$ *DSM 13258* | *Muricauda lutimaris*$^T$ KCTC 22173 |
|---|---|---|---|---|
| Straight-chain | | | | |
| $C_{14:0}$ | 0.5 | 0.12 | 0.82 | 0.08 |
| $C_{16:0}$ | 4.6 | 0.29 | 1.1 | 0.03 |
| $C_{17:0}$ | 2.44 | 0.1 | 0.53 | 0.14 |
| Branched | | | | |
| Iso-C14:$_0$ | 4.5 | 6.62 | 6.1 | 6.5 |
| Iso-C17:$_0$ | 0.34 | 0.11 | 0.11 | 0.12 |
| iso-$C_{16:0}$ | 6.5 | 8.2 | 7.5 | 8.6 |
| anteiso A-$C_{15:0}$ | 23.5 | 37.17 | 34.38 | 37.49 |
| anteiso-$C_{17:0}$ | 0.5 | 0.56 | 0.52 | 0.6 |
| Iso-C15:$_0$ | 23 | 34.81 | 32.13 | 34.86 |
| Unsaturated | | | | |
| $C_{15:1\ w6c}$ | 0.7 | 0.22 | 0.2 | 0.23 |
| $C_{16:1\ w5c}$ | 1.47 | 1.2 | 1.3 | 1.6 |
| $C_{17:1w8c}$ | 2.6 | 0.11 | 0.17 | 0.15 |
| $C_{17:1w6c}$ | 38.98 | 1.52 | 2.55 | 2.08 |
| $C_{18:1w5c}$ | 0.52 | 0.56 | 0.6 | 0.45 |
| $C_{18:1\ w7c\ 11\text{-}methyl}$ | 6.20 | 0.22 | 0.38 | 0.32 |

| | | | | |
|---|---|---|---|---|
| | | | | |

#### 4.3.3.4 Genotypic properties

##### 4.3.3.4.1 G+C content

Strain *Muricauda gelenilca* GB37 has a G+C content of 44%.

##### 4.3.3.4.2 16S rRNA gene sequence analysis

The full 16S rRNA gene sequence was determined for 1,472 bases of *Muricauda gelenilca* GB37$^T$ (MK367393) as indicated in Appendix 5. The strain GB37 presents the highest 16S rRNA gene sequence similarity to *Muricauda aquimarina* JCM 11811$^T$ (98.01%), *Muricauda ruestringensis DSM 13258$^T$* (97.41%) and *Muricauda lutimaris* KCTC 22173$^T$ (97.19%)

##### 4.3.3.4.3 Phylogenetic analysis for strain *Muricauda gelenilca* GB37

The nearly complete sequence of the candidate strain GB37 (1,472 bp; GenBank accession no. MK367393) was examined for phylogenetic analysis. All published type strains of the genus *Muricauda* were included in the construction of the phylogenetic dendrogram indicated in Figure 4.9. The phylogenetic tree displayed the candidate strain GB37 is the member of the genus *Muricauda* within the family *Flavobacteriaceae* which belongs to the same clade with the closest neighbour strain*, Muricauda aquimarina* JCM 11811$^T$. This affiliation was confirmed with both minimum-evolution and neighbour-joining (Appendix 5) algorisms.

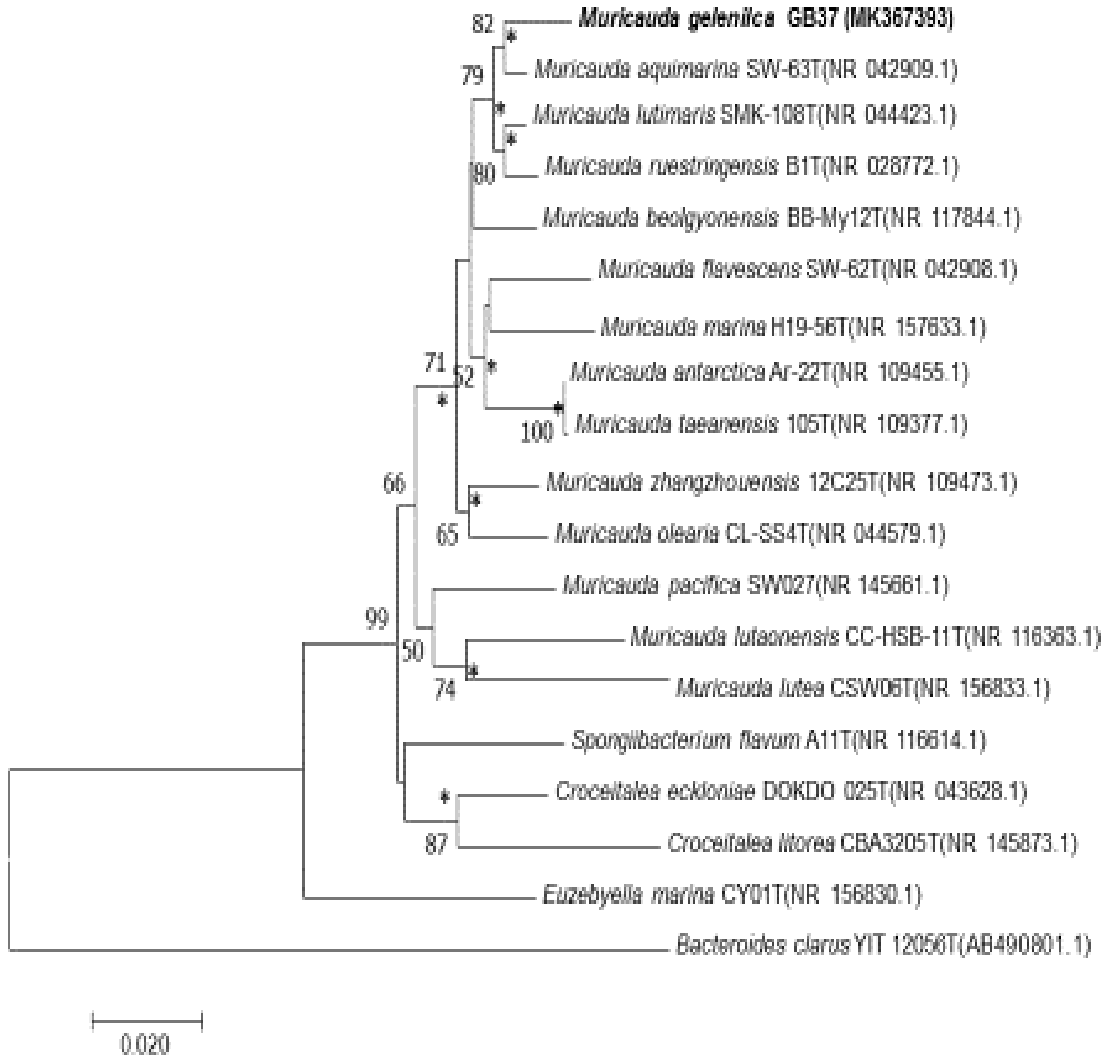



Figure 4.9 Phylogenetic tree analysis of strain GB37. Minimum-Evolution tree showing the relationship of strain GB37 with related members of the genus *Muricauda.* The asterisk indicates branches of the tree that were also identified by other algorithms. The numbers at the node indicated the levels of bootstrap support based on 1000 resampled datasets. The scale bar represents changes per nucleotide. Bacterroides clarus YIT as an outgroup.

### 4.3.3.5 Differentiation of strain GB37 with its closest strains

The results of polyphasic studies displayed the candidate strain GB37 was unique compared to the validly published type strains of the genus *Muricauda*. The physical and biological properties that differentiated strain GB37 with the closest species and phylogenetic neighbour, *Muricauda aquimarina* JCM 11811$^T$ was indicated in Table 4.6. The candidate strain GB37 differed from *Muricauda aquimarina* JCM 11811$^T$ with the negative reaction of acid production from L-arabinose, D-cellobiose, D-glucose, L-rhamnose, sucrose, D-xylose, D-mannitol, and D-sorbitol;

negative for starch degradation; negative for TSI and sensitivity to antimicrobial mupirocin. Unlike *Muricauda aquimarina, Muricauda gelenilca* GB37 showed growth in presence of glucose, alanine, and arginine; acid production from myo-inositol; positive for assimilation of sodium citrate, oxidase test, degradation of L-tyrosine and xanthine, and golden yellow colonies in SYP medium.

DNA-DNA relatedness of *Muricauda gelenilca* GB37 with its closest type strain of *Muricauda aquimarina* was assessed by using the optical renaturation method. The results indicated strain *Muricauda gelenilca* GB37 displayed a DNA-DNA relatedness of 44.4% with *Muricauda aquimarina.* The experiments were conducted twice which resulted in 45.3% and 43.5% of DNA-DNA relatedness in the first and second run, respectively. In the same manner strain, *Muricauda gelenilca* GB37 resulted in a DNA-DNA similarity of 38.3% (duplicate run of 38 and 38.6%) with *Muricauda ruestringensis*.

The polyphasic taxonomy indicates the strain *Muricauda gelenilca* GB37 is a novel species of the genus *Muricauda* and the name *Muricauda gelenilca* sp.nov. was proposed.

The polyphasic taxonomy indicates the strain Gb37 is a novel species of the genus *Muricauda* and the name *Muricauda gelenilca* sp.nov. was proposed.

#### 4.3.3.6 Description of novel strain Muricauda gelenilca Gb37$^T$

The candidate strain GB37 is Gram-negative, non-motile, non-spore-forming rod with a length of 3 to 6µm, a width of 0.2–4 µm. It had good growth in SYP and MA media with the production of circular, shiny, golden yellow colour colonies about 1-1.5 mm diameter within 4 days of incubation at 27$^o$C in aerobic conditions. They grew optimally at temperature ranges of 15-30$^o$C. Their minimum growth temperature was 10$^o$C and a maximum of 30$^o$C. They grew well at pH of 7-8 but not in pH below 6. The strain grew well in media prepared with seawater than water containing NaCl. Their growth was optimal in 1-5% (w/v) NaCl and growth observed until 15% (w/v) of NaCl. The strain didn't grow in the absence of NaCl on medium and at anaerobic incubation. The following chemical structure identified: Mk-6 menaquinone, LLDAP, rhamnose (cell wall sugar), phospholipid types of Phosphatidylethanolamine, Phosphatidylserine, phosphatidic acid and phosphatidylcholine. The major fatty acids of iso-C15 : 0, iso-C15: 1 and iso-C17 : 0 3-OH. They showed a positive reaction to acid production from Myo-inositol,

assimilation of sodium citrate, oxidase test, degradation of L-tyrosine, and xanthine. Other phenotypic properties were indicated in Table 4.6. The DNA G+C content was 44 mol%.

The type strain, Gb37$^T$, was isolated from a marine sponge *Chondrosida* sp.(Gb 08) collected from Glenelg block, South Australia.

### 4.3.3.7 Affiliation of novel strain Muricauda gelenilca GB37$^T$ with other members of Muricauda

The genus *Muricauda* was proposed by Bruns *et al.* (2001) with the single species *Muricauda ruestringensis.* Phylogenetically, the genus Muricauda belongs to family *Flavobacteriaceae* within the Cytophaga–Flavobacterium–Bacteroides (CFB) complex (Bruns et al., 2001). They are Gram-negative, non-motile, non-spore former and facultative anaerobic bacteria. The genus also characterized by several amounts of branched and straight-chain fatty acids and a G+C content of 41–45 mol%. They have LL-DAP and major menaquinone of MK-6. The candidate strain GB37 displayed also the same chemical profiles with the reference strains and their categorization in this genus is valid (Bruns *et al.*, 2001; Yoon *et al.*, 2005; Yoon *et al.*, 2008).

During write up of this thesis, the genus *Muricauda* consists of 13 species with validly published names and as indicated in Table 4.4.4, all are identified from various environmental sources. Comparison of 16S rRNA gene sequence analysis of *Muricauda gelenilca* GB37 with other members of the genus *Muricauda* showed that the strain has the highest percentage similarity with *Muricauda aquimarina* (98.01%), *Muricauda ruestringensis* (97.41%) and *Muricauda luminaries* (97.19%). They also share sequence similarity with the rest of the members with percentages less than 97%. The phylogenetic analysis indicated the strain Gb37 appeared in the same clade with *Muricauda aquimarina* with a higher bootstrap value of 82. However, the strain Gb37 belonged to different clusters with the other closely related strain of *Muricauda ruestringensis* which is in a cluster containing *Muricauda lutimaris,* which showed similarity of 97.19% (Table 4.8).

Table 4.8 List of validly published species of genus *Muricauda*

| Isolate names | Sources | % similarity | References |
|---|---|---|---|
| *Muricauda aquimarina* sp. nov. | Salt Lake | 98.01 | (Yoon *et al.*, 2005) |
| *Muricauda ruestringensis* sp. nov. | Coastal sediment | 97.41 | (Bruns *et al.*, 2001) |
| *Muricauda lutimaris* sp. nov. | Tidal flat of the Yellow Sea | 97.19 | (Yoon *et al.*, 2008) |
| *Muricauda zhangzhouensis* sp. nov. | Mangrove sediment | 96.76 | (Yang *et al.*, 2013) |
| *Muricauda beolgyonensis* sp. nov. | Tidal flat | 96.76 | (Lee *et al.*, 2012) |
| *Muricauda olearia* sp. nov. | Oil contaminated seawater | 96.41 | (Hwang *et al.*, 2009) |
| *Muricauda flavescens* sp. nov. | Salt Lake | 96.17 | (Yoon *et al.*, 2005) |
| *Muricauda antarctica* sp. nov. | Antarctic seawater | 95.70 | (Wu *et al.*, 2013) |
| *Muricauda taeanensis* sp. nov. | Tidal flat | 95.58 | (Kim *et al.*, 2013) |
| *Muricauda marina* sp. nov. | Marine snow of Yellow Sea | 95.44 | (Su *et al.*, 2017) |
| *Muricauda pacifica* sp. nov. | Surface seawater | 93.65 | (Zhang *et al.*, 2015) |
| *Muricauda lutaonensis* sp. nov. | Coastal hot spring | 93.41 | (Arun *et al.*, 2009) |
| *Muricauda lutea* sp. nov. | Surface seawater | 91.95 | (Wang *et al.*, 2017) |

### 4.3.4 *Mycolicibacterium rapidicum* strain LC1

#### 4.3.4.1 Morphological properties

The morphological characteristics of *Mycolicibacterium rapidicum* LC1 were assessed by comparing with its closest type strain of *Mycolicibacterium iranicum* JCM 17461 on three media. The strain LC1 showed morphological belongs to the genus *Mycolicibacterium* with the production of orange, acid-fast positive, Gram-positive, non-motile and non-spore former rod-shaped bacterium. The cultural characteristics of the candidate strain LC1 compared to type strain were indicated in Table 4.9.

Table 4.9 Morphological characteristics of *Mycolicibacterium rapidicum* LC1 in five media compared with the closest type strain.

| Media | *Mycolicibacterium rapidicum* LC1 | | *Mycolicibacterium iranicum*$^{T}$ JCM 17461 | |
|---|---|---|---|---|
| | *Growth* | *Colony colour* | *Growth* | *Colony colour* |
| TSA | Good | Orange | Good | Orang |
| SYP | Good | Orang | Poor | Orang |
| MA | Poor | Orang | Good | Orang |

### 4.3.4.2 Physiological and biochemical properties

Side by side comparison of strain *Mycolicibacterium rapidicum* LC1 with type strains was assessed for various physical and biochemical properties as indicated in Table 4.10. The strain LC1 produced acid from L-arabinose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannose, sucrose, D-trehalose, d-xylose, D-mannitol, and myo-inositol but not from D-cellobiose, D-melezitose, Methylα-D-glucoside, L-rhamnose, lactose, and D-sorbitol. The strain did not decompose adenine, hypoxanthine, starch but they degraded xanthine, L-tyrosine, casein and urea, and produced catalase. The candidate strain could assimilate acetate and propionate but not benzoate, malate, tartrate, and citrate. The candidate strain demonstrated the ability to grow NaCl concentration of 1-15%, pH of 7-8 and temperature of 10-37$^{o}$C.

Both strains *Mycolicibacterium rapidicum* LC1 and *Mycolicibacterium iranicum* JCM 17461 were positive for growth at temperature ranges of 27-37$^{o}$C, pH of 6-8, NaCl concentrations of 1-5%, and acid production from L-arabinose, D-fructose, D-glucose, D-mannose, sucrose, D-trehalose and D-xylose. They also positive for sodium propionate assimilation, catalase production, susceptibility to rifampicin, vancomycin, colistin sulphate and clindamycin, and produce orange colour colony in SYP. They were negative for growth at pH of 4, NaCl concentration of 20%, acid production from D-melezitose, L-rhamnose and lactose, degradation of starch, assimilation of sodium benzoate, sodium malate and sodium tartrate, $H_2S$ production, and assimilation of adenine and hypoxanthine.

#### 4.3.4.3 Chemical properties

*Mycolicibacterium rapidicum* LC1 presented LL DAP, and cell wall sugers of arabinose, galactose and rhamnose. Phosphatidylethanolamine, Phosphatidylserine, phosphatidic acid, and phosphatidylinositolmanosides were the common phospholipids. The major menaquinones were MK-8($H_2$) and MK-9($H_2$)**.** Mycolic acid RF values of 0.8, 1, 2.2, 2.6 and 3.5 compared to RF values of 1, 2.2 and 3.5 of the type strain. The major cellular fatty acids in comparison with the type strain were presented in Table 4.11. The major fatty acid types of the reference strains and the candidate strain were qualitatively like but quantitatively different.

Table 4.10 Phenotypic properties of strain *Mycolicibacterium rapidicum* LC1 that differentiate them with related type strains

| Characteristics | *Mycolicibacterium rapidicum* LC1 | *Mycolicibacterium iranicum*$^T$ JCM 17461 | *Mcolicibacterium houstonense*$^T$ ATCC 49403 | *Mcolicibacterium poriferae*$^T$ ATCC 35087 |
|---|---|---|---|---|
| Acid production from: | | | | |
| D-cellobiose | - | + | NR | NR |
| D-galactose | + | - | NR | NR |
| D-maltose | + | - | NR | NR |
| Methylα-D-glucoside | - | + | NR | NR |
| L-rhamnose | - | + | NR | NR |
| *myo*-inositol | + | - | NR | NR |
| D-mannitol | - | + | NR | NR |
| D-sorbitol | - | + | NR | NR |
| TSI fermentation | Yellow/red, no $H_2S$ | red/red, no $H_2S$ | NR | NR |
| nitrate reduction | + | - | NR | NR |
| pyrazinamidase activity | + | - | +* | -* |
| arylsulfatase (3 days) | + | - | +* | +* |
| Growth on MacConkey agar without crystal violet | - | + | +* | -* |

+ Positive for tested characteristics; - Negative for tested characteristics; R resistance; S susceptible; * Data obtained from other study (Shojaei *et al.*, 2013); NR: Not reported.

Table 4.10 (Continued)

| Characteristics | *Mycolicibacterium rapidicum* LC1 | *Mycolicibacterium iranicum*$^{T}$ JCM 17461 | *Mcolicibacterium houstonense*$^{T}$ ATCC 49403 | *Mcolicibacterium poriferae*$^{T}$ ATCC 35087 |
|---|---|---|---|---|
| Growth at: 10°C-15°C | + | - | NR | NR |
| Growth at: 42°C | - | + | +* | -* |
| Salt tolerance: 10-15% | + | - | NR | NR |
| Growth at pH 5 | - | + | NR | NR |
| Decomposition of urea | + | - | -* | +* |
| xanthine | + | - | NR | NR |
| L-tyrosine | + | - | NR | NR |
| Degradation of casein | + | - | NR | NR |
| sodium acetate | + | - | NR | NR |
| sodium citrate | - | + | NR | NR |
| mupirocin(5µg)<br>chloramphenicol(30µg)<br>gentamicin(10µg) | R<br>S<br>S | S<br>R<br>R | NR<br>NR<br>NR | NR<br>NR<br>NR |

+ Positive for tested characteristics; - Negative for tested characteristics; R resistance; S susceptible; * Data obtained from other study (Shojaei *et al.*, 2013); NR: Not reported.

Table 4.11 FAME patterns of strain *Mycolicibacterium rapidicum* LC1 in comparison with type strains

| Fatty acid | *Mycolicibacterium rapidicum* LC1 | *Mycolicibacterium iranicum*$^T$ JCM 17461 | *Mcolicibacterium houstonense*$^T$ ATCC 49403 | *Mcolicibacterium poriferae*$^T$ ATCC 35087 |
|---|---|---|---|---|
| $C_{14:0}$ | 2.34 | 5.04 | 7* | 4* |
| $C_{16:0}$ | 4.42 | 7.49 | 21* | 15* |
| $C_{17:1w7c}$ | 30.14 | 25.34 | 7* | 17* |
| $C_{18:1w9c}$ | 4.33 | 7.49 | 18* | 11* |
| TBSA 10Me C18:0 | 2.40 | 3.09 | 2* | 3* |
| Summed feature 3($C_{16:1\ w7c}$/ $C_{16:1\ w6c}$) | 4.02 | 6.68 | NR | NR |
| Summed feature 7(un 18.846/$c_{19:1\ w6cc}$) | 51.47 | 42.34 | NR | NR |
| Summed feature 8($C_{18:1\ w7c}$) | - | 0.76 | NR | NR |

*: data obtained from other study (Shojaei *et al.*, 2013); NR: Not reported.

### 4.3.4.4 Genotypic properties

#### 4.3.4.4.1 G+C content

*Mycolicibacterium rapidicum* LC1 has a G+C content of 64.50%

#### 4.3.4.4.2 16S rRNA gene sequence analysis

The full 16S rRNA gene sequence was determined for 1,452 bases of *Mycolicibacterium rapidicum* LC1$^T$ (MK358953) as indicated in Appendix 5. The strain LC1 presents the highest 16S rRNA gene sequence similarity to *Mycolicibacterium iranicum* JCM 17461$^T$ (98.2%), *Mycolicibacterium houstonense* ATCC 49403 $^T$ (96.92%) and *Mycolicibacterium poriferae* ATCC35087 $^T$ (96.63%).

4.3.4.4.3 Phylogenetic analysis for strain Mycolicibacterium rapidicum LC1

The nearly complete sequence of strain *Mycolicibacterium rapidicum* LC1 (1,452 bp; GenBank accession no. MK358953) was examined for phylogenetic analysis. Some of the closest published type strains of the genus *Mycolicibacterium* were included in the construction of the phylogenetic dendrogram indicated in Figure 4.10. The phylogenetic tree displayed the strain LC1 is the member of the genus *Mycolicibacterium* within the family *Mycobacteriaceae* which belongs to the same clade with the closest neighbour strain*, Mycolicibacterium iranicum* JCM 17461$^{T}$. This affiliation was confirmed with both neighbour-joining and maximum-parsimony (Appendix 5) algorithms.

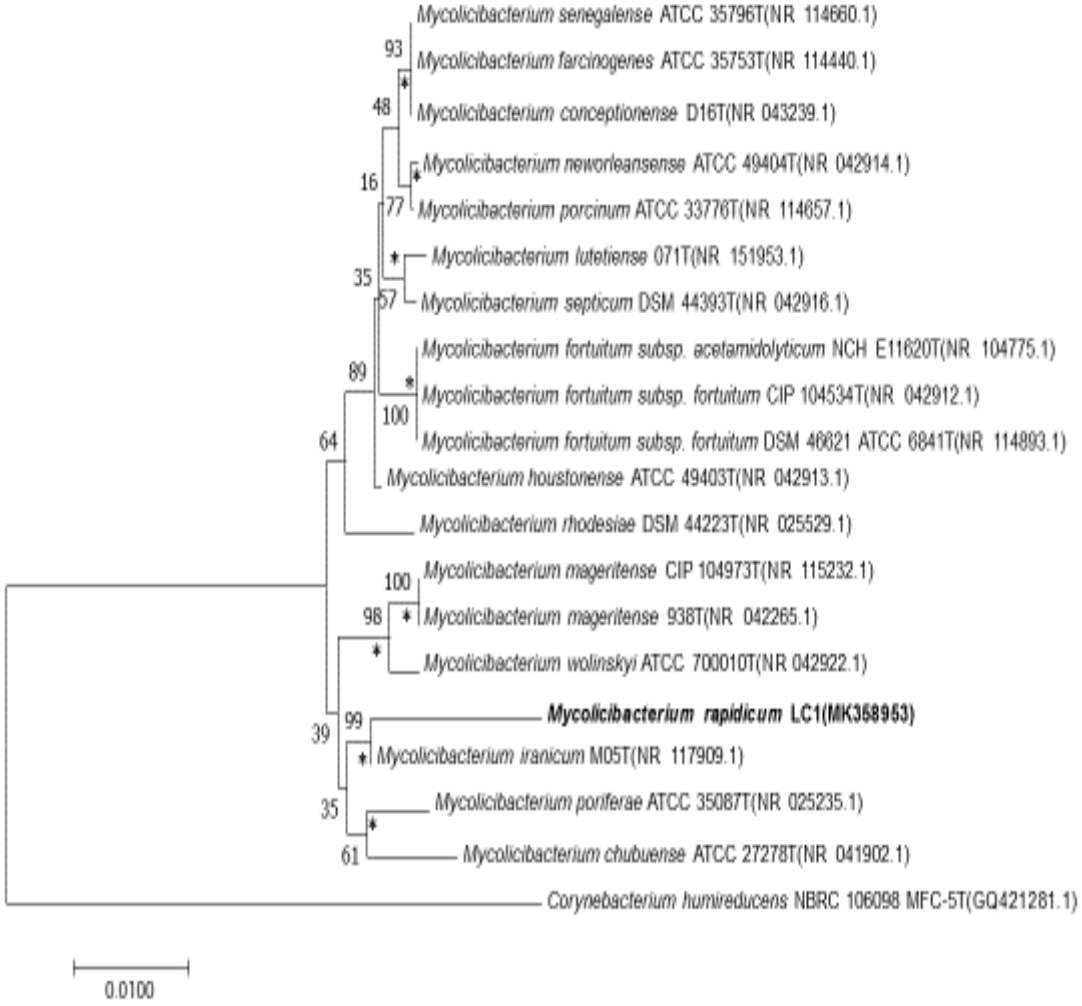



Figure 4.10 Phylogenetic tree analysis of strain *Mycolicibacterium rapidicum* LC1. Neighbour-joining tree showing the relationship between strains LC1 with related members of the genus *Mycolicibacterium*. The asterisk indicates branches of the tree that were also identified by other algorithms. The numbers at the node indicated the levels of bootstrap support based on 1000 resampled datasets. The scale bar represents changes per nucleotide.

### 4.3.4.5 Differentiation of strain *Mycolicibacterium rapidicum* LC1 with its closest strain

Phenotypic and genotypic studies revealed the candidate strain LC1 was different compared to the validly published type stain of the genus *Mycolicibacterium*. The physical and biological properties that differentiated strain LC1 with the closest species and phylogenetic neighbour, *Mycolicibacterium iranicum* JCM 17461$^T$ was indicated in Table 4.10. The candidate strain LC1 differed from *Mycolicibacterium iranicum* JCM 17461$^T$ with a negative reaction of acid production from D-cellobiose, methylα-D-glucoside, myo-inositol, and D-mannitol; negative for sodium citrate assimilation, sensitivity to antimicrobial mupirocin, and growth at pH of 5. Unlike *Mycolicibacterium iranicum* JCM 17461$^T$ strain LC1 showed a positive reaction to acid production from D-galactose, D-maltose, L-rhamnose, and D-sorbitol. Furthermore, strain LC1 showed a positive reaction for TSI, degradation of casein, decomposition of urea, assimilation of sodium acetate, sensitivity to gentamicin, and growth at 10-15$^o$C and salt concentrations of 10-15%.

DNA-DNA relatedness of strain *Mycolicibacterium rapidicum* LC1 with the closest type strain of *Mycolicibacterium iranicum* JCM 17461$^T$ was assessed and the result indicated strain LC1 displayed DNA-DNA relatedness of 34.5% with *Mycolicibacterium iranicum* JCM 17461$^T$. (The experiments were conducted twice which resulted in 33.5% and 35.5% of DNA-DNA relatedness in the first and second run, respectively).

The polyphasic taxonomy indicates the candidate strain LC1 is a novel species of the genus *Mycolicibacterium* and the name *Mycolicibacterium rapidicum* sp.nov. was proposed.

### 4.3.4.6 Description of novel *Mycolicibacterium rapidicum* LC1$^T$

The candidate strain LC1 is acid-fast positive, Gram-positive, non-motile and non-spore former rod-shaped bacterium with a length of 2 to 3 µm and width of 1.5 µm. Their growth was good in SYP and TSA media with the production of circular, shiny, orange colour colonies about 1-1.5 mm diameter within 3 days of incubation at a temperature of 30$^o$C in aerobic conditions. They grow optimally at temperatures of 15-37$^o$C. Their minimum growth temperature was 10$^o$C and a maximum of 37$^o$C. They grew well at pH of 7-8 but not at a pH below 6. The strain grew well in media prepared with or without seawater. Their growth was optimal in 1-5% (w/v) NaCl and

growth were observed until 15% (w/v) of NaCl. The following chemical structures were identified: MK-8($H_2$) and MK-9($H_2$) menaquinone, LLDAP, arabinose, galactose and rhamnose cell wall sugars, phospholipid types of Phosphatidylethanolamine, Phosphatidylserine, phosphatidic acid and phosphatidylinositolmanosides, and the major fatty acids of $C_{14:0}$, $C_{16:0}$, $C_{17:1w7c}$, $C_{18:1w9c}$ and TBSA 10Me C18:0. The DNA G+C content was 64.5 mol%. The strain LC1 showed a positive reaction to acid production from D-galactose, D-maltose, L-rhamnose and, D-sorbitol; positive reaction for TSI, degradation of casein, decomposition of urea and assimilation of sodium acetate. Other phenotypic properties are indicated in Table 4.10.

The type strain, *Mycolicibacterium rapidicum* LC1$^T$, was isolated from a marine sponge *Aplysilla sulfurea* (RB 16) collected from Rapid Bay, South Australia.

### 4.3.4.7 Affiliation of novel *Mycolicibacterium rapidicum* strain LC1$^T$ with other members of Mycolicibacterium

Phylogenetic analysis based on 16S rRNA gene sequences showed that the genus *Mycolicibacterium* is a member of the family *Mycobacteriaceae*. They are Gram-positive, acid-fast positive, non-motile, non-spore former and facultative anaerobic bacteria. The fatty acid profile is characterized by large amounts of branched and straight-chain fatty acids. The DNA G+C content is 60–72 mol% (Shojaei *et al.*, 2013).

During write up of this thesis, the genus *Mycolicibacterium* consists of 44 species with validly published names and as indicated in Table 4.12, all are identified from various environmental sources. Comparison of 16S rRNA gene sequence analysis of LC1 with other members of the genus *Mycolicibacterium* showed that the strain has the highest percentage similarity with *Mycolicibacterium iranicum* JCM 17461$^T$ (98.2%), *Mcolicibacterium houstonense* ATCC 49403$^T$ (96.92%) and *Mcolicibacterium poriferae* ATCC 35087$^T$ (96.63%). They also share sequence similarity with the rest of the members with percentages less than 96.63%. The phylogenetic analysis indicated the candidate strain LC1 appeared in the same clade with *Mycolicibacterium iranicum* JCM 17461$^T$ with a higher bootstrap value of 99. However, the candidate strain LC1 belonged to different clusters with the other closely related strain of *Mcolicibacterium poriferae* ATCC 35087$^T$ placed in the same cluster containing *Mycolicibacterium chubuense* ATCC 27278T, which showed a similarity of 96.49% (Table 4.12).

Table 4.12 List of validly published species of genus *Mycolicibacterium*

| Isolate names | Sources | % similarity | References |
|---|---|---|---|
| *Mycobacterium iranicum sp. nov.* | Clinical specimens | 98.2 | (Shojaei *et al.*, 2013) |
| *Mycolicibacterium houstonense* | Clinical specimens | 96.92 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium conceptionense* | Clinical specimens | 96.64 | (Adekambi *et al.*, 2006) |
| *Mycolicibacterium poriferae* | Marine sponges | 96.63 | (Tortoli, 2018) |
| *Mycolicibacterium senegalense* | Clinical specimens | 96.62 | (Turenne *et al.*, 2001) |
| *Mycobacterium lutetiense* sp. nov | Water | 96.57 | (Konjek *et al.*, 2016) |
| *Mycolicibacterium farcinogenes* | Clinical specimens | 96.55 | (Tortoli, 2018) |
| *Mycolicibacterium wolinskyi* | Clinical specimens | 96.51 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium mageritense* | Clinical specimens | 96.51 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium fortuitum subsp.acetamidolyticum* | Clinical specimens | 96.50 | (Katahira *et al.*, 2016) |
| *Mycolicibacterium fortuitum subsp. Fortuitum* | Clinical specimens | 96.50 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium chubuense* | Clinical specimens | 96.49 | (Turenne *et al.*, 2001) |
| *Mycolicibacterium Porcinum* | Clinical specimens | 96.44 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium neworleansense* | Clinical specimens | 96.37 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium septicum* | Clinical specimens | 96.30 | (Adekambi & Drancourt, 2004) |
| *Mycolicibacterium rhodesiae* | Clinical specimens | 96.23 | (Tortoli, 2018) |

## 4.3.5 Characterization of a halotolerant fungus from a marine sponge

***Prefix: This section of the thesis published as "Yitayal S. Anteneh, Melissa H. Brown and Christopher M.M. Franco. Characterization of a halotolerant fungus from a marine sponge. BioMed Research International Volume 2019, Article ID 3456164, 9 pages. https://doi.org/10.1155/2019/3456164.***

***After publication, only lipid production and profile from the strain in Section 4.3.5.2.6 was added and the rest overlap with publication.***


## Abstract

**Introduction:** Marine sponges have established symbiotic interactions with many microorganisms including fungi. Most of the studies so far have focussed on the characterization of sponge-associated bacteria and archaea with only a few reports on sponge-associated fungi. During the isolation and characterization of bacteria from marine sponges of South Australia, we observed multiple types of fungi. One isolate, in particular, was selected for further investigation due to its unusually large size and being chromogenic. Here, we report on the investigations on the physical, morphological, chemical, and genotypic properties of this yeast-like fungus.

**Methods and Materials:** Sponge samples were collected from South Australian marine environments, and microbes were isolated using different isolation media under various incubation conditions. Microbial isolates were identified based on morphology, staining characteristics, and their 16S rRNA or ITS/28S rRNA gene sequences.

**Results** Twelve types of yeast and fungal isolates were detected together with other bacteria and one of these fungi measured up to 35 µm in diameter with a unique chromogen compared to other fungi. Depending on the medium type, this unique fungal isolate appeared as yeast-like fungi with different morphological forms. The isolate can ferment and assimilate nearly all the tested carbohydrates. Furthermore, it tolerated a high concentration of salt (up to 25%) and a range of pH and temperature. ITS and 28S rRNA gene sequencing revealed a sequence similarity of 93% and 98%, respectively, with the closest genera of *Eupenidiella*, *Hortaea,* and *Stenella*.

**Conclusions:** Based on its peculiar morphology, size, and genetic data, this yeast-like fungus possibly constitutes a new genus and the name *Magnuscella marinae*, gen nov., sp. nov., is proposed. This study is the first of its kind for the complete characterization of a yeast-like fungus from marine sponges. This novel isolates developed a symbiotic interaction with living hosts, which was not observed with other reported closest genera (they exist in a saprophytic relationship). The observed unique size and morphology may favour this new isolate to establish symbiotic interactions with living hosts.


Keywords: Yeast like fungus, Marine sponges, South Australia

### 4.3.5.1 Introduction

Fungi contribute a large share of the microbial community on earth (Mueller & Schmit, 2007), and participate largely in organic matter decomposition, nutrient recycling and symbiotic interactions with other living forms (Rodriguez *et al.*, 2004). About 1.5 million species of fungi are distributed worldwide and most of the current knowledge about them has originated from cultivable representatives, primarily from terrestrial environments (Hawksworth, 2001; Naim *et al.*, 2017). Unlike their terrestrial counterparts, little is known about the diversity of the fungal community in the marine environment (Jones & Pang, 2012). In the marine environments, several families of fungi exist, which contribute about 0.6 % of the total fungal community in the world (Richards *et al.*, 2012; Richards *et al.*, 2015). There have been attempts to isolate fungi from different marine habitats including seawater (Kis-Papo *et al.*, 2003), sea sediments (Singh *et al.*, 2011) and very hypoxic deeper parts of the oceans (Bass *et al.*, 2007), where these species have been in association with different marine plants and animals such as algae (Loque *et al.*, 2010), corals (Amend *et al.*, 2012) and sea fans (Toledo-Hernández *et al.*, 2008).

In addition to the above-mentioned habitats, marine sponges can also provide a home for fungi. About 50% of the mass of marine sponges are microorganisms which is many times more compared to those found in seawater (Wang, 2006). These sponges have established close associations with a range of prokaryotic and eukaryotic microbes (Hentschel *et al.*, 2006; Taylor *et al.*, 2007; Taylor *et al.*, 2007; Gao *et al.*, 2008; Liu *et al.*, 2010; Ding *et al.*, 2011; Schmitt *et al.*, 2012; Simister *et al.*, 2012;.Webster & Taylor, 2012). So far, most studies have focussed on the characterization of sponge-associated bacteria and archaea (Simister *et al.*, 2012; Taylor *et al.*, 2007). In contrast, information about sponge-associated fungi is very limited (Webster & Taylor, 2012). Fungi are ubiquitous and it is easy to isolate them from the inner tissue of the sponges (König *et al.*, 2006; Wang, 2006). However, most of the fungal isolation studies from sponges emphasised on detection and characterization of their compounds as fungi are a major producer of novel marine metabolites (Jensen, 2002; Bugni & Ireland, 2004; Wang, 2006; Blunt *et al.*, 2015).

Hundreds of fungal strains, representing three phyla of *Ascomycota*, *Zygomycota*, and *Mitosporic*, have been isolated from marine sponges ( Höller *et al.*, 2000; Bugni & Ireland, 2004; Li & Wang, 2009). Those studies mostly characterized fungi based on their gene profile,

morphology, physical properties or chemical characteristics (Chen *et al.*, 2012; Laich *et al.*, 2013), and no single study has attempted the complete characterization of fungal isolates from sponges.

While studying the diversity of bacteria in marine sponges of South Australia, 12 types of fungal strains were detected. One of these presented a characteristic yeast-like and filamentous appearance, large size, and unique colour compared to the rest of the group. This section addressed the morphological, physical, chemical, and genotypic properties of this yeast-like fungus. As far as we know, this is the first study to report the complete characterization of a sponge-associated yeast-like fungus from the marine environment of South Australia.

#### 4.3.5.2 Materials and Methods

##### 4.3.5.2.1 Sponge sample collection, processing, and microbial isolation

The methods for sponge samples collection, processing, and yeast isolation were similar to the methods mentioned in section 2.2.1.

##### 4.3.5.2.2 Purification of isolates

Once a week, microbial colonies that emerged were picked individually, streaked onto fresh medium and incubated again at 27$^{o}$C for 2 weeks. Purified microorganisms were stored in sterile 50% (w/v) glycerol at -20$^{o}$C (Laich *et al.*, 2013).

##### 4.3.5.2.3 Morphological and molecular identification

The existence of yeast-like fungi was identified primarily by microscopic observation of the yeast form using a wet mount and lactophenol cotton blue stain preparations (Basava *et al*.; 2016). Further morphological identification was attained by growing them on standard fungal media of malt extract agar (MEA) (Al-Enazi *et al*., 2018), cornmeal agar (CMA) (Nerurkar *et al*.,2014), potato dextrose agar (PDA) (Chen *et al*., 2012; Calabon *et al*.,2018) and Saboraud dextrose agar (SDA) (Das *et al*.,2010). All identification media were supplied from Oxoid. Colony shape, size, colour, and nature of hyphae, growth rate, and features of conidia were considered for morphological identification.

Sequence-based phylogenetic analysis was employed for molecular identification of the isolates. DNA was extracted using cetyltrimethylammonium bromide (CTAB), as described in a previous

study (Kurtzman & Robnett, 1998). The 28S rRNA gene was amplified and sequenced using LROR (ACCCGCTGAACTTAAGC) and LR5 (TCCTGAGGGAAACTTCG) primers. Similarly, the internal transcribed spacer region gene ( ITS) was amplified and further sequenced using primers set of ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). All amplification reactions were carried out in a Swift Thermal Cycler (Esco GB Ltd), with a reaction cycle of 95$^{o}$C for 10 min, 35 cycles of 94$^{0}$C for 1min, 52$^{o}$C for 1min and 72$^{o}$C for 2min, followed by a cycle of 72$^{o}$C for 10 min and 12$^{o}$C cooling. The PCR products were detected by electrophoresis on 1% (w/v) agarose gels and following cleaning of the products, they were sequenced at Macrogen, South Korea. The nucleotide sequences were compared with the GenBank database using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were initially aligned using the multiple alignment program CLUSTAL W version 2.0 (Larkin *et al.*, 2007) and phylogenetic trees were constructed using the neighbour-joining method (based on 1000 bootstrap iterations) with MEGA version 7 (Tamura *et al.*, 2011).

#### 4.3.5.2.4 Physical Characterization

Temperature, pH and NaCl tolerance of the isolates were examined using the PDA medium. A 1.5 mm diameter agar block was cut from the three-day-old culture grown on PDA and inoculated on freshly prepared PDA medium for the following assays. Plates for the temperature tolerance test were incubated at 5$^{o}$C, 15$^{o}$C, 20$^{o}$C, 25$^{o}$C, 30$^{o}$C, 37$^{o}$C for 3 weeks, while those for the acid tolerance (pH of 4, 5, 6, 7 and 8) and NaCl tolerance (1, 2, 3, 4, 5, 10, 15, 25 and 30% NaCl) were incubated at 27$^{o}$C for 3 weeks (Chen *et al.*, 2012). Each treatment was carried out in quadruplicate and the mean diameter of the colony growth was used for data analysis.

#### 4.3.5.2.5 Biochemical Characterization

##### 4.3.5.2.5.1 Carbohydrate assimilation

Growth on the following D-carbohydrates was assessed: inositol, glucose, maltose, mannose, sucrose, fructose, meleanzoite, trehalose, raffinose, cellobiose, lactose, and xylose. A 20 % solution of each carbohydrate was prepared in 10x concentrate of yeast nitrogen base (Difco) (8.04 g/120 ml of RO) and filter sterilized (Minisart®, 0.22µm) (Huppert *et al.*, 1975). Basal agar medium was prepared by dissolving 6.7 g of Bacto Yeast Nitrogen Base and 20 g of high-grade agar in 1 litre of RO water. A 24-48 h old culture was used to prepare a yeast suspension in 2 ml

of MilliQ water and mixed with 18 ml of molten agar and placed into a 90 mm Petri plate. Following solidification, 50 µl of each carbohydrate was placed into 12 well (6 mm diameter) and the plate was incubated for three weeks. Dense growth around the well was assigned as positive for assimilation for the particular sugar (Kurtzman *et al.*, 2010).

#### 4.3.5.2.5.2 Carbohydrate Fermentation

The following D-carbohydrates were tested: glucose, maltose, sucrose, trehalose, lactose, and galactose with a basal inorganic nitrogen medium containing, per litre: $(NH_4)_2HPO_4$ 1 g, KCl 0.2 g, $MgSO_4.7H_2O$ 0.2 g, agar 15 g. Fifteen millilitres of 0.04% bromocresol purple medium was added, per liter, as a pH indicator. After autoclaving, the medium was transferred to sterilized 10 ml tubes and a 1% final concentration filter-sterilized carbohydrate was added. A 24-48-hour old culture from PDA was streaked into agar tubes with carbohydrates and with no carbohydrates as a negative control. The assay was performed in duplicate and the inoculated media were incubated for 2 weeks at 27$^oC$. The medium changed to yellow when it was acidic and was recorded as a positive result (Saitou *et al*.,1987; Yarrow,1998).

### 4.3.5.2.6 Lipid production

#### 4.3.5.2.6.1 Culture maintenance, biomass production, and harvesting

The strain *Magnuscella marinae* is tested for its biocatalyst role for the production of lipid from glucose. The isolate was maintained in Glucose yeast peptone (GYP) consisting of (g/l): glucose 5, yeast extract 2, peptone 2 and agar 10 in sterile seawater at pH of 6.5 and temperature of 25$^oC$. The isolate subculture every week. Two loopful of colonies were cultivated in 50 mL GYP medium containing glucose (5 g/L), yeast extract (2 g/L), mycological peptone (2 g/L), sterilized seawater at pH 6.5 for 48 h. Five percent of 2 days old culture was transferred in fresh 50 ml GYP medium with the same composition and incubated at 25°C, 150 rpm for four days for lipid production as indicated in previous studies (Gupta *et al.*, 2013; Gupta *et al.*, 2016). The biomass was harvested by centrifugation of the culture at 10,000 x g for 15 min and then freeze-dried until analysis

#### 4.3.5.2.6.2 Fatty acid extraction, esterification, and GC analysis

Fatty acid extraction, esterification, and GC analysis were done following previous methods (Gupta *et al.*, 2012a; Gupta *et al.*, 2012b; Gupta *et al.*, 2013). In brief, 5 mg of freeze-dried biomass was placed in a test tube and lipid extraction was followed with solvent mixtures of chloroform: methanol (2:1). The top part was collected and dried using nitrogen gas. The total lipid was measured gravimetrically. For conversion of lipid into FAMEs, 1 ml of toluene was added which is followed by the addition of 200 µl internal standard of methyl nonadecanoate (C19:0) and 200 µl of butylated hydroxytoluene. Once 2 ml acidic methanol added, the mixture was incubated overnight at 50$^{o}$C. The next day, FMAEs were extracted into hexane and the hexane layer then removed and dried using sodium sulphate. Then the dried FAMEs were analysed using Shimadzu GC 2090N. The system contained a capillary column of FAMEWAX with a size of 30 m x 0.32 mm internal diameter equipped with flame ionisation detector (FID) and connected to a BID 2030 unit (Shimadzu) for identification of fatty acid methyl esters (FAME) (split injection, 1/100). It used helium as a carrier gas with a flow rate of 50 µl/min. The oven program was programmed at 170°C and ramped to 200°C (at a rate of 5°C per min) and final increment to 240°C (at a rate of 10°C per min) and held at 240°C for 10 min. The detector temperature was held at 240°C. The total run time was 20 min. 1 µl of sample volume was injected. The peaks for FAMEs were identified by comparing the retention data of authentic standards (Sigma Aldrich CRM47885). Chemstation chromatography software (Agilent Technologies, US) was used for peaks quantification. ***Lipid GC profile and analysis was done by Dr Adarsha Gupta***.

#### 4.3.5.3 Results and Discussion

##### 4.3.5.3.1 Morphological

Twelve Isolates of yeast and fungi were cultured, and one of them is characterized in detail in this paper. The morphological characteristics of this fungus were assessed by culturing it onto four common fungal identification media. They grew very slowly in all media with an average colony diameter of 13 mm after 20 days of incubation. In the first three weeks of incubation, the colonies appeared as yeast (wet, non-filamentous), thereafter the colonies advanced to dry and mouldy forms. As indicated in Figure 4.11, the nature of the colonies varies depending on the medium type. Pronounced colony growths were observed in all media apart from those cultured onto CMA. Even though growths were marked on PDA, MEA and SDA media, significant variations were observed on the appearance of the colonies, in particular, the front view of the colonies.

The front and the reverse views of the colonies are among the many factors considered for standard fungal identification and categorization (Calabon *et al*., 2018). Our finding underscored the requirement for more than one medium for morphological categorization of fungi because data from a single medium is not conclusive. At the early stage of growth, in all media, the colonies were smooth, wet, and pale brown to black in colour. Gradually, except for those growing on CMA, the colonies developed aerial mycelium and appeared as green, brown and black filamentous fungi. This type of incubation period dependent morphological dimorphism is not reported in studies describing fungi from marine sponges (Saitou *et al*.,1987; Calabon *et al*., 2018) and other sources (Chen *et al*., 2012). This form of transformation is not the same as temperature-dependent fungal dimorphism which is commonly observed in some fungal species (Boyce and Andrianopoulos, 2015). This is an important evolutionary observation of the isolate and further study is required to understand the mechanisms behind this morphological variation and the specific role of morphological forms in fungi biology and their interaction with sponges.

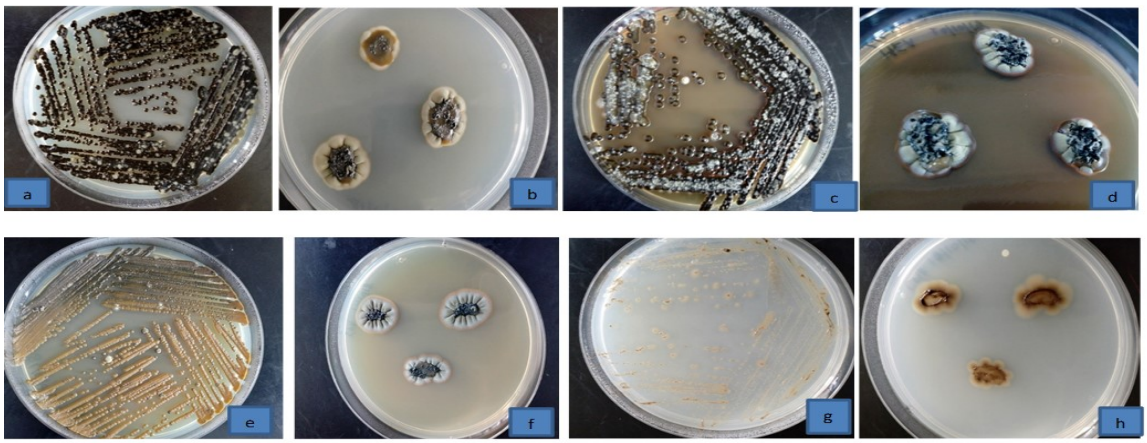



Figure 4.11 Morphological characteristics of two views of the isolate grown on four different media. **a** & **b** potato dextrose agar (PDA), yeast forms were globose, shiny, dark green and turned to black, produced flat greenish-grey spores surrounding green centre; **c** & **d** malt extract agar (MEA), yeast forms were globose, shiny, black, produced raised light green spores surrounding black hole; **e** & **f** Saboraud Dextrose agar (SDA), yeast forms were globose, not shiny, light brown and turned to dark brown to black, produced raised grey spores with black centre; **g** & **h** corn meal agar (CMA),yeast forms grew poor, with light brown color, no spore.

Lactophenol cotton blue preparations from the yeast-like form revealed elliptical conidia occurring in single, bicellular and short chains of spindle-shaped blastospores (Figure 4.12). Their size varied from 6-35 µm in diameter. Similar preparations from mycelia showed branched, thick-walled, smooth and aseptate hyphae with an aggregated mass of conidia. Conidiogenous cells were observed within the hyphae forming chains of conidia. Compared to related fungal strains,

this isolate displayed unique features including aseptate hyphae, spindle-shaped conidia and large size of the yeast form (Höller *et al*., 2004; Crous *et al*., 2009a; Crous *et al*., 2009b; Chen *et al*., 2012). Phenotypic observation of morphological forms such as the nature of hyphae and conidia are the most commonly considered characteristics for Ascomycota taxonomy-the fungal phylum to which this isolate belongs (Guarro *et al*., 1999).

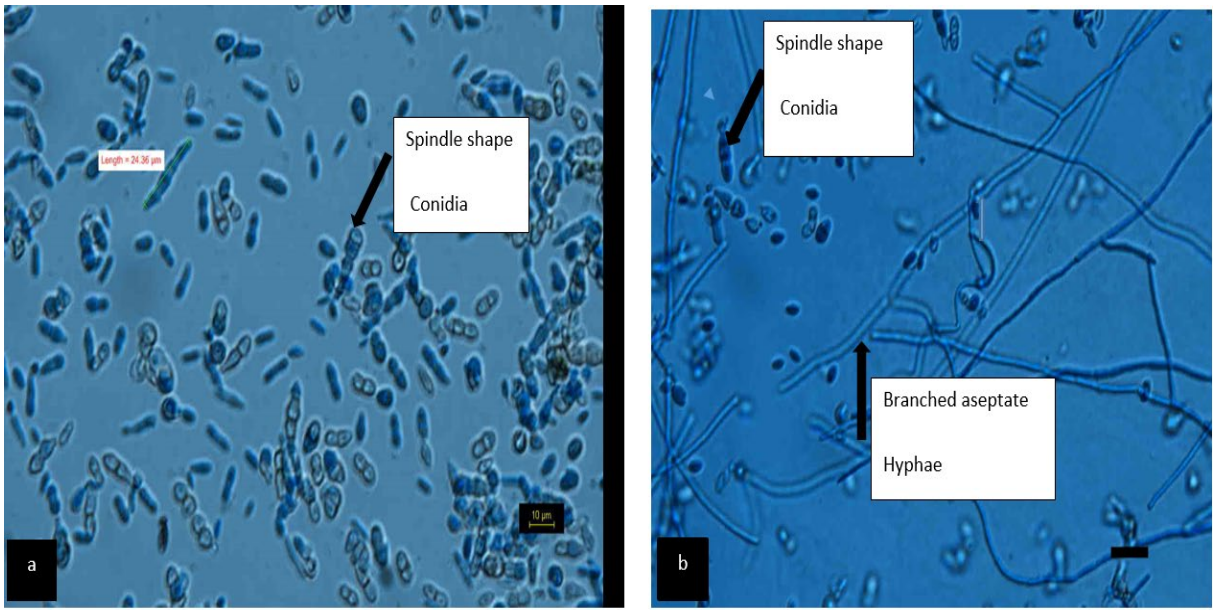



Figure 4.12 Morphological characteristics of the isolate after 3 weeks of incubation. **a** lactophenol cotton blue preparation from yeast-like colonies; **b** preparation from mycelium under 40x magnification and the scale bar of 10 µm.

#### 4.3.5.3.2 Phylogenetic analysis

Genetic identification of the isolate was achieved through the sequencing of the ITS region (MK55958) and the 28S rRNA gene (MK409743). BLASTN analysis of the ITS sequence revealed the most closely related strains were *Eupenidiella venezuelensis* (93%), *Hortaea thailandica* (89%), and *Stenella araguta* (89%), which was supported by the phylogenetic tree (Figure 4.13). In the same manner, a BLASTN of the 28S rRNA showed a close relationship with the same species but with percentage sequence similarities of 98%, 97%, and 96.5%, respectively. These data were also supported by the phylogenetic tree in Figure 4.14. Considering the genetic data, we assigned the name *Magnuscella marinae* gen. nov., sp. nov., for this isolate.

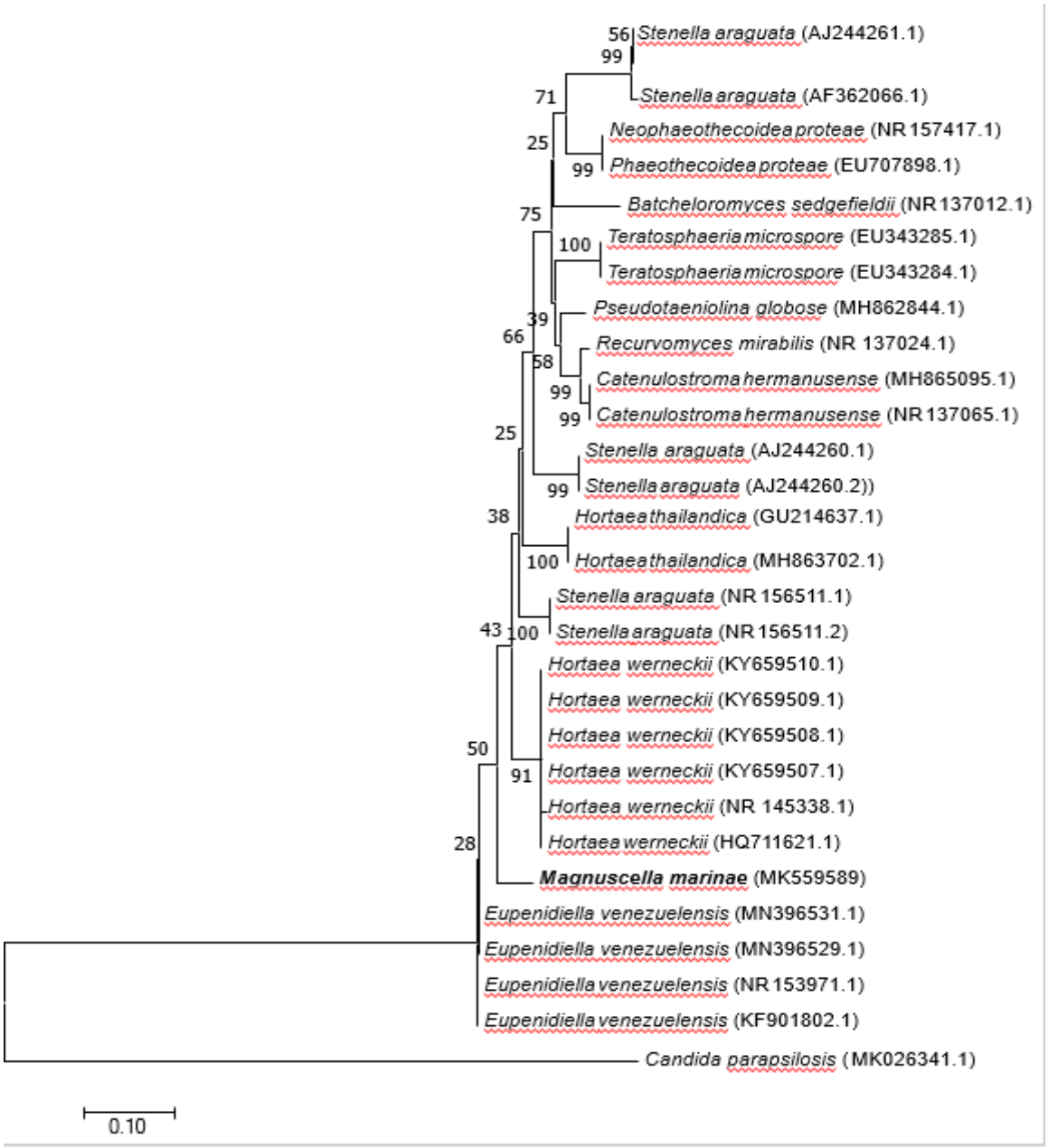



Figure 4.13 Phylogenetic analysis of the *Magnuscella marinae* inferred from ITS region gene. Evolutionary relationships were assessed using the Neighbor-Joining method (Saitou & Nei, 1987) and bootstrap values (>50 %) are shown above the branch. *Candida parapsilosis* was used as an outgroup. Bar 10 % sequence divergence (Kumar *et al.*, 2016).

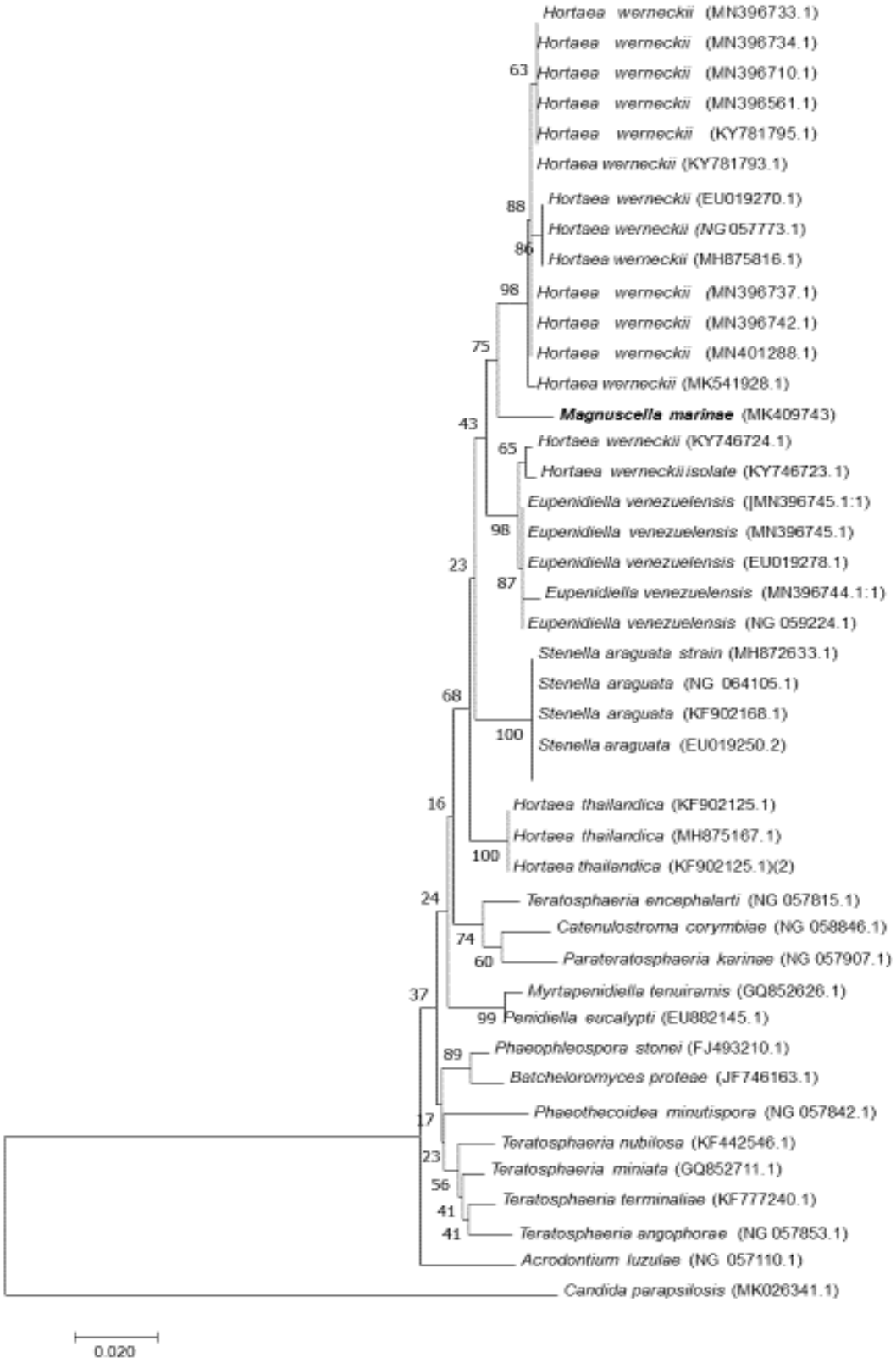



Figure 4.14 Phylogenetic analysis of the *Magnuscella marinae* inferred from 28S rRNA gene. Evolutionary relationships were assessed using the Neighbor-Joining method (Saitou & Nei, 1987) and bootstrap values (>50 %) are shown above the branch. *Candida parapsilosis* was used as an outgroup. Bar 2% sequence variation(Kumar *et al.*, 2016).

The fungal species which are most closely related to *Magnuscella marinae* belongs to phylum Ascomycota, class Dothideomycetes, order Capnodiales and family either Teratosphaeria (such as genus *Eupenidiella and Hortaea*) ( Crous *et al.*, 2007; Chen *et al.*, 2012) or Mycosphaerellaceae (such as genus *Stenella*) (Aguilera-Cogley *et al.*, 2017). These two families are generally characterized by their widespread existence as saprophytes, opportunistic human pathogens and phytopathogens (Crous *et al.*, 2007; Crous *et al.*, 2009). The filamentous form produces branched, septate, chromogenic hyphae, which measure about 2–6 µm in diameter (Crous *et al.*, 2009). Conidiogenous cells exist as integral or terminal parts of the hyphae appear as subcylindrical or slightly swollen tips ( Crous *et al.*, 2007; Chen *et al.*, 2012). It is important to determine whether this new genus is pathogenic to mammals or plants are given its taxonomic relatedness to known pathogens. The existence of various fungi in marine sponges is documented. However, none of the most common phylogenetically related species to this isolate were reported from a marine sponge, most of them were obtained from the terrestrial environment and plants.

#### 4.3.5.3.3 Physical characterization

The yeast-like fungus *Magnuscella marinae* was capable of growing at temperatures ranging from 5-30$^{o}$C, with optimum growth seen at 25$^{o}$C (Figure 4.15 a). At lower and higher temperatures, the fungus grew slowly with limited hyphae or mycelia production. The fungus grew well within pH ranges of 4-8, with somewhat higher average growth observed at pH 5 (Figure 4.15 b). The fungus tolerated up to 25% NaCl (Figure 4.15 c). It was also observed that growth was highly restricted in medium devoid of NaCl (data not presented) indicating the fungus requires some degree of salt to grow, a trait common to marine microorganisms. The observed survival under harsh conditions might favor them to survive in the highly fluctuating marine environments, including within sponges. The isolate could be a potential candidate to study survival mechanisms.

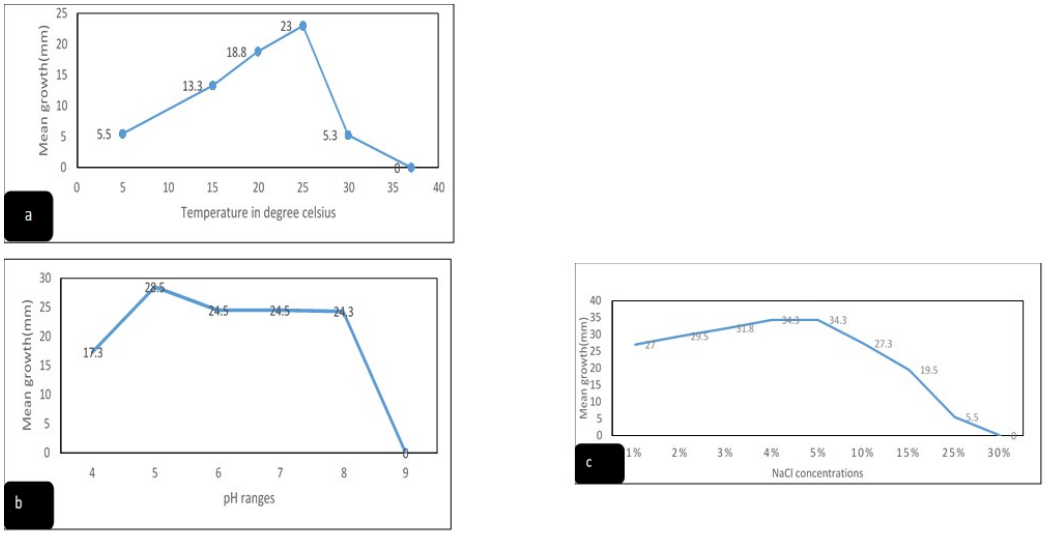



Figure 4.15 Fungal growth after 3 weeks of incubation for different conditions. **a**, **b**, **c** growth at different ranges temperature, pH and NaCl concentration.

Similar to our findings, some related species in genus *Hortaea* are known for their ability to tolerate a wide range of temperature, pH and salt concentrations. These species have been widely studied to reveal the mechanisms behind survival at different harsh environmental conditions. Several unique pathways have been identified that could attribute this adaptation (Chen *et al.*, 2012). Like the genus *Hortaea, Magnuscella marinae* can be used as a potential model organism to assess the mechanisms behind survival in different harsh environmental conditions including high salt concentrations.

#### 4.3.5.3.4 Biochemical characteristics

As indicated in Table 4.13, the isolate displayed the capability of fermenting almost all the tested carbohydrates apart from lactose. Also, they were able to utilize about two-thirds of the tested sugars. No data was available in the literature regarding carbohydrate assimilation and fermentation profiles for the closest phylogenetically related species. The results of this study can be used as baseline for future related studies. The ability of *Magnuscella marinae* to assimilate and ferment several carbohydrates make them an ideal candidate organism for industrial fermentations if they do not possess pathogenic determinants (Gibson *et al*., 2008).

Table 4.13 D-carbohydrates fermentation and assimilation pattern of the isolate.

| Carbohydrates | fermentation | assimilation |
|---|---|---|
| Glucose | ✓ | ✓ |
| Maltose | ✓ | ✓ |
| Sucrose | ✓ | ✓ |
| Trehalose | ✓ | ✓ |
| Lactose | X | X |
| Galactose | ✓ | ND |
| Inositol | ND | X |
| Mannose | ND | ✓ |
| Fructose | ND | X |
| Meleanzoite | ND | ✓ |
| Raffinose | ND | ✓ |
| Cellobiose | ND | X |
| Xylose | ND | ✓ |

ND: Not done

Since the sponge environment is unique, *Magnuscella marinae* has likely adapted some type of relationship with its host and other microorganisms present in the sponge tissue. Their ability to utilize a range of carbohydrates might help them to survive in a competitive environment like sponges, as their metabolic diversity provides for the uptake of an alternative nutrient source in the absence of others. Apart from their importance to fungi, this metabolic diversity together with their large size could make *Magnuscella marinae* as a potential biological catalyst or feedstock in the fermentation industry. Furthermore, the isolate could be a model organism to study the effect of cell size on the efficiency of cellular processes and whether a large cell size offers any advantages for bioprocessing or as a host for recombinant protein production.

4.3.5.3.5 Lipid production

Marine microbes including yeast have been reported for their ability to produce medically useful fats (Gupta *et al.*, 2012b). These microbes generally known as oleaginous organisms able to accumulating fats and yeasts are by far noticeable fats accumulators with fast growth rates compared to other microbes (Meng *et al.*, 2009). We tested the ability of *Magnuscella marinae* to synthesise lipids from glucose medium and the GC peaks of the lipid extracts displayed in Figure 4.16 and the analysis of the peaks resulted in different types of lipids as indicated in Table 4.14. The GC results indicated over 80% of the total fatty acids produced by the strain were C16, C18:1, and C18:2. The most common fatty acids were palmitic acid, oleic acid, and linoleic acid. Related types of fatty acids with C16, C18 and their polyunsaturated forms were reported from different yeast strains ( Li *et al.*, 2007; Gupta *et al.*, 2012b). The observed biocatalytic effect of *Magnuscella marinae* will encourage to screen of this strain for several applications.

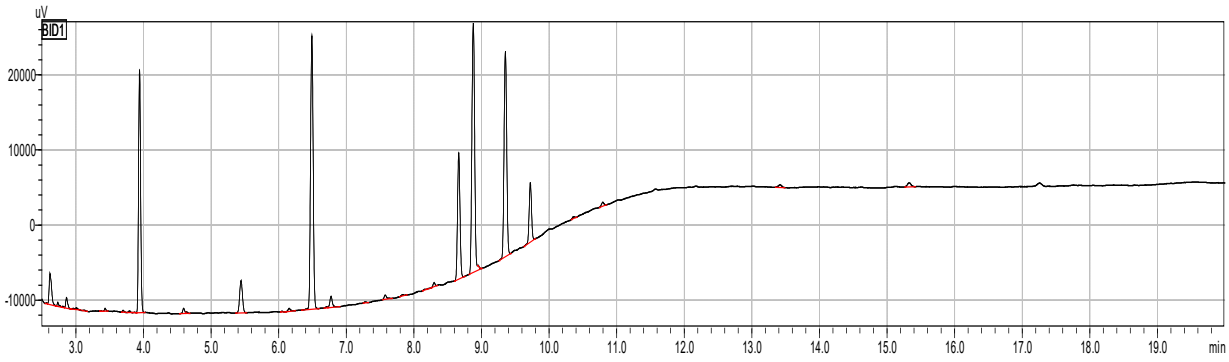



Figure 4.16 GC peaks of lipid produced with the help of *Magnuscella marinae*.

Table 4.14 The percentage distribution of fatty acids from *Magnuscella marinae*.

| Fatty acid methyl esters | % Total fatty acids (%TFA) | std dev |
|---|---|---|
| C12:0 Methyl laurate | 0.05 | 0.03 |
| C14:0 Methyl myristate | 0.45 | 0.01 |
| C14:1 Methyl myristoleate | - | - |
| C15:0 Methyl Pentadecanoate | 3.61 | 0.14 |
| C15:1 Methyl Pentadecenoate | - | - |
| C16:0 Methyl palmitate | 29.89 | 0.03 |
| C16:1 Methyl palmitoleate | 1.00 | 0.17 |
| C17:0 Methyl heptadecanoate | 0.52 | 0.06 |
| C17:1n7 cis-10-Heptadecanoic acid methyl ester | - | - |
| C18:0 Methyl stearate | 12.72 | 0.23 |
| C18:1 Methyl oleate | 28.31 | 0.01 |
| C18:1 Methyl vaccenate | 0.04 | 0.01 |
| C18:2 Methyl linoleate | 22.35 | 0.23 |
| C19:0 Methyl nonadecanoate | 6.24 | 0.36 |
| C18:3 Methyl linolenate | 0.48 | 0.00 |
| C20:0 Methyl arachidate | 0.38 | 0.02 |
| C20:3n6 cis-8,11,14-Eicosatrienoic acid methyl ester | - | - |
| C20:4 Methyl arachidonate | - | - |
| C20:3n3 Methyl 11,14,17-eicosatrienoate | - | - |
| C20:5 Methyl eicosapentaenoate | - | - |
| C22:5 DPA | - | - |
| C24:0 Methyl lignocerate | 0.87 | 0.00 |
| C22:6 Methyl docosahexaenoate | - | - |

### 4.3.5.4 Conclusions

This study described for the first time complete phenotypic and genotypic characterization of a yeast-like fungus from pink unidentified marine sponge from Rapid Bay, South Australia. This study could be the baseline for future comparative studies. Marked genotypic variations accompanied with unique properties such as big size, aseptate hypha, and spindle-shaped conidia compared to the most closely related species, the isolate was considered to be a candidate for a novel genus which has been named Magnuscella from *Magnus,* meaning large (Latin) and Cell (English), with the species named *marinae*, denoting the source which was a marine sponge.

None of the closely related species reported from marine sponges, most exist as a saprophytic. However, this new taxon isolate evolves within the livening macroorganism. This is an important evolutionary observation where fungi previously believe to survive on dead organic matters, can

now be indicated to interact and survive within a host probably as a symbiont. This new taxon widens the existing knowledge on the ecology of marine fungi. Furthermore, the unusual big size and metabolic versatility of the new isolate could be a significant evolutionary phenomenon that contributes to their existence in a competitive environment like sponges. This new taxon will create an opportunity to investigate further for evolutionary changes that promote their survival within harsh environmental conditions such as high salt.

# Chapter Five: Antimicrobial activities of selected bacterial isolates

## 5.1 Introduction

Excessive and misuse of antimicrobial agents has been attributed to the emergence of multidrug-resistant microorganisms and the morbidity and mortality associated with these microbes are increased as most of the current antibiotics are not effective to eliminate them (Singer *et al.*, 2003; Magiorakos *et al.*, 2012; Aminov, 2010). These multidrug-resistant microorganisms associated morbidity and mortality demand a redoubling of efforts to find new effective antimicrobials from various sources. Even though there are few potential options for antibiotic treatment such as passive immunization or phage therapy (Aminov, 2010; Monk *et al*., 2010), the conventional approaches depend on the discovery and development of newer, more effective antibiotics.

The discoveries of antibiotics that are in use today have originated from limited sources, mainly from soil Actinomyces. In the past 20+ years, extensive examination of this ecological niche with several types of advanced technologies did not prove in the discovery of a new antimicrobial class for human therapeutic use (Aminov, 2010). As a result, different approaches have been considered to uncover novel antibiotics and one of the approaches are searching for antibiotics from less common ecological niches such as the marine environment (Hughes and Fenical, 2010; Rahman *et al*., 2010).

Approximately 50% of new drugs in the antibiotic pipeline have originated from natural products, including marine microorganisms and in the past few decades these marine products, including microorganisms and their associated bioactive products, have been the subject of studies (Webster & Taylor, 2012). Among many marine organisms known to produce bioactive compounds, marine sponges contribute to more than 250 new compounds on an annual basis (Blunt & Copp, 2007).

Marine sponges are one of the earliest living forms on earth and their amount and diversity are significantly higher compared to other living forms in the marine environment. (Maloof *et al.*, 2010; Van Soest *et al.*, 2012). Microbes surviving in extremely nutrient-poor and antagonistic environments, such as marine sponges, frequently produce a variety of secondary metabolites to overcome the negative effect of the surrounding environment (Mearns-Spragg *et al.*, 1998; Selvin *et al.*, 2012).

Cultivation of sponges at an industrial scale for mass production of active compounds has not yet been achieved though there have been recent attempts to use resin to capture sponge metabolites from sponge kept alive in an aquarium (Mehbub *et al*., 2018). On the other hand, if compounds are produced by sponge-associated microbes, the scale-up is relatively easy to handle. Therefore, this section aimed to screen selected bacterial isolates presented in Chapter 3 for antimicrobial activities against human pathogenic bacteria and fungi. This section also investigated the effect of media and incubation time for the optimal production of antibiotics from selected bacterial isolates. Finally, this section tried to characterize a potential novel antibiotic.

## 5.2 Materials and Methods

### 5.2.1 Antibiotic production and screening

#### 5.2.1.1 Bacterial strains

One hundred and sixty-nine bacterial strains were selected for the screening of antimicrobial activity. These strains were selected from each morphological form within the 38 RFLP patterns to represent the 21 genera identified. Based on this there were *Streptomyces* (n=72)*, Bacillus* (n=26), *Kocuria* (n=9), *Sulfitobacter* (n=8), *Rhodococcus* (n=8), *Microbacterium* (n=7), *Micrococcus* (n=6), *Falsibacillus* (n=5), *Fictibacillus* (n=5), *Limimaricola* (n=4), *Pseudomonas* (n=3), *Gordonia* (n=3), *Pseudonocardia* (n=2), *Staphylococcus* (n=2), *Rhodovulum* (n=2), Isoptericola (n=2), *Janibacter*, *Pseudoalteromonas*, *Leisingera*, *Mycolicibacterium* and *Muricauda* each (n=1). The full list of the bacteria is provided in Table 5.1.

Table 5.1 Bacterial isolates screened for antimicrobial activities

| Genus | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups |
|---|---|---|---|---|---|---|---|---|
| *Streptomyces* | RB19 | 31 | RB116 | 31 | RBYA7 | 31 | RB26 | 32 |
| | RB29 | 31 | RB117 | 31 | RBYA8 | 31 | RB27 | 32 |
| | RB31 | 31 | RB119 | 31 | RBYA13 | 31 | RB28 | 32 |
| | RB45 | 31 | RB124 | 31 | RBYA20 | 31 | RB30 | 32 |
| | RB46 | 31 | RB134 | 31 | RBYA21 | 31 | RB32 | 32 |
| | RB49 | 31 | RB135 | 31 | RBYA22 | 31 | RB33 | 32 |
| | RB53 | 31 | RB140 | 31 | RBYA24 | 31 | RB34 | 32 |
| | RB57 | 31 | RB145 | 31 | RBYA27 | 31 | RB35 | 32 |
| | RB60 | 31 | RB146 | 31 | RBYA28 | 31 | RB36 | 32 |
| | RB65 | 31 | RB147 | 31 | RBYA32 | 31 | RB54 | 32 |
| | RB66 | 31 | RB150 | 31 | RBLC14 | 37 | RB111 | 32 |
| | RB67 | 31 | RB151 | 31 | Rb47 | 38 | RB124 | 32 |
| | RB69 | 31 | RB152 | 31 | RB15 | 32 | RB131 | 32 |
| | RB70 | 31 | RB154 | 31 | RB20 | 32 | RB144 | 32 |
| | RB74 | 31 | RB155 | 31 | RB21 | 32 | RB243 | 32 |
| | RB76 | 31 | RB158 | 31 | RB22 | 32 | | |
| | RB112 | 31 | YA1 | 31 | RB23 | 32 | | |
| | RB114 | 31 | YA2 | 31 | RB24 | 32 | | |
| | RB115 | 31 | YA3 | 31 | RB25 | 32 | | |
| *Bacillus* | GB21 | 1 | RB199 | 5 | GB66 | 5 | L30 | 5 |
| | RB1 | 7 | RB13 | 7 | RB90 | 7 | RB92 | 7 |
| | RB98 | 7 | RB99 | 7 | RB141 | 7 | RB160 | 7 |
| | RB178 | 7 | RB193 | 7 | GB2 | 7 | GB25 | 7 |
| | GB48 | 7 | GB52 | 7 | GBYA14 | 7 | GBYA15 | 7 |
| | GBYA24 | 7 | LC2 | 7 | RB97 | 13 | GB14 | 13 |
| | RBYA37 | 35 | GB3 | 36 | | | | |

Table 5.1 (Continued)

| Genus | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups |
|---|---|---|---|---|---|---|---|---|
| *Kocuria* | RB9 | 21 | RB10 | 21 | RB11 | 21 | RB16 | 21 |
| | RB44 | 21 | RBYA2 | 31 | RB17 | 33 | RB100 | 33 |
| | RB107 | 34 | | | | | | |
| *Sulfitobacter* | GB40 | 2 | GB42 | 2 | GB49 | 2 | RB58 | 2 |
| | GB6 | 26 | GB26 | 27 | GB39 | 27 | GB20 | 28 |
| *Rhodococcus* | GB24 | 12 | GB63 | 12 | RB163 | 12 | RBYAN | 12 |
| | RBLC21 | 12 | RB75 | 12 | RB82 | 12 | RBYA12 | 12 |
| *Microbacterium* | RB197 | 20 | RB206 | 20 | GB56 | 20 | RBA4 | 20 |
| | RB4 | 20 | RB4 | 20 | RB2 | 20 | | |
| *Micrococcus* | RB5 | 23 | LC12 | 24 | RBYA34 | 26 | RBYA39 | 26 |
| | RB166 | 29 | GB9 | 29 | | | | |
| *Falsibacillus* | RB184 | 10 | GB74 | 10 | GB75 | 10 | GBYA47 | 10 |
| | RBLC16 | 10 | | | | | | |
| *Fictibacillus* | RB189 | 9 | GB73 | 9 | GB72 | 9 | RBYAC | 9 |
| | RBLC7 | 9 | | | | | | |
| *Limimaricola* | RB6 | 6 | GB11 | 4 | GB45 | 6 | GB69 | 6 |
| *Gordonia* | RB180 | 15 | RB194 | 15 | GB17 | 15 | | |
| *Pseudomonas* | RB161 | 14 | RB198 | 14 | GB4 | 14 | | |
| *Isoptericola* | RB202 | 19 | L40 | 19 | | | | |

Table 5.1 (Continued)

| Genus | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups | Strain name | RFLP groups |
|---|---|---|---|---|---|---|---|---|
| *Pseudonocardia* | RB78 | 30 | YA11 | 30 | | | | |
| *Rhodovulum* | GB76 | 11 | GB79 | 11 | | | | |
| *Staphylococcus* | RB176 | 18 | L11 | 18 | | | | |
| *Janibacter* | GB23 | 1 | | | | | | |
| *Leisingera* | RB86 | 16 | | | | | | |
| *Muricauda* | GB37 | 3 | | | | | | |
| *Mycolicibacterium* | RBLC1 | 17 | | | | | | |
| *Pseudoalteromonas* | RB122 | 25 | | | | | | |

A total of 38 RFLP groups generated with restriction enzymes which comprise of 383 bacterial strains (Chapter 3). From each RFLP group, representative bacterial isolates selected for Antimicrobial activity. For some RFLP groups, all bacterial isolates screened as their number was low. A large number of bacterial isolates from the genus *Streptomyces* were included as this genus was the most dominant in the isolation study.

#### 5.2.1.2 Agar-based antimicrobial production

Selected bacterial isolates were sub-cultured in duplicate onto ISP2 medium for Actinobacteria and brain heart infusion agar for other bacteria. The plates were incubated for 15 days for Actinobacteria and 5 days for other bacterial species at 27$^{o}$C in an aerobic environment. After the designated incubation periods, a 6 mm plug of each plate of the bacterium was cut out and screened for activity. The second plates were used to obtain an extract: the full plate containing the culture and the agar medium was chopped and placed into a tube containing 40 ml methanol. The tube was shaken for 6 hrs and then the contents were centrifuged at 30,000 x g for 10 min. The supernatant was collected and screened for the activities.

#### 5.2.1.3 Evaluation of primary media for antimicrobial production

The ability of selected bacteria to produce antimicrobial activity was tested by growing them on five media: yeast extract peptone, potato dextrose agar, starch yeast peptone agar, mannitol soya bean agar, and ISP2. Each strain was inoculated in the same manner as described above and the methanol extracts were screened for antibiotic activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA).

#### 5.2.1.4 Antimicrobial production in liquid fermentation

Antimicrobial production in liquid media was done following the previous method by Kaewkla,(2009). The bacterial strains were cultured in 50 ml of IM22 seed medium (composition g/l: glucose- 15, Soyatone-15, Pharmamedia-5, $CaCO_3$-2, and NaCl- 5) in 250 ml baffled Erlenmeyer flasks and incubated at 27$^{O}$C with shaking at 150 rpm for 3 days. Two and a half milliliters of well-grown seed medium was inoculated into 50 ml of four types of production media 1) SI (per l; sucrose 20 g, $CaCO_3$ 2.5 g, $KNO_3$ 1 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g); 2) ISP2 (malt extract 10 g, yeast extract 4 g, glucose 4 g); 3) F26 (glucose 20g, soybean flour 10 g, $CaCO_3$ 4 g, $COCl_2.6H_2O$ 1 mg), and 4) MS (mannitol 20 g, soybean flour 20 g) in 250 ml baffled Erlenmeyer flasks and incubated for 7 days. Every day, 1 ml of the culture was collected, centrifuged at 30,000 x g for 10 min and the supernatant was screened for antimicrobial activities.

### 5.2.2 Bioassay

#### 5.2.2.1 Test organisms

Two types of bacterial strains were used for antibacterial screening. One category consists of reference bacterial strains of *Escherichia coli* (JCM 109) and *Staphylococcus aureus* (ATCC 29213). The rest are clinical isolates comprised of resistant bacteria, *Pseudomonas aeruginosa* 06348315, methicillin-resistant *S. aureus* (MRSA) 03120385, and non-resistant bacterial isolates of *Streptococcus pyogenes* and *Salmonella typhimurium*. Fungal clinical strains of *Candida albicans*, *Trichophyton rubrum*, *Trichophyton interdigitalis*, *Sacclophosis* sp. and *Microsporum gypsum* were also included as test organisms. All clinical bacterial strains and *Candida albicans* were obtained from the Clinical Microbiology and Infectious Diseases Department, Flinders Medical center, and the four fungal species were provided by SA Pathology, South Australia. Antimicrobial profile of the two resistant bacterial strains was provided by the department and its indicated in Table 5.2.

Table 5.2 The antibiotic inhibition profile of the resistant strains

| Strains | Resistant | Intermediate resistance | Susceptible |
|---|---|---|---|
| *Pseudomonas aeruginosa* | amikacin, cefepime, cefotaxime, cetazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin, | aztreonam | Colistin |
| MRSA | ciprofloxacin, erythromycin, flucoxacillin, fusidic acid, gentamicin, penicillin, rifampicin, trimethoprim | Not reported | doxycycline, linezpoid, mupirocin vancomycin |

#### 5.2.2.2 Antibacterial screening

Antibiotic assay medium (AAM: Oxoid) was used for the antibacterial activity assay and the bacterial strains primarily screened for activity using plug assay method as done previously by

Kaewkla,(2009). Test organisms were grown in tryptone soy broth (TSB: Oxoid) at 37$^{o}$C by shaking at 150 rpm overnight. The growth was assessed by measuring optical density (OD) at 600 nm and an OD of 0.25 of test organisms was added to AAM at the ratio of 1 ml per 25 ml of the medium which was then poured into a 9 cm Petri plate and left a while to completely solidify. A 6 mm plug of 14 days (Actinobacteria) and 5 days (other bacterial strains) culture were cut out and placed on the surface of AAM medium containing test organisms to check for the activity. A maximum of 10 plugs of bacterial strains per plate was placed. The culture was incubated overnight at 37$^{o}$C and the diameter of the zone of inhibition was measured in millimetre using ruler, and those with a zone of inhibition greater than 10 mm consider active. Bacteria-free 6 mm plug was used as negative control and vancomycin (200 ug/ml) and ciprofloxacin (200 ug/ml) as positive controls for Gram-positive and Gram-negative bacteria, respectively.

To confirm the activity shown by the plug assay method, the methanol extracts of the crude compound from each active bacterial strain were tested for activity (see section 5.2.1.2 for extract production). In this method, 50 µl crude methanol extract was placed into a 6 mm diameter well (the well was made using 6 mm cork borer) on the AAM containing test organisms. The culture incubated overnight at 37$^{o}$C and the zone of inhibition was measured as described above. Methanol was used as a negative control and vancomycin (200 ug/ml) and ciprofloxacin (200 ug/ml) as positive controls for Gram-positive and Gram-negative bacteria, respectively. All screening was done in duplicate and the mean zone of inhibition calculated and the results were roundoff to the nearest whole numbers.

A similar approach was followed for the screening of anti-*Candida* activities where Sabouraud broth and Sabouraud agar (Oxoid) used for seed medium and antibiotic assays, respectively. Amphotericin B (100 U) was used as positive control while strain-free medium plug and methanol used as a negative control. All tests were carried out in duplicate.

#### 5.2.2.3 Antifungal screening

A modified cross-streaking method was used for antifungal screening (Lertcanawanichakul & Sawangnop, 2008; Velho-Pereira & Kamat, 2011). Four pathogenic dermatophytic fungal species of *T. rubrum*, *T. interdigitalis*, *Sacclophosis* sp. and *M. gypsum* were grown on Potato Dextrose agar (PDA, Oxoid) at pH 6 for 7 days. At the same time, a heavy inoculum of bacteria was streaked

at the edge of the media (maximum of three bacteria per plate) as indicated in Figure 5.1 and incubated until good growth was observed. A 6 mm plug of well-grown test fungi (one per plate) was cut out and placed in the centre of the plate (perpendicular to each bacterium streak) and incubated for two weeks. Similarly, a plug of fungus was placed onto the medium without bacteria as a negative control. Results were recorded as follows: very strong inhibition (+4), when the growth of the fungi away from the streaks was > 20 mm; strong inhibition(+3), when the growth of the fungi away from the streaks was 15-20 mm; moderate inhibition (+2), at 10-14 mm or less; weak (+1), 10 mm or less; negative (-), no difference compared to the negative control.

Removed due to copyright restriction

Figure 5.1 Top view of a plate with streaked bacteria with a pathogenic fungal plug in the middle.

### 5.2.2.4 Antimicrobial production in different media

In the first stage, eleven bacterial strains with zones of inhibition >18 mm against susceptible and methicillin-resistant strains of *S. aureus* were selected and their antimicrobial production was investigated using five primary production media. Each bacterium was inoculated into 250 ml baffled Erlenmeyer flasks containing 50 ml of each medium in duplicate. The culture was incubated for 2 weeks at 27$^{o}$C on a shaker at 150 rpm and sampled daily. After centrifugation at 30,000 x g for 10 min, 50 µl of the supernatant was tested for antibiotic activities in duplicate and the mean zone of inhibition was used for analysis.

In the second stage, three of the most active bacterial strains (RB 27, RB53 and RB 154) against *S. aureus* strains were further analysed for their ability to produce antibiotics in different submerged production media. Two loopfuls of each bacterium from a 7 day-old HPDA medium were inoculated into 50 ml of IM22 seed medium in 250 ml baffled Erlenmeyer flasks and incubated at 27$^{O}$C with shaking at 150 rpm for 3 days. 2.5 ml from seed medium was used to inoculate 50 ml each of SI, ISP2, F26, and MS in 250 ml baffled Erlenmeyer flask and incubated for 7 days with shaking at 150 rpm at 27$^{O}$C. Each medium was inoculated in triplicate and each

day 1 ml of the culture was collected, centrifuged at 30,000 x g for 10 min and 50 µl of the supernatant was tested for their activities for 7 consecutive days and the zone of inhibition was recorded for each day.

### 5.2.2.5 Large scale antimicrobial production from RB27

Several batches of flask fermentations were designed to collect a total of 5 litres of ferment from strain RB27. This strain was selected due to presenting consistent and stable antimicrobial production with a large zone of inhibition. A similar protocol as described in Section 5.2.1.4 and 5.2.2.4 was followed using seed culture with IM22 before transferring into several flasks with MS medium. The activity in each flask was screened as described in Section 5.2.2.4 and those flasks which showed activity were centrifuged and the supernatant collected for further purification.

### 5.2.2.6 Preliminary investigation of extract from RB 27

Initial screening of possible solvents for purification of the extract was tested against chloroform, butanol and ethyl acetate. Two ml of the extract was mixed with equal and two-volume of each of the solvents. After the mixture was settled at room temperature, the solvent containing layer and water reach portions were tested for activities. The extract was also checked for its stability at room temperature. About 5 ml of the extracts was left at a temperature for two months and every week the activity was measured. Furthermore, the portion of the extract was assessed for solubility in methanol and water.

### 5.2.2.7 Bioautogram testing of extract from RB27

Fifty microliters of the broth extract from RB 27 were spotted onto a silica gel 60 $F_{254}$ TLC plates (Merck). The plates were placed in a TLC tank containing different eluting solvent systems. One plate in each of butanol; chloroform: methanol: water (7:7:3); chloroform: methanol (7:7); and pyridine: 6 m HCl: RO water: methanol (10:4:26:80) solvent system-and run until the solvent reached 1 cm from the top of the TLC plates. After drying, the plates were observed at 254 and 365 nm and the position of the different compounds marked with a pencil. The area of the TLC plates which contained the compound was cut out and placed onto a AAM that contains *S. aureus* as described in Section 5.2.2.2. The TLC plates were left on the agar medium for 1 hr to allow the

compound to diffuse into the plate and then the plates were removed from the medium. Finally, the plates were incubated overnight at 37$^{o}$C and the zones of inhibition were recorded.

### 5.2.2.8 Purification strategies

Data from preliminary investigation and bioautogram indicated the extract from strain RB 27 is more water-soluble.This water-soluble antibiotic was purified in contrast to the usual purification protocols which depend on the isolation of only pure compounds out of the crude extracts. Here, we removed different junks sequentially with the help of various techniques. The following protocols were used for purification. The collected supernatant in Section 5.2.2.5 was first mixed with water-saturated butanol which split the supernatant to an organic layer and water-soluble portions. Both fractions were tested for antibacterial activity and the organic layer was discarded as there was no observable effect. After the residual solvent had evaporated off, the water-soluble portion was passed through an amberlite XAD-4 column (resins help for isolating and concentrating organic compounds from water) and both the spent and the eluate were tested and only the spent (the more water-soluble portion) was active. The spent was freeze-dried to remove all liquid and then washed with methanol to remove any solvent-soluble material. In the final step, an off-white powder was dissolved in water and passed through a thin long LH20 (Sephadex), molecular sieve, which separate sample components by partition between the stationary and mobile phases. The column was a 50 ml burette which allowed for better separation. Water was used to eluent components from LH20 and the fractions were collected with multiple tubes with 1 ml volume. Each fraction was tested for activity as described in Section 5.2.2.2 against *S. aureus,* and those showed inhibition were collected in one tube. Finally, the active compound was freeze-dried and stored at -20$^{o}$C until further experimentation. The complete protocols from production to the last step are diagrammatically present in Figure 5.2.

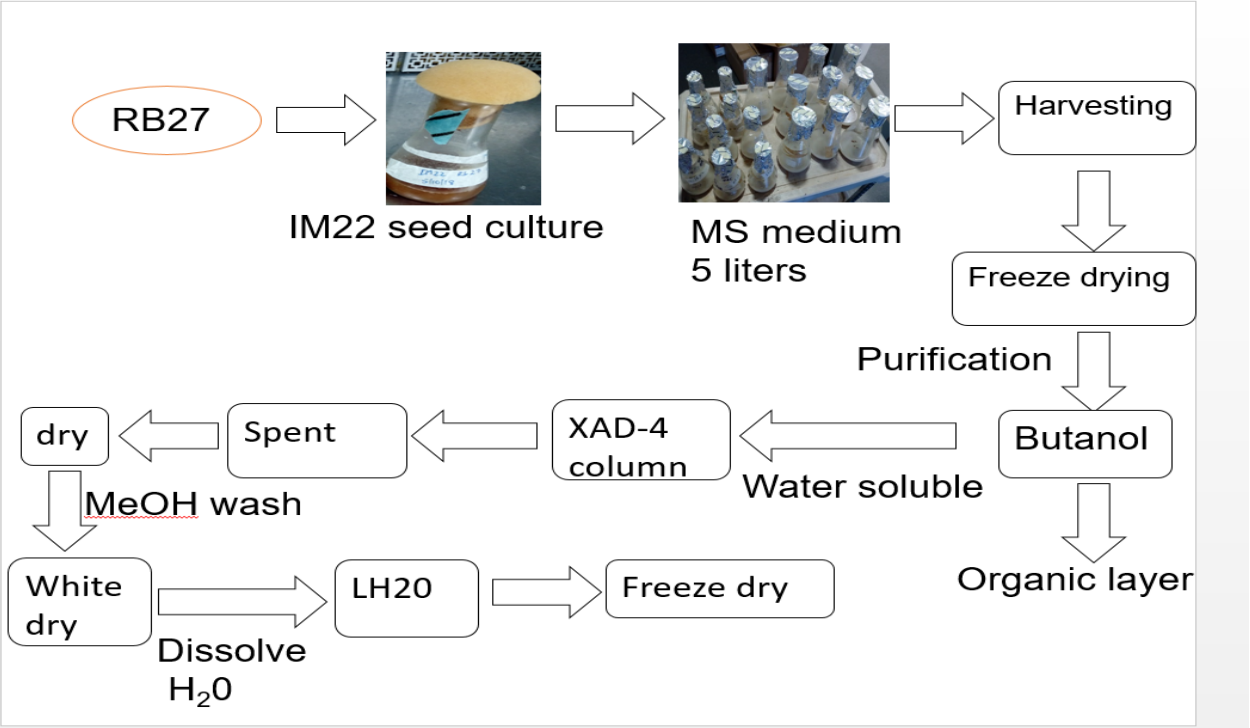



Figure 5.2 Diagrammatic representation of extract purification from the strain RB27

### 5.2.3 Results

#### 5.2.3.1 Overall antimicrobial activities

A total of 169 nine representative bacterial isolates were selected proportionally from each RFLP patterns and tested for their antimicrobial activities against Gram-negative bacteria: *S. typhimurium*, *P. aeruginosa and E. coli*; Gram-positive bacteria: *S. pyogenes*, *S. aureus*, and MRSA; *C. albicans* and dermatophytic fungi: *T. rubrum*, *T. interdigitalis*, *Sacclophosis* sp., and *M. gypsum.* Examples of the zone of inhibitions are shown in Figure 5.3. About 42.6% of the tested bacterial strains displayed antimicrobial activity in at least against one of the tested pathogens but none of them showed activities against any of the Gram-negative bacteria or *S. pyogenes* (Table 5.3).

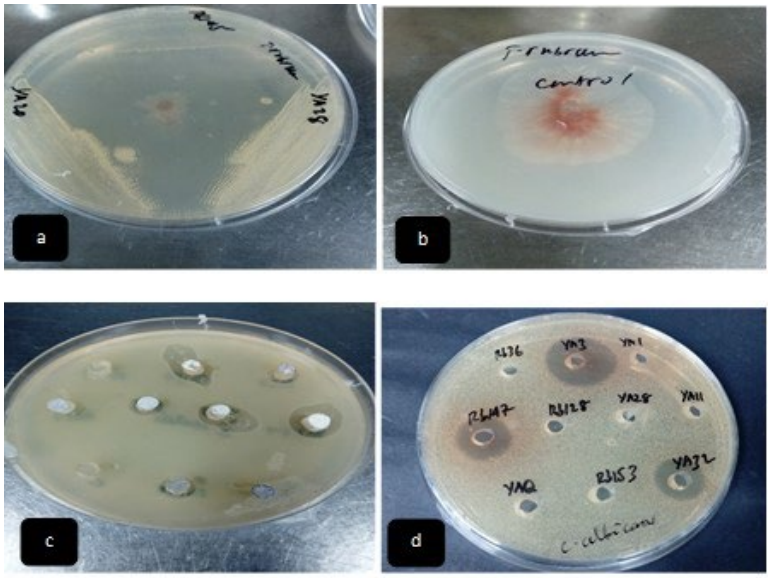



Figure 5.3 Antimicrobial activities of some of the bacterial strains after 2 weeks of incubation. **a** *T.rubrum* grew far from the bacteria strains; **b** *T. ruburum* control; **c** Plugs of bacteria against *S. aureus* where some showed zone of inhibition; **d** Some bacterial strains showed anti-*candida* activities.

Table 5.3 Bacterial isolates with antimicrobial activities

| Strains name | RFLP group | genus | *S. aureus* (SF) | *S. aureus* (LF) | MRSA (SF) | MRSA (LF) | *C. albicans* (SF) | *C. albicans* (LF) | *T. rubrum* | *T. interdigitalis* | *Sacclophosis sp.* | *M. gypsum* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RB28 | 32 | *Streptomyces* | 19 | 21 | 16 | 18 | - | - | - | - | +3 | +3 |
| RB30 | 32 | *Streptomyces* | 16 | 19 | 16 | 19 | - | - | - | - | - | - |
| RB34 | 32 | *Streptomyces* | 17 | 20 | - | - | 17 | 19 | +2 | +2 | +2 | +4 |
| RB35 | 32 | *Streptomyces* | 18 | 20 | 17 | 20 | - | - | - | - | - | - |
| RB36 | 32 | *Streptomyces* | 16 | 16 | 17 | 20 | - | - | - | +2 | +2 | - |
| RB45 | 31 | *Streptomyces* | 21 | 23 | 18 | 19 | - | - | +1 | +2 | +2 | +2 |
| RB46 | 31 | *Streptomyces* | 16 | 20 | - | - | 17 | 21 | +3 | +2 | +3 | - |
| RB49 | 31 | *Streptomyces* | 17 | 21 | 17 | 19 | - | - | - | - | - | - |
| RB53 | 31 | *Streptomyces* | 19 | 22 | 18 | 21 | - | - | - | - | - | - |
| RB54 | 32 | *Streptomyces* | 18 | 20 | 17 | 19 | 17 | 20 | +2 | +3 | - | - |
| RB57 | 31 | *Streptomyces* | 16 | 19 | 16 | 18 | - | - | - | - | - | - |
| RB60 | 31 | *Streptomyces* | 18 | 21 | 17 | 20 | - | - | - | - | - | - |
| RB65 | 31 | *Streptomyces* | 16 | 19 | 17 | 22 | - | - | - | - | - | - |
| RB66 | 31 | *Streptomyces* | 17 | 22 | - | - | 16 | 18 | +3 | +3 | +1 | +3 |
| RB67 | 31 | *Streptomyces* | 17 | 21 | - | - | 16 | 18 | +3 | +3 | +3 | +3 |
| RB70 | 31 | *Streptomyces* | 16 | 18 | - | - | - | - | +3 | +1 | +3 | +2 |
| RB74 | 31 | *Streptomyces* | 17 | 21 | - | - | 17 | 20 | +2 | +1 | - | - |
| RB76 | 31 | *Streptomyces* | 18 | 22 | 17 | 22 | - | - | - | - | - | - |
| RBYA1 | 31 | *Streptomyces* | 18 | 22 | 18 | 21 | 18 | 20 | +3 | +3 | +3 | +3 |
| RBYA13 | 31 | *Streptomyces* | 18 | 21 | 18 | 18 | 16 | 19 | +3 | +3 | +3 | +2 |
| RBYA18 | 31 | *Streptomyces* | - | - | - |  | 17 | 19 | - | - | - | - |
| RBYA2 | 31 | *Streptomyces* | 18 | 21 | 18 | 20 | - | - | - | - | +1 | +1 |
| RBYA20 | 31 | *Streptomyces* | 16 | 21 | - | 16 | 18 | 22 | +4 | +4 | +4 | +4 |
| RBYA21 | 31 | *Streptomyces* | 19 | 21 | 19 | 21 | 17 | 20 | +3 | +3 | +3 | +2 |
| RBYA22 | 31 | *Streptomyces* | 18 | 18 | 19 | 21 | - | - | +2 | +2 | +1 | +2 |
| RBYA27 | 31 | *Streptomyces* | 16 | 18 | 16 | 17 | 18 | 21 | - | - | - | - |
| RBYA28 | 31 | *Streptomyces* | 18 | 24 | 18 | -20 | - | - | +4 | +4 | +4 | +4 |
| RBYA3 | 31 | *Streptomyces* | 16 | 19 | 18 | 25 | 19 | 26 | +4 | +3 | +2 | +4 |
| RBYA32 | 31 | *Streptomyces* | 18 | 22 | 18 | 19 | 16 | 19 | +4 | +3 | +2 | +3 |
| RBYA7 | 31 | *Streptomyces* | - | - | - | - | 16 | 18 | +3 | +3 | +3 | +3 |
| RBYA8 | 31 | *Streptomyces* | - | - | - | - | 16 | 18 | - | - | - | - |
| RB58 | 2 | *Sulfitobacter* | 17 | 20 | - | - | 17 | 19 | - | - | - | - |
| RB115 | 31 | *Streptomyces* | 17 | 20 | - | - | 16 | 18 | +2 | +1 | - | - |
| RB118 | 32 | *Streptomyces* | 18 | 19 | - | - | 16 | 19 | +2 | - | - | - |
| RB119 | 31 | *Streptomyces* | 17 | 20 | 16 | 18 | - | - | - | - | - | - |

Table 5.3 (Continued)

| Strains name | RFLP group | genus | *S. aureus* (SF) | *S. aureus* (LF) | MRSA (SF) | MRSA (LF) | *C. albicans* (SF) | *C. albicans* (LF) | *T. rubrum* | *T. interdigitalis* | *Sacclophosis sp.* | *M. gypsum* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RB131 | 32 | *Streptomyces* | 20 | 22 | 17 | 18 | 19 | 21 | +4 | +1 | +3 | +3 |
| RB134 | 31 | *Streptomyces* | 16 | 21 | 16 | 18 | - | - | - | - | - | - |
| RB135 | 31 | *Streptomyces* | 16 | 16 | - | - | - | - | - | - | +3 | - |
| GB48 | 7 | *Bacillus* | 16 | 19 | - | - | 17 | 21 | - | +3 | +1 | - |
| RBL30 | 7 | *Bacillus* | 18 | 21 | - | - | 16 | 18 | - | +2 | - | - |
| GBYA15 | 7 | *Bacillus* | 16 | 20 | - |  | - | - | - | - | +2 | +1 |
| RB98 | 7 | *Bacillus* | - | - | - | - | - | - | - | - | +2 | +1 |
| RBLC7 | 9 | *Fictibacillus* | - | - | - |  | 19 | 21 | - | +2 | - | - |
| GB17 | 15 | *Gordonia* | 16 | 19 | 16 | 18 | - | - | - | - | - | - |
| RB71 | 33 | *Kocuria* | 19 | 21 | - | - | 16 | 20 | - | - | - | +2 |
| RB16 | 21 | *Kocuria* | 19 | 19 | - | - | - | - | - | - | - | - |
| RBYA34 | 26 | *Micrococcus* | 16 | 19 | - |  | 16 | 18 | - | - | +2 | - |
| RB2 | 20 | *Microbacterium* | - | - | - | - | 18 | - | - | - | +3 | - |
| RBYA11 | 30 | *Pseudonocardia* | 18 | 20 | - |  | 16 | 18 | +1 | - | - | +2 |
| RB144 | 32 | *Streptomyces* | - | 18 | 22 | - | 18 | 20 | +2 | +2 | +2 | +3 |
| RB145 | 31 | *Streptomyces* | - | 18 | 21 | 18 | 23 | - | - | +1 | +2 | +1 |
| RB146 | 31 | *Streptomyces* | - | 19 | 21 | - | 17 | 20 | - | +3 | +3 | +4 |
| RB147 | 31 | *Streptomyces* | - | 16 | 18 | 17 | 19 | 16 | - | +3 | +2 | - |
| RB15 | 32 | *Streptomyces* | - | 18 | 20 | - | - | - | - | - | - | +2 |
| RB150 | 31 | *Streptomyces* | - | 17 | 20 | - | 17 | 20 | - | - | - | +2 |
| RB151 | 31 | *Streptomyces* | - | - | - | - | 18 | 21 | - | +2 | +2 | +1 |
| RB152 | 31 | *Streptomyces* | - | 17 | 20 | - | - | - |  | - | - | - |
| RB154 | 31 | *Streptomyces* | - | 18 | 26 | 16 | 18 | 19 | - | +3 | +3 | +2 |
| RB155 | 31 | *Streptomyces* | - | - | - | - | 16 | 20 | - | - | - | - |
| RB158 | 31 | *Streptomyces* | 16 | 20 | - | - | - | -- | - | - | - | - |
| RB19 | 31 | *Streptomyces* | 18 | 18 | 18 | 19 | - | - | +2 | +3 | - | - |
| RB21 | 32 | *Streptomyces* | 18 | 26 | 14 | 18 | 16 | 20 | - | - | - | - |
| RB22 | 32 | *Streptomyces* | 16 | 19 | 16 | 20 | - | - | - | - | - | +2 |
| RB23 | 32 | *Streptomyces* | 18 | 21 | 16 | 22 | - | - | - | - | - | - |
| RB25 | 32 | *Streptomyces* | 16 | 18 | 18 | 21 | - | - | - | - | - | - |
| RB26 | 32 | *Streptomyces* | 18 | 21 | - | - | 18 | 21 | - | - | - | - |
| RB27 | 32 | *Streptomyces* | 21 | 26 | 21 | 24 | - | - | - | - | - | - |
| RB112 | 31 | *Streptomyces* | 18 | 21 | - | - | - | - | - | +2 | - | - |
| RB47 | 38 | *Streptomyces* | 16 | 16 | - | - | - | - | - | - | - | - |
| RB114 | 31 | *Streptomyces* | 17 | 20 | - | - | - | - | - | - | - | - |

The antimicrobial profile of 70 strains which showed inhibitory activities against resistant and non-resistant strains of *S.auerus* and five fungal strains as presented in the zone of inhibition (mm). All the tested bacteria isolates did not show any activities against *S. typhimurium*, *P. aeruginosa and E. coli*, and *S. pyogenes* and the data not presented in the table. For two strains of *S.auerus and C. albicans antimicrobial from* SF: Solid fermentation and; LF: Liquid fermentation were tested. + indicated the degree of the zone of inhibition as described in Section 5.2.2.3. (-) indicated absence of activities.

As indicated in Table 5.4, out of the 21 genera included in the screening 10 of them displayed antimicrobial activities. Six of the active genera belong to phylum Actinobacteria and two each from phylum Firmicutes and Proteobacteria. Specifically, 58 bacterial isolates out of 72 from genus *Streptomyces*; 1 out of 3 from genus *Gordonia*;.3 out of 26 from genus *Bacillus*; 1 out of 5 from genus Fictibacillus; 1 out of 8 from genus *Sulfitobacter*; 2 out of 9 from genus *Kocuria*; 1 out of 2 from genus *Pseudonocardia*;.1 out of 7 from genus *Microbacterium*; 1 out of 4 from genus *Limimaricola*; and 1 out of 6 from genus *Micrococcus* showed overall activities at least against one of the tested organisms. Antimicrobial activities were not observed from bacteria isolates belong to *Janibacter*, *Muricauda*, *Staphylococcus*, *Falsibacillus*, *Rhodovulum*, *Mycolicibacterium, Rhodococcus*, *Pseudomonas*, *Leisingera*, *Isoptericola*, *and Pseudoalteromonas.*

Table 5.4 Antimicrobial activity of 169 bacterial strains from 21 genera

| Strains | Active | Inactive | Total tested |
|---|---|---|---|
| *Streptomyces* | 58 | 14 | 72 |
| *Gordonia* | 1 | 2 | 3 |
| *Bacillus* | 3 | 23 | 26 |
| *Fictibacillus* | 1 | 4 | 5 |
| *Sulfitobacter* | 1 | 7 | 8 |
| *Kocuria* | 2 | 7 | 9 |
| *Pseudonocardia* | 1 | 1 | 2 |
| *Microbacterium* | 1 | 6 | 7 |
| *Limimaricola* | 1 | 3 | 4 |
| *Janibacter* | 0 | 1 | 1 |
| *Muricauda* | 0 | 1 | 1 |
| *Staphylococcus* | 0 | 2 | 2 |
| *Micrococcus* | 1 | 5 | 6 |
| *Falsibacillus* | 0 | 5 | 5 |
| *Rhodovulum* | 0 | 2 | 2 |
| *Mycolicibacterium* | 0 | 1 | 1 |

| | | | |
|---|---|---|---|
| *Rhodococcus* | 0 | 8 | 8 |
| *Pseudomonas* | 0 | 3 | 3 |
| *Leisingera* | 0 | 1 | 1 |
| *Isoptericola* | 0 | 2 | 2 |
| *Pseudoalteromonas* | 0 | 1 | 1 |
| Total | 70 | 99 | 169 |

As indicated in Figure 5.4, different ranges of antimicrobial activities have been observed against two bacterial and five fungal strains. Thirty-seven percent of the bacterial isolates had inhibitory activity against non-resistant *S. aureus* and 21% against MRSA. Antifungal activities of 27% against C. albicans; 17% against *T. rubrum*; 19% against *T. interdigitalis*; 18% against *M. gypsum*; and 18% against *Sacclophosis sp.*(Figure 5.4 **a**). Figure 5.4 **b** shows out of 70 bacterial strains with antimicrobial activities against two bacterial and five fungal strains, only 7.bacteria were active only against a single pathogen whereas most of them displayed wider ranges of inhibitory activities.

Specific analysis of antimicrobial activities revealed 38% of the total activities was attributed to actinobacteria with *Streptomyces* being the most common genus., with 80% of the tested strains in this genus producing antibiotic activity against the test microbes; while 11 out of 21 genera were inactive. The phylogenetic tree of the active strains are shown in figures 5.5 and the tree was constructed using the method described in Section 4.2.4.1.

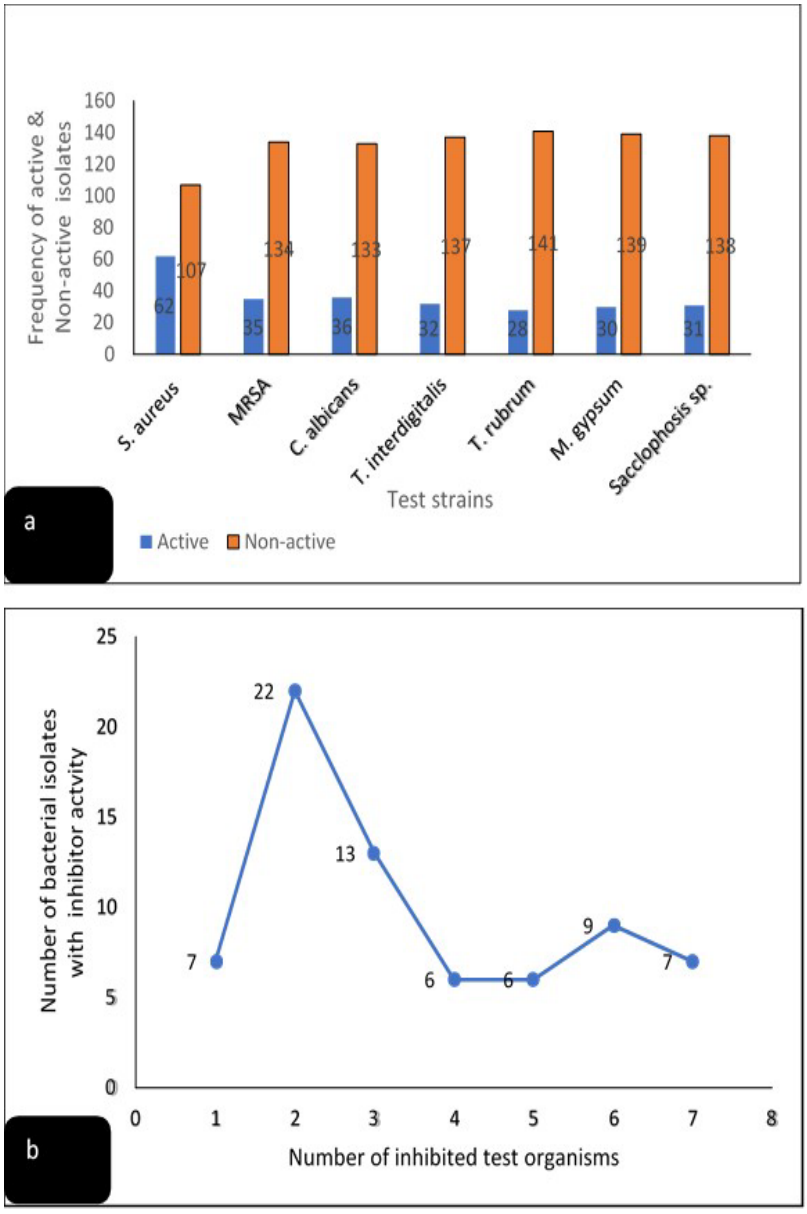
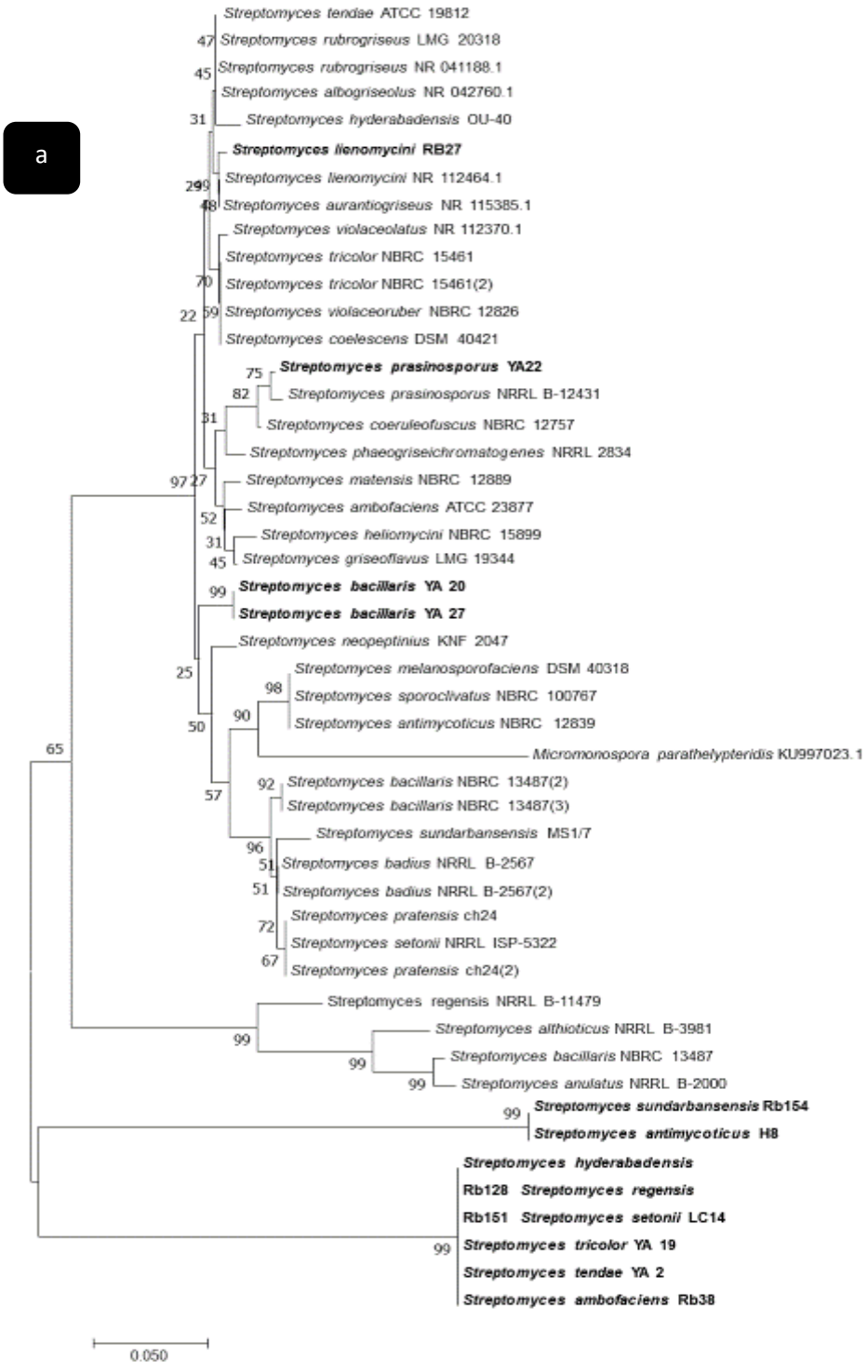
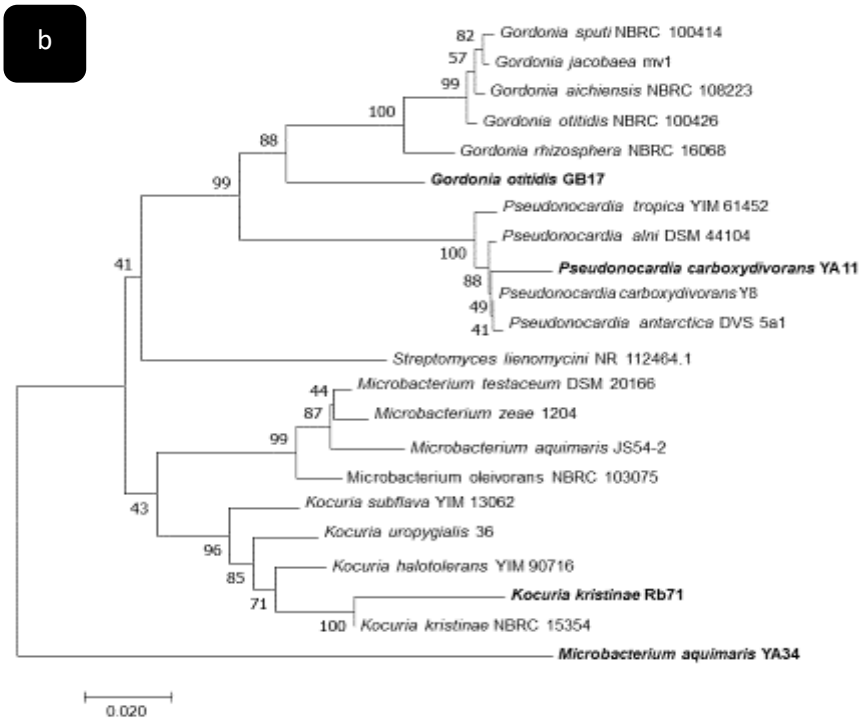



Figure 5.4 Ranges of antimicrobial activities of 169 bacterial strains. **a** the antimicrobial activities of bacterial strains for specific pathogens; **b** the range of antimicrobial activities of 70 active bacterial isolates, where the number.in the x-axis indicated the number of test organisms inhibited by specific bacterial strains and the y-axis indicated the number of bacteria isolates which showed activities against one or more of the test organisms.

a


Streptomyces tendae ATCC 19812
Streptomyces rubrogriseus LMG 20318
Streptomyces rubrogriseus NR 041188.1
Streptomyces albogriseolus NR 042760.1
Streptomyces hyderabadensis OU-40
Streptomyces lienomycini RB27
Streptomyces lienomycini NR 112464.1
Streptomyces aurantiogriseus NR 115385.1
Streptomyces violaceolatus NR 112370.1
Streptomyces tricolor NBRC 15461
Streptomyces tricolor NBRC 15461(2)
Streptomyces violaceoruber NBRC 12826
Streptomyces coelescens DSM 40421
Streptomyces prasinosporus YA22
Streptomyces prasinosporus NRRL B-12431
Streptomyces coeruleofuscus NBRC 12757
Streptomyces phaeogriseichromatogenes NRRL 2834
Streptomyces matensis NBRC 12889
Streptomyces ambofaciens ATCC 23877
Streptomyces heliomycini NBRC 15899
Streptomyces griseoflavus LMG 19344
Streptomyces bacillaris YA 20
Streptomyces bacillaris YA 27
Streptomyces neopeptinius KNF 2047
Streptomyces melanosporofaciens DSM 40318
Streptomyces sporoclivatus NBRC 100767
Streptomyces antimycoticus NBRC 12839
Micromonospora parathelypteridis KU997023.1
Streptomyces bacillaris NBRC 13487(2)
Streptomyces bacillaris NBRC 13487(3)
Streptomyces sundarbansensis MS1/7
Streptomyces badius NRRL B-2567
Streptomyces badius NRRL B-2567(2)
Streptomyces pratensis ch24
Streptomyces setonii NRRL ISP-5322
Streptomyces pratensis ch24(2)
Streptomyces regensis NRRL B-11479
Streptomyces althioticus NRRL B-3981
Streptomyces bacillaris NBRC 13487
Streptomyces anulatus NRRL B-2000
Streptomyces sundarbansensis Rb154
Streptomyces antimycoticus H8
Streptomyces hyderabadensis
Rb128 Streptomyces regensis
Rb151 Streptomyces setonii LC14
Streptomyces tricolor YA 19
Streptomyces tendae YA 2
Streptomyces ambofaciens Rb38
0.050



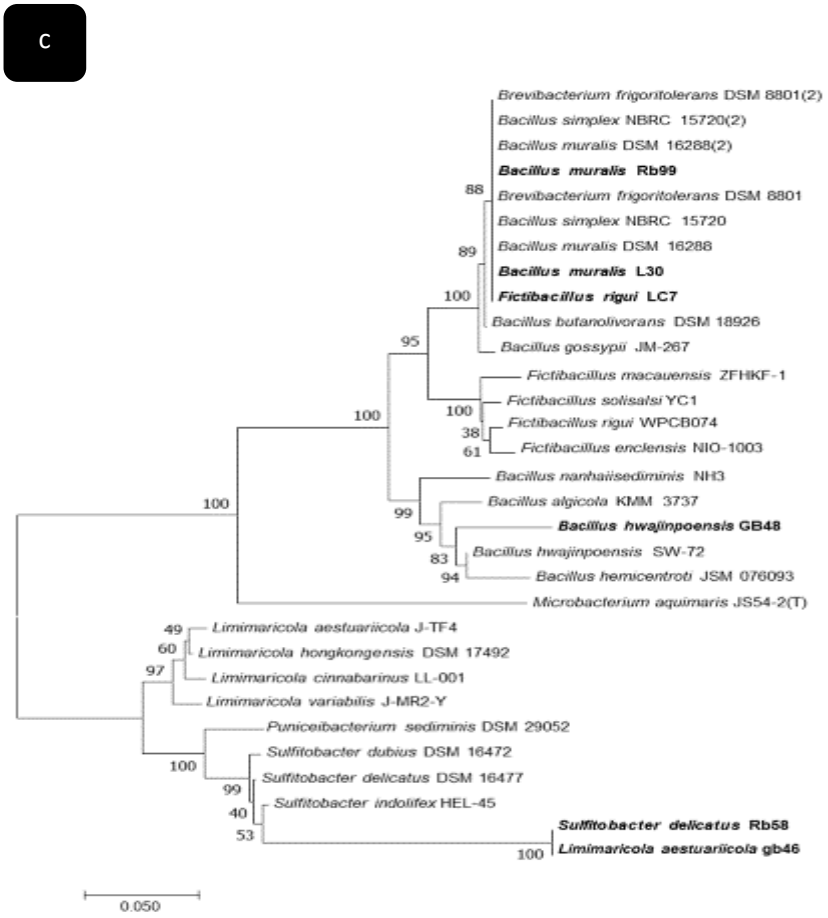




Figure 5.5 Phylogenetic tree of bacterial strains with antimicrobial activities. Neighbour-joining tree showing the relationship between representative sequence from each active bacterial genera. The numbers at the node indicated the levels of bootstrap support based on 1000

resampled. datasets. The scale bar represents changes per nucleotide. **a** phylogenetic tree of *Streptomyces* spp.; **b** Non-*Streptomyces* Actinobacteria*;* **c** bacteria other than Actinobacteria. The bold in each tree represents the closest active strains and the rest are sequence from databases.

### 5.2.3.2 Antimicrobial production in different media

Eleven bacterial strains with a large zone of inhibition to non-resistant and multidrug-resistant strains of *S.auerus* were selected and their antimicrobial production investigated using five primary isolation media. The findings revealed a marked variation of antimicrobial production by specific strains depending on the media type. ISP2 was the best medium as it promoted at least some degree of antimicrobial production by all strains. Though the number of strains producing antimicrobials in MS medium was low compared to ISP2, a significant zone of inhibition was produced. A medium like YP didn't support antimicrobial production while only a negligible amount of production was supported by the SYP medium (Table 5.5).

Table 5.5 Antimicrobial production of selected bacterial strains using different media

| Strains | Media | | | | |
|---|---|---|---|---|---|
| | YPA | PDA | SYP | MS | ISP2 |
| RB27 | - | 14 | - | 26 | 26 |
| RBYA28 | - | - | - | - | 24 |
| RBYA 13 | - | - | - | - | 21 |
| RBYA 1 | - | - | 14 | 18 | 16 |
| RBYA 21 | - | - | 14 | - | 21 |
| RB154 | - | - | 10 | 21 | 26 |
| RB53 | - | 16 | - | 29 | 19 |
| RBYA32 | - | 14 | - | 18 | 22 |
| RB19 | - | - | - | - | 16 |
| RBYA2 | - | 14 | - | 20 | 18 |
| RBYA22 | - | - | - | - | 16 |

*The antimicrobial production of 12 bacteria strains on five media: YPA-Yeast extract peptone; PDA-Potato dextrose agar; SYP-Starch yeast extract peptone; MS- Mannitol soybean; ISP2-International Streptomyces medium 2.Th zone of inhibition present in millimetre where "-" indicated no zone of inhibition.

### 5.2.3.3 Antimicrobial production using four production media

Three of the most active bacterial strains (RB27, RB53 and RB154) against both strains of *S. aureus* were further analysed for their ability to produce antibiotics using four different production media (ISP2, SI, F26, and MS) for 7 days. The zone of inhibition was measured in mm (Table 5.6). The results indicated that MS and ISP2 media were supported for antimicrobial production by the three strains as demonstrated by a zone of inhibition ranging from 13 to 29

mm. However, SI and F26 media did not support production by the strain RB53. Moreover, the zone of inhibitions resulted from SI and F26 media were low compared to the other two media. Starin RB27 produced a larger zone of inhibition in all media, particularly in MS medium, compared to the other two strains. If antimicrobial produced, most of them appeared after three days of incubation and the maximum zone of inhibition observed by day 7 of incubation.

Table 5.6 Antimicrobial production of three selected strains in four media for 7 days

| Strains | Media | Extract day1 | | Day2 | | Day3 | | Day 4 | | Day 5 | | Day 6 | | Day 7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SA | MRSA | SA | MRSA | SA | MRSA | SA | MRSA | SA | MRSA | SA | MRSA | SA | MRSA |
| RB154 | ISP2 | 13 | 13 | 13 | 13 | 16 | 14 | 16 | 11 | 11 | 8 | 9 | 8 | 18 | 10 |
| | SI | 14 | 17 | 17 | 18 | 17 | 19 | 13 | 19 | 13 | 13 | 13 | 16 | 13 | 13 |
| | MS | 14 | 14 | 15 | 17 | 13 | 17 | 12 | 12 | 13 | 17 | 13 | 17 | 13 | 17 |
| | F26 | 16 | 17 | 18 | 20 | 18 | 19 | 19 | 18 | 17 | 19 | 18 | 20 | 18 | 20 |
| RB27 | ISP2 | - | - | 23 | 19 | 27 | 23 | 23 | 20 | 24 | 21 | 23 | 20 | 26 | 19 |
| | SI | - | - | 14 | - | 12 | - | 14 | 13 | 17 | 15 | 17 | 15 | 17 | 15 |
| | MS | - | - | 27 | 26 | 27 | 27 | 28 | 29 | 28 | 28 | 28 | 26 | 29 | 28 |
| | F26 | - | - | 21 | 15 | 21 | 17 | 23 | 18 | 23 | 17 | 23 | 20 | 24 | 19 |
| RB53 | ISP2 | - | - | 16 | - | 17 | - | 18 | 10 | 18 | 10 | 18 | 10 | 18 | 10 |
| | SI | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | MS | - | - | - | - | 22 | 19 | 22 | 18 | 22 | 21 | 22 | 19 | 22 | 19 |
| | F26 | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

### 5.2.3.4 Preliminary investigation of extract from RB 27

Results for the preliminary investigation of extract from RB 27 revealed that the extract was not purified with butanol, chloroform, and ethyl acetate as the solvents reach portions of the mixture did not show any inhibitory activity. Furthermore, the extract was completely dissolved in water but not in methanol. It was also discovered the extract maintain its activity at room temperature for more than a month.

### 5.2.3.5 Bioautogram of the purified compound from strain RB27

The bioautogram of the purified compound from strain RB27 was determined using TLC with different solvent systems as indicated in Section 5.2.2.6. The results indicated, except for pyridine: 6m HCl: RO water: methanol, the other solvents (chloroform: methanol: water, chloroform: methanol, and butanol) were not able to move the compound up as only a zone of inhibition was observed at the site where the extract was spotted (Base) (Figure 5.6).

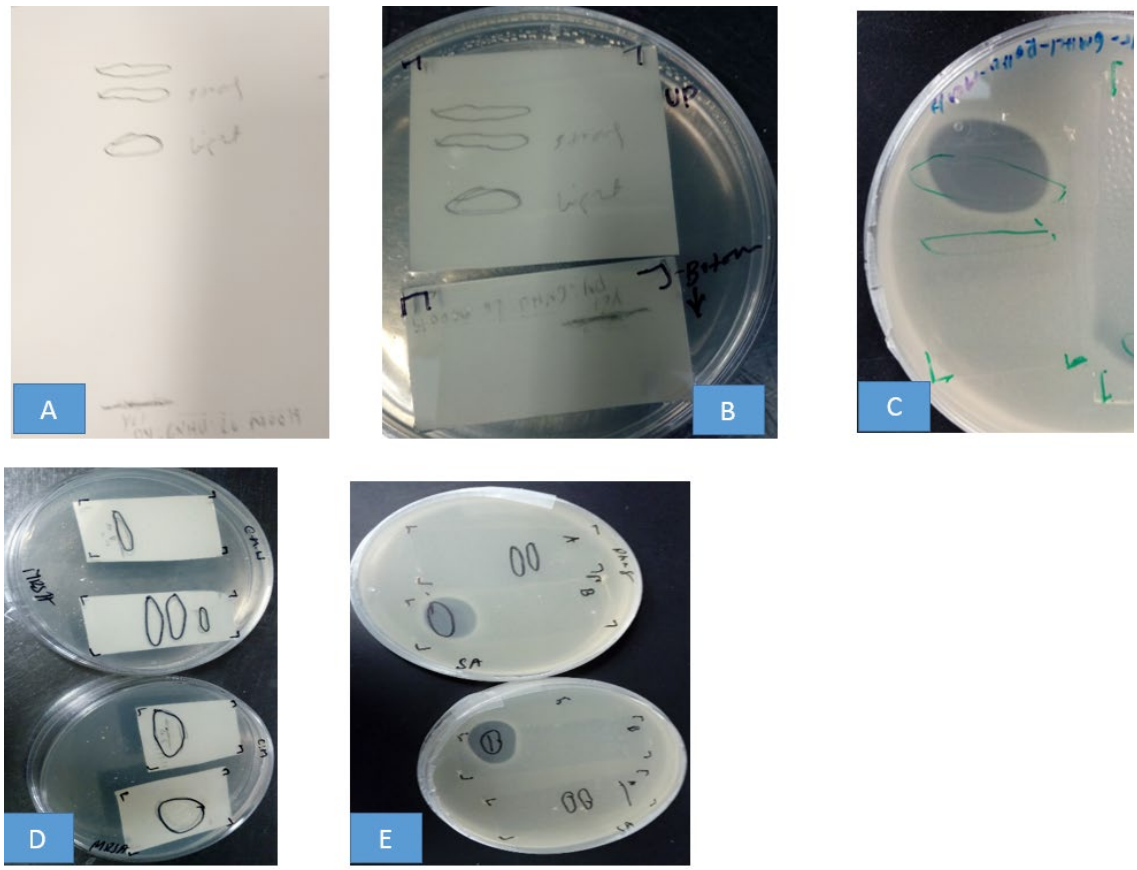



Figure 5.6 Bioautogram of RB27 extract using different solvents. **a-c** steps for bioautogram using pyridine: 6m HCl: RO water: methanol solvents where the active compound moved up; **d**, **e** bioautogram using chloroform: methanol: water and chloroform: methanol where only the base showed activities.

### 5.2.3.6 Accurate mass determination of the extract from RB27

Five milligrams of the extract was submitted to Flinders University Analytical Centre to accurately determine its mass using High Definition Mass Spectrometer. The extract was dissolved in 15 ml of acetonitrile and subjected to mass analysis. These spectra are presented in Figure 5.7. All of the spectra of the higher peaks were checked for their agreement with a set of known contaminant peaks such as peaks for different detergents, poly ethyl glycol, saponin, MS medium, and many more and most except peak 685.4340 were identified as common contaminants. The potential formula for peaks 685.4340 was assessed using single mass analysis and the results were indicated in Table 5.7.

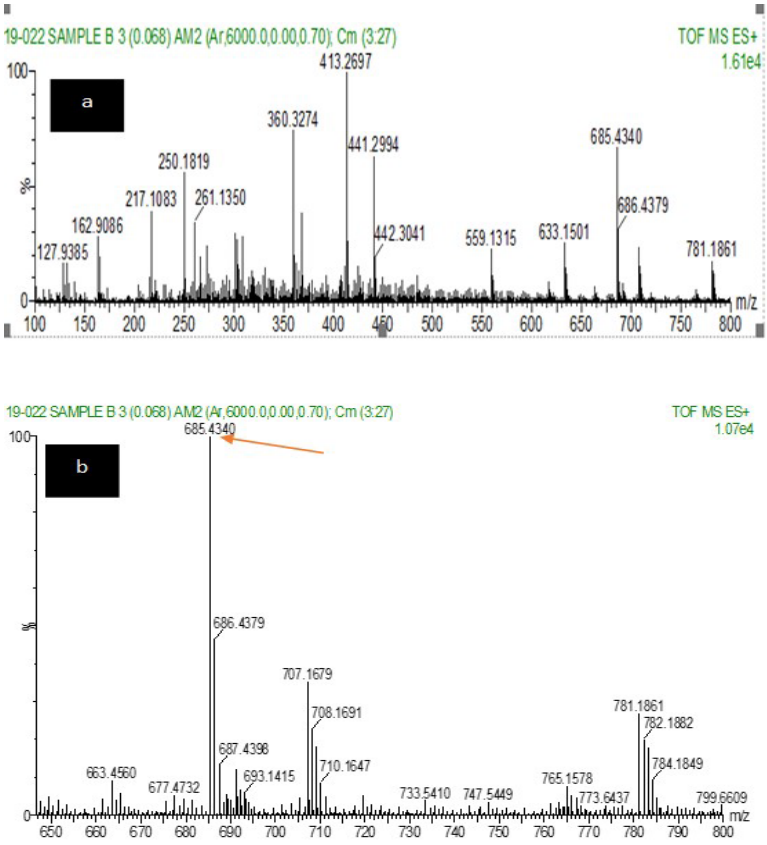



Figure 5.7 Mass spectra of HDMS$^{TOF\ MS\ ES+}$. **a** mass scan of the extract from 100 to 800 Daltons; **b** zooms of mass scan 650 to 800 Daltons. The arrow points the peak of the compound of interest as none of the internal contaminant peaks much with it.

Table 5.7 Possible formula for the compound

| Mass | Calc. Mass | mDa | PPM | DBE | i-FIT | i-FIT (Norm) | Formula |
|---|---|---|---|---|---|---|---|
| 685.434 | 685.4339 | 0.1 | 0.1 | 1.5 | 39.3 | 7.2 | C31 H66 N4 O8 P S |
| | 685.4338 | 0.2 | 0.3 | 14.5 | 42.3 | 10.2 | C42 H61 N4 S2 |
| | 685.4342 | -0.2 | -0.3 | 20.5 | 34.4 | 2.2 | C42 H53 N8 O |
| | 685.4343 | -0.3 | -0.4 | 0.5 | 38.3 | 6.1 | C33 H73 N2 O2 P4 S |
| | 685.4337 | 0.3 | 0.4 | 9.5 | 36.3 | 4.1 | C41 H67 O2 P2 S |
| | 685.4337 | 0.3 | 0.4 | -1.5 | 41.3 | 9.1 | C23 H62 N10 O11 P |
| | 685.4344 | -0.4 | -0.6 | 5.5 | 41.6 | 9.5 | C34 H67 N6 P2 S2 |
| | 685.4336 | 0.4 | 0.6 | 11.5 | 36.6 | 4.4 | C34 H57 N10 O3 S |
| | 685.4335 | 0.5 | 0.7 | 6.5 | 38.2 | 6.1 | C33 H63 N6 O5 P2 |
| | 685.4346 | -0.6 | -0.9 | 10.5 | 34.2 | 2.1 | C39 H62 N2 O6 P |

* Single Mass Analysis of the extract with search elements of C: 0-100 H: 0-1000 N: 0-10 O: 0-50 P: 0-10 S: 0-2. A total of 43785 formula(e) evaluated with 48 results within limits (all results (up to 1000) for each mass) with Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0 and number of isotope peaks used for i-FIT = 3.

### 5.2.3.7 NMR analysis of the extract

Forty milligrams of purified extract from strain RB27 was submitted to Associate professor Michael Perkins Chemistry laboratory (Flinders University, College of Science and Engineering) for $^{1}$H Nuclear magnetic resonance spectroscopy (NMR) analysis for structural identification of the compound of interest. A maximum of 20 mg of the extract was dissolved in 500 µl of Deuterated dimethyl sulfoxide (DMSO-d6). NMR analyses were performed using Bruker 600 MHz spectrometer, equipped with 5 mm inverse multinuclear probe, 5 mm triple resonance (HCN) probe, variable temperature, z-gradients and autosampler which is optimized for $^{1}$H and $^{13}$C detection. 1H NMR spectra were acquired at 400 MHz. Standard Bruker software was used to execute recording of 1 and 2 dimensional. All resonance bands were referenced to tetramethylsilane (TMS) internal standard (Dona *et al.*, 2016). The 1 and 2-dimensional $^{1}$H-NMR spectra are shown in Figures 5.8. These $^{1}$H spectra of the extract from strain RB27 were checked for their identity with the reference spectra in databases and the search showed none of them matched .

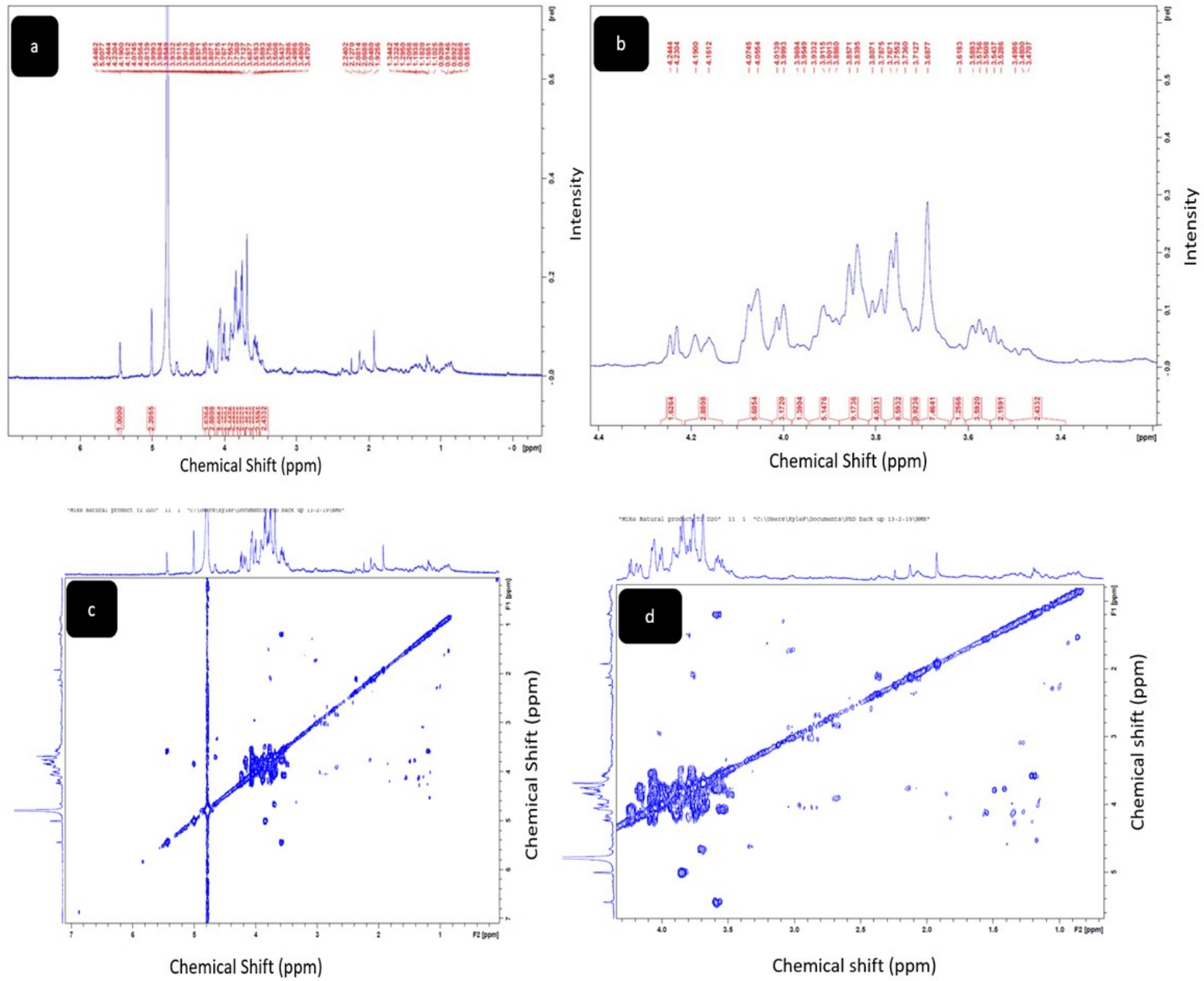



Figure 5.8 $^{1}$H-NMR spectra of extract from strain RB27 in DMSO-d6. **a** 1 dimensional 1H spectrum; **b** expanded 1 dimensional 1H spectrum; **c & d** 2D correlation spectroscopy (COSY) ($^{1}$H, $^{1}$H)-NMR spectrum where the horizontal axis is defined as F2 (direct dimension) and the vertical axis as F1 (indirect dimension).

### 5.2.3.8 Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentration (MBC) analysis of the active compound from Rb27

The purified extract from bacterial strain RB27 was assessed for the minimum concentrations to inhibit and kill different human pathogenic bacteria.

#### 5.2.3.8.1 Test microorganisms

MIC and MBC of the compound from strain RB27 were analysed against 16 human pathogenic strains obtained for the Clinical Microbiology Unit, Flinders Medical Centre. The antimicrobial profile of some of these strains also provided alongside the strains and it is presented in Table 5.8.

Table 5.8 List of bacteria tested for MIC and MBC

| Strains | Antimicrobial resistance pattern | Strains | Antimicrobial resistance pattern |
|---|---|---|---|
| *Streptococcus agalactiae* | ND | *Fictibacillus arsenicus* | ND |
| *Enterococcus faecalis* | ND | *Staphylococcus capitis* | ND |
| *Klebsiella pneumoniae* | ND | *Stenotrophomonas maltophilia* | ND |
| *Corynebacterium sp.* | ND | *Corynebacterium striatum* | ND |
| *Pseudomonas aeruginosa* | Resistant: Ampicillin, Augmentin, Cotrimoxzole<br><br>Sensitive: Gentamycin, ciprofloxacin, Cephepime | *Streptococcus pyogenes* | Sensitive: Penicillin, clindamycin, Doxycycline, erythromycin |
| *Staphylococcus aureus* | Resistance: Penicillin, ampicillin | *Methicillin-resistant Staphylococcus aureus* | Resistance: Ciprofloxacin, erythromycin, Flucoxacillin, Fusidic acid, Gentamicin, |

| | | | |
|---|---|---|---|
| | Sensitive: erythromycin, clindamycin, Doxycycline | | Penicillin, Rifampicin, Trimethoprim<br><br>Sensitive: Doxycycline linezolid mupirocin vancomycin |
| *Escherichia coli* | ND | *Bacillus cereus* | ND |
| *Micrococcus sp.* | ND | *Staphylococcus epidermidis* | ND |

ND: not done

### 5.2.3.8.2 Standardization of inoculum

Two loopful of each bacterium was inoculated in TSB broth and incubated overnight at 37°C. The OD at 600 nm was measured for each and a 10-fold serial dilution of $10^{-1}$-$10^{-10}$ was prepared and 10 µl from each dilution was spotted in two places on the TSA medium. After overnight incubation at 37°C, the CFU of each bacterium was calculated by the Miles & Misra (1938) method as shown in Figure 5.9 below. The final CFU was adjusted to be 1x $10^6$/ml.

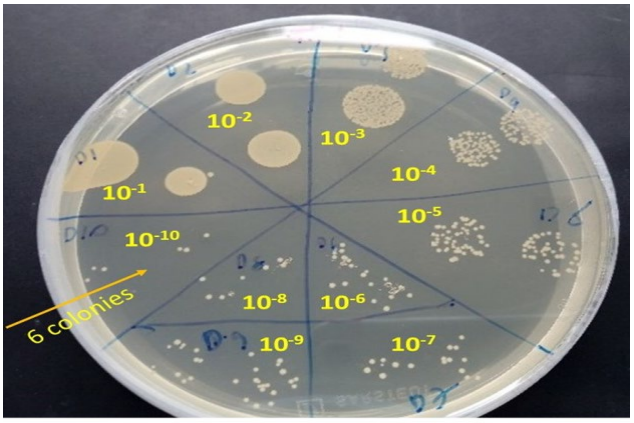



Figure 5.9 CFUcount by the Miles & Misra technique. The arrow indicated 6 countable colonies at $10^{-10}$ dilution which resulted in 6x$10^{10}$ CFU/10µl, and this concentration adjusted to 1x$10^{10}$ CFU/ml. This final standardized CFU was used MIC and MBC.analysis.

5.2.3.8.3 MIC analysis

The MIC of the extract analysed using sterilized MHB. The stock of the extract was prepared by diluting 20 mg of the dried powder in 1800 µl of MHB. From this stock, 900 µl final volume of double dilutions of 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.313 mg/ml, 0.156 mg/ml, 0.078 mg/ml, and 0.039 (9 tubes) were prepared. In each concentration, 100 µl of $1x10^6$ CFU/ml of the bacterium (standardized in Section 5.2.3.7.2) was added and the inoculum was incubated 18 hrs at 37°C. At the same time bacteria free for each concentration was incubated as a negative control. For all 16 pathogenic bacterial strains, a total of 144 tubes were analysed. The MIC was reported as the last dilution with no visible growth of bacterium as indicated by the absence of any turbidity as compared to a medium without bacterial strains as a negative control.

5.2.3.8.4 MBC analysis

To obtain the MBC a reduction in CFU of 3 log values is measured by spotting 10 µl of 1 in 10 diluted sample from all tubes without visible turbidity onto TSA medium, and the plate incubated overnight at 37°C. The lowest concentration which resulted in no bacterial growth was reported as the MBC. The results of MIC and MBC are presented in Table 5.9. and Figure 5.10 presents MBC.

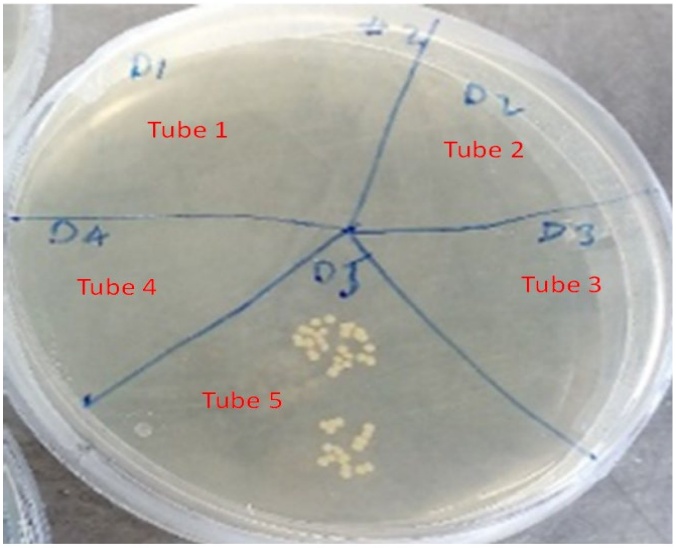



Figure 5.10 MBC determination. Tube1-5 are tubes with no visible growth in MIC and only Tube 5 showed colonies. The MBC is the concentration represented by Tube 4.

Table 5.9 MIC and MBC values of the compound against 16 types of bacteria

| Strains | MIC (mg/ml) | MBC (mg/ml) |
|---|---|---|
| *Methicillin-resistant Staphylococcus aureus* | 1.25 | 2.5 |
| *Staphylococcus aureus* | 0.625 | 1.25 |
| *Enterococcus faecalis* | 0.625 | 1.25 |
| *Corynebacterium striatum* | 1.25 | 2.5 |
| *Streptococcus agalactiae* | 1.25 | 2.5 |
| *Staphylococcus capitis* | 0.3125 | 1.25 |
| *Klebsiella pneumoniae* | >10 mg/ml | >10 mg/ml |
| *Corynebacterium sp.* | >10 mg/ml | >10 mg/ml |
| *Pseudomonas aeruginosa* | >10 mg/ml | >10 mg/ml |
| *Escherichia coli* | >10 mg/ml | >10 mg/ml |
| *Micrococcus sp.* | >10 mg/ml | >10 mg/ml |
| *Bacillus cereus* | >10 mg/ml | >10 mg/ml |
| *Staphylococcus epidermidis* | >10 mg/ml | >10 mg/ml |
| *Fictibacillus arsenicus* | >10 mg/ml | >10 mg/ml |
| *Stenotrophomonas maltophilia* | >10 mg/ml | >10 mg/ml |
| *Streptococcus pyogenes* | >10 mg/ml | >10 mg/ml |

### 5.2.4 Discussion

The main aim of the work described in this chapter was to establish marine sponges associated bacteria from South Australia as an alternative source of antimicrobial agents. A total of 169 bacterial isolates were screened for antimicrobial activities and about 42% displayed different degrees of activities against a set of human pathogens. This percentage of overall antimicrobial activity was well within the range found in other comparable studies (Abdelmohsen *et al.*, 2010; Flemer *et al.*, 2012; Graça *et al.*, 2013; Cita *et al.*, 2017), although here a higher percentage was observed compared to other related studies (Hentschel *et al.*, 2001; Zhang *et al.*, 2009; Kuo *et al.*, 2019). General antimicrobial activity profiles of 10-50 % were reported with various studies (Hentschel *et al.*, 2001; Kennedy *et al.*, 2009; Santos *et al.*, 2010; Flemer *et al.*, 2012) though. factors such as the number of strains tested and assay conditions may affect the reported overall antimicrobial percentages (Kennedy *et al.*, 2009).

A specific analysis of the antimicrobial profile varies from study to study. For example, in line with our study, a lack of activity against Gram-negative bacteria was reported by Abdelmohsen *et al*. (2010), however, other studies have seen activity against Gram-negative bacteria (Devi *et al.*, 2010; Santos *et al.*, 2010; Graça *et al.*, 2013; Graça *et al.*, 2015; Cita *et al.*, 2017; Matobole *et al.*, 2017). Antifungal activities, on the other hand, were reported by Kuo *et al*. (2019) and Flemer *et al.* (2012) but not by Graca *et al*. (2013) and Hentschel *et al*. (2001). These variations in the range of antimicrobial activities could be due to the difference of the sponges surrounding environment which favours the development of specific defence mechanisms over others. As many as 90% of bacterial isolates with antimicrobial activities were able to inhibit the growth of two or more of the tested pathogens. These findings may indicate that marine environments, especially marine sponges, could be unique environments that demand the symbionts to develop multiple forms of survival strategies, including antibiotics to win the competition.

Ten of the 21 genera showed antibiotic activity including *Streptomyces, Gordonia, Kocuria, Pseudonocardia, Microbacterium, Micrococcus*, *Bacillus, Fictibacillus, Sulfitobacter,* and *Limimaricola*. The phylogenetic analysis of the active bacterial isolates indicated that most of them belong to phylum Actinobacteria particularly the genus *Streptomyces* which are known for their ability to produce various types of biologically active compounds (Bull & Stach, 2007; Iniyan *et al.*, 2019; Babadi *et al.*, 2020). While this finding was supported by similar studies (Kennedy *et*

*al.*, 2009; Abdelmohsen *et al.*, 2010; Kuo *et al.*, 2019), lower percentages of antimicrobial activities by phylum Actinobacteria were reported compared to other phyla (Flemer *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*, 2015).

Many of the antimicrobial active genera reported in this study have been described as having antimicrobial activities. In addition to *Streptomyces* (Selvin *et al.*, 2004; Kennedy *et al.*, 2009; Abdelmohsen *et al.*, 2010; Kuo *et al.*, 2019), antimicrobial activity has also been reported for bacteria of the genus *Bacillus* (Kennedy *et al.*, 2009; Devi *et al.*, 2010; Santos *et al.*, 2010; Flemer *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*, 2015; Kuo *et al.*, 2019), *Micrococcus* (Hentschel *et al.*, 2001; Graça *et al.*, 2013; Palomo *et al.*, 2013; Cita *et al.*, 2017), *Microbacterium* (Lang *et al*., 2004; Abdelmohsen *et al.*, 2010; Margassery *et al.*, 2012; Graça *et al.*, 2013), *Gordonia* (Graça *et al.*, 2013), *Sulfitobacter* (Kennedy *et al.*, 2009), *Kocuria (*Palomo *et al.*, 2013). As far as we know, antimicrobial activities due to *Pseudonocardia, Fictibacillus*, and *Limimaricola* have not been previously reported, though phylogenetically, the genus *Fictibacillus* is highly related to *Bacillus* and genus *Limimaricola* with *Sulfitobacter*. However, genus *Pseudonocardia* is distinct from other genera in the same or different phyla. This observation indicated that the search for new antimicrobials should also consider less common and unexplored genera.

The striking observation of, this and related studies, indicated the non-consistent distribution of genera showing antimicrobial activities. Some genera such as *Pseudovibrio*, *Pseudoalteromonas*, *Vibrio*, *Bacillus*, and *Proteus* were the most common antimicrobial producing bacterial isolates (Hentschel *et al.*, 2001; Flemer *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*, 2015), which were not observed in our study. Several factors may attribute to this variation. First, specific genus/genera may dominate in some geographical areas and isolated in higher amount compared to a less common one. The other factor may be methodological. Here, studies which screened only a few representatives from each group could miss an active strain, as those strain which are look-alike not always produced the same metabolites. The third factor could be the expert effect. Researchers may have different levels of skill for identification of one genus over the other and this lead the incorporation of one genus more in the study over the other, personal bias effect. Whether it was a real difference or personal factors, the observed genera variations in terms of activities among studies encourage to search for more active genera as alternative sources of antimicrobials.

The importance of medium composition and time of incubation for bioactive compounds productions was acknowledged in this study and this determined the outcome of screening results. We tested 12 highly active bacterial strains for antimicrobial production in different media. Media like ISP2 and MS support antimicrobial production by most of the tested strains. As indicated in Chapter 3 and other related studies (Abdelmohsen *et al.*, 2010; Sipkema *et al.*, 2011), bacterial isolation is determined by media selection. Likewise, the importance of media for bioactive compound production has been reported in several studies (Huck *et al.*, 1991; Wang *et al.*, 2008; Flemer *et al.*, 2012; Sethi *et al.*, 2013; Vijayakumari *et al.*, 2013). All of these studies recognized the importance of media for the isolation of larger amounts and proportions of bacterial isolates with bioactivities.

This study not only identified the importance of media for primary screening but also large scale liquid state production of bioactive compounds. Here, not only the media but also the time of incubation influenced production levels. Most antimicrobial activities were observed after three-days of fermentation from the three-day incubation seed medium and the activities increased in terms of inhibition until seven days. Graça *et al.* (2013) reported the importance of media and the proper time for antimicrobial production in liquid state fermentation. A recent review also indicated the importance of medium optimization (pH, temperature, agitation speed, incubation period, medium composition) for optimum yield of fermentation products (Singh *et al.*, 2017).

Due to time limitations, this study purified one active compound from strain RB27 which showed a strong inhibition against resistant ***Staphylococcus aureus***. 16S rRNA sequence analysis of the strain found the isolate belongs to genus Streptomyces with 99.83% similarity with *Streptomyces lienomycini* LMG 20091$^{T}$. The compound was not dissolved in methanol and highly water-soluble. We used an unusual method of purification by removing impurities with sequential treatment as the common solvents were not effective to purify the extract. This approach produced an almost pure compound and could be used in future similar studies for purification of water-soluble compounds.

Though still not confirmed, data from NMR and accurate mass were compared with the online database and the result indicated the compound could be novel. The compound has a molecular weight of about 683 daltons which possibly contained 3 to 4 sugar molecules with OH/NH groups as observed in chemical shifts around 4 Mhz. The activity of the compound is tested against 16

human pathogenic bacteria and have a MIC (mg/ml) of 0.3125 to 1.25 and MBC (mg/ml) of 1.25 to 2.5 for MRSA*, S. aureus, E. faecalis, C. striatum, S. agalactiae* and *S. capitis,* and MIC and MBC> 10 mg/ml for the rest. This initial observation encourages to purify more compounds from several strains which showed antibacterial and antifungal activities. The result also indicated the possibility of discovering new compounds not only from novel bacteria but also from known bacteria from marine sponges, as the environment may force the bacteria to produce bioactive compounds.

### 5.2.5 Conclusions

Considerable numbers and diversity of the bacterial population were isolated from different sponge species of South Australian marine environments. Quite a remarkable percentage of the selected isolates displayed antimicrobial activities against *S. aureus, C. albicans*, *T. rubrum*, *T. interdigitalis*, *M. gypsum*, and *Sacclophosis* sp. Bacteria under the phylum Actinobacteria, particularly *Streptomyces,* showed marked antimicrobial activities compared to other isolates. Antimicrobial production with strains depends on media ingredients and incubation time. One isolation of the antibiotic compounds was scaled up and subsequently purified. It is a water-soluble compound and accurate mass and NMR characterization of active extract from *Streptomyces lienomycini* Rb27 strain resulted in a potentially new compound consisting of 20 to 30 carbons and a considerable number of OH/NH groups. This finding encourages further compound characterization from active extracts for the search of candidate novel compounds. The overall findings indicated a marine environment of South Australia could be a good source of marine sponges having diverse bacteria with antimicrobial activities and further studies in the area may result in novel bacteria strains with new antimicrobial agents.

# Chapter Six: Major findings and Future directions

## 6.1 Major findings of the project

The major aims of this study were to characterise culturable sponge-associated bacterial diversity and their antimicrobial activities from South Australian marine environments. The hypothesis of the study was proven as the sponges of South Australia were the source of a highly diverse bacterial population with potential novelty and antimicrobial activities against bacteria, yeast, and fungi. The main findings of this thesis are discussed below.

### 6.1.1 The application of morphological characteristics and 16S rRNA gene PCR RFLP for the rapid categorization of bacteria from sponges

To characterize a large number of bacterial isolates obtained in this study, initial categorization was based on microscopic properties followed by morphological similarities allowed the removal of duplicates. Finally, restriction enzyme digestion of 16S rRNA gene PCR products of the categorized bacterial isolates using two enzymes enabled us to reduce the number of bacteria to be sequenced. This approach helped to categorize the bacteria into four phyla and 21 genera. This methodology had the additional benefit saving time and resources rather than using a genotypic approach to bacterial identification, and the efficiency of this approach for rapid bacterial categorization was. acknowledged by Cook and Meyers, (2003), Gernert *et al.* (2005), and Zhang *et al.* (2006).

### 6.1.2 Marine sponges of South Australia are sources of highly diverse and novel bacteria

South Australian marine environments are a rich source of highly abundant and endemic sponge species. However, there is little information about the symbiont bacteria associated with these sponges. The approaches used in this study, culturing of the multiple numbers of plates, placing a large fraction of sponge samples and long periods of incubation (unlike to other related studies), may be contributed for isolation of a large number of bacterial populations with various diversity. This study reported about 57% uncommon genera including *Fictibacillus, Falsibacillus, Isoptericola, Limimaricola, Muricuda, Mycolicibacterium, Janibacter, Microbacterium, Leisingera, Rhodovulum, Sulfitobacter,* and *Pseudonocardia,* where half of these uncommon genera described for the first time from marine sponges. Several related studies reported uncommon bacteria genera from marine sponges (Lafi *et al.*, 2005; Kennedy *et al.*, 2009; Abdelmohsen *et al.*, 2010; Li *et al.*, 2011; Flemer *et al.*, 2012; Margassery *et al.*, 2012; Graça *et al.*, 2013; Graça *et al.*,

2015; Ellis *et al.*, 2017; Indraningrat *et al.*, 2019; Kuo *et al.*, 2019), but none report the amount this study reported. Incubating culture for longer period was found as one of the determinant factors for isolation of rare bacteria (Davis *et al.*, 2011; Kaewkla and Franco, 2013; Pulschen *et al.*, 2017) and its importance further strengthen by failure to report slow-growing Actinobacteria by Bibi *et al.*, (2018), where the culture incubate for short duration. Plating large volume of the original samples coupled with longer incubation expected to increase the chance of more bacteria isolation, though the former observation was not supported with related studies.

The further genotypic analysis supported a significant percentage (29%) of sequenced bacterial isolates could be a potential candidate for subsequent novelty studies. The polyphasic studies of the selected bacterial isolates confirmed three novel bacterial species: *Isoptericola rapidicum* L4O, *Muricauda gelenilca* GB37, and *Mycolicibacterium rapidicum* LC1. Taking into consideration that only a small portion of the bacterial isolates (16%) were sequenced, the actual number of potential novel isolates could be more than what is reported here. Several scholars such as Abdelmohsen *et al.* (2010), Ahn *et al.* (2011), and Afonso de Menezes *et al.* (2017) reported rare and novel bacteria from marine sponges and signify the source, marine sponges, is an important area to concentrate for search the search of novel microbes that could be a source for new metabolites.

The most significant observation is more than half of the isolates belong to phylum Actinobacteria consisting of 9 genera (42.8%). Moreover, they isolated more from Rapid Bay compared to Glenelg Block, and genus *Streptomyces* only isolated from the former site. Since these groups of bacteria are known for their secondary metabolite production with multiple biological activities, finding them in high numbers signifies the host sponges could be a potential target for the discovery of novel biologically active metabolites. Considering the observed chemical variation among sponges of South Australia as stated by Sorokin *et al.* (2007) and Sorokin & Currie, (2008), it is reasonable to observe disparity among symbionts, which could be influenced by the nature of the hosts. Observation from several related studies ( Hentschel *et al.*, 2001; Lafi *et al.*, 2005;.Kennedy *et al.*, 2009; Margassery *et al.*, 2012; Flemer *et al.*, 2012; Bibi *et al.*, 2018; Kuo *et al.*, 2019) indicated the composition of bacteria population significantly varied, and thus require further investigation to reveal the reason behind this variation.

### 6.1.3 Isolation of bacterial genera not reported by Next generation sequencing

Comparison of culture-dependent and culture-independent bacterial profile for one sponge species revealed an interesting profile. Though numerous genera which were reported by NGS were not cultured, culture-dependent approach resulted in a significant number of bacteria genera not observed by NGS including *Gordonia*, *Kocuria*, *Isoptericola*, *Janibacter*, *Micrococcus*, *Mycolicibacterium*, *Rhodococcus*, *Muricauda*, *Bacillus*, and *Sulfitobacter*. Only 6 genera reported by culturing approach were also observed by NGS. This is one of the areas which need further investigation why NGS failed to report all genera isolated by culture-dependent approach.

### 6.1.4 The role of media, sponge sources, incubation period and NaCl for isolation of highly diverse bacteria from marine sponges

This study identified media-dependent variation in bacterial abundance and diversity. Low nutrient containing medium such as SWA medium yielded only a limited isolate with low diversity. The importance of trace elements in the medium was acknowledged by isolation of high number and diversity of bacteria using ASP and HV media compared to MA, TSA, SYP, and NA. This indicated in addition to common nutrients such as source of carbon and nitrogen, media with various supplements are essential to improve the yield of bacteria isolated from marine sponges.

One of the striking observations of this study is the huge difference in genus diversity among sponge samples collected from the two sources. Isolates from Rapid Bay were dominated by Actinobacteria and genera such as *Streptomyces, Rhodococcus, Mycolicibacterium, Janibacter, Muricauda, Isoptericola,* and *Pseudoalteromonas* were only isolated from this site. Apart from *Fictibacillus*, all the isolated genera from Glenelg were also detected from Rapid Bay. The finding may indicate the importance of source selection before doing large scale studies. It would also be a neat ecological study to determine the cause of the differences in the diversity between the two sampling sites. Furthermore, significant variations in genera were observed within different sponge species from the same location as well as among the same species from the same site. For example, sponge *Aplysilla sulfurea* (RB 16) displayed significant genera diversity compared to other sponge species from the same location. Similarly, sponge GB 08 (*Chondrosida* sp.) showed more genera diversity compared to the other two species of *Chondrosida* spp. (GB 21 and GB023) which were collected from a similar site.

This study showed the importance of maintaining a sufficiently long incubation period to reflect the true picture of the bacterial population in marine sponges. The finding indicated that if the isolation plates were incubated for a short period (less than a month), less than half of the genera in sponge would be identified, mostly dominated by non-actinobacteria genera. Most of the less common bacterial genera, including two of the three novel bacteria reported in this study, were isolated after one month of incubation and incubation periods of at least nine weeks were necessary to isolate all possible genera.

Although all the bacteria were isolated in the presence of at least 2% NaCl or ½ strength seawater, when tested for growth in the presence of NaCl at least 58% were able to grow in its absence. For the isolation of bacteria, better bacterial growth was observed in natural seawater compared to water containing NaCl. This indicated the presence of some factors beyond saltiness which determine the successful isolation of bacteria from marine sponges.

### 6.1.5 Some of the strains could produce antimicrobials effective against human pathogens

This study showed several bacterial isolates (41% of those screened) produce metabolites active against bacteria and fungi. The antimicrobial activity was not evenly observed across the genera and the genus *Streptomyces* spp. were the predominant producers of antibiotics. 73.6% of the tested *Streptomyces* showed activities against susceptible *S. aureus*, 44.4% against MRSA, 48.6% anti-candida and 50% against at least one dermatophytic fungus. Media selection is essential for antibiotic production as some media, such as MS and ISP2, favour production while SI and YEA did not. Furthermore, this study identified that antibiotic production started after a defined period of incubation. Most of them produce antibiotics after 3 days employing a seed medium to inoculate the production medium and the activities increased until seven days of incubation. This signifies the importance of having correct medium ingredients and incubation times for optimum production of antibiotics. Purification and identification of a compound from *Streptomyces* strain RB27 indicated the antibiotic could be novel. Further study is required to formulate its complete structure and analysis of more strains for the search of novel antibiotics.

## 6.2 Future directions

### 6.2.1 Identifying factors that determine variation in bacterial diversity among sponges

This study reported a marked difference in the diversity of bacteria among sponge samples collected from different sites. It is worthwhile to look for specific factors within sponges that favour more diversity of their bacteria such as the presence of molecules and the source of nutrient in those sponge which support higher diversity. This will be achieved by a side-by-side comparison of sponge with high and low diversity for their chemical compositions. Once the chemical composition identified, further study should follow to determine which chemical influence more for bacteria diversity. The findings will help to design better isolation strategies to increase the chance of isolating more diverse bacteria from marine sponges.

### 6.2.2 Fungal diversity from a marine sponge of South Australia

A potential new fungal genus was identified during the course of this study despite the use of antifungal antibiotics in the isolation media. Of the 12 fungal morphological forms, the one which showed a large cell type was found to be a novel genus. This data indicated the sponge samples from the area could be a source of different fungal species. Since most studies focus on the diversity of bacteria from sponges, it would be advisable to consider fungal diversity in sponges as fungi are a good source of secondary metabolites with various biological activities (Jensen, 2002; Bugni & Ireland, 2004; Wang, 2006; Blunt *et al.*, 2015). This fungal study should consider different factors affecting diversity and complete polyphasic taxonomy of the novel isolates which can be used as a baseline for further related studies.

### 6.2.3 More novel species

The preliminary 16S rRNA gene analysis of selected bacterial isolates in this study indicated many bacterial isolates could be potential candidates for novelty. The study characterised three novel species(two Actinobacteria and one Bacteroidetes) to confirm this observation. There are other bacteria isolates in Table 3.3 Chapter 3 (9 Actinobacteria, 3 Firmicutes, and 4 Proteobacteria) that require further characterization to confirm their novelty. This is essential because the chance of getting biologically active compounds is higher from new isolates as observed by Abdelmohsen *et al.* (2010).

### 6.2.4 Comparison with 'molecular' diversity

In chapter 3, four bacterial phyla and 21 genera from sponges samples from two sites were described. Sponge microbiome study with next generation sequencing from these sites revealed the presence of a larger diversity in the sponge community (Yang *et al*., 2019a). It identified bacterial genera that are culturable but were not successfully cultured in this study, therefore, better isolation strategies need to be developed. This includes the incorporation of molecules and nutrients available in sponge into the medium, long time of incubation and culturing of bacteria in a liquid medium. This study observed genera which were successfully isolated failed to be observed by NGS. Further baseline studies need to be undertaken to understand the discrepancies between isolation and NGS.

### 6.2.5 Screening bacteria for various biological activities

This study reported a considerable number of bacterial isolates with anti-bacterial and anti-fungal activities. For example, colleagues in my department screened some bacterial strains from this study for herbicidal and anticancer activity. Further detailed analysis is required to provide insight into their antimicrobial, anticancer and enzymatic activities. In addition, bacterial strains should be screened for biocatalyst where Actinobacteria demonstrated to act as whole-cell biocatalyst (Anteneh & Franco, 2019). To support this role further, this study demonstrated lipid production with the help of fungus (Section 4.3.5.3.5).

### 6.2.6 Purification and structural elucidation of antimicrobial agents

It was decided to purify the antibiotic from a single strain (RB27) based on its activity against MRSA. Several strains also showed inhibitory activities against bacteria and dermatophytic fungi these require further purification and structural determination to check their novelty. The chances of getting new compounds are higher these days from marine environments compared to the terrestrial samples.

### 6.2.7 Comparison study for a compound profile between sponge host and symbiont bacteria

Cultivation of sponges in the laboratory is more difficult compared to symbiont microbes. It was hypothesized that most of the metabolites reported from sponges are derived from the symbiont (Chapter 1 section 1.4). It would be of interest to compare the metabolite profile from the host

sponge and symbiont side by side. This should help to reach a decision on which to select, sponge or symbiont microbes, for future metabolite search studies.

# 7 References

Acharya, T. (2015). Oxidase test: Principle Procedure and oxidase positive organisms.

Abdelmohsen, U. R., Bayer, K., & Hentschel, U. (2014). Diversity, abundance and natural products of marine sponge-associated actinomycetes. *Nat Prod Rep,* 31(3), 381-399.

Abdelmohsen, U. R., Pimentel-Elardo, S. M., Hanora, A., Radwan, M., Abou-El-Ela, S. H., Ahmed, S., & Hentschel, U. (2010). Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated Actinomycetes. *Mar Drugs,* 8(3), 399-412.

Abdjul, D. B., Yamazaki, H., Ukai, K., & Namikoshi, M. (2015). Two new indole derivatives from a marine sponge *Ircinia* sp. collected at Iriomote Island. *J. Nat. Med.,* 69(3), 416-420.

Adekambi, T., Berger, P., Raoult, D., & Drancourt, M. (2006). *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. *Int J Syst Evol Microbiol,* 56(1), 133-143.

Adekambi, T., & Drancourt, M. (2004). Dissection of phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sodA*, *recA* and *rpoB* gene sequencing. *Int J Syst Evol Microbiol,* 54(6), 2095-2105.

Afonso de Menezes, C. B., Afonso, R. S., Souza, W. R., Parma, M., Melo, I. S., Zucchi, T. D., & Fantinatti-Garboggini, F. (2017). *Williamsia spongiae* sp. nov., an Actinomycete isolated from the marine sponge *Amphimedon viridis*. *Int J Syst Evol Microbiol,* 67(5), 1260-1265.

Aguilera-Cogley, V. A., Berbegal, M., Català, S., Brentu, F. C., Armengol, J., & Vicent, A. (2017). Characterization of *Mycosphaerellaceae* species associated with citrus greasy spot in Panama and Spain. *PloS one, 12*(12), e0189585.

Ahn, J., Park, J., McConnell, J. A., Ahn, Y., & Häggblom, M. M. (2011). *Kangiella spongicola* sp. nov., a halophilic marine bacterium isolated from the sponge *Chondrilla nucula*. *Int J Syst Evol Microbiol, 61*(4), 961-964.

Alain, K., & Querellou, J. (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles,* 13(4), 583-594.

Amend, A. S., Barshis, D. J., & Oliver, T. A. (2012). Coral-associated marine fungi form novel lineages and heterogeneous assemblages. *ISME Jj,* 6(7), 1291.

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Front Microbiol,* 1, 134.

Anteneh, Y. S., & Franco, C. M. M. (2019). Whole cell actinobacteria as biocatalysts. *Front. Microbiol.,* 10(77).

Arthur, M., & Courvalin, P. (1993). Genetics and mechanisms of glycopeptide resistance in enterococci. *Antimicrob. Agents Chemother.*, 37(8), 1563.

Arun, A. B., Chen, W. M., Lai, W. A., Chao, J. H., Rekha, P. D., Shen, F. T., & Young, C. C. (2009). *Muricauda lutaonensis* sp. nov., a moderate thermophile isolated from a coastal hot spring. *Int J Syst Evol Microbiol,* 59(11), 2738-2742.

Ayyad, S.-E. N., Katoua, D. F., Alarif, W. M., Sobahi, T. R., Aly, M. M., Shaala, L. A., & Ghandourah, M. A. (2015). Two new polyacetylene derivatives from the Red Sea sponge *Xestospongia* sp. *Zeitschrift für Naturforschung C,* 70(11-12), 297-303.

Babadi, Z. K., Sudarman, E., Ebrahimipour, G. H., Primahana, G., Stadler, M., & Wink, J. (2020). Structurally diverse metabolites from the rare actinobacterium *Saccharothrix xinjiangensis*. *J. Antibiot.,* 73(1), 48-55.

Bae, J.-W., Rhee, S.-K., Park, J. R., Kim, B.-C., & Park, Y.-H. (2005). Isolation of uncultivated anaerobic thermophiles from compost by supplementing cell extract of *Geobacillus toebii* in enrichment culture medium. *Extremophiles,* 9(6), 477-485.

Baig, U., Dahanukar, N., Shintre, N., Holkar, K., Pund, A., Lele, U., Watve, M. (2020). Phylogenetic diversity and activity screening of cultivable actinobacteria isolated from marine sponges and associated environments from the western coast of India. *bioRxiv*, 2020.2001.2009.901108.

Bass, D., Howe, A., Brown, N., Barton, H., Demidova, M., Michelle, H.,& Willcock, S. (2007). Yeast forms dominate fungal diversity in the deep oceans. *Proc. R. Soc. B,* 274(1629), 3069-3077.

Bayer, K., Schmitt, S., & Hentschel, U. (2008). Physiology, phylogeny and in situ evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environ Microbiol,* 10(11), 2942-2955.

Bell, J. J. (2008). The functional roles of marine sponges. Estuar Coast Shelf Sci*, 79*(3), 341-353.

Berd, D. (1973). Laboratory identification of clinically important aerobic actinomycetes. *Appl Microbiol,* 25(4), 665-681.

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. *Experientia,* 52(7), 716-722.

Bhalla, G. S., Sarao, M. S., Kalra, D., Bandyopadhyay, K., & John, A. R. (2018). Methods of phenotypic identification of non-tuberculous mycobacteria. *Pract. Lab. Med., 12*, e00107-e00107.

Bibi, F., Alvi, S. A., Al-Sofyani, A., Yasir, M., Kensarah, E. A., & Azhar, E. I. (2018). Two marine sponges-associated cultivable bacteria: Diversity and biological activities. *Genet. Mol. Biol.,* 17(2).

Bibi, F., Faheem, M., Azhar, E. I., Yasir, M., Alvi, S. A., Kamal, M. A., & Naseer, M. I. (2017). Bacteria from marine sponges: A source of new drugs. *Curr. Drug Metab.,*18(1), 11-15.

Bloom, B. R. (1994). Tuberculosis: pathogenesis, protection, and contro*l*: ASM press.

Blunt, J. W., Carroll, A. R., Copp, B. R., Davis, R. A., Keyzers, R. A., & Prinsep, M. R. (2018). Marine natural products. *Nat Prod Rep,* 35(1), 8-53.

Blunt, J. W., Copp, B. R., Hu, W.-P., Munro, M., Northcote, P. T., & Prinsep, M. R. (2009). Marine natural products. *Nat. Prod. Rep.,* 26(2), 170-244.

Blunt, J. W., Copp, B. R., Hu, W.-P., Munro, M., Northcote, P. T., & Prinsep, M. R. (2007). Marine natural products. *Nat. Prod. Rep.,* 24(1), 31-86.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., & Prinsep, M. R. (2015). Marine natural products. *Nat. Prod. Rep.,* 32(2), 116-211.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., & Prinsep, M. R. (2013). Marine natural products. *Nat. Prod. Rep, 30*(2), 237-323.

Blunt, J. W., Copp, B. R., Munro, M. H., Northcote, P. T., & Prinsep, M. R. (2004). Marine natural products. *Nat. Prod. Rep.,* 21(1), 1-49.

Bollmann, A., Lewis, K., & Epstein, S. S. (2007). Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Appl. Environ. Microbiol.,* 73(20), 6386-6390.

Bollmann, A., Palumbo, A. V., Lewis, K., & Epstein, S. S. (2010). Isolation and physiology of bacteria from contaminated subsurface sediments. *Appl. Environ. Microbiol.,* 76(22), 7413-7419.

Bose, U., Ortori, C. A., Sarmad, S., Barrett, D. A., Hewavitharana, A. K., Hodson, M. P., Shaw, P. N. (2017). Production of N-acyl homoserine lactones by the sponge-associated marine actinobacteria *Salinispora arenicola* and *Salinispora pacifica*. *FEMS microbiology letters, 364*(2).

Brady, S. F., Simmons, L., Kim, J. H., & Schmidt, E. W. (2009). Metagenomic approaches to natural products from free-living and symbiotic organisms. *Nat Prod Rep,* 26(11), 1488-1503.

Brenner, D. J., Fanning, G. R., Rake, A. V., & Johnson, K. E. (1969). Batch procedure for thermal elution of DNA from hydroxyapatite. *Anal Biochem*, *28*, 447-459.

Bruns, A., Cypionka, H., & Overmann, J. (2002). Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. *Appl. Environ. Microbiol.,* 68(8), 3978-3987.

Bruns, A., Rohde, M., & Berthe-Corti, L. (2001). *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. *Int J Syst Evol Microbiol,* 51(6), 1997-2006.

Bugni, T. S., & Ireland, C. M. (2004). Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod. Rep.,* 21(1), 143-163.

Bull, A. T., & Stach, J. E. (2007). Marine actinobacteria: new opportunities for natural product search and discovery. *Trends* Microbiol.,15(11), 491-499.

Burja, A. M., & Hill, R. T. (2001). Microbial symbionts of the Australian Great Barrier reef sponge, *Candidaspongia flabellata*. *Hydrobiologia,* 461(1-3), 41-47.

Button, D., Schut, F., Quang, P., Martin, R., & Robertson, B. R. (1993). Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl. Environ. Microbiol.,* 59(3), 881-891.

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., & Knight, R. (2010). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics,* 26(2), 266-267.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J.,Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci., 108* (Supplement 1), 4516-4522.

Chaiya, L., Matsumoto, A., Wink, J., Inahashi, Y., Risdian, C., Pathom-Aree, W., & Lumyong, S. (2019). *Amycolatopsis eburnea* sp. nov., an actinomycete associated with arbuscular mycorrhizal fungal spores. *Int J Syst Evol Microbiol,* 69(11), 3603-3608.

Chambers, H. F., & Deleo, F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol*., 7(9), 629-641.

Chen, J., Xing, X.-K., Zhang, L.-C., Xing, Y.-M., & Guo, S.-X. (2012). Identification of *Hortaea werneckii* Isolated from mangrove plant *Aegicerascomiculatum* based on morphology and rDNA sequences. *Mycopathologia,* 174(5-6), 457-466.

Cheng, Z.-B., Xiao, H., Fan, C.-Q., Lu, Y.-N., Zhang, G., & Yin, S. (2013). Bioactive polyhydroxylated sterols from the marine sponge *Haliclona crassiloba*. *Steroids,* 78(14), 1353-1358.

Choi, K. D., Lee, G. E., & Park, J. S. (2018). *Aquimarina spongiicola* sp. nov., isolated from spongin. *Int J Syst Evol Microbiol,* 68(4), 990-994.

Cita, Y. P., Suhermanto, A., Radjasa, O. K., & Sudharmono, P. (2017). Antibacterial activity of marine bacteria isolated from sponge *Xestospongia testudinaria* from Sorong, Papua. *Asian Pac. J. Trop. Biomed.,* 7(5), 450-454.

Claverias, F. P., Undabarrena, A., Gonzalez, M., Seeger, M., & Camara, B. (2015). Culturable diversity and antimicrobial activity of Actinobacteria from marine sediments in Valparaiso bay, Chile. *Front Microbiol,* 6, 737.

Coenye, T., & Vandamme, P. (2004). Use of the genomic signature in bacterial classification and identification. *Syst Appl Microbiol,* 27(2), 175-185.

Cook, A. E., & Meyers, P. R. (2003). Rapid identification of filamentous actinomycetes to the genus level using genus-specific 16S rRNA gene restriction fragment patterns. *Int J Syst Evol Microbiol,* 53(6), 1907-1915.

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *Int. J. Med. Microbio*l*,* 303(6-7), 287-292.

Croué, J., West, N. J., Escande, M.-L., Intertaglia, L., Lebaron, P., & Suzuki, M. T. (2013). A single betaproteobacterium dominates the microbial community of the crambescidine-containing sponge Crambe crambe. *Sci. Rep.,* 3, 2583.

Crous, P. W., Braun, U., Schubert, K., & Groenewald, J. Z. (2007). Delimiting *Cladosporium* from morphologically similar genera. S*tud Mycol.,* 58, 33-56.

Crous, P. W., Schoch, C., Hyde, K., Wood, A., Gueidan, C., De Hoog, G., & Groenewald, J. (2009a). Phylogenetic lineages in the Capnodiales. S*tud Mycol.,* 64, 17-47.

Crous, P. W., Summerell, B. A., Carnegie, A. J., Wingfield, M. J., & Groenewald, J. Z. (2009b). Novel species of *Mycosphaerellaceae* and *Teratosphaeriaceae*. *Persoonia,* 23, 119-146.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., & Lewis, K. (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem*. Biol*.,* 17(3), 254-264.

Dai, H.-F., Edrada, R. A., Ebel, R., Nimtz, M., Wray, V., & Proksch, P. (2005). Norlanostane triterpenoidal saponins from the marine sponge *Melophlus sarassinorum*. *J. Nat. Prod.,* 68(8), 1231-1237.

Damiani, G., Bandi, C., Sironi, M., Magrassi, L., & Fani, R. (1997). CircumVent® thermal cycle sequencing of eubacterial 16S ribosomal DNAs. *NEB Transcript, 8*, 15.

Dantas, G., & Sommer, M. O. (2012). Context matters—the complex interplay between resistome genotypes and resistance phenotypes. *Curr Opin Microbiol,* 15(5), 577-582.

Davies, J., & Davies, D. (2010). Resistance origins and evolution of antibiotic. Microbiology and Molecular Biology reviews*. Microbiol Mol Biol Rev,* 74(3), 417-433.

Davis, K. E., Joseph, S. J., & Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl Environ Microbiol,* 71(2), 826-834.

Davis, K. E., Sangwan, P., & Janssen, P. H. (2011). Acidobacteria, Rubrobacteridae and Chloroflexi are abundant among very slow-growing and mini-colony-forming soil bacteria. *Environ. Microbiol.,* 13(3), 798-805.

de Goeij, J. M., van den Berg, H., van Oostveen, M. M., Epping, E. H., & Van Duyl, F. C. (2008). Major bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavity sponges. *Mar. Ecol.,* 357, 139-151.

De Ley, J., Cattoir, H., & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem,* 12(1), 133-142.

de Menezes, C. B., Tonin, M. F., Silva, L. J., de Souza, W. R., Parma, M., de Melo, I. S.,& Fantinatti-Garboggini, F. (2015). *Marmoricola aquaticus* sp. nov., an actinomycete isolated from a marine sponge. *Int J Syst Evol Microbiol,* 65(7), 2286-2291.

Debitus, C., Guella, G., Mancini, I., Waikedre, J., Guemas, J.-P., Nicolas, J. L., & Pietra, F. (1998). Quinolones from a bacterium and tyrosine metabolites from its host sponge, *Suberea creba* from the Coral Sea. *J. Mar. Biotechnol.,* 6(3), 136-141.

Devi, P., Wahidullah, S., Rodrigues, C., & Souza, L. D. (2010). The Sponge-associated Bacterium *Bacillus licheniformis* SAB1: A Source of Antimicrobial Compounds. *Mar. Drugs,* 8(4), 1203-1212.

Ding, B., Yin, Y., Zhang, F., & Li, Z. (2011). Recovery and phylogenetic diversity of culturable fungi associated with marine sponges *Clathrina luteoculcitella* and *Holoxea* sp. in the South China Sea. *Mar. Biotechnol.,* 13(4), 713-721.

Dona, A. C., Kyriakides, M., Scott, F., Shephard, E. A., Varshavi, D., Veselkov, K., & Everett, J. R. (2016). A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments. *Comput Struct Biotechnol J,* 14, 135-153.

Doroghazi, J. R., & Metcalf, W. W. (2013). Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes. *BMC genomics, 14*, 611-611.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science,* 308(5728), 1635-1638.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics, 26*(19), 2460-2461.

Ekiz, G., Hames, E. E., Demir, V., Can, F., Yokes, M. B., Uzel, A., & Bedir, E. (2014). Cultivable sponge-associated *Actinobacteria* from coastal area of Eastern Mediterranean Sea. *Adv Microbiol,* 4, 306-316.

El-Gendy, M. M., & El-Bondkly, A. M. (2010). Production and genetic improvement of a novel antimycotic agent, saadamycin, against dermatophytes and other clinical fungi from endophytic *Streptomyces* sp. Hedaya48. *J Ind Microbiol Biotechnol,* 37(8), 831-841.

Ellis, G. A., Thomas, C. S., Chanana, S., Adnani, N., Szachowicz, E., Braun, D. R., Bugni, T. S. (2017). Brackish habitat dictates cultivable Actinobacterial diversity from marine sponges. *PloS one,* 12(7), e0176968.

Engelbrektson, A., Kunin, V., Wrighton, K. C., Zvenigorodsky, N., Chen, F., Ochman, H., & Hugenholtz, P. (2010). Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *ISME J,* 4(5), 642.

Ereskovsky, A. V., Gonobobleva, E., & Vishnyakov, A. (2005). Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini Johnston* (Porifera, Demospongiae, Halisarcida). *Mar. Biol.,* 146(5), 869-875.

Erwin, D. H., Laflamme, M., Tweedt, S. M., Sperling, E. A., Pisani, D., & Peterson, K. J. (2011). The Cambrian conundrum: early divergence and later ecological success in the early history of animals. *Science,* 334(6059), 1091-1097.

Fajardo, A., Martínez-Martín, N., Mercadillo, M., Galán, J. C., Ghysels, B., Matthijs, S., & Martínez, J. L. (2008). The neglected intrinsic resistome of bacterial pathogens. *PloS one*, 3(2).

Faulkner, D. J. (2002). Marine natural products. *Nat Prod Rep,* 19(1), 1-48.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* 39(4), 783-791.

Ferrari, B. C., Binnerup, S. J., & Gillings, M. (2005). Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. *Appl. Environ. Microbiol.,* 71(12), 8714-8720.

Ferrari, B. C., & Gillings, M. R. (2009). Cultivation of fastidious bacteria by viability staining and micromanipulation in a soil substrate membrane system. *Appl Environ Microbiol,* 75(10), 3352-3354.

Ferrari, B. C., Winsley, T., Gillings, M., & Binnerup, S. (2008). Cultivating previously uncultured soil bacteria using a soil substrate membrane system. *Nat Protoc,* 3(8), 1261-1269.

Fieseler, L., Horn, M., Wagner, M., & Hentschel, U. (2004). Discovery of the novel candidate phylum “Poribacteria” in marine sponges. *Appl Environ Microbiol,* 70(6), 3724-3732.

Fishovitz, J., Hermoso, J. A., Chang, M., & Mobashery, S. (2014). Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *IUBMB life,* 66(8), 572-577.

Flatt, P. M., Gautschi, J. T., Thacker, R. W., Musafija-Girt, M., Crews, P., & Gerwick, W. H. (2005). Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea* (*Lamellodysidea*) *herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis.*Mar. Biol*., 147(3), 761-774.

Flemer, B., Kennedy, J., Margassery, L., Morrissey, J., O'Gara, F., & Dobson, A. (2012). Diversity and antimicrobial activities of microbes from two Irish marine sponges, *Suberites carnosus* and *Leucosolenia* sp. *J. Appl. Microbiol.,* 112(2), 289-301.

Foster, T. J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future prospects. *FEMS Microbiol.Rev.,* 41(3), 430-449.

Freeman, M. F., Gurgui, C., Helf, M. J., Morinaka, B. I., Uria, A. R., Oldham, N. J., & Piel, J. (2012). Metagenome mining reveals polytheonamides as posttranslationally modified ribosomal peptides. *Science,* 338(6105), 387-390.

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 Microbiol Ecol,* 38(2-3), 105-113.

Fróes, A. M., Freitas, T. C., Vidal, L., Appolinario, L. R., Leomil, L., Venas, T.,& Thompson, C. C. (2018). Genomic attributes of novel symbiont *Pseudovibrio brasiliensis* sp. nov. Isolated from the sponge *Arenosclera brasiliensis*. *Front Mar Sci,* 5, 81.

Fuerst, J. A. (2014). Diversity and biotechnological potential of microorganisms associated with marine sponges. *Appl Microbiol Biotechnol,* 98(17), 7331-7347.

Galand, P. E., Casamayor, E. O., Kirchman, D. L., & Lovejoy, C. (2009). Ecology of the rare microbial biosphere of the Arctic Ocean. *PNAS USA,* 106(52), 22427-22432.

Gandhimathi, R., Arunkumar, M., Selvin, J., Thangavelu, T., Sivaramakrishnan, S., Kiran, G. S., Natarajaseenivasan, K. (2008). Antimicrobial potential of sponge associated marine actinomycetes. *Med. Mycol. J.,* 18(1), 16-22.

Gao, Z., Li, B., Zheng, C., & Wang, G. (2008). Molecular detection of fungal communities in the Hawaiian marine sponges *Suberites zeteki* and *Mycale armata*. *Appl Environ Microbiol,* 74(19), 6091-6101.

Gavrish, E., Bollmann, A., Epstein, S., & Lewis, K. (2008). A trap for in situ cultivation of filamentous *actinobacteria*. *J. Microbiol. Methods,* 72(3), 257-262.

Gazave, E., Lapébie, P., Renard, E., Vacelet, J., Rocher, C., Ereskovsky, A. V.,& Borchiellini, C. (2010). Molecular phylogeny restores the supra-generic subdivision of homoscleromorph sponges (Porifera, Homoscleromorpha). *PloS one,* 5(12), e14290.

Gernert, C., Glöckner, F. O., Krohne, G., & Hentschel, U. (2005). Microbial diversity of the freshwater sponge *Spongilla lacustris*. *Microb. Ecol.,* 50(2), 206-212.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., & Swings, J. (2005). Opinion: Re-evaluating prokaryotic species. *Nat. Rev. Microbiol.,* 3(9), 733-739.

Gillespie, D. E., Brady, S. F., Bettermann, A. D., Cianciotto, N. P., Liles, M. R., Rondon, M. R.,& Handelsman, J. (2002). Isolation of antibiotics turbomycin a and B from a metagenomic library of soil microbial DNA. *Appl Environ Microbiol,* 68(9), 4301-4306.

Gillis, M., De Ley, J. & De Cleene, M. (1970). The determination of molecular weight of bacterial genome DNA from renaturation rates. *Eur J Biochem* 12, 143–153.

Giovannoni, S., & Stingl, U. (2007). The importance of culturing bacterioplankton in the 'omics' age. *Nat. Rev. Microbiol.,* 5(10), 820-826.

Gogineni, V., & Hamann, M. T. (2018). Marine natural product peptides with therapeutic potential: Chemistry, biosynthesis, and pharmacology. *Biochim Biophys Acta Gen Subj,* 1862(1), 81-196.

Goldberg, S. R., Haltli, B. A., Correa, H., & Kerr, R. G. (2018). Description of *sansalvadorimonas verongulae* gen. nov., sp. nov., a gammaproteobacterium isolated from the marine sponge Verongula gigantea. *Int J Syst Evol Microbiol,* 68(6), 2006-2014.

Goodfellow, M., & Fiedler, H.-P. (2010). A guide to successful bioprospecting: informed by actinobacterial systematics. *Antonie Van Leeuwenhoek,* 98(2), 119-142.

Goodfellow, M., Manfio, G., & Chun, J. (1997). Towards a practical species concept for cultivable bacteria.

Gordon, R. E., Barnett, D. A., Handerhan, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the Nocardin strain. *Int J Syst Bacteriol* 24, 54–63

Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., & Tiedje, J. M. (2007). DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol,* 57(1), 81-91.

Graça, A. P., Bondoso, J., Gaspar, H., Xavier, J. R., Monteiro, M. C., de la Cruz, M., & Lage, O. M. (2013). Antimicrobial activity of heterotrophic bacterial communities from the marine sponge *Erylus discophorus* (*Astrophorida*, *Geodiidae*). *PloS one,* 8(11), e78992.

Graça, A. P., Viana, F., Bondoso, J., Correia, M. I., Gomes, L., Humanes, M., & Lage, O. M. (2015). The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge *Erylus deficiens* (*Astrophorida*, *Geodiidae*). *Front. Microbiol.,* 6, 389.

Groth, I., Schumann, P., Schutze, B., Gonzalez, J. M., Laiz, L., Saiz-Jimenez, C., & Stackebrandt, E. (2005). *Isoptericola hypogeus* sp. nov., isolated from the Roman catacomb of Domitilla. *Int J Syst Evol Microbiol,* 55(4), 1715-1719.

Guan, T. W., Teng, Y., Yang, L. L., Zhang, X. P., & Che, Z. M. (2013). *Isoptericola salitolerans* sp. nov., a halotolerant filamentous actinobacterium isolated from a salt lake, China. *Extremophiles,* 17(3), 471-476.

Gupta, A., Barrow, C. J., & Puri, M. (2012a). Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. *Biotechnol. Adv,* 30(6), 1733-1745.

Gupta, A., Singh, D., Barrow, C. J., & Puri, M. (2013). Exploring potential use of Australian thraustochytrids for the bioconversion of glycerol to omega-3 and carotenoids production. *Biochem. Eng. J., 78*, 11-17.

Gupta, A., Singh, D., Byreddy, A. R., Thyagarajan, T., Sonkar, S. P., Mathur, A. S., Puri, M. (2016). Exploring omega-3 fatty acids, enzymes and biodiesel producing thraustochytrids from Australian and Indian marine biodiversity. *Biotechnol. J,* 11(3), 345-355.

Gupta, A., Vongsvivut, J., Barrow, C. J., & Puri, M. (2012b). Molecular identification of marine yeast and its spectroscopic analysis establishes unsaturated fatty acid accumulation. *J. Biosci. Bioeng,* 114(4), 411-417.

Gutleben, J., Loureiro, C., Ramírez Romero, L. A., Shetty, S., Wijffels, R. H., Smidt, H., & Sipkema, D. (2020). Cultivation of Bacteria From *Aplysina aerophoba*: Effects of Oxygen and Nutrient Gradients. *Frontiers in Microbiology, 11*(175).

Haber, M., Shefer, S., Giordano, A., Orlando, P., Gambacorta, A., & Ilan, M. (2013a). *Aureivirga marina* gen. nov., sp. nov., a marine bacterium isolated from the Mediterranean sponge *Axinella verrucosa*. *Int J Syst Evol Microbiol,* 63(3), 1089-1095.

Haber, M., Shefer, S., Giordano, A., Orlando, P., Gambacorta, A., & Ilan, M. (2013b). *Luteivirga sdotyamensis* gen. nov., sp. nov., a novel bacterium of the phylum *Bacteroidetes* isolated from the Mediterranean sponge *Axinella polypoides*. *Int J Syst Evol Microbiol,* 63(3), 939-945.

Hacker, J., Hentschel, U., Friedrich, A. B., Fischer, I., & Proksch, P. (2001). Temporal variation of the microbial community associated with the mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol Ecol,* 38(2-3), 105-113.

Hagiwara, K., Garcia Hernandez, J. E., Harper, M. K., Carroll, A., Motti, C. A., Awaya, J., & Wright, A. D. (2015). Puupehenol, a potent antioxidant antimicrobial meroterpenoid from a Hawaiian deep-water *Dactylospongia* sp. sponge. *J. Nat. Prod.,* 78(2), 325-329.

Hamaki, T., Suzuki, M., Fudou, R., Jojima, Y., Kajiura, T., Tabuchi, A., & Shibai, H. (2005). Isolation of novel bacteria and actinomycetes using soil-extract agar medium. J *Biosci Bioeng,* 99(5), 485-492.

Hawksworth, D. L. (2001). The magnitude of fungal diversity: the 1· 5 million species estimate revisited. *Mycol. Res.,* 105(12), 1422-1432.

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. *Appl Environ Microbiol,* 68(9), 4431-4440.

Hentschel, U., Piel, J., Degnan, S. M., & Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nat. Rev. Microbiol.,* 10(9), 641-654.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., & Hacker, J. (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiol Ecol,* 35(3), 305-312.

Hentschel, U., Usher, K. M., & Taylor, M. W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiol Ecol,* 55(2), 167-177.

Hewavitharana, A., Shaw, P., Ng, Y., & Fuerst, J. (2009). Simple screening method for staurosporine in bacterial cultures using liquid chromatography-tandem mass spectrometry. *J Bioanal Biomed,* 1(1), 001-004.

Hicks, N., Liu, X., Gregory, R., Kenny, J., Lucaci, A., Lenzi, L., & Duncan, K. R. (2018). Temperature driven changes in benthic bacterial diversity influences biogeochemical cycling in coastal sediments. *Front Microbiol,* 9(1730).

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

Hoffmann, F., Larsen, O., Thiel, V., Rapp, H. T., Pape, T., Michaelis, W., & Reitner, J. (2005). An anaerobic world in sponges. *Geomicrobiol. J.,* 22(1-2), 1-10.

Hoffmann, M., Monday, S. R., Allard, M. W., Strain, E. A., Whittaker, P., Naum, M., & Brown, E. W. (2012). *Vibrio caribbeanicus* sp. nov., isolated from the marine sponge *Scleritoderma cyanea*. *Int J Syst Evol Microbiol,* 62(8), 1736-1743.

Hoffmann, F., Røy, H., Bayer, K., Hentschel, U., Pfannkuchen, M., Brümmer, F., & De Beer, D. (2008). Oxygen dynamics and transport in the Mediterranean sponge *Aplysina aerophoba*. *Mar. Biol,* 153(6), 1257-1264.

Hoffmann F, Radax R,Woebken D et al (2009) Complex nitrogen cycling in the sponge *Geodia barretti*. *Environ Microbiol* 11:2228–2243.

Höller, U., Wright, A. D., Matthee, G. F., Konig, G. M., Draeger, S., Hans-Jürgen, A., & Schulz, B. (2000). Fungi from marine sponges: diversity, biological activity and secondary metabolites. *Mycol. Res.,* 104(11), 1354-1365.

Hooper, J. N. (2000). Sponguide: guide to sponge collection and identification: Queensland museum.

Hoppers, A., Stoudenmire, J., Wu, S., & Lopanik, N. B. (2015). Antibiotic activity and microbial community of the temperate sponge, *Haliclona* sp. *J Appl Microbiol,* 118(2), 419-430.

Hooper, J., & van Soest, R. (2002). *Systema Porifera: A Guide to the Classification of Sponges* (Vol. 18).

Hopwood, D. A. (2007). *Streptomyces* in nature and medicine*:* the antibiotic makers: Oxford University Press.

Hu, G. P., Yuan, J., Sun, L., She, Z. G., Wu, J. H., Lan, X. J.,& Chen, S. P. (2011). Statistical research on marine natural products based on data obtained between 1985 and 2008. *Mar Drugs,* 9(4), 514-525.

Huang, X., Zhou, S., Huang, D., Chen, J., & Zhu, W. (2016). *Streptomyces spongiicola* sp. nov., An actinomycete derived from marine sponge. *Int J Syst Evol Microbiol,* 66(2), 738-743.

Huang, Z., Sheng, X. F., Zhao, F., He, L. Y., Huang, J., & Wang, Q. (2012). *Isoptericola nanjingensis* sp. nov., a mineral-weathering bacterium. *Int J Syst Evol Microbiol,* 62(4), 971-976.

Huck, T. A., Porter, N., & Bushell, M. E. (1991). Positive selection of antibiotic-producing soil isolates. *Microbiology,* 137(10), 2321-2329.

Hughes, C. C., & Fenical, W. (2010). Antibacterials from the sea. *Chemistry,* 16(42), 12512-12525.

Huppert, M., Harper, G., Sun, S. H., & Delanerolle, V. (1975). Rapid methods for identification of yeasts. *J Clin Microbiol,* 2(1), 21-34.

Huss, V. A., Festl, H., & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol, 4*(2), 184-192.

Hwang, C. Y., Kim, M. H., Bae, G. D., Zhang, G. I., Kim, Y. H., & Cho, B. C. (2009). *Muricauda olearia* sp. nov., isolated from crude-oil-contaminated seawater, and emended description of the genus *Muricauda*. *Int J Syst Evol Microbiol,* 59( 8), 1856-1861.

Ilias, M., Ibrahim, M. A., Khan, S. I., Jacob, M. R., Tekwani, B. L., Walker, L. A., & Samoylenko, V. (2012). Pentacyclic ingamine alkaloids, a new antiplasmodial pharmacophore from the marine sponge Petrosid Ng5 Sp5. *Planta medica,* 78(15), 1690-1697.

Indraningrat, A. A. G., Micheller, S., Runderkamp, M., Sauerland, I., Becking, L. E., Smidt, H., & Sipkema, D. (2019). Cultivation of sponge-associated bacteria from *Agelas sventres* and *Xestospongia muta* collected from different depths. *Mar. Drugs,* 17(10), 578.

Indraningrat, A. A. G., Smidt, H., & Sipkema, D. (2016). Bioprospecting sponge-associated microbes for antimicrobial compounds. *Mar. Drugs,* 14(5), 87.

Ingham, C. J., Sprenkels, A., Bomer, J., Molenaar, D., van den Berg, A., van Hylckama Vlieg, J. E., & de Vos, W. M. (2007). The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. *Proc Natl Acad Sci USA,* 104(46), 18217-18222.

Iniyan, A. M., Sudarman, E., Wink, J., Kannan, R. R., & Vincent, S. G. P. (2019). Ala-geninthiocin, a new broad spectrum thiopeptide antibiotic, produced by a marine *Streptomyces* sp. ICN19. *J. Antibiot,* 72(2), 99-105.

Jackson, C. R., Randolph, K. C., Osborn, S. L., & Tyler, H. L. (2013). Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol,* 13(1), 274.

Jackson, S. A., Kennedy, J., Morrissey, J. P., O'Gara, F., & Dobson, A. D. W. (2015). *Maribacter spongiicola* sp. nov. and *Maribacter vaceletii* sp. nov., isolated from marine sponges, and emended description of the genus *Maribacter*. *Int J Syst Evol Microbiol,* 65(7), 2097-2103.

Jang, H., Yang, S. H., Seo, H. S., Lee, J. H., Kim, S. J., & Kwon, K. K. (2015). *Amphritea spongicola* sp. nov., isolated from a marine sponge, and emended description of the genus *Amphritea*. *Int J Syst Evol Microbiol,* 65(6), 1866-1870.

Jayatilake, G. S., Thornton, M. P., Leonard, A. C., Grimwade, J. E., & Baker, B. J. (1996). Metabolites from an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *J Nat Prod,* 59(3), 293-296.

Jensen, P. (2002). Secondary metabolites from marine fungi. *Fungi in marine environments*, 293-315.

Jensen, P. R., Williams, P. G., Oh, D.-C., Zeigler, L., & Fenical, W. (2007). Species-specific secondary metabolite production in marine actinomycetes of the genus *Salinispora*. *Appl Environ Microbiol,* 73(4), 1146-1152.

Jeong, S. H., Yang, S. H., Jin, H. M., Kim, J. M., Kwon, K. K., & Jeon, C. O. (2013). *Microbulbifer gwangyangensis* sp. nov. and *Microbulbifer pacificus* sp. nov., isolated from marine environments. *Int J Syst Evol Microbiol,* 63(4), 1335-1341.

Jiang, S., Sun, W., Chen, M., Dai, S., Zhang, L., Liu, Y.,& Li, X. (2007). Diversity of culturable Actinobacteria isolated from marine sponge *Haliclona* sp. *Antonie Van Leeuwenhoek,* 92(4), 405-416.

Jones, E. G., Hyde, K. D., & Pang, K. L. (Eds.). (2014). Freshwater fungi: and fungal-like organisms. Walter de Gruyter GmbH & Co KG.

Kaeberlein, T., Lewis, K., & Epstein, S. S. (2002). Isolating" uncultivable" microorganisms in pure culture in a simulated natural environment. *Science,* 296(5570), 1127-1129.

Kaewkla.O (2009). Isolation and identification of endophytic actinobacteria from Australian native plants and their antimicrobial activity. PhD thesis, The Flinders University, Adelaide.

Kaewkla, O., & Franco, C. M. M. (2016). *Kribbella pittospori* sp. nov., an endophytic actinobacterium isolated from the surface-sterilized stem of an Australian native apricot tree, Pittosporum angustifolium. *Int J Syst Evol Microbiol,* 66(6), 2284-2290.

Kaewkla, O., & Franco, C. M. (2013). Rational approaches to improving the isolation of endophytic actinobacteria from Australian native trees. *Microb Ecol,* 65(2), 384-393.

Kaewkla, O., Thamchaipinet, A., & Franco, C. M. M. (2017). *Micromonospora terminaliae* sp. nov., an endophytic actinobacterium isolated from the surface-sterilized stem of the medicinal plant Terminalia mucronata. *Int J Syst Evol Microbiol,* 67(2), 225-230.

Kämpfer, P., Glaeser, S. P., Busse, H. J., Abdelmohsen, U. R., Ahmed, S., & Hentschel, U. (2015). *Actinokineospora spheciospongiae* sp. nov., isolated from the marine sponge *Spheciospongia vagabunda*. *Int J Syst Evol Microbiol,* 65(3), 879-884.

Kämpfer, P., Glaeser, S. P., Busse, H. J., Abdelmohsen, U. R., & Hentschel, U. (2014). *Rubrobacter aplysinae* sp. nov., isolated from the marine sponge *Aplysina aerophoba*. *Int J Syst Evol Microbiol,* 64(3), 705-709.

Kämpfer, P., Glaeser, S. P., Kloepper, J. W., Hu, C.-H., McInroy, J. A., Martin, K., & Busse, H.-J. (2016). *Isoptericola cucumis* sp. nov., isolated from the root tissue of cucumber (*Cucumis sativus*). *Int J Syst Evol Microbiol,* 66(8), 2784-2788.

Kaur, I., Das, A. P., Acharya, M., Klenk, H. P., Sree, A., & Mayilraj, S. (2012). *Planococcus plakortidis* sp. nov., isolated from the marine sponge *Plakortis simplex* (Schulze). *Int J Syst Evol Microbiol,* 62(4), 883-889.

Kaur, N., Rajendran, M. K., Kaur, G., & Shanmugam, M. (2014). *Isoptericola rhizophila* sp. nov., a novel actinobacterium isolated from rhizosphere soil. *Antonie Van Leeuwenhoek,* 106(2), 301-307.

Katahira, K., Ogura, Y., Gotoh, Y., & Hayashi, T. (2016). Draft genome sequences of five rapidly growing *Mycobacterium* Species, *M. thermoresistibile*, *M. fortuitum* subsp. *acetamidolyticum*, *M. canariasens*e, *M. brisbanense*, and *M. novocastrense*. *Genome Announc,* 4(3).

Kennedy, J., Baker, P., Piper, C., Cotter, P. D., Walsh, M., Mooij, M. J.,& Dobson, A. D. (2009). Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. *Mar Biotechnol,* 11(3), 384-396.

Kennedy, J., Margassery, L. M., O'Leary, N. D., O'Gara, F., Morrissey, J., & Dobson, A. D. W. (2014). *Aquimarina amphilecti* sp. nov., isolated from the sponge *Amphilectus fucorum*. *Int J Syst Evol Microbiol,* 64(2), 501-505.

Khan, S. T., Tamura, T., Takagi, M., & Shin-Ya, K. (2010). *Streptomyces tateyamensis* sp. nov., *Streptomyces marinus* sp. nov. and *Streptomyces haliclonae* sp. nov., isolated from the marine sponge *Haliclona* sp. *Int J Syst Evol Microbiol,* 60(12), 2775-2779.

Kim, T. K., Garson, M. J., & Fuerst, J. A. (2005). Marine actinomycetes related to the '*Salinospora*'group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environ. Microbiol.,* 7(4), 509-518.

Kim, J. M., Jin, H. M., & Jeon, C. O. (2013). *Muricauda taeanensis* sp. nov., isolated from a marine tidal flat. *Int J Syst Evol Microbiol,* 63(7), 2672-2677.

Kisand, V., & Wikner, J. (2003). Combining culture-dependent and-independent methodologies for estimation of richness of estuarine bacterioplankton consuming riverine dissolved organic matter. *Appl. Environ. Microbiol.,* 69(6), 3607-3616.

Kis-Papo, T., Oren, A., Wasser, S., & Nevo, E. (2003). Survival of filamentous fungi in hypersaline Dead Sea water. *Microb Ecol,* 45(2), 183-190.

Konjek, J., Souded, S., Guerardel, Y., Trivelli, X., Bernut, A., Kremer, L., & Heym, B. (2016). *Mycobacterium lutetiense* sp. nov., *Mycobacterium montmartrense* sp. nov. and *Mycobacterium arcueilense* sp. nov., members of a novel group of non-pigmented rapidly growing mycobacteria recovered from a water distribution system. *Int J Syst Evol Microbiol,* 66(9), 3694-3702.

König, G. M., Kehraus, S., Seibert, S. F., Abdel-Lateff, A., & Müller, D. (2006). Natural products from marine organisms and their associated microbes. *ChemBioChem,* 7(2), 229-238.

Konstantinidis, K. T., & Tiedje, J. M. (2007). Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. *Curr Opin Microbiol,* 10(5), 504-509.

Köpke, B., Wilms, R., Engelen, B., Cypionka, H., & Sass, H. (2005). Microbial diversity in coastal subsurface sediments: a cultivation approach using various electron acceptors and substrate gradients. *Appl. Environ. Microbiol.,* 71(12), 7819-7830.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol,* 33(7), 1870-1874.

Kunin, V., Copeland, A., Lapidus, A., Mavromatis, K., & Hugenholtz, P. (2008). A bioinformatician's guide to metagenomics. *Microbiol Mol Biol Rev,* 72(4), 557-578.

Kuo, J., Yang, Y. T., Lu, M. C., Wong, T. Y., Sung, P. J., & Huang, Y. S. (2019). Antimicrobial activity and diversity of bacteria associated with Taiwanese marine sponge Theonella swinhoei. *Ann. Microbiol*., 69(3), 253-265.

Kurtzman, C. P., Fell, J. W., Boekhout, T., & Robert, V. (2010). Methods for isolation, phenotypic characterization and maintenance of yeasts *The Yeasts (Fifth Edition)* (pp. 87-110): Elsevier.

Kurtzman, C. P., & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek,* 73(4), 331-371.

Kurup, P., & Schmitt, J. (1973). Numerical taxonomy of Nocardia. *Can. J. Microbiol.,* 19(8), 1035-1048.

Kwon, H. C., Kauffman, C. A., Jensen, P. R., & Fenical, W. (2006). Marinomycins A-D, antitumor-antibiotics of a new structure class from a marine actinomycete of the recently discovered genus "marinispora". *J Am Chem Soc,* 128(5), 1622-1632.

Lafi, F. F., Garson, M. J., & Fuerst, J. A. (2005). Culturable bacterial symbionts isolated from two distinct sponge species (*Pseudoceratina clavata* and *Rhabdastrella globostellata*) from the Great Barrier Reef display similar phylogenetic diversity. *Microb. Ecol*., *50*(2), 213-220.

Laich, F., Vaca, I., & Chavez, R. (2013). *Rhodotorula portillonensis* sp. nov., a basidiomycetous yeast isolated from Antarctic shallow-water marine sediment. *Int J Syst Evol Microbiol,* 63(10), 3884-3891.

Laport, M. S., Santos, O. C., & Muricy, G. (2009). Marine sponges: potential sources of new antimicrobial drugs. *Curr Pharm Biotechnol,* 10(1), 86-105.

Landwehr, W., Kampfer, P., Glaeser, S. P., Ruckert, C., Kalinowski, J., Blom, J., . . . Wink, J. (2018). Taxonomic analyses of members of the Streptomyces cinnabarinus cluster, description of *Streptomyces cinnabarigriseus* sp. nov. and *Streptomyces davaonensis* sp. nov. *Int J Syst Evol Microbiol, 68*(1), 382-393.

Lane, D. (1991). 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*, 115-175.

Larkin, M. A., Blackshields, G., Brown, N., Chenna, R., McGettigan, P. A., McWilliam, H.,& Lopez, R. (2007). Clustal W and Clustal X version 2.0. *J*. *Bioinform,* 23(21), 2947-2948.

Lazzarini, A., Cavaletti, L., Toppo, G., & Marinelli, F. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie Van Leeuwenhoek,* 78(3-4), 399-405.

Lee, G. E., Im, W. T., & Park, J. S. (2018). *Bacillus spongiae* sp. nov., isolated from sponge of Jeju Island. *J Microbiol ,* 56(4), 217-222.

Lee, Y. K., Lee, J.-H., & Lee, H. K. (2001). Microbial symbiosis in marine sponges. *J Microbiol ,* 39(4), 254-264.

Lee, O. O., Lai, P. Y., Wu, H., Zhou, X., Miao, L., Wang, H., & Qian, P. Y. (2012a). *Marinobacter xestospongiae* sp. nov., isolated from the marine sponge *Xestospongia testudinaria* collected from the Red sea. *Int J Syst Evol Microbiol,* 62(8), 1980-1985.

Lee, S. Y., Park, S., Oh, T. K., & Yoon, J. H. (2012b). *Muricauda beolgyonensis* sp. nov., isolated from a tidal flat. *Int J Syst Evol Microbiol,* 62(5), 1134-1139.

Lee, O. O., Wang, Y., Yang, J., Lafi, F. F., Al-Suwailem, A., & Qian, P. Y. (2011). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *Isme j,* 5(4), 650-664.

Lehmann, K. B., & Neumann, R. O. (1896). *Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik*. München: J.F. Lehmann.

Lertcanawanichakul, M., & Sawangnop, S. (2008). A comparison of two methods used for measuring the antagonistic activity of *Bacillus* species. *Walailak* J. Sci. & *Tech*., 5(2), 161-171.

Li, C.-Q., Liu, W.-C., Zhu, P., Yang, J.-L., & Cheng, K.-D. (2011). Phylogenetic diversity of bacteria associated with the marine sponge *Gelliodes carnosa* Collected from the Hainan Island Coastal Waters of the South China Sea. *Microbial ecology,* 62(4), 800-812.

Li, H., Luo, Y.-F., Williams, B. J., Blackwell, T. S., & Xie, C.-M. (2012). Structure and function of OprD protein in *Pseudomonas aeruginosa*: from antibiotic resistance to novel therapies. *Int. J. Med. Microbiol,* 302(2), 63-68.

Li, Q., & Wang, G. (2009). Diversity of fungal isolates from three Hawaiian marine sponges. *Microbiol. Res*., 164(2), 233-241.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P.,& Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature,* 517(7535), 455-459.

Liu, H.-B., Lauro, G., O'Connor, R. D., Lohith, K., Kelly, M., Colin, P., & Bewley, C. A. (2017). Tulongicin, an antibacterial tri-indole alkaloid from a deep-water *Topsentia* sp. *sponge*. *J. Nat. Prod*., 80(9), 2556-2560.

Liu, T., Wu, S., Zhang, R., Wang, D., Chen, J., & Zhao, J. (2019). Diversity and antimicrobial potential of Actinobacteria isolated from diverse marine sponges along the Beibu Gulf of the South China Sea. *FEMS Microbiology Ecology, 95*(7).

Liu, W., Li, C., Zhu, P., Yang, J., & Cheng, K. (2010). Phylogenetic diversity of culturable fungi associated with two marine sponges: *Haliclona simulans* and *Gelliodes carnosa*, collected from the Hainan Island coastal waters of the South China Sea. *Fungal Divers.,* 42(1), 1-15.

Loque, C. P., Medeiros, A. O., Pellizzari, F. M., Oliveira, E. C., Rosa, C. A., & Rosa, L. H. (2010). Fungal community associated with marine macroalgae from antarctica. *Polar Biol.,* 33(5), 641-648.

Lu, Z., Koch, M., Harper, M. K., Matainaho, T. K., Barrows, L. R., Van Wagoner, R. M., & Ireland, C. M. (2013). Plakinamine M, a steroidal alkaloid from the marine sponge Corticium sp. *J. Nat. Prod.,* 76(11), 2150-2152.

Magiorakos, A. P., Srinivasan, A., Carey, R., Carmeli, Y., Falagas, M., Giske, C.,& Olsson-Liljequist, B. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect ,* 18(3), 268-281.

Maloof, A. C., Rose, C. V., Beach, R., Samuels, B. M., Calmet, C. C., Erwin, D. H.,& Simons, F. J. (2010). Possible animal-body fossils in pre-Marinoan limestones from South Australia. *Nat. Geosci.,* 3(9), 653.

Marcus, N. H., Pomponi, S., Rhines, P., Tester, P., & Vena, J. (1999). Marine derived pharmaceuticals and related bioactive compounds. *Monsoons to Microbes: Understanding the Oceanss Role in Human Health*, p 7186.

Margassery, L. M., Kennedy, J., O'Gara, F., Dobson, A. D., & Morrissey, J. P. (2012). Diversity and antibacterial activity of bacteria isolated from the coastal marine sponges *Amphilectus fucorum* and *Eurypon major*. *Lett Appl Microbiol,* 55(1), 2-8.

Martínez, J. L. (2019). Ecology and evolution of chromosomal gene transfer between environmental microorganisms and pathogens. *Microbial Transmission*, 139-160.

Martínez, J. L., Coque, T. M., & Baquero, F. (2015). What is a resistance gene? Ranking risk in resistomes. *Nat.Rev. Microbiol.,* 13(2), 116-123.

Matobole, R. M., van Zyl, L. J., Parker-Nance, S., Davies-Coleman, M. T., & Trindade, M. (2017a). Antibacterial activities of bacteria isolated from the marine sponges *Isodictya compressa* and *Higginsia bidentifera* collected from Algoa Bay, South Africa. *Mar. Drugs,* 15(2), 47.

Mearns-Spragg, A., Bregu, M., Boyd, K., & Burgess, J. (1998). Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett Appl Microbiol,* 27(3), 142-146.

Meesala, S., Singh, R., Watve, M. G., & Naik, D. G. (2018). A new antibacterial imidazole from the marine sponge *Iricinia fusca*. *Indian J Nat Prod Resour ,* 9(1), 75-78.

Mehbub, M. F., Lei, J., Franco, C., & Zhang, W. (2014). Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. *Mar. Drugs,* 12(8), 4539-4577.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, M. (2009). Biodiesel production from oleaginous microorganisms. *Renew.Energy, 34*(1), 1-5.

Mesbah, M., Premachandran, U., & Whitman, W. (1989). Precise measurement of the G+C content of Deoxyribonucleic Acid by high-performance liquid chromatography. *Int J Syst Bacteriol, 39*, 159-167.

Miles, A. A., Misra, S., & Irwin, J. (1938). The estimation of the bactericidal power of the blood. *Epidemiol. Infect.,* 38(6), 732-749.

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in *Enterococci*. *Expert* Rev Anti Infect Ther*.,* 12(10), 1221-1236.

Minnikin, D. E., Alshamaony, L., & Goodfellow, M. (1975). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin-layer chromatographic analysis of whole-organism methanolysates. *Microbiology*, 88(1), 200-204.

Mitra, S., Nishijima, M., Kasai, H., Yokota, A., & Yoon Dr, J. (2012). *Spongitalea numazuensis* gen. nov., sp. nov., a new marine bacterium of the family *Flavobacteriaceae* isolated from an unidentified marine sponge. *J Gen Appl Microbiol.,* 58(5), 379-386.

Moellering, R. C., Jr. (2011). Discovering new antimicrobial agents. *Int J Antimicrob Agents,* 37(1), 2-9.

Mohamed, G. A., Abd-Elrazek, A. E., Hassanean, H. A., Youssef, D. T., & van Soest, R. (2014). New compounds from the Red Sea marine sponge *Echinoclathria gibbosa*. *Phytochem. Lett.,* 9, 51-58.

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(1), 38.

Monk, A. B., Rees, C. D., Barrow, P., Hagens, S., & Harper, D. R. (2010). Bacteriophage applications: where are we now?. *Lett Appl Microbiol*, 51(4), 363-369.

Moore, E. R., Mihaylova, S. A., Vandamme, P., Krichevsky, M. I., & Dijkshoorn, L. (2010). Microbial systematics and taxonomy: relevance for a microbial commons. *Res Microbiol,* 161(6), 430-438.

Mueller, G. M., & Schmit, J. P. (2007). Fungal biodiversity: what do we know? What can we predict? *Biodivers Conserv.,* 16(1), 1-5.

Müller, W., & Müller, I. (2003a). Analysis of the sponge [Porifera] gene repertoire: implications for the evolution of the metazoan body plan *Sponges (Porifera)* (pp. 1-33): Springer.

Müller, W. E., & Müller, I. M. (2003b). Origin of the metazoan immune system: identification of the molecules and their functions in sponges. *Integr Comp Biol.,* 43(2), 281-292.

Müller, W., Zahn, R., Kurelec, B., Lucu, C., Müller, I., & Uhlenbruck, G. (1981). Lectin, a possible basis for symbiosis between bacteria and sponges. *J. Bacteriol,* 145(1), 548-558.

Müller, W., Li, J., Schröder, H., Qiao, L., & Wang, X. (2007). The unique skeleton of siliceous sponges (Porifera; Hexactinellida and Demospongiae) that evolved first from the Urmetazoa during the Proterozoic: a review.

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiol. Spectr.,* 4(2), 10.1128.

Murali Krishna Kumar, M., Devilal Naik, J., Satyavathi, K., Ramana, H., Raghuveer Varma, P., Purna Nagasree, K.,& Venkata Rao, D. (2014). Denigrins A–C: new antitubercular 3, 4-diarylpyrrole alkaloids from *Dendrilla nigra*. *Nat Prod Res.,* 28(12), 888-894.

Muscholl-Silberhorn, A., Thiel, V., & Imhoff, J. F. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microb. Ecol.,* 55(1), 94-106.

Nadell, C. D., Xavier, J. B., & Foster, K. R. (2008). The sociobiology of biofilms. *FEMS Microbiol Lett.,* 33(1), 206-224.

Naim, M. A., Smidt, H., & Sipkema, D. (2017). Fungi found in Mediterranean and North Sea sponges: how specific are they? *PeerJ,* 5, e3722.

Nelson, M. C., Morrison, H. G., Benjamino, J., Grim, S. L., & Graf, J. (2014). Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. *PloS one, 9*(4).

Newman, D. J. (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery? *J. Med. Chem.,* 51(9), 2589-2599.

Newman, D. J., & Hill, R. T. (2006). New drugs from marine microbes: the tide is turning. *J Ind Microbiol Biot.,* 33(7), 539-544.

Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'connor, P.,& Epstein, S. (2008). Short peptide induces an “uncultivable” microorganism to grow in vitro. *Appl. Environ. Microbiol.,* 74(15), 4889-4897.

Nichols, S. A. (2005). An evaluation of support for order-level monophyly and interrelationships within the class Demospongiae using partial data from the large subunit rDNA and cytochrome oxidase subunit I. *Mol Phylogenet Evol, 34*(1), 81-96.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.,* 67(4), 593-656.

Nikaido, H., & Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *BBA*-Proteins *Proteom.,* 1794(5), 769-781.

Nishijima, M., Adachi, K., Katsuta, A., Shizuri, Y., & Yamasato, K. (2013). *Endozoicomonas numazuensis* sp. nov., a gamma proteobacterium isolated from marine sponges, and emended description of the genus *Endozoicomonas Kurahashi* and *Yokota* 2007. *Int J Syst Evol Microbiol,* 63(2), 709-714.

Nouioui, I., Rückert, C., Willemse, J., van Wezel, G. P., Klenk, H. P., Busche, T. & Zotchev, S. B. (2017). *Actinoalloteichus fjordicus* sp. nov. isolated from marine sponges: phenotypic, chemotaxonomic and genomic characterisation. *Antonie van Leeuwenhoek,* 110(12), 1705-1717.

O'Halloran, J., Barbosa, T., Morrissey, J., Kennedy, J., O'Gara, F., & Dobson, A. (2011). Diversity and antimicrobial activity of *Pseudovibrio* spp. from Irish marine sponges. *J. Appl. Microbiol.,* 110(6), 1495-1508.

O'Halloran, J. A., Barbosa, T. M., Morrissey, J. P., Kennedy, J., Dobson, A. D., & O'Gara, F. (2013). *Pseudovibrio axinellae* sp. nov., isolated from an Irish marine sponge. *Int J Syst Evol Microbiol,* 63(1), 141-145.

Oclarit, J. M., Okada, H., Ohta, S., Kaminura, K., Yamaoka, Y., Iizuka, T.,& Ikegami, S. (1994). Anti-bacillus substance in the marine sponge, *Hyatella* species, produced by an associated *Vibrio* species bacterium. *Microbios,* 78(314), 7-16.

Ogawara, H. (2016). Self-resistance in *Streptomyces*, with special reference to β-Lactam antibiotics. *Molecules,* 21(5), 605.

Ohno, M., Shiratori, H., Park, M.-J., Saitoh, Y., Kumon, Y., Yamashita, N.,& Beppu, T. (2000). *Symbiobacterium thermophilum* gen. nov., sp. nov., a symbiotic thermophile that depends on co-culture with a *Bacillus* strain for growth. *Int J Syst Evol Microbiol,* 50(5), 1829-1832.

Olson, J. B., Harmody, D. K., Bej, A. K., & McCarthy, P. J. (2007). *Tsukamurella spongiae* sp. nov., a novel actinomycete isolated from a deep-water marine sponge. *Int J Syst Evol Microbiol,* 57(7), 1478-1481.

Olson, J. B., & McCarthy, P. J. (2005). Associated bacterial communities of two deep-water sponges. *Aquat Microb Ecol,* 39(1), 47-55.

Onuki, H., & Kamino, K. (2000). Bacterial growth stimulation with exogenous siderophore and synthetic N-acyl homoserine lactone autoinducers under iron-limited and low-nutrient conditions. *Appl. Environ. Microbiol.,* 66(7), 2797-2803.

Palmieri, C., Varaldo, P. E., & Facinelli, B. (2011). *Streptococcus suis*, an emerging drug-resistant animal and human pathogen. *Front Microbiol.,* 2, 235.

Palomo, S., González, I., de la Cruz, M., Martín, J., Tormo, J., Anderson, M.,& Genilloud, O. (2013). Sponge-derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic kocurin. *Mar. Drugs,* 11(4), 1071-1086.

Park, S., Yokota, A., Itoh, T., & Park, J. S. (2011). *Sphingomonas jejuensis* sp. nov., isolated from marine sponge *Hymeniacidon flavia*. *J Microbiol,* 49(2), 238-242.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev,* 18(4), 657-686.

Payne, D. J., Gwynn, M. N., Holmes, D. J., & Pompliano, D. L. (2007). Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov,* 6(1), 29-40.

Peterson, E., & Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Front Microbiol.,* 9(2928).

Piel, J. (2009). Metabolites from symbiotic bacteria. *Nat Prod Rep,* 26(3), 338-362.

Pimentel-Elardo, S. M., Scheuermayer, M., Kozytska, S., & Hentschel, U. (2009). *Streptomyces axinellae* sp. nov., isolated from the Mediterranean sponge *Axinella polypoides* (Porifera). *Int J Syst Evol Microbiol,* 59(6), 1433-1437.

Pimentel-Elardo, S. M., Tiro, L. P., Grozdanov, L., & Hentschel, U. (2008). *Saccharopolyspora cebuensis* sp. nov., a novel actinomycete isolated from a Philippine sponge (Porifera). *Int J Syst Evol Microbiol,* 58(3), 628-632.

Prakash, O., Verma, M., Sharma, P., Kumar, M., Kumari, K., Singh, A.,& Khanna, M. (2007). Polyphasic approach of bacterial classification—an overview of recent advances. *Indian J Microbiol,* 47(2), 98-108.

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PloS one, 5*(3).

Projan, S. J. (2003). Why is big Pharma getting out of antibacterial drug discovery? *Curr Opin Microbiol,* 6(5), 427-430.

Proksch, P., Putz, A., Ortlepp, S., Kjer, J., & Bayer, M. (2010). Bioactive natural products from marine sponges and fungal endophytes. P*hytochem Rev.,* 9(4), 475-489.

Pulschen, A. A., Bendia, A. G., Fricker, A. D., Pellizari, V. H., Galante, D., & Rodrigues, F. (2017). Isolation of unculturedbBacteria from antarctica using long incubation periods and low nutritional media. *Front. Microbiol,* 8(1346).

Radjasa, O. K., Vaske, Y. M., Navarro, G., Vervoort, H. C., Tenney, K., Linington, R. G., & Crews, P. (2011). Highlights of marine invertebrate-derived biosynthetic products: Their biomedical potential and possible production by microbial associants. *Bioorg Med Chem,* 19(22), 6658-6674.

Ramirez, M. S., & Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug Resist Updat,* 13(6), 151-171.

Ramasamy, D., Mishra, A. K., Lagier, J.-C., Padhmanabhan, R., Rossi, M., Sentausa, E.,& Fournier, P.-E. (2014). A polyphasic strategy incorporating genomic data for the taxonomic description of novel bacterial species. *Int J Syst Evol Microbiol,* 64(2), 384-391.

Rahman, H., Austin, B., Mitchell, W. J., Morris, P. C., Jamieson, D. J., Adams, D. R., Spragg, A. M., and Schweizer, M. (2010). Novel anti-infective compounds from marine bacteria. *Mar. Drugs*, 8, 498–518.

Rappé, M. S., Connon, S. A., Vergin, K. L., & Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature,* 418(6898), 630.

Raslan, A. E., Radwan, M. M., Ahmed, S. A., Nafady, A. M., Zaki, M. A., Wanas, A. S.,& ElSohly, M. A. (2018). Monanchoramides A–D, ceramides from the marine sponge *Monanchora clathrata* with cytotoxic activity. *Phytochem. Lett.,* 23, 83-89.

Reichelt, J. L., & Baumann, P. (1974). Effect of sodium chloride on growth of heterotrophic marine bacteria. *Arch. Microbio*l.*,* 97(1), 329-345.

Reimer, A., Blohm, A., Quack, T., Grevelding, C. G., Kozjak-Pavlovic, V., Rudel, T.,& Abdelmohsen, U. R. (2015). Inhibitory activities of the marine streptomycete-derived compound SF2446A2 against *Chlamydia trachomatis* and *Schistosoma mansoni*. *J Antibiot,* 68(11), 674-679.

Reveillaud J, Maignien L, Eren AM, Huber JA, Apprill A, Sogin ML, Vanreusel A (2014) Host-specificity among abundant and rare taxa in the sponge microbiome. *ISME J* 8:1198–1209

Richards, T. A., Jones, M. D., Leonard, G., & Bass, D. (2012). Marine fungi: their ecology and molecular diversity. A*nnu Rev Mar Sc*i. *,* 4, 495-522.

Richards, T. A., Leonard, G., Mahé, F., del Campo, J., Romac, S., Jones, M. D.,& Massana, R. (2015). Molecular diversity and distribution of marine fungi across 130 European environmental samples. *Proc. R. Soc. B,* 282(1819), 20152243.

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol.Lett.,* 245(2), 195-203.

Rodriguez, R. J., Redman, R. S., & Henson, J. M. (2004). The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitig Adapt Strat Gl.,* 9(3), 261-272.

Rosselló-Mora, R. (2006). DNA-DNA reassociation methods applied to microbial taxonomy and their critical evaluation. In *Molecular identification, systematics, and population structure of prokaryotes* (pp. 23-50). Springer, Berlin, Heidelberg

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol.,* 4(4), 406-425.

Santos-Gandelman, J. F., Giambiagi-deMarval, M., Oelemann, W. M., & Laport, M. S. (2014). Biotechnological potential of sponge-associated bacteria. *Curr Pharm Biotechnol,* 15(2), 143-155.

Santos, O. C., Pontes, P. V., Santos, J. F., Muricy, G., Giambiagi-deMarval, M., & Laport, M. S. (2010). Isolation, characterization and phylogeny of sponge-associated bacteria with antimicrobial activities from Brazil. *Res Microbiol,* 161(7), 604-612.

Schellenberg, J., Busse, H. J., Hardt, M., Schubert, P., Wilke, T., Kämpfer, P., & Glaeser, S. P. (2017). *Winogradskyella haliclonae* sp. nov., isolated from a marine sponge of the genus *Haliclona*. *Int J Syst Evol Microbiol,* 67(12), 4902-4910.

Schellenberg, J., Busse, H. J., Hardt, M., Schubert, P., Wilke, T., Kämpfer, P., & Glaeser, S. P. (2018). Proposal of *Litorimonas haliclonae* sp. nov., isolated from a marine sponge of the genus *Haliclona*. *Int J Syst Evol Microbiol,* 68(3), 835-843.

Schindler, B. D., & Kaatz, G. W. (2016). Multidrug efflux pumps of Gram-positive bacteria. *Drug Resist Updat,* 27, 1-13.

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N.,& Vacelet, J. (2012). Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges. *ISME J,* 6(3), 564.

Schneemann, I., Kajahn, I., Ohlendorf, B., Zinecker, H., Erhard, A., Nagel, K.,& Imhoff, J. F. (2010b). Mayamycin, a cytotoxic polyketide from a *Streptomyces* strain isolated from the marine sponge *Halichondria panicea*. *J. Nat. Prod.,* 73(7), 1309-1312.

Schneemann, I., Nagel, K., Kajahn, I., Labes, A., Wiese, J., & Imhoff, J. F. (2010a). Comprehensive investigation of marine Actinobacteria associated with the sponge *Halichondria panicea*. *Appl Environ Microbiol,* 76(11), 3702-3714.

Schneemann, I., Ohlendorf, B., Zinecker, H., Nagel, K., Wiese, J., & Imhoff, J. F. (2010c). Nocapyrones A− D, γ-pyrones from a *Nocardiopsis* strain isolated from the marine sponge *Halichondria panicea*. *J. Nat. Prod.,* 73(8), 1444-1447.

Schwartz, I., Haber, M., Ilan, M., Keren, R., & Lavy, A. (2014). Implementing sponge physiological and genomic information to enhance the diversity of its culturable associated bacteria. *FEMS Microbiol Ecol.,* 87(2), 486-502.

Schwarz, S., Kehrenberg, C., Doublet, B., & Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol.*Rev*.,* 28(5), 519-542.

Selvin, J., Gandhimathi, R., Kiran, G. S., Priya, S. S., Ravji, T. R., & Hema, T. A. (2009). Culturable heterotrophic bacteria from the marine sponge *Dendrilla nigra*: isolation and phylogenetic diversity of actinobacteria. Helgol. *Mar. Res.,* 63(3), 239-247.

Selvin, J., Kennedy, J., Lejon, D. P., Kiran, G. S., & Dobson, A. D. (2012). Isolation identification and biochemical characterization of a novel halo-tolerant lipase from the metagenome of the marine sponge Haliclona simulans. *Microb Cell Fact.,* 11(1), 72.

Sethi, S., Kumar, R., & Gupta, S. (2013). Antibiotic production by microbes isolated from soil. Int. *J Pharm Sci Rev Res, 4*(8), 2967.

Shen, S., Liu, D., Wei, C., Proksch, P., & Lin, W. (2012). Purpuroines A–J, halogenated alkaloids from the sponge *Iotrochota purpurea* with antibiotic activity and regulation of tyrosine kinases. *Bioorg Med Chem,* 20(24), 6924-6928.

Shojaei, H., Daley, C., Gitti, Z., Hashemi, A., Heidarieh, P., Moore, E. R., & Tortoli, E. (2013). *Mycobacterium iranicum* sp. nov., a rapidly growing scotochromogenic species isolated from clinical specimens on three different continents. *Int J Syst Evol Microbiol,* 63(4), 1383-1389.

Silva, F. S., Souza, D. T., Zucchi, T. D., Pansa, C. C., de Figueiredo Vasconcellos, R. L., Crevelin, E. J.,& Melo, I. S. (2016). *Streptomyces atlanticus* sp. nov., a novel actinomycete isolated from marine sponge *Aplysina fulva* (Pallas, 1766). *Antonie Van Leeuwenhoek,* 109(11), 1467-1474.

Simister, R. L., Deines, P., Botté, E. S., Webster, N. S., & Taylor, M. W. (2012). Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. *Environ. Microbiol.,* 14(2), 517-524.

Simu, K., & Hagström, Å. (2004). Oligotrophic bacterioplankton with a novel single-cell life strategy. *Appl. Environ. Microbiol.,* 70(4), 2445-2451.

Singer, R. S., Finch, R., Wegener, H. C., Bywater, R., Walters, J., & Lipsitch, M. (2003). Antibiotic resistance—the interplay between antibiotic use in animals and human beings. *Lancet Infect Dis,* 3(1), 47-51.

Singh, M. P., Kong, F., Janso, J. E., Arias, D. A., Suarez, P. A., Bernan, V. S.,& Greenstein, M. (2003). Novel α-pyrones produced by a marine *Pseudomonas* sp. F92S91. *J Antibiot,* 56(12), 1033-1044.

Singh, P., Raghukumar, C., Verma, P., & Shouche, Y. (2011). Fungal community analysis in the deep-sea sediments of the Central Indian Basin by culture-independent approach. *Microb Ecol,* 61(3), 507-517.

Singh, S. B., & Pelaez, F. (2008). Biodiversity, chemical diversity and drug discovery. In *Natural compounds as drugs volume I* (pp. 141-174). Birkhäuser Basel.

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., & Tripathi, C. K. M. (2017). Strategies for Fermentation Medium Optimization: An In-Depth Review. *Front. Microbiol.,* 7(2087).

Sipkema, D., Schippers, K., Maalcke, W. J., Yang, Y., Salim, S., & Blanch, H. W. (2011). Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona* (*gellius*) sp. *Appl Environ Microbiol,* 77(6), 2130-2140.

Song, J., Oh, H.-M., & Cho, J.-C. (2009). Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean. *FEMS Microbiol Lett,* 295(2), 141-147.

Sorokin, S. J., & Currie, D. R. (2008). The distribution and diversity of sponges in Spencer Gulf. *Report to Nature Foundation SA Inc. SARDI Aquatic Sciences Publication*, 001153-001151.

Sorokin, S., Fromont, J., & Currie, D. (2007). Demosponge Biodiversity in the Benthic Protection Zone of the Great Australian Bight. *T Roy Soc South Aust., 131*(2), 192-204.

Souza, D. T., da Silva, F. S. P., da Silva, L. J., Crevelin, E. J., Moraes, L. A. B., Zucchi, T. D., & Melo, I. S. (2017). *Saccharopolyspora spongiae* sp. nov., a novel actinomycete isolated from the marine sponge *Scopalina ruetzleri* (Wiedenmayer, 1977). *Int J Syst Evol Microbiol,* 67(6), 2019-2025.

Speitling, M., Smetanina, O. F., Kuznetsova, T. A., & Laatsch, H. (2007). Bromoalterochromides A and A', unprecedented chromopeptides from a marine *Pseudoalteromonas maricaloris* strain KMM 636 T. *J Antibiot,* 60(1), 36.

Stackebrandt, E. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol. Today*, 33, 152-155.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C.,& Trüper, H. G. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol,* 52(3), 1043-1047.

Stackebrandt, E., & Goodfellow, M. (1991). *Nucleic acid techniques in bacterial systematics*. Chichester; New York: Wiley.

Stackebrandt, E., Schumann, P., & Cui, X. L. (2004). Reclassification of Cellulosimicrobium variabile Bakalidou et al. 2002 as *Isoptericola variabilis* gen. nov., comb. nov. *Int J Syst Evol Microbiol,* 54(Pt 3), 685-688.

Stager, C. E., Erikson, E., & Davis, J. R. (1983). Rapid method for detection, identification, and susceptibility testing of enteric pathogens. *J. Clin. Microbiol.,* 17(1), 79-84.

Stefani, F. O., Bell, T. H., Marchand, C., de la Providencia, I. E., El Yassimi, A., St-Arnaud, M., & Hijri, M. (2015). Culture-dependent and-independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. *PloS one,* 10(6).

Steinert, G., Whitfield, S., Taylor, M. W., Thoms, C., & Schupp, P. J. (2014). Application of diffusion growth chambers for the cultivation of marine sponge-associated bacteria. *Mar Biotechnol,* 16(5), 594-603.

Stevenson, B. S., Eichorst, S. A., Wertz, J. T., Schmidt, T. M., & Breznak, J. A. (2004). New strategies for cultivation and detection of previously uncultured microbes. *Appl. Environ. Microbiol.,* 70(8), 4748-4755.

Stewart, E. J. (2012). Growing unculturable bacteria. *J. Bacteriol,* 194(16), 4151-4160.

Stierle, A. C., Cardellina, J. H., 2nd, & Singleton, F. L. (1988). A marine *Micrococcus* produces metabolites ascribed to the sponge Tedania ignis. *Experientia,* 44(11-12), 1021.

Stingl, U., Cho, J.-C., Foo, W., Vergin, K. L., Lanoil, B., & Giovannoni, S. J. (2008). Dilution-to-extinction culturing of psychrotolerant planktonic bacteria from permanently ice-covered lakes in the McMurdo Dry Valleys, Antarctica. *Microb Ecol,* 55(3), 395-405.

Stott, M. B., Crowe, M. A., Mountain, B. W., Smirnova, A. V., Hou, S., Alam, M., & Dunfield, P. F. (2008). Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. Environ. *Microbiol.,* 10(8), 2030-2041.

Strobel, G., Daisy, B., Castillo, U., & Harper, J. (2004). Natural products from endophytic microorganisms. *J Nat Prod,* 67(2), 257-268.

Su, Y., Yang, X., Wang, Y., Liu, Y., Ren, Q., & Zhang, X. H. (2017). *Muricauda marina* sp. nov., isolated from marine snow of Yellow Sea. *Int J Syst Evol Microbiol,* 67(7), 2446-2451.

Sun, J., Deng, Z., & Yan, A. (2014). Biochemical and Biophysical Research Communications Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. *Biochem. Biophys. Res. Commun,* 453, 254-267.

Sun, S., Canning, C. B., Bhargava, K., Sun, X., Zhu, W., Zhou, N.,& Zhou, K. (2015). Polybrominated diphenyl ethers with potent and broad spectrum antimicrobial activity from the marine sponge *Dysidea*. *Bioorg Med Chem Lett, 25*(10), 2181-2183.

Sun, W., Dai, S., Jiang, S., Wang, G., Liu, G., Wu, H., & Li, X. (2010a). Culture-dependent and culture-independent diversity of Actinobacteria associated with the marine sponge Hymeniacidon perleve from the South China Sea. *Antonie Van Leeuwenhoek, 98*(1), 65-75.

Sun, W., Zhang, F., He, L., Karthik, L., & Li, Z. (2015). Actinomycetes from the South China Sea sponges: isolation, diversity, and potential for aromatic polyketides discovery. *Front Microbiol,* 6, 1048.

Supong, K., Suriyachadkun, C., Suwanborirux, K., Pittayakhajonwut, P., & Thawai, C. (2013a). *Verrucosispora andamanensis* sp. nov., isolated from a marine sponge. *Int J Syst Evol Microbiol,* 63(11), 3970-3974.

Supong, K., Suriyachadkun, C., Pittayakhajonwut, P., Suwanborirux, K., & Thawai, C. (2013b). *Micromonospora spongicola* sp. nov., an actinomycete isolated from a marine sponge in the Gulf of Thailand. *J Antibiot,* 66(9), 505-509.

Suzumura, K., Yokoi, T., Funatsu, M., Nagai, K., Tanaka, K., Zhang, H., & Suzuki, K. (2003). YM-266183 and YM-266184, novel thiopeptide antibiotics produced by *Bacillus cereus* isolated from a marine sponge. Structure elucidation. *J Antibiot,* 56(2), 129-134.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol,* 10(3), 512-526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol,* 28(10), 2731-2739.

Tanaka, N., Kusama, T., Takahashi-Nakaguchi, A., Gonoi, T., Fromont, J., & Kobayashi, J. i. (2013). Nagelamides X–Z, dimeric bromopyrrole alkaloids from a marine sponge *Agelas* sp. *Org. Lett.,* 15(13), 3262-3265.

Tanaka, Y., Hanada, S., Manome, A., Tsuchida, T., Kurane, R., Nakamura, K., & Kamagata, Y. (2004). *Catellibacterium nectariphilum* gen. nov., sp. nov., which requires a diffusible compound from a strain related to the genus *Sphingomonas* for vigorous growth. *Int J Syst Evol Microbiol,* 54(3), 955-959.

Tawfike, A., Attia, E. Z., Desoukey, S. Y., Hajjar, D., Makki, A. A., Schupp, P. J., Abdelmohsen, U. R. (2019). New bioactive metabolites from the elicited marine sponge-derived bacterium *s* sp. nov. *AMB Express, 9*(1), 12.

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(3), 187.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007b). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol. Mol. Biol. Rev.,* 71(2), 295-347.

Taylor, M. W., Schupp, P. J., Dahllöf, I., Kjelleberg, S., & Steinberg, P. D. (2004a). Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environmental microbiology,* 6(2), 121-130.

Taylor, M. W., Schupp, P. J., Baillie, H. J., Charlton, T. S., de Nys, R., Kjelleberg, S., & Steinberg, P. D. (2004b). Evidence for acyl homoserine lactone signal production in bacteria associated with marine sponges. *Appl Environ Microbiol,* 70(7), 4387-4389.

Taylor, P. L., & Wright, G. D. (2008). Novel approaches to discovery of antibacterial agents. *Anim Health Res Rev,* 9(2), 237-246.

Thakur, A. N., Thakur, N. L., Indap, M. M., Pandit, R. A., Datar, V. V., & Muller, W. E. (2005). Antiangiogenic, antimicrobial, and cytotoxic potential of sponge-associated bacteria. *Mar Biotechnol,* 7(3), 245-252.

Thawai, C., Rungjindamai, N., Klanbut, K., & Tanasupawat, S. (2017). *Nocardia xestospongiae* sp. nov., isolated from a marine sponge in the Andaman Sea. *Int J Syst Evol Microbiol,* 67(5), 1451-1456.

Thiel, V., Neulinger, S. C., Staufenberger, T., Schmaljohann, R., & Imhoff, J. F. (2007). Spatial distribution of sponge-associated bacteria in the Mediterranean sponge *Tethya aurantium*. *FEMS Microbiol Ecol,* 59(1), 47-63.

Thoms C, Horn M, Wagner M, Hentschel U, Proksch P (2003) Monitoring microbial diversity and natural product profiles of the sponge Aplysina cavernicola following transplantation. Mar Biol 142:685–692.

Thomas, T. R. A., Kavlekar, D. P., & LokaBharathi, P. A. (2010). Marine drugs from sponge-microbe association—A Review. *Mar. Drugs,* 8(4), 1417-1468.

Tilvi, S., Rodrigues, C., Naik, C., Parameswaran, P., & Wahidhulla, S. (2004). New bromotyrosine alkaloids from the marine sponge *Psammaplysilla purpurea*. *Tetrahedron,* 60(45), 10207-10215.

Tindall, B. J., Rossello-Mora, R., Busse, H. J., Ludwig, W., & Kampfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *Int J Syst Evol Microbiol,* 60(1), 249-266.

Toledo-Hernández, C., Zuluaga-Montero, A., Bones-González, A., Rodriguez, J., Sabat, A., & Bayman, P. (2008). Fungi in healthy and diseased sea fans (Gorgonia ventalina): is *Aspergillus sydowii* always the pathogen? *Coral Reefs,* 27(3), 707-714.

Torres, Y. R., Berlinck, R. G., Magalhães, A., Schefer, A. B., Ferreira, A. G., Hajdu, E., & Muricy, G. (2000). Arenosclerins A− C and Haliclonacyclamine E, new tetracyclic alkaloids from a Brazilian endemic haplosclerid Sponge *Arenosclera b rasiliensis*. *J. Nat. Prod.,* 63(8), 1098-1105.

Tortoli, E. (2018). Commentary: Phylogenomics and comparative genomic studies robustly support division of the genus *Mycobacterium* into an emended genus *Mycobacterium* and four novel genera. *Front Microbiol,* 9, 2065-2065.

Trieber, C. A., Burkhardt, N., Nierhaus, K. H., & Taylor, D. E. (1998). Ribosomal protection from tetracycline mediated by Tet (O): Tet (O) interaction with ribosomes is GTP-dependent. *Biol. Chem.,* 379(7), 847-856.

Tripp, H. J., Kitner, J. B., Schwalbach, M. S., Dacey, J. W., Wilhelm, L. J., & Giovannoni, S. J. (2008). SAR11 marine bacteria require exogenous reduced sulphur for growth. *Nature,* 452(7188), 741.

Tseng, M., Liao, H. C., Chiang, W. P., & Yuan, G. F. (2011). *Isoptericola chiayiensis* sp. nov., isolated from mangrove soil. *Int J Syst Evol Microbiol,* 61(7), 1667-1670.

Turenne, C. Y., Tschetter, L., Wolfe, J., & Kabani, A. (2001). Necessity of quality-controlled 16S rRNA gene sequence databases: identifying nontuberculous *Mycobacterium* species. *J Clin Microbiol,* 39(10), 3637-3648.

Udwary, D. W., Zeigler, L., Asolkar, R. N., Singan, V., Lapidus, A., Fenical, W., & Moore, B. S. (2007). Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proc Natl Acad Sci,* 104(25), 10376-10381.

Van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., & Aarts, H. J. (2011). Acquired antibiotic resistance genes: an overview. *Front Microbiol,* 2, 203.

Van Netten, P., Mossel, D., & Van de Moosdijk, A. (1987). Rapid detection of excessive cfu counts of Enterobacteriaceae, *S. aureus* and yeasts by nitrate reduction/glucose dissimilation (NGD) monitoring in selective media. *J. Food Sci.,* 52(3), 751-756.

Van Soest, R., Boury-Esnault, N., Hooper, J., Rützler, K. d., De Voogd, N., Alvarez de Glasby, B., & Schoenberg, C. (2012). World porifera database. *The World Register of Marine Species (WoRMS). Available online: http://www. marinespecies. org/porifera*.

Van Soest, R. W., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., De Voogd, N. J.,& Hooper, J. N. (2012). Global diversity of sponges (Porifera). *PloS one,* 7(4), e35105.

Velho-Pereira, S., & Kamat, N. M. (2011). Antimicrobial screening of actinobacteria using a modified cross-streak method. *Indian J. Pharm. Sci.,* 73(2), 223-228.

Vickers, N. J. (2017). Animal Communication: When I'm Calling You, Will You Answer Too? *Curr Biol,* 27(14), R713-R715.

Vijayakumari, S. J., Sasidharannair, N. K., Nambisan, B., & Mohandas, C. (2013). Optimization of media and temperature for enhanced antimicrobial production by bacteria associated with Rhabditis sp. *Iran* J Microbiol *, 5*(2), 136-141.

Visamsetti, A., Ramachandran, S. S., & Kandasamy, D. (2016). *Penicillium chrysogenum* DSOA associated with marine sponge (*Tedania anhelans*) exhibit antimycobacterial activity. *Microbiol. Res.,* 185, 55-60.

Waksman, S. A., Reilly, H. C., & Johnstone, D. B. (1946). Isolation of Streptomycin-producing Strains of *Streptomyces griseus*. *J. Bacteriol,* 52(3), 393-397.

Wang, G. (2006). Diversity and biotechnological potential of the sponge-associated microbial consortia. *J Ind Microbiol Biotechnol,* 33(7), 545-551.

Wang, Y. H., Feng, J. T., Zhang, Q., & Zhang, X. (2008). Optimization of fermentation condition for antibiotic production by *Xenorhabdus nematophila* with response surface methodology. ***J****. Appl. Microbiol., 104*(3), 735-744.

Wang, Y., Yang, X., Liu, J., Wu, Y., & Zhang, X. H. (2017). *Muricauda lutea* sp. nov., isolated from seawater. *Int J Syst Evol Microbiol,* 67(4), 1064-1069.

Webster, N., & Hill, R. (2001a). The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. *Mar. Biol.,* 138(4), 843-851.

Webster, N. S., Luter, H. M., Soo, R. M., Botté, E. S., Simister, R. L., Abdo, D., & Whalan, S. (2013). Same, same but different: symbiotic bacterial associations in GBR sponges. *Front Microbiol,* 3, 444.

Webster, N. S., & Taylor, M. W. (2012). Marine sponges and their microbial symbionts: love and other relationships. *Environ Microbiol,* 14(2), 335-346.

Webster, N.S., Taylor, M.W., Behnam, F., Lu¨cker, S., Rattei, T., Whalan, S., Horn, M. and Wagner, M. (2010) Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environ Microbiol* 12, 2070–2082.

Webster, N. S., Wilson, K. J., Blackall, L. L., & Hill, R. T. (2001b). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl Environ Microbiol,* 67(1), 434-444.

Wei, X., Nieves, K., & Rodríguez, A. D. (2010). Neopetrosiamine A, biologically active bis-piperidine alkaloid from the Caribbean sea sponge *Neopetrosia proxima*. *Bioorg Med Chem Lett,* 20(19), 5905-5908.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrob Agents Chemother,* 39(3), 577.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol, 173*(2), 697-703.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications,* 18(1), 315-322.

Whitman, W. B., Coleman, D. C., & Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc Natl Acad Sci ,* 95(12), 6578-6583.

Wilkinson, C. (1978). Microbial associations in sponges. I. Ecology, physiology and microbial populations of coral reef sponges. *Mar. Biol.,* 49(2), 161-167.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol,* 5(3), 175-186.

Wu, Y., Li, W.-J., Tian, W., Zhang, L.-P., Xu, L., Shen, Q.-R., & Shen, B. (2010). *Isoptericola jiangsuensis* sp. nov., a chitin-degrading bacterium. *Int J Syst Evol Microbiol,* 60(4), 904-908.

Wu, Y. H., Yu, P. S., Zhou, Y. D., Xu, L., Wang, C. S., Wu, M.,& Xu, X. W. (2013). *Muricauda antarctica* sp. nov., a marine member of the *Flavobacteriaceae* isolated from Antarctic seawater. *Int J Syst Evol Microbiol,* 63(9), 3451-3456.

Xi, L., Ruan, J., & Huang, Y. (2012). Diversity and biosynthetic potential of culturable actinomycetes associated with marine sponges in the China Seas. *Int J Mol Sci,* 13(5), 5917-5932.

Yang, C., Li, Y., Guo, Q., Lai, Q., Wei, J., Zheng, T., & Tian, Y. (2013). *Muricauda zhangzhouensis* sp. nov., isolated from mangrove sediment. *Int J Syst Evol Microbiol,* 63(6), 2320-2325.

Yang, S. H., Seo, H. S., Lee, J. H., Kim, S. J., & Kwon, K. K. (2015). *Kiloniella spongiae* sp. nov., isolated from a marine sponge and emended description of the genus Kiloniella Wiese et al. 2009 and Kiloniella laminariae. *Int J Syst Evol Microbiol,* 65(1), 230-234.

Yang, Q., Franco, C. M., Lin, H.-W., & Zhang, W. (2019b). Untapped sponge microbiomes: structure specificity at host order and family levels. *FEMS Microbiol. Ecol.,* 95(9), fiz136.

Yang, Q., Franco, C. M., Sorokin, S. J., & Zhang, W. (2017). Development of a multilocus-based approach for sponge (phylum Porifera) identification: refinement and limitations. *Sci. Rep.,* 7, 41422.

Yang, Q., Franco, C. M., & Zhang, W. (2015). Sponge-associated actinobacterial diversity: validation of the methods of actinobacterial DNA extraction and optimization of 16S rRNA gene amplification. *Appl Microbiol Biotechnol,* 99(20), 8731-8740.

Yang, Q., Franco, C. M. M., & Zhang, W. (2019a). Uncovering the hidden marine sponge microbiome by applying a multi-primer approach. *Scientific reports, 9*(1), 6214.

Yashiro, E., Spear, R., & McManus, P. (2011). Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *J Appl Microbiol,* 110(5), 1284-1296.

Yoon, E.-J., Goussard, S., Touchon, M., Krizova, L., Cerqueira, G., Murphy, C., Courvalin, P. (2014). Origin in *Acinetobacter guillouiae* and dissemination of the aminoglycoside-modifying enzyme Aph (3')-VI. *MBio, 5*(5), e01972-01914.

Yoon, J. H., Lee, M. H., Oh, T. K., & Park, Y. H. (2005). *Muricauda flavescens* sp. nov. and *Muricauda aquimarina* sp. nov., isolated from a salt lake near Hwajinpo Beach of the East Sea in Korea, and emended description of the genus Muricauda. *Int J Syst Evol Microbiol,* 55(3), 1015-1019.

Yoon, J. H., Kang, S. J., Jung, Y. T., & Oh, T. K. (2008). *Muricauda lutimaris* sp. nov., isolated from a tidal flat of the Yellow Sea. *Int J Syst Evol Microbiol,* 58(7), 1603-1607.

Yoon, B. J., & Oh, D. C. (2011a). *Formosa spongicola* sp. nov., isolated from the marine sponge *Hymeniacidon flavia*. *Int J Syst Evol Microbiol,* 61(2), 330-333.

Yoon, B. J., & Oh, D. C. (2012). *Spongiibacterium flavum* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from the marine sponge *Halichondria oshoro*, and emended descriptions of the genera *Croceitalea* and *Flagellimonas*. *Int J Syst Evol Microbiol,* 62(5), 1158-1164.

Yoon, J., Adachi, K., & Kasai, H. (2016a). *Spongiiferula fulva* gen. nov., sp. nov., a bacterium of the family *Flavobacteriaceae* Isolated from a Marine Sponge. *Curr. Microbiol.,* 73(1), 15-21.

Yoon, J., Adachi, K., & Kasai, H. (2016b). *Spongiimicrobium salis* gen. nov., sp. nov., a bacterium of the family Flavobacteriaceae isolated from a marine sponge. *Arch Microbiol,* 198(7), 663-670.

Yoon, J., Adachi, K., Kasai, H., & Lee, M. K. (2014). S*pongiivirga citrea* gen. nov. sp. nov. a new marine bacterium of the family *Flavobacteriaceae* isolated from a marine sponge. *Curr. Microbiol.,* 70(1), 51-57.

Yoon, J., Jang, J. H., & Kasai, H. (2013). *Spongiimonas flava* gen. nov., sp. nov., a new member of the family *Flavobacteriaceae* isolated from an unidentified marine sponge. *Anton Leeuw Int J G.,* 103(3), 625-633.

Yoon, J., Oku, N., Park, S., Kasai, H., & Yokota, A. (2011b). *Porifericola rhodea* gen. nov., sp. nov., a new member of the phylum Bacteroidetes isolated by the bait-streaked agar technique. *Anton Leeuw Int J G.,* 100(1), 145-153.

Yoon, J. H., Schumann, P., Kang, S. J., Jung, S. Y., & Oh, T. K. (2006). *Isoptericola dokdonensis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol,* 56(12), 2893-2897.

Youssef, D. T., Badr, J. M., Shaala, L. A., Mohamed, G. A., & Bamanie, F. H. (2015). Ehrenasterol and biemnic acid; new bioactive compounds from the Red Sea sponge *Biemna ehrenbergi*. *Phytochem. Lett.,* 12, 296-301.

Yurkov, V., Stackebrandt, E., Holmes, A., Fuerst, J. A., Hugenholtz, P., Golecki, J.,Drews, G. (1994). Phylogenetic positions of novel aerobic, bacteriochlorophyll a-containing bacteria and description of *Roseococcus thiosulfatophilus* gen. nov., sp. nov., *Erythromicrobium ramosum* gen. nov., sp. nov., and *Erythrobacter litoralis* sp. nov. *Int J Syst Bacteriol, 44*(3), 427-434.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M., & Keller, M. (2002). Cultivating the uncultured. *Proc Natl Acad Sci,* 99(24), 15681-15686.

Zhang, H., Lee, Y. K., Zhang, W., & Lee, H. K. (2006). Culturable actinobacteria from the marine sponge *Hymeniacidon perleve*: isolation and phylogenetic diversity by 16S rRNA gene-RFLP analysis. *Antonie Van Leeuwenhoek,* 90(2), 159-169.

Zhang, L., Xi, L., Ruan, J., & Huang, Y. (2012). *Micromonospora yangpuensis* sp. nov., isolated from a sponge. *Int J Syst Evol Microbiol,* 62(2), 272-278.

Zhang, W., Li, Z., Miao, X., & Zhang, F. (2009). The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China Sea sponges. *Mar Biotechnol,* 11(3), 346.

Zhang, Y. Q., Schumann, P., Li, W. J., Chen, G. Z., Tian, X. P., Stackebrandt, E., & Jiang, C. L. (2005). *Isoptericola halotolerans* sp. nov., a novel actinobacterium isolated from saline soil from Qinghai Province, north-west China. *Int J Syst Evol Microbiol,* 55(5), 1867-1870.

Zhang, Z., Gao, X., Qiao, Y., Wang, Y., & Zhang, X. H. (2015). *Muricauda pacifica* sp. nov., isolated from seawater of the South Pacific Gyre. *Int J Syst Evol Microbiol,* 65(11), 4087-4092.

Zheng, L., Chen, H., Han, X., Lin, W., & Yan, X. (2005). Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge *Hymeniacidon perleve*. *World J Microbiol Biotechnol,* 21(2), 201-206.

Zhuang, L., Lin, B., Qin, F., & Luo, L. (2018). *Zhouia spongiae* sp. nov., isolated from a marine sponge. *Int J Syst Evol Microbiol,* 68(7), 2194-2198.

# 8 Appendices

## Appendix 1 Microscopic Staining procedures

Content removed for privacy reasons

## Appendix 2 Phospholipid detection sprays

Content removed for privacy reasons

Appendix 3 Descriptions of bacteria isolates from marine sponges from Glenelg and Rapid Bay beachs of South Australia

Content removed for privacy reasons

**Appendix 4. List of 383 bacterial isolates by incubation condition and RFLP patterns.**

Content removed for privacy reasons

Appendix 5: partial and full 16S rRNA gene sequence of selected bacteria isolates from each RFLP patteren and phyolgentic tree for three novel bacteria.

Content removed for privacy reasons

Appendix 6 Growth potential of 383 bacteria isolates at different ranges of temperature

Content removed for privacy reasons

Appendix 7 Growth potential of 383 bacteria at different concentration of NaCl

Content removed for privacy reasons

## Appendix 8 Sample biochemical reactions

Content removed for privacy reasons

**Appendix 9 The genera profile of NGS data from three set of primers**

Content removed for privacy reasons