ABSTRACT

Microbial populations in human inhabited coastal environment are subject to disturbance and human activity. Thus, the diversity of microbes in this environment is often interesting because of the challenges that the microbial population faced. In this study, actinobacteria was isolated from intertidal sediment samples and *Padina antillarum* (Dictyotales, Phaeophyceae) from Tanjung Tuan (Cape Rachado), Port Dickson, Malaysia. Isolates were subjected to polymerase chain reaction (PCR) screening specific for *Streptomycetaceae* and *Micromonosporaceae*, prior to characterization by phenotypic characterisation and its genotyping for the purpose of clustering. Molecular identification by 16S rRNA gene sequence revealed five families and nine genera, namely *Streptomycetaceae*, *Micromonosporaceae*, *Pseudonocardiaceae*, *Nocardiaceae* and *Tsukamurellaceae*. The presence of a relatively higher number of isolates belonging to *Streptomycetaceae* and *Micromonosporaceae* is as expected. However, two endophytic isolates PE32 and PE37 have been identified to be closely related to the clinical actinobacteria *Nocardia nova* (99.9% pairwise similarity) and *Williamsia muralis* (99.4% pairwise similarity), respectively. Isolate PE36 could be a novel species within the genus *Prauserella*, with 96.6% pairwise similarity to the closest identification, *Prauserella marina*. Additional characterization to identify potentially bioactive isolates were carried out to detect ketoacyl synthase and methyl- transferase domain of malonyl -type I polyketide synthases (PKSI) and adenylation domains in non- ribosomal peptide synthetase (NRPS) . Isolates were also evaluated for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* by resazurin based microtitre assay. The presence of the target genes and detectable antibacterial activity showed a high correlation. Additionally isolate SE31 was wholly genome sequenced.

ABSTRAK

Populasi mikrob dalam persekitaran pantai yang didiami manusia adalah tertakluk kepada gangguan dan aktiviti manusia. Dengan itu, kepelbagaian mikrob dalam persekitaran ini sering menarik kerana cabaran yang dihadapi. Dalam kajian ini, aktinobakteria telah dipencilkan daripada sampel sedimen intertidal dan *Padina antillarum* (Dictyotales, *Phaeophyceae*) dari Tanjung Tuan (Cape Rachado), Port Dickson, Malaysia. Terikan telah tertakluk kepada pemeriksaan tindak balas rantai polymerase (PCR) spesifik untuk *Streptomycetaceae* dan *Micromonosporaceae,* sebelum pencirian melalui ciri fenotip (warna) dan ciri genotip (PCR turutan jujukan berulangan) bagi tujuan pengelompokan. Pengenalpastian molekular melalui gen 16S rRNA mendedahkan lima famili dan sembilan genus, iaitu *Streptomycetaceae* (*Streptomyces*), *Micromonosporaceae* (*Micromonospora* dan *Verrucosispora*), *Pseudonocardiaceae* (*Prauserella*, *Pseudonocardia* dan *Sciscionella*), *Nocardiaceae* (*Nocardia* dan *Williamsia*) dan *Tsukamurellaceae* (*Tsukamurella*). Kehadiran bilangan strain relatif yang lebih tinggi oleh *Streptomycetaceae* dan *Micromonosporaceae* adalah seperti yang dijangkakan. Walau bagaimanapun, dua terikan endofitik PE32 dan PE37 telah dikenalpasti sebagai mikrob klinikal iaitu *Nocardia nova* (99.9%) dan *Williamsia muralis* (99.4%). Terikan PE36 berkemungkinan spesies novel dalam genus *Prauserella* (96.6%). Pencirian tambahan untuk mengenalpasti terikan yang berpotensi bioaktif telah dilakukan dengan primer untuk mengesan ‘synthase ketoacyl’ dan domain ‘metil-transferase malonyl’ dalam ‘polyketide synthases’ jenis-I (PKSI) dan domain ‘adenylation’ dalam synthetase peptida bukan ribosom (NRPS). Terikan juga dinilai untuk aktiviti antibakteria terhadap *Escherichia coli*, *Staphylococcus aureus* dan *Bacillus subtilis* melalui pengujian resazurin-microtiter. Kehadiran gen sasaran dan aktiviti antibakteria yang dikesan menunjukkan korelasi yang tinggi. Tambahan pula, turutan jujukan genom terikan SE31 telah diperolehi.

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CHAPTER 1

1.1 Introduction

Historically, actinobacterial population were generally thought to only occur in terrestrial environment and actinobacteria exist in marine environments generally as dormant spores (Goodfellow & Haynes, 1984). However, subsequent research has revealed that a great diversity and abundance of actinobacteria are in the marine environments (Tan *et al.*, 2005; Vikineswary *et al.*, 2005; Abbas, 2006; Subramani & Aalbersberg, 2012; Eom *et al.*, 2013). Even though studies indicate that major populations of marine actinobacteria reside in marine sediments (Jensen *et al.*, 2005; Maldonado *et al.*, 2009), however, increasing studies on marine actinobacteria isolated from marine macroorganisms (Ismet *et al.*, 2004; Vikineswary, *et al.*, 2005; Zheng *et al.*, 2005; Kanagasabhapathy *et al.*, 2006; Lam, 2006; Zhang *et al.*, 2008) have display highly evolved marine adaptations. Transient marine actinobacteria that grow on the surfaces of marine macroorganisms survive in a highly volatile and competitive environment where space and access to nutrients are limited (Zheng, *et al.*, 2005; Kanagasabhapathy, *et al.*, 2006; Jensen & Lauro, 2008; Carvalho & Fernandes, 2010). As a result, these epiphytic and endophytic actinomycetes have been acknowledged to be of higher percentage of compound-producing isolates than that observed in free-living marine environments (Zheng, *et al.*, 2005). Consequently, isolation and cultivation of a broad range of taxa are needed to assess the chemical and genetic diversity of marine actinobacteria (Goodfellow & Fiedler, 2010) and hence their full potential as a source of novel metabolites. Continuous studies on marine actinobacteria have revealed many new chemical entities and bioactive metabolites (Waldron *et al.*, 2000; Strobel & Daisy, 2003; Tormo *et al.*, 2003; Bull *et al.*, 2005; Jensen, *et al.*, 2005; Zheng, *et al.*, 2005; Lam, 2006; Baltz, 2007; Bull & Stach, 2007; Laidi *et al.*, 2008; Lin *et al.*, 2009; Goodfellow & Fiedler, 2010; Schneemann *et al.*, 2010).

However, the current downward trends of novel compound discovery and the decreasing number of approved compound for medical use has been daunting. Compounds that were previously isolated are incessantly recurring, rendering novel compound discovery sporadic. If, and when, novel compounds accomplished detailed laboratorial setting, it will proceed to compound development and clinical trials, many which would not be approved for medical use (Ratti & Trist, 2001; Scheffler *et al.*, 2013). Despite confronted with such despair outlook, current trend in exploration and exploitation have been a continuous undertaking with the marine inhabitant and its environment focused as new and rich reservoir for novel compounds. Marine microbial compounds have been elucidated with different approaches to discover either a novel compound or variant of a poorly explored compound (Donadio *et al.*, 2010). Among the microbial candidates sourced, actinobacteria remain the forerunner for novel compounds due to its diversity and ability to produce bioactive compounds as 70% of the known secondary metabolites are produced by actinobacteria (Subramani & Aalbersberg, 2012).

1.2 Objectives

1. To isolate and characterize actinobacteria from marine sediments and *Padina antillarum* using morphology and molecular method.
2. To screen the actinobacteria isolates for antibacterial activity and assess the biosynthetic potential for antibacterial activity.

CHAPTER 2

2.1 Bioactive Marine Actinobacteria

The class Actinobacteria contains the order Actinomycetales and several deep-branching and sometimes uncultured taxonomic groups, for example, the subclass Acidimicrobida (Stackebrandt *et al.*, 1997). On the other hand, Actinomycetes are actually members of the order Actinomycetales belonging to the class Actinobacteria, containing many readily cultivated and among the different genera and families belonging to the order Actinomycetales, the very prolific genus *Streptomyces*, the family *Micromonosporaceae* (mainly *Micromonospora* and *Actinoplanes*), the family *Pseudonocardiaceae* (mainly *Amycolatopsis*, *Saccharopolyspora* and *Saccharothrix*), the family *Thermomonosporaceae* (mainly *Actinomadura*), the family *Nocardiaceae* (*Nocardia* and related genera), and the family *Streptosporangiaceae* (mainly *Streptosporangium*) (Lazzarini *et al.*, 2000). Marine actinobacteria, strictly speaking, should only include members of the taxonomic group indigenous to the marine environment (Ward & Bora, 2006) but, since it is difficult to establish that they are indigenous to the marine environment (Tsueng & Lam, 2008; Imada *et al.*, 2010; Khan *et al.*, 2010), hence marine actinobacteria also includes actinobacteria isolated or detected in the marine environment.

Diverse actinobacteria have been sourced for microbial antibiotics, mainly from the genus *Streptomyces* (Jensen & Lauro, 2008) and marine actinobacteria are a growing source for novel biodiscovery (Bull *et al.*, 2000; Lam, 2006; Baltz, 2007; Bull & Stach, 2007). Baltz (2007) contended that the increasing recognitions for novel natural product are simply due to the complexity to synthesis clinically important antibiotics by combinatorial chemistry.

Though the study of marine actinobacteria is still developing, the advent and developments in molecular biology and genomics has greatly enhance the systematics, ecological and evolutionary studies of marine actinobacteria (Bull, *et al.*, 2005), and hence directing and improving the bioactive screening programmes (Jensen, *et al.*, 2005; Goodfellow & Fiedler, 2010).

Studies on marine macroorganisms (Ismet, *et al.*, 2004; Vikineswary, *et al.*, 2005; Zheng, *et al.*, 2005; Kanagasabhapathy, *et al.*, 2006; Lam, 2006; Zhang, *et al.*, 2008) have revealed that marine actinobacteria display highly evolved marine adaptations which include the requirement of seawater and/or sodium salt for growth. Essentially, marine actinomycetes is associated with the requirement of seawater and/or sodium/potassium salt for growth. However, the specific requirement of halophilic conditions, which has been identified as a primary characteristic of marine microorganisms, has been topics of much debate (Ismet, *et al.*, 2004; Tsueng & Lam, 2008; Imada, *et al.*, 2010; Khan, *et al.*, 2010) as the quantity, stability, and uniqueness of the salinity requirement are not established. However, there are reports that the production of secondary metabolites by obligate marine actinobacteria are only triggered under halophilic conditions (Okami *et al.*, 1976; Imada *et al.*, 2007; Tsueng & Lam, 2008). However, the production of salinosporamide B by *Salinispora tropica*, is significantly higher in the high-sodium-salt-formulation media, while, the production of salinosporamide A does not require high halophilic media formulation (Tsueng & Lam, 2008).

In a general microbiological marine survey, a non-selective media for marine actinobacteria would yield many non-actinobacterial isolates (Muscholl-Silberhorn *et al.*, 2008). However, a more refined isolation and cultivation approach, would yield more

actinobacterial isolates but, until recently, most marine actinobacterial isolates have been restricted to the genera *Micromonospora – Rhodococcus – Streptomyces* (Maldonado, *et al.*, 2009). Furthermore, phylogenetic analysis of DNA fragments recovered from DGGE bands revealed that most of the actinobacteria were uncultured and were quite different from known culturable isolates, such as members of the genera *Micromonospora*, *Rhodococcus*, and *Streptomyces* (Yoshida *et al.*, 2008). These results suggest that the marine environment is still an attractive site for discovering new marine actinobacterial populations.

Consequently, isolation and cultivation of a broad range of taxa are needed to assess the chemical and genetic diversity of marine actinobacteria (Goodfellow & Fiedler, 2010) and hence their full potential as a source of novel metabolites. The manipulation of growth conditions and selective isolation for specific taxa is used as a common strategy to improve the quantities and spectra of metabolites (Tormo, *et al.*, 2003). Due to taxonomical studies, it is now relatively easier to detect rare and uncommon actinobacteria due to the increasing availability of sound classifications based on the integrated use of genotypic and phenotypic data (Bull, *et al.*, 2000). However, the genotypic and phenotypic aspects of marine actinobacteria must be first understood before the potential of these bacteria to produce new metabolites can be fully appreciated (Jensen, *et al.*, 2005; Imada, *et al.*, 2010; Khan, *et al.*, 2010).

2.2 Seaweed as Sources of Bioactive Compounds

Seaweeds have traditionally used supplementary diet for indigenous people. However, studies have shown that seaweeds as source of bioactive compounds and produce a great variety of metabolites characterized by a broad spectrum of biological activities (Chew *et al.*, 2008; Vallinayagam *et al.*, 2009) including antimicrobial

properties (Smit, 2004; Engel *et al.*, 2007; Puglisi *et al.*, 2007; Kandhasamy & Arunachalam, 2008; Shanmughapriya *et al.*, 2008; Ibtissam *et al.*, 2009; Rajasulochana *et al.*, 2009; Vallinayagam, *et al.*, 2009).

Many bioactive compounds may have evolved due to ecological pressures (the competition for space and the maintenance of clean thallus surfaces, grazing pressures by herbivores, tolerance to dangerous levels of sunlight or UV-B radiation, desiccation during exposure at low tide or highly saline waters and conditions resulting from thallus breakage and wound formation) acting on seaweeds (Strobel & Daisy, 2003; Smit, 2004). Furthermore, exposure to different microbial assemblages may also affect the production of specific antimicrobial metabolites through induction (Puglisi, *et al.*, 2007). Consequently, the concentrations of antimicrobial metabolites may vary seasonally and geographically.

However, Puglisi, *et al.* (2007) deliberate on the function of antimicrobial metabolites in active extracts, even though they observed a consistency in the activities against relevant marine pathogens and saprophytes. Further studies have support the notion that marine plants maintain chemical defenses (Smit, 2004; Engel, *et al.*, 2007; Puglisi, *et al.*, 2007; Kandhasamy & Arunachalam, 2008; Shanmughapriya, *et al.*, 2008; Ibtissam, *et al.*, 2009; Rajasulochana, *et al.*, 2009; Vallinayagam, *et al.*, 2009), however, marine plants crude extract are complex mixtures of primary and secondary metabolites, including fatty acids that can exhibit antimicrobial activities, and, given that most marine plants contain epiphytic and endophytic microorganisms, it is not possible to rule out that microbial metabolites may contribute to the overall activity of the extracts tested (Engel, *et al.*, 2007).

Furthermore, many sessile marine plants have defense mechanisms against fouling by possible utilization of metabolites that may influence the settlement, growth and survival of other microorganisms potentially able to attach on their surface. However, Kanagasabhapathy, *et al.* (2006) observed that some algae and animals lacking chemical and physical defenses, such as surface shedding, relies on metabolites produced by surface-associated bacteria as their defense against fouling.

Henceforth pre-existing metabolites or bioactive compounds from the seaweeds would influence the microbial community within its micro-environment. Consequently, it is speculated that actinobacteria from seaweeds would have adaptation to compete for nutrient and space despite the pre-existing metabolites or bioactive compounds from the seaweeds.

2.3 Type-I polyketide synthases (PKSI) and non-ribosomal peptide synthetase (NRPS) gene fragments in Actinobacteria

There are three major classes of PKS systems, arranged by their mode of synthesis and structural type of product. One unifying theme in the diverse polyketide family of metabolites is their biosynthesis through the sequential condensation of small carboxylic acids, reminiscent of the synthesis of fatty acids in bacteria, lower and higher eukaryotes (Hopwood & Sherman, 1990). Type I PKSs in bacteria are multi-enzyme complexes that are organized into individual, linear modules, each of which is responsible for a single, specific chain elongation process and post condensation modification of the resulting β-carbonyl (Staunton & Weissman, 2001).

Non-ribosomally produced peptide metabolites are large, multifunctional enzyme complexes, that build growing chains from individually selected building blocks which

display a remarkable spectrum of activities and are extremely important in pharmaceuticals applications, as some of which are the clinically useful peptides such as the antibiotics vancomycin and penicillin, the immunosuppressive agent cyclosporine and the antitumor compound bleomycin (Jiménez *et al.*, 2010). Compounds synthesized by NRPSs can be distinguished by the presence of non-proteinogenic branched D-amino acids, which are often cyclic in structure (Walsh *et al.*, 2001; Mootz *et al.*, 2002).

A lot of new information in the biosynthetic chemical processes has been discovered over the past 20 years by sequence-based approaches with new biosynthetic chemistry discovered through the continued exploitation of information from genome sequencing projects (Jiménez, *et al.*, 2010). The annotation of newly discovered gene clusters would then complements the biochemical and bioassay data, enabling manipulation of culturing conditions to stimulate expression on previously undetected metabolite (Minowa *et al.*, 2007; Jiménez, *et al.*, 2010; Winter *et al.*, 2011; Schulze *et al.*, 2013; Zhu *et al.*, 2013). Metabolites prediction through genome mining of *Salinispora tropica* leads to Salinilactam A (Udwary *et al.*, 2007), and likewise, genome mining of two different *Streptomyces* strains that have similar biosynthetic gene cluster leads to the isolation and identification of three new polyketide (Banskota *et al.*, 2006).

Though metagenomic analysis has reveals diverse biosynthetic gene clusters (Schirmer *et al.*, 2005; Minowa, *et al.*, 2007; Pang *et al.*, 2008), however, Baltz (2007) argued that metagenomic approaches has failed so far to uncover substantially new antibiotics as the problem lies largely with technical limits, even though approaches through metagenomic analysis have revealed diverse biosynthetic gene clusters (Schirmer, *et al.*, 2005; Minowa, *et al.*, 2007; Pang, *et al.*, 2008). In the case where the biosynthetic gene cluster was obtained from metagenomic approaches, then, heterologous

expression of the gene cluster in a suitable host for compound production would pose a major hurdle, as only with the implementation of a genetically similar host strain would maximize the potential for productive transcription, translation and finally, metabolite production (Fortman & Sherman, 2005). The construction of such expression library is possible and heterologous expression has been achieved from entire biosynthetic cluster of the actinobacteria (Martinez *et al.*, 2004; Komatsu *et al.*, 2010). This approach would circumvents the need for numerous fermentations manipulation to obtain the compound of interest (Martinez*, et al.*, 2004; Pimentel Elardo, 2008). For instance, the discovery of turbomycin A and B from metagenomic library expressed by a non-actinomycete host have shown that such undertaking is viable (Gillespie *et al.*, 2002). Nonetheless such successful undertakings have been few and far between (Baltz, 2007, 2008).

Hence, simply surveying microbes for genes encoding large PKSI and NRPS can only be helpful for determining a possible potential of the sample (Ayuso *et al.*, 2005; Ostash *et al.*, 2005; Savic & Vasiljevic, 2006; Baltz, 2007).

Genome-wide analysis of biosynthetic genes, on the other hand, are already fostering new methods for predicting secondary metabolite production thereby maximizing opportunities for drug discovery (Minowa*, et al.*, 2007; Goodfellow & Fiedler, 2010). There is a great diversity of polyketide synthase and non-ribosomal peptide synthetase biosynthetic pathways in actinobacteria (Ayuso*, et al.*, 2005) as the majority of actinobacteria derived compounds are shown to be complex polyketides and non-ribosomal peptides (Donadio *et al.*, 2007; Schneemann*, et al.*, 2010). Simply because none of the other taxonomic groups devote high percentages of the coding capacity to PKS and NRPS functions as in actinobacteria (Baltz, 2008), and this further emphasizes the rationale to focus antibiotic discovery efforts on actinobacteria.

It can be assumed that a genome with a higher number of biosynthetic gene clusters is more likely to result in a positive hit in a PKSI/NRPS screening approach. Therefore, positive results in a PCR-based screening not only provide evidence of the production of corresponding metabolites, but also may indicate the existence of further metabolic pathways of secondary metabolite synthesis (Ayuso, *et al.*, 2005; Ostash, *et al.*, 2005; Savic & Vasiljevic, 2006; Schneemann, *et al.*, 2010). However, the lack of detectable gene fragments does not definitely prove the absence of the respective biosynthetic gene clusters as there are also other metabolites and other biosynthetic pathways that exist as reflected in actinobacterial genomes (Schneemann, *et al.*, 2010).

2.4 Actinobacterial Studies in Malaysia

Malaysia, has large actinobacterial diversity with an under-explored potential, however, Numata and Nimura (2003) noted that the limitation imposes by Convention on Biological Diversity limits the participations of foreign private institutions in actinobacterial research here.

But having said that, local researchers have carried out studies on Malaysian actinobacteria in natural environments over the years, though studies have been mainly focused on diversity survey; mountain range in Sabah (Lo *et al.*, 2002), mangrove soils and its macroorganisms (Tan, *et al.*, 2005; Vikineswary, *et al.*, 2005), medicinal plants, (Zin *et al.*, 2007), agricultural soils (Jeffrey, 2008), and rhizosphere soils (Ting *et al.*, 2009), leaf litters (Muramatsu *et al.*, 2011), or for phylogenetic comparative studies (Muramatsu *et al.*, 2003; Muramatsu, 2008). The studies, mostly, concluded a high diversity of actinobacteria, but with a dominant Streptomycetes population.

Survey of potential bioactive actinobacteria for antibacterial (Jeffrey, 2008; Ting, *et al.*, 2009), antifungal (Ismet, *et al.*, 2004; Zin, *et al.*, 2007; Jeffrey, 2008; Muramatsu, *et al.*, 2011), and anticancer (Lo, *et al.*, 2002; Kamal *et al.*, 2009) was also carried out, some showing promising results that warrant further research.

CHAPTER 3

3.1 Materials

Artificial sea water (ASW)

Artificial sea water with a pH of 8.2 – 8.4 and salinity of 31.0ppt was prepared by dissolving 33.3g of commercial artificial sea salt (Red Sea, United States of America) in one litre of type III reverse osmosis water.

Modified Bennett's agar, MBA [modified from Bennett's agar (Jones, 1949)]

Beef extract: 1g, glycerol: 10g, casitone: 2g, yeast extract: 1g, agar: 12g in 1L of ASW

Modified Bennett's medium, MBM [modified from Bennett's agar (Jones, 1949)]

Beef extract: 1g, glycerol: 10g, casitone: 2g, yeast extract: 1g in 1L of ASW

One-tenth strength modified Bennett's agar, 1/10MBA [modified from Bennett's agar (Jones, 1949)]

Beef extract: 0.1g, glycerol: 1g, casitone: 0.2g, yeast extract: 0.1g, agar: 12g in 1L of ASW

One-tenth strength modified Bennett's agar with 2% Gelatin, MBG [modified from Bennett's agar (Jones, 1949)]

Beef extract: 0.1g, glycerol: 1g, casitone: 0.2g, yeast extract: 0.1g, gelatine: 20g, agar: 12g in 1L of ASW

Modified organic agar, MOA [modified from Organic Medium 79 (Atlas, 2004)]

Dextrose: 1g, peptone: 1g, casein hydrolysate peptone: 0.2g, yeast extract: 0.2g, agar: 12g, sodium chloride (NaCl): 20g in 1L of distilled water

Modified starch-casein agar, MSC [modified from starch-casein agar (Kuster & Williams, 1964)]

Soluble starch: 10g, casein hydrolysate peptone: 0.3g, potassium nitrate: 2g, NaCl: 2g, monopotassium phosphate: 2g, agar: 12g in 1L of ASW

Padina extract agar, PEA [modified method of extract preparation (Atlas, 2004)]

In every 100ml of ASW, 10g of air-dried *Padina sp* specimen was extracted with a juicer. Agar (1.2g) was then added to every 100ml of *Padina* extract solution

Modified Zhang's medium, M1 [modified from Zhang's medium (Zhang *et al.*, 2006)]

Soluble starch: 10g, yeast extract: 4 g, peptone: 2 g, agar: 12g in 1L of ASW

Modified Zhang's medium, M2 [modified from Zhang's medium (Zhang, *et al.*, 2006)]

Glycerol: 6ml, arginine: 1g, dipotassium phosphate (K_2HPO_4): 1g, magnesium sulphate ($MgSO_4$): 0.5g, agar: 12g, in 1L of ASW

Modified Zhang's medium, M4 [modified from Zhang's medium (Zhang, *et al.*, 2006)]

L-asparagine: 0.1 g, K_2HPO_4: 0.5g, $MgSO_4$: 0.1g, peptone: 2g, sodium propionate: 4g, agar: 12g in 1L of ASW

Modified Zhang's medium, M7 [modified from Zhang's medium (Zhang et al., 2006)]

Peptone: 2g, L-asparagine: 0.1 g, sodium propionate: 4g, K_2HPO_4: 0.5g, $MgSO_4$: 0.1g, glycerol: 5g, agar: 12g in 1L of ASW

Modified Zhang's medium, M8 [modified from Zhang's medium (Zhang, *et al.*, 2006)]

Yeast extract: 4g, soluble starch: 15g, K_2HPO_4: 1g, $MgSO_4$: 0.1g, agar: 12g in 1L of ASW

Modified AFMS agar, AFMS [modified from AFMS medium (Monciardini *et al.*, 2002)]

Glucose: 20g, yeast extract: 2g, Soybean flour: 8g, calcium carbonate ($CaCO_3$): 4g, agar: 18g in 1L of ASW

Modified International Streptomyces Project medium 2, GYMS [modified from International Streptomyces Project medium 2, ISP2 (Shirling & Gottlieb, 1966)]

Yeast Extract: 4g, malt extract: 10g, dextrose: 4g, agar: 12g in 1L of ASW

Modified International Streptomyces Project medium 3, ISP3 [modified from International Streptomyces Project medium 3 (Shirling & Gottlieb, 1966)]

Difco Oatmeal Agar: 72.5g in 1L of ASW

Modified International Streptomyces Project medium 4, ISP4 [modified from International Streptomyces Project medium 4 (Shirling & Gottlieb, 1966)]

Difco ISP Medium 4: 37g in in 1L of ASW

Modified Waksman medium [modified from Waksman's Glucose Agar (Waksman, 1967)]

Glucose: 20g, beef extract: 5g, peptone: 5g, yeast extract: 3g, $CaCO_3$: 3g, agar: 12g in 1L of ASW

Muller-Hinton agar

Muller Hinton Broth: 21g, agar: 12g in 1L of type III reverse osmosis water

Normal saline

Sodium chloride: 8g in 1L of type III reverse osmosis water

SB buffer (Brody & Kern, 2004)

Preparation of 20X SB Buffer stock solution

4g of sodium hydroxide pellets were dissolved in 450mL of type III reverse osmosis water under constant agitation with magnetic stirrer. The pH was then adjusted to 8.5 with boric acid. Total volume of the solution was then topped up to 500mL with type III reverse osmosis water.

Preparation of 1X SB Buffer working solution

Stock solution of 20X SB Buffer was diluted to 1X with type III reverse osmosis water.

3.2 Collection and preparation of samples

Specimens of seaweed, *Padina antillarum* (Dictyotales, *Phaeophyceae*) and its surrounding soil sediments were collected in March 2008 from the intertidal beach of Tanjung Tuan, Malacca (2° 24' N, 101° 51' E) during receding tide.

Seaweed specimens were placed in zip-lock plastic bags containing natural seawater, soil sediments were pooled in 50ml sterile plastic collection tubes. All samples were transported to the laboratory soonest.

In the laboratory, seaweed specimens were rinsed under running tap water, then rinsed three times with sterile ASW and kept at 4ºC until further use.

A portion of the soil sediments were spread unto sterile glass petri dishes and left to air-dry at room temperature. The rest of the portion were and kept at 4ºC until further use.

3.3 Isolation of actinobacteria

Air-dried soil sediment (5g) was 10-fold serially diluted with sterile ASW to 10^{-8}, whereas, 5g of *Padina antillarum* leaves were bead-beat for 3 minutes and the homogenate was 10-fold serially diluted with sterile ASW to 10^{-8}.

Dilution was plated in triplicates onto MBA, 1/10MBA, MBG, MOA, MSC, PEA, M1, M2, M4, M7 and M8. All media were adjusted to pH 8.0 ± 0.2 (soil pH: 8.0) prior to autoclaving at 121ºC for 15mins. Media were then supplemented with potassium dichromate, nystatin and/or cycloheximide (50µg/ml each) after autoclaving to control fungal growth.

Growth of actinobacteria was promoted by adopting the one of following strategies: additional supplement of nalidixic acid (25µg/ml) into media, or prepared dilution were incubated for 60ºC for 15mins prior to plating. Both strategies were employed to reduce the number of non-actinobacteria.

Plates were incubated at 24ºC for 3 weeks. Enumeration (CFU/g) of total bacteria and putative actinobacteria were carried out on the seventh, fourteenth, and twenty-first day during incubation.

Putative actinobacteria then were purified onto MBA and the isolation media which the isolates were selected. Plates were incubated at 24ºC. Medium that gives better growth rate will be used for subsequent culturing.

Purified actinobacterial isolates were kept as slant culture and as 20% (w/v) glycerol stock. The slant cultures were stored at room temperature and kept in dark while 20% (w/v) glycerol stocks were kept in freezer at -20ºC and -80ºC.

All isolates were then clustered based colour-grouping of isolates were observed from AFMS, GYMS, ISP3, ISP4, MBA and Waksman media, after 14 days incubation. Colour grouping data was converted to binary data prior to clustering. Hierarchical cluster analysis (Method: Between group linkage; Measure: Binary, Coefficients: Simple matching, Jaccard, and Dice) was done using Statistical Product and Service Solutions (SPSS) version 16.0 (IBM, United States of America).

3.4 Salinity test

Salinity tolerance test as modified from method established by Kutzner (1981) was conducted by incorporating 2%, 10%, and 15% (w/v) of sodium chloride (NaCl) into MBA2 (as MBA but instead of ASW, ultrapure water was used). Isolates were also plated on MBA2 as control. Plates were observed after 3, 5, 7 and 14 days of incubation at 28ºC.

3.5 Antibacterial Assay

Purified isolates were grown on MBM for 10 days. Supernatant (centrifugation at 9000g for 30 minutes) was filtered sterilized (cellulose acetate membrane filter, pore size: 0.2µm, Sartorius, Germany) and freeze-dried prior to reconstitution in ultrapure water at the concentration of 1g/ml. The concentrated and sterilized supernatant were then tested against *Escherichia coli* M2, , *Staphylococcus aureus* M33 and *Bacillus subtilis* M57 in resazurin microtiter assay (REMA) (Sarker *et al.*, 2007). Antibiotic stock of nalidixic acid, penicillin G, ampicillin, neomycin and streptomycin (500mg/ml) was prepared. All bacterial strains were obtained from culture collection of Institute of Biological Sciences (Microbiology), Faculty of Science, University of Malaya. Each bacterial strain was first grown overnight on Mueller-Hinton agar at 37ºC. Pure colonies were then suspended in 1ml of sterile normal saline. The optical density of the suspension was then recorded. The actual bacterial counts in the suspension were enumerated with haemocytometer. Suspensions with different optical density were used for enumeration of the bacterial count to obtain standard curves which were plotted with optical density of the suspension against the actual bacterial count. Bacterial suspension (5×10^5 bacteria/ml) was then used for REMA. The 0.1% (w/v) resazurin solution was prepared by dissolving resazurin powder (Aldrich, United States of America) in ultrapure water. Resazurin solution was then sterilized by filtration through a 0.2µm membrane. The resazurin solution is prepared fresh when needed. The 96-well microtiter plates were prepared under aseptic conditions

in a biosafety cabinet (AirStream, Esco, Singapore). REMA were then used to test the concentrated (100mg/ml) and sterilized supernatant. Supernatants were tested in triplicates. Supernatant that shows bioactivity against either *E. coli* M2, *S. aureus* M33 and *B. subtilis* M57 were then used for a subsequent screening employing REMA to determine the minimum inhibitory concentration (MIC) against the respective bacterial strains.

The initial screening with REMA was set up with a final volume of 150µl in each well containing 75µl of double strength Mueller-Hinton Broth, 30µl of bacterial suspension (5 X 10^5 bacteria/ml), 15µl of supernatant stock (1g/ml) or 15µl of antibiotic stock (500mg/ml), 15µl of 0.1% resazurin solution and 15µl of ultrapure water.

The subsequent screening with REMA was set up with a final volume of 150µl in each well containing 75µl of two times strength Mueller-Hinton Broth, 30µl of bacterial suspension (5 X 10^5 bacteria/ml), 15µl of 10-fold sequentially diluted stock (initial final concentration of 100mg/ml) or 15µl of 10-fold sequentially diluted antibiotic stock (initial final concentration of 50mg/ml), 15µl of 0.1% resazurin solution and 15µl of ultrapure water.

All plates were incubated at 37°C for 18 hours. Bioactivity was determined visually when the colour of resazurin solution changes from blue to purple or pink.

3.6 Molecular Characterization

3.6.1 Total genomic DNA extraction

Pure cultures of purified isolates grown on MBA at 28°C were used for total genomic DNA extraction using NucleoSpin® Tissue extraction kit (Macherey-Nagel, Germany). Bacterial biomass was suspended in 180µl pre-lysis buffer (Buffer T1) supplemented with lysozyme (20mg/ml) and incubated for one hour at 37°C. Suspensions were then incubated overnight at 56°C after the addition of 25µl Proteinase K (20mg/ml). Complete lysis of the suspension was obtained after the addition of 200µl lysis buffer (Buffer B3) and incubation at 70°C for 10 minutes. Binding condition of the lysates was adjusted with addition of 200µl molecular graded ethanol (Merck, Germany). Pure genomic DNA was eluted from the binding silica membrane after filtration and washing. Concentration of the eluted genomic DNA was determined with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States of America). Genomic DNA was also examined for integrity and quality with 0.8% (w/v) agarose gel electrophoresis, ran for 30mins at 100V in SB buffer. The genomic DNA was stored at -20°C until use.

3.6.2 Gene fragment screening

Genotyping with *Streptomycetaceae* and *Micromonosporaceae* specific 16S rRNA gene amplification

All isolates were screened with primer pairs for *Streptomycetaceae* and *Micromonosporaceae* specific 16S rRNA gene fragment (Monciardini, *et al.*, 2002). Family-specific 16S rRNA gene fragment amplifications were first optimized using Veriti® Thermal Cycler (Applied Biosystems, United States of America) in a final volume of 20µl containing 20ng of genomic DNA, 10µl of 2X MyTaq^{TM} Mix (Bioline,

United Kingdom), and 0.4µl of 10µM of each primer. Annealing temperature, that yielded the expected size of amplification product from the isolates previously identified with 16S rRNA, was selected as annealing temperature for subsequent amplification. Amplified products were examined with 1.0% (w/v) agarose gel electrophoresis, ran for 45mins at 100V in SB buffer.

Streptomycetaceae specific amplification

PCR primers: Sm6F (5'- GGTGGCGAAGGCGGA -3');

Sm5R (5'- GAACTGAGACCGGCTTTTTGA -3')

Expected size of amplification product: 600bp

Amplifications profile: initial denaturation of 5 minutes at 95ºC, followed by 35 cycles of 45s at 95ºC, 2 minutes at 68ºC (optimization step: 65ºC, 66ºC, 68ºC and 70ºC) and 1 minute at 72ºC, and final 10 minutes incubation at 72ºC.

Micromonosporaceae specific amplification

PCR primers: M2F (5'- SAGAAGAAGCGCCGGCC -3');

A3R (5'- CCAGCCCCACCTTCGAC -3')

Expected size of amplification product: 1000bp

Amplifications profile: initial denaturation of 5 minutes at 95ºC, followed by 35 cycles of 45s at 95ºC, 2 minutes at 69ºC (optimization step: 68ºC, 68.5ºC, 69ºC and 70ºC) and 1 minute at 72ºC, and final 10 minutes incubation at 72ºC.

PKSI and NRPS gene fragment screening

All isolates were also screened with degenerate primers for PKSI ketoacyl synthase and methyl-malonyl transferase domains and NRPS adenylation domain (Ayuso, *et al.*, 2005). Gene fragment amplifications was carried out using Swift^{TM} Maxi

Thermal Cycler (Esco, Singapore) in a final volume of 50µl containing 50ng of genomic DNA, 10µl of 5X Green GoTaq® Flexi Buffer (Promega, United States of America), 3µl of 25mM magnesium chloride, 1µl of 40mM dNTP Mix (Promega, United States of America), 1µl of 10µM of each primer and 0.15µl of GoTaq® Flexi DNA Polymerase (Promega, United States of America). Amplified products were examined with 1.0% (w/v) agarose gel electrophoresis ran for 45mins at 100V in SB buffer.

PKSI amplification

PCR primers: K1F (5'-TSAAGTCSAACATCGGBCA-3');

M6R (5'-CGCAGGTTSCSGTACCAGTA-3')

Expected size of amplification product: 1200bp-1400bp

Amplifications profile: initial denaturation of 5 minutes at 95ºC, followed by 35 cycles of 30s at 95ºC, 2 minutes at 55ºC and 4 minutes at 72ºC, and final 10 minutes incubation at 72ºC.

NRPS amplification

PCR primers: A3F (5'-GCSTACSYSATSTACACSTCSGG-3');

A7R (5'-SASGTCVCCSGTSCGGTAS-3')

Expected size of amplification product: 700bp-800bp

Amplifications profile: initial denaturation of 5 minutes at 95ºC, followed by 35 cycles of 30s at 95ºC, 2 minutes at 59ºC and 4 minutes at 72ºC, and final 10 minutes incubation at 72ºC.

3.6.3 Repetitive Sequence-derived Genotyping

Repetitive sequence-derived amplification using the BOXA1R primer was used to generate genomic fingerprints of all the isolates. Genomic fingerprint amplifications was first optimized using Veriti® Thermal Cycler (Applied Biosystems, United States of America) in a final volume of 25µl containing 25ng of genomic DNA, 5µl of 5X Green GoTaq® Flexi Buffer (Promega, United States of America), 1.5µl of 25mM magnesium chloride, 0.5µl of 40mM dNTP Mix (Promega, United States of America), 2.5µl of 20µM BOXA1R primer and 0.075µl of GoTaq® Flexi DNA Polymerase (Promega, United States of America) with four randomly selected isolates. Annealing temperature that yielded clear banding patterns of amplification products was repeated three times to check for reproducibility. Genomic fingerprint of all isolates were then generated at least twice. Amplified products were examined with 2.0% (w/v) agarose gel electrophoresis ran for 90mins at 100V in SB buffer.

PCR primer: BOXA1R (5'- CTACGGCAAGGCGACGCTGACG -3');
Amplifications profile: initial denaturation of 7 minutes at 95ºC, followed by 35 cycles of 1 minute at 95ºC, 1 minute at 55ºC (optimization step: 55ºC, 58ºC, 60ºC and 65ºC) and 8 minutes at 72ºC, and final 15 minutes incubation at 72ºC.

All fingerprinting patterns obtained from repetitive sequence-derived amplification using the BOXA1R primer were also converted to binary data to be used for hierarchical cluster analysis (Method: Between group linkage; Measure: Binary, Coefficients: Simple matching, Jaccard, and Dice) using SPSS version 16.0.

3.6.4 16S rRNA gene amplification, sequencing and analysis

Selected representative isolates were subjected to 16S rRNA gene amplification for the purpose of identification. 16S rRNA gene fragment amplifications was carried out using Swift^{TM} Maxi Thermal Cycler (Esco, Singapore) in a final volume of 50µl containing 50ng of genomic DNA, 10µl of 5X Green GoTaq® Flexi Buffer (Promega, United States of America), 3µl of 25mM magnesium chloride, 1µl of 40mM dNTP Mix (Promega, United States of America), 1µl of 10µM of each primer and 0.15µl of GoTaq® Flexi DNA Polymerase (Promega, United States of America).

PCR primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3');

1492R (5'-TACGGYTACCTTGTTACGACTT-3')

Expected size of amplification product: 1500bp

Full amplifications profile: initial denaturation of 5 minutes at 95ºC, followed by 35 cycles of 45s at 95ºC, 1 minute at 55ºC and 1 minutes at 72ºC, and final 10 minutes incubation at 72ºC.

The expected 1500bp amplified 16S rRNA gene fragment was purified using a NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany) prior to sequencing. The purified product were examined with 1.2% (w/v) agarose gel electrophoresis, ran for 60mins at 100V in SB buffer. Concentration of the purified product was determined with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States of America). Sequencing of the amplified products was carried out by commercial sequencing provider 1st BASE (BASE Life Sciences Holdings, Malaysia) using BigDye® Terminator chemistry on the Applied Biosystems 3730xl DNA Analyser.

The 16S rRNA sequences were visually checked using Sequence Scanner Software version 1.0 (Applied Biosystems, United States of America) and at least 600bp were aligned with comparative sequences of reference type-strains retrieved from the GenBank database through the EzTaxon server (Kim *et al.*, 2012) using MEGA 5.0 (Tamura *et al.*, 2011).

CHAPTER 4

4.1 Isolation of actinobacteria

A total of 62 putative actinobacterial isolates were obtained from specimens of seaweed, *Padina antillarum* (31 isolates) and its surrounding soil sediments (31 isolates) from different media (Table 4.1, Appendix A) with highest number of soil isolates from MBA and highest number of seaweed isolates from MOA.

Table 4.1: Number of putative actinobacteria isolated with different media.

Media	*Padina antillarum*	Soil
MBA	2	13
$\frac{1}{10}$MBA	1	1
MBG	0	7
MOA	9	7
MSC	1	2
PEA	8	1
M1	3	0
M2	2	0
M4	2	0
M7	1	0
M8	2	0

All isolates were first clustered into either *Streptomycetaceae* or *Micromonosporaceae* based on family specific PCR screening (Figures 4.1 and 4.2). Isolates that are not tested positive were clustered as a third group. This clustering is in line with other reports that also suggest that *Micromonospora* spp. and *Streptomyces* spp. are extensively found at shore and aquatic environments (Bredholt *et al.*, 2008; Maldonado, *et al.*, 2009). Twenty eight isolates (25 isolates from sediment, 3 from *Padina*) were positive for *Streptomycetaceae* specific amplification, 19 (3 isolates from sediment, 16 from *Padina*) were positive for *Micromonosporaceae* specific amplification, while the other 15 isolates (3 isolates from sediment, 12 from *Padina*) were negative for both *Streptomycetaceae* and *Micromonosporaceae* specific amplification.

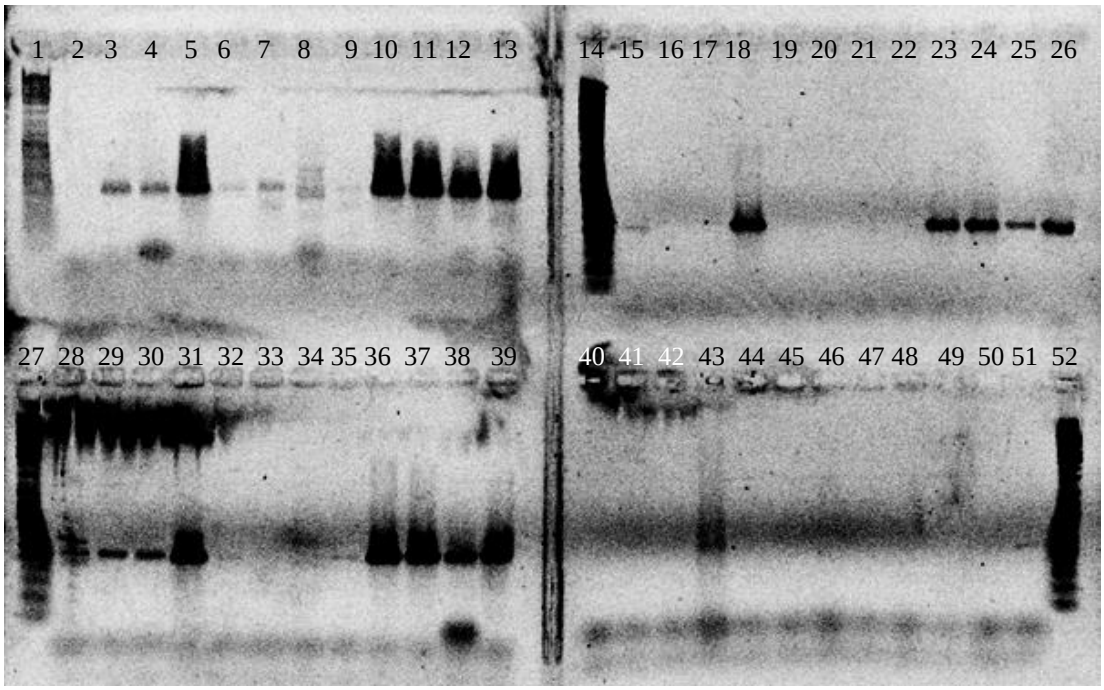


Figure 4.1: *Streptomycetaceae*-specific amplification with expected size of amplification product of 600bp. Amplification profiles with different annealing temperature (65ºC, 66ºC, 68ºC and 70ºC) to select for the optimum temperature that would yield expected amplification product. Samples selected as control were previously identified by 16S rRNA gene sequences.

Lane				Samples	16S rRNA gene sequence identification to family
Annealing temperature:					
65°C	68°C	66°C	70°C		
1	14	27	52	Gene Ruler DNA Ladder Mix, ready-to-use (Thermo Fisher Scientific Inc., United States of America)	
2	15	28	40	PE 4	*Micromonosporaceae*
3	16	29	41	PE 14	*Micromonosporaceae*
4	17	30	42	PE 17	*Micromonosporaceae*
5	18	31	43	PE 28	*Streptomycetaceae*
6	19	32	44	PE 31	*Pseudonocardiaceae*
7	20	33	45	PE 32	*Nocardiaceae*
8	21	34	46	PE 36	*Pseudonocardiaceae*
9	22	35	47	PE 37	*Nocardiaceae*
10	23	36	48	SE 1	*Streptomycetaceae*
11	24	37	49	SE 30	*Streptomycetaceae*
12	25	38	40	SE 42	*Streptomycetaceae*
13	26	39	51	SE 43	*Streptomycetaceae*

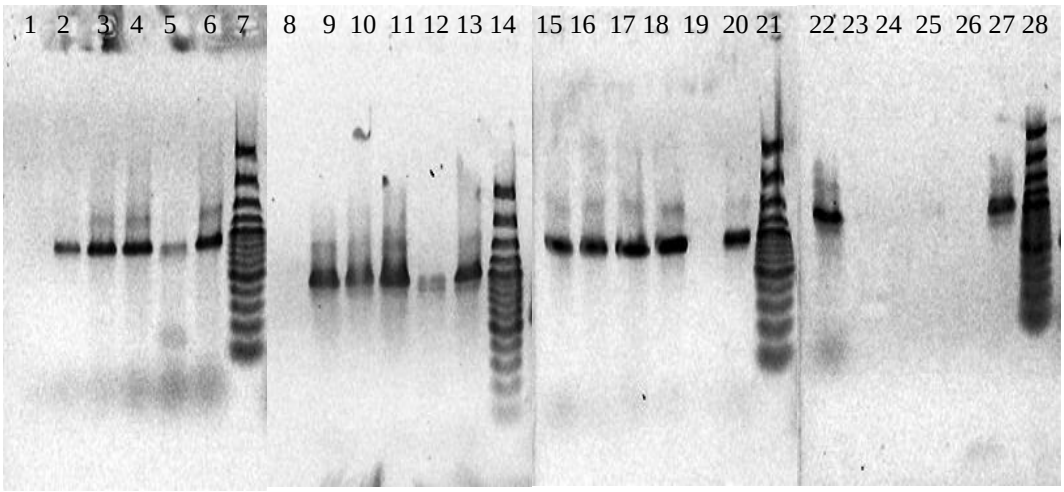


Figure 4.2: *Micromonosporaceae*-specific amplification with expected size of amplification product of 1000bp. Amplification profiles with different annealing temperature (68ºC, 68.5ºC, 69ºC and 70ºC) to select for the optimum temperature that would yield expected amplification product. Samples used as control were previously identified by 16S rRNA gene sequencing.

Lane				Samples	16S rRNA gene sequence identification to family
Annealing temperature:					
68°C	68.5°C	69°C	70°C		
1	8	15	22	PE 14	*Micromonosporaceae*
2	9	16	23	PE 17	*Micromonosporaceae*
3	10	17	24	PE 25	*Micromonosporaceae*
4	11	18	25	PE 29	*Micromonosporaceae*
5	12	19	26	PE 31	*Pseudonocardiaceae*
6	13	20	27	PE 35	*Micromonosporaceae*
7	14	21	28	Gene Ruler 100bp Plus DNA Ladder, ready-to-use (Thermo Fisher Scientific Inc., United States of America)	

The colour grouping as delineation method based on colour observed on AFMS (Figure 4.3), GYMS (Figure 4.4), ISP3 (Figure 4.5), ISP4 (Figure 4.6), MBA (Figure 4.7) and Waksman (Figure 4.8) media was carried out. Each medium presented a dissimilar colour-type: AFMS (14 colour-types), GYMS (12 colour-types), ISP3 (9 colour-types), ISP4 (10 colour-types), MBA (14 colour-types) and Waksman (13 colour-types). Each isolate was scored for 'absence/presence' in each colour-type. Combination of colour-types has clustered members of *Streptomycetaceae* into 7 colour-groups, members of *Micromonosporaceae* into 5 colour-groups and as for the rest of the isolates into 8 colour-groups (Table 4.2) based on corresponding rescaled distance estimated from hierarchical cluster analysis (Method: Between group linkage; Measure: Binary, Coefficients: Simple matching, Jaccard, and Dice).

Concurrently to the colour grouping, isolates were also clustered based on fingerprinting patterns obtained from repetitive sequence-derived amplification (Figure 4.9). Likewise, members of *Streptomycetaceae* were clustered into 13 fingerprinting patterns, members of *Micromonosporaceae* into 9 fingerprinting patterns and as for the rest of the isolates into 10 fingerprinting patterns (Table 4.3).

The grouping results obtained by phenotypic colour grouping and genotypic fingerprinting revealed positive correlation ($n = 61$; correlation co-efficient: $r = 0.989$; $p < 0.0001$). Furthermore, representative member of each grouping were identified on the basis of pairwise similarity of the 16S rRNA gene sequences (Table 4.4). However, isolate PE22, which was identified to be closely related to *Bacillus vietnamensis*, were then excluded from subsequent analysis.

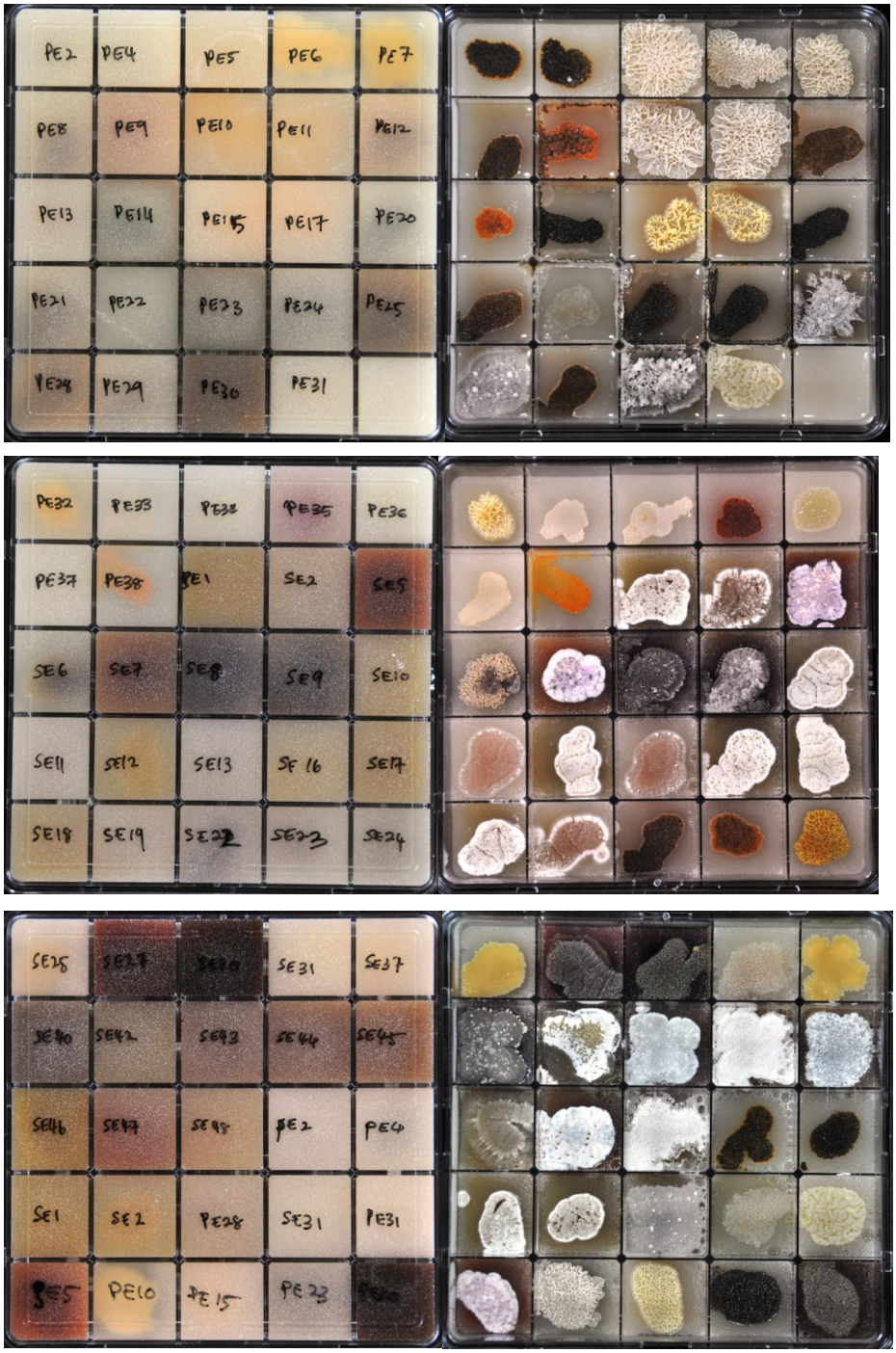


Figure 4.3: Pure culture of actinobacterial isolates inoculated onto AFMS. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

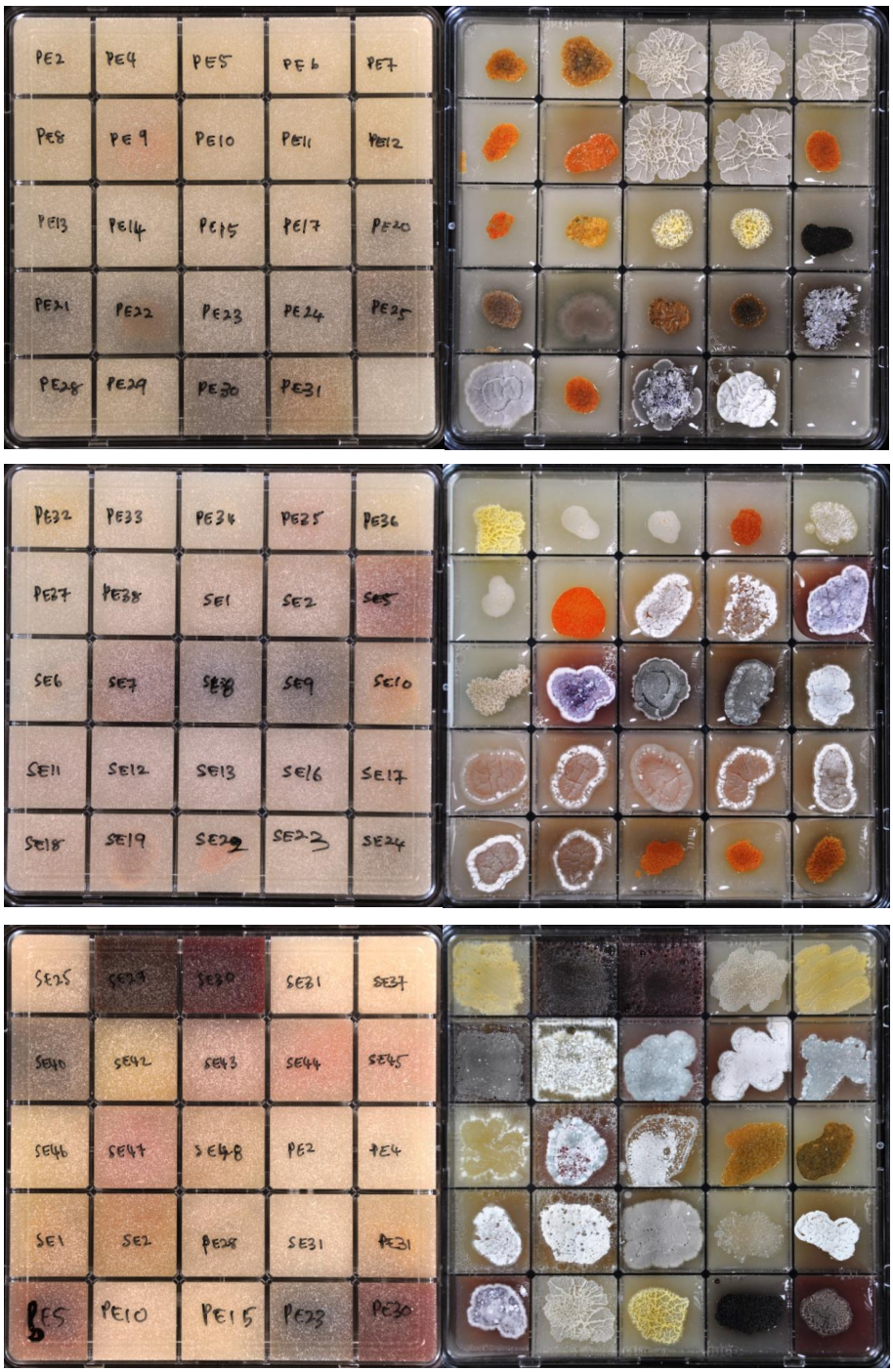


Figure 4.4: Pure culture of actinobacterial isolates inoculated onto GYMS. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

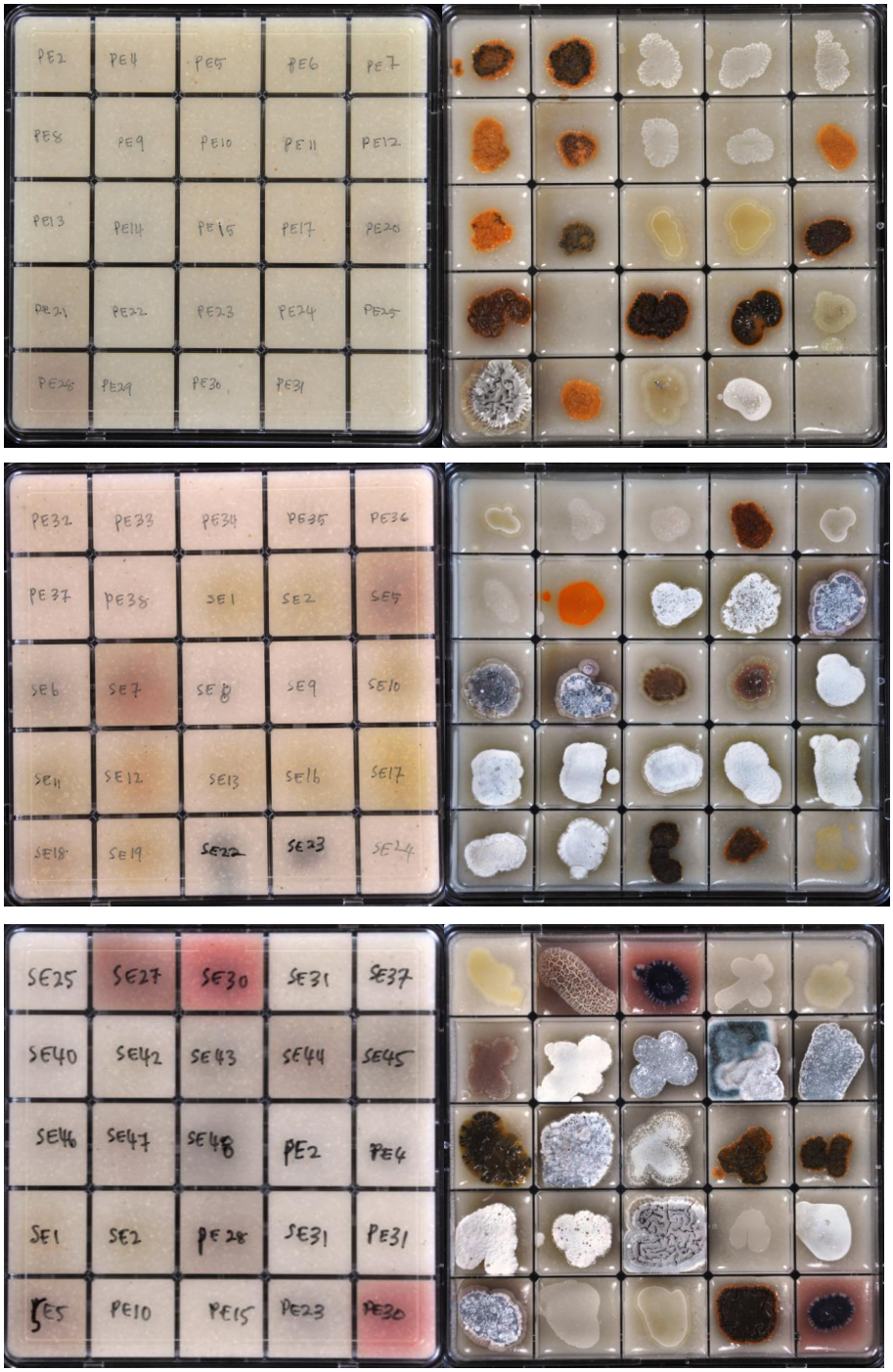


Figure 4.5: Pure culture of actinobacterial isolates inoculated onto ISP3. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

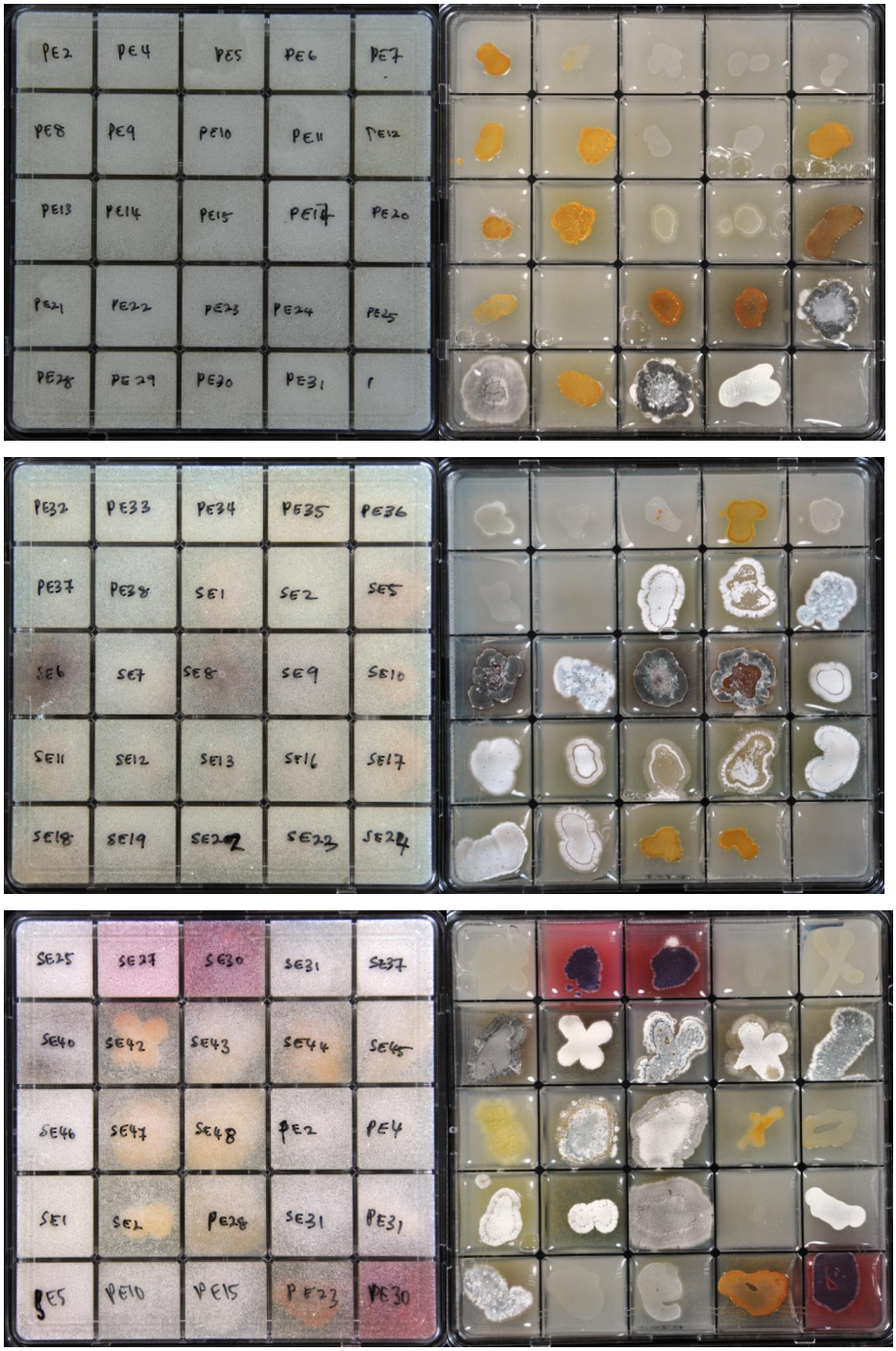


Figure 4.6: Pure culture of actinobacterial isolates inoculated onto ISP4. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

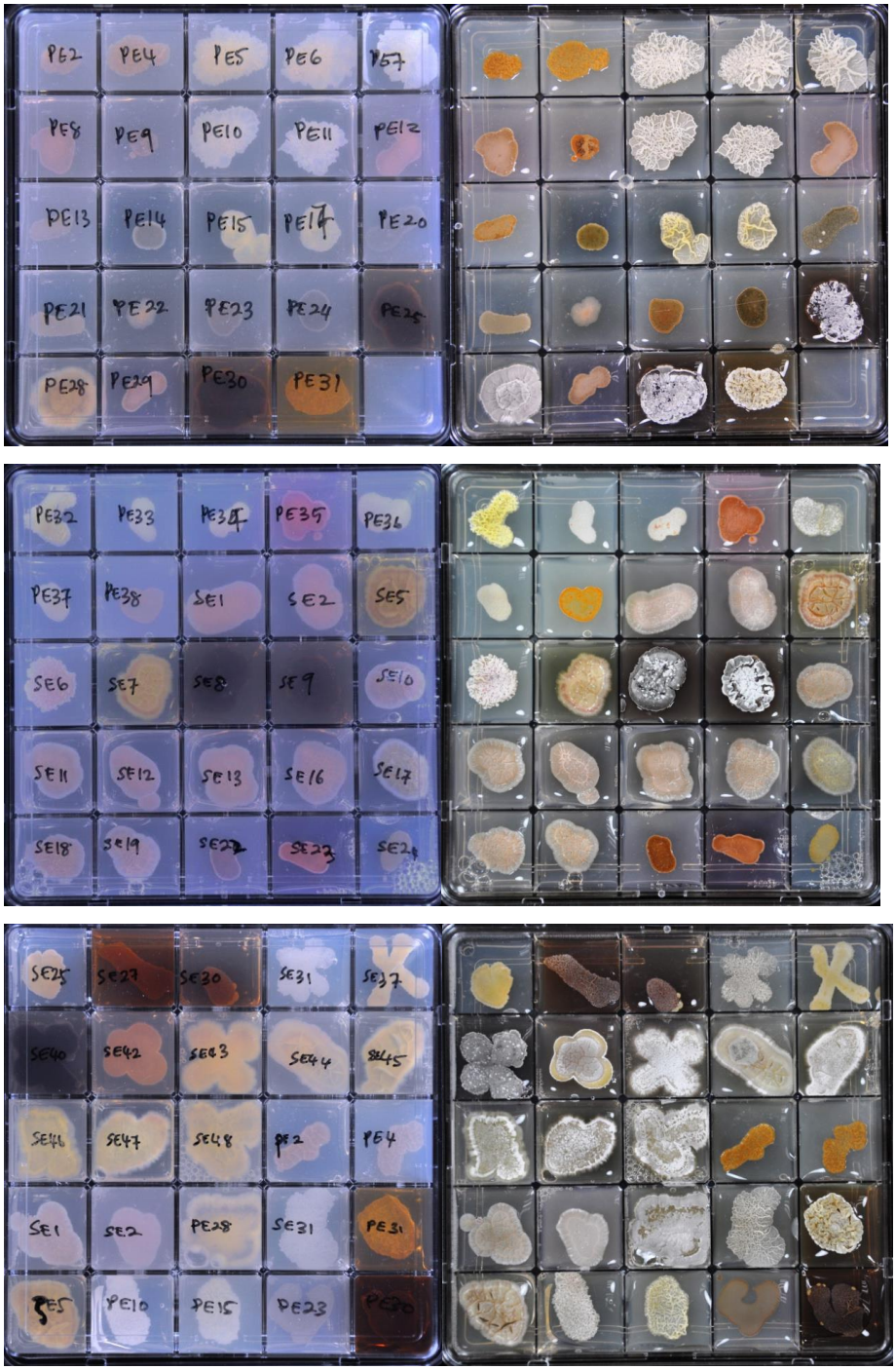


Figure 4.7: Pure culture of actinobacterial isolates inoculated onto MBA. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

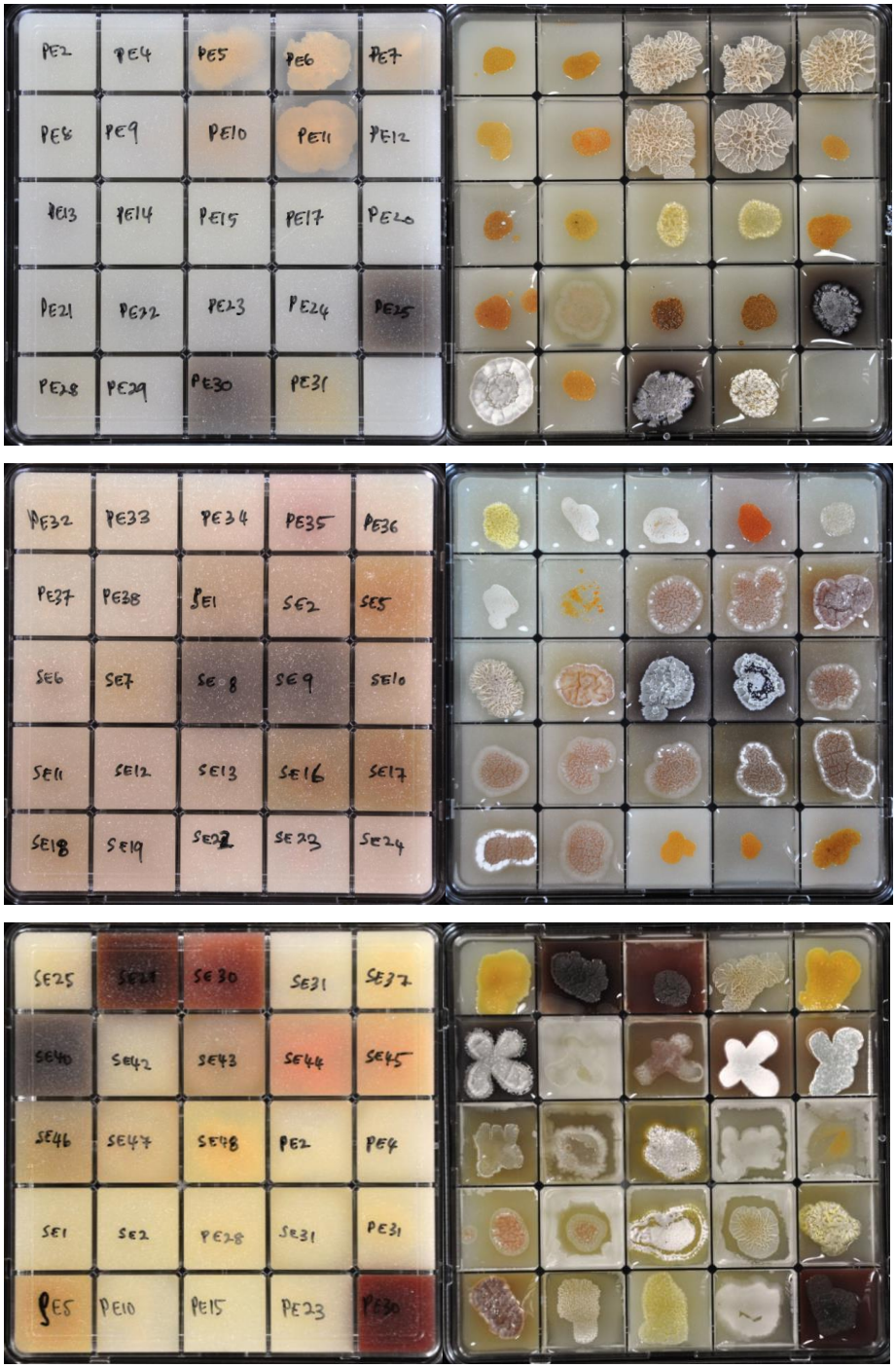


Figure 4.8: Pure culture of actinobacterial isolates inoculated onto Waksman. Isolates were grouped based on colour of the aerial (right) and substrate (left) mycelia. Diffusible pigment when present was also recorded.

Table 4.2: Colour-grouping of isolates based on colour of aerial mycelium, substrate mycelium and pigmentation colour when grown on AFMS, GYMS, ISP3, ISP4, MBA and Waksman media.

Cluster	Colour-group	Isolates[a]
Streptomycetaceae	1	SE43[b], SE44, SE45, SE46[b], SE47, SE48 [b]
(Rescaled distance: <20)	2	SE1[b], SE2, SE10, SE11[b], SE12, SE13, SE16, SE17, SE18, SE19[b], SE42[b]
	3	SE5[b], SE7
	4	SE6[b]
	5	PE25, PE30[b], SE8, SE9[b], SE40[b]
	6	PE28[b]
	7	SE27, SE30[b]
Micromonosporaceae	1	PE2, PE4[b], PE8, PE9, PE12, PE29[b], PE35[b], SE23[b]
(Rescaled distance: <5)	2	PE38[b]
	3	PE13, PE14[b], PE20[b], PE21, PE23[b], PE24, SE22
	4	SE24[b]
	5	PE15, PE17[b]
Non-*Streptomycetaceae*	1	SE25, SE37[b]
and	2	PE32[b]
Non-*Micromonosporaceae*	3	PE36[b]
(Rescaled distance: <5)	4	SE31[b]
	5	PE31[b]
	6	PE5[b], PE6[b], PE7, PE10[b], PE11
	7	PE33, PE34, PE37[b]
	8	PE22[b]

[a] Prefix PE denotes isolates from *Padina antillarum*; and SE denotes isolates from soil sediments

[b] Selected isolates identified with 16S rRNA gene sequences

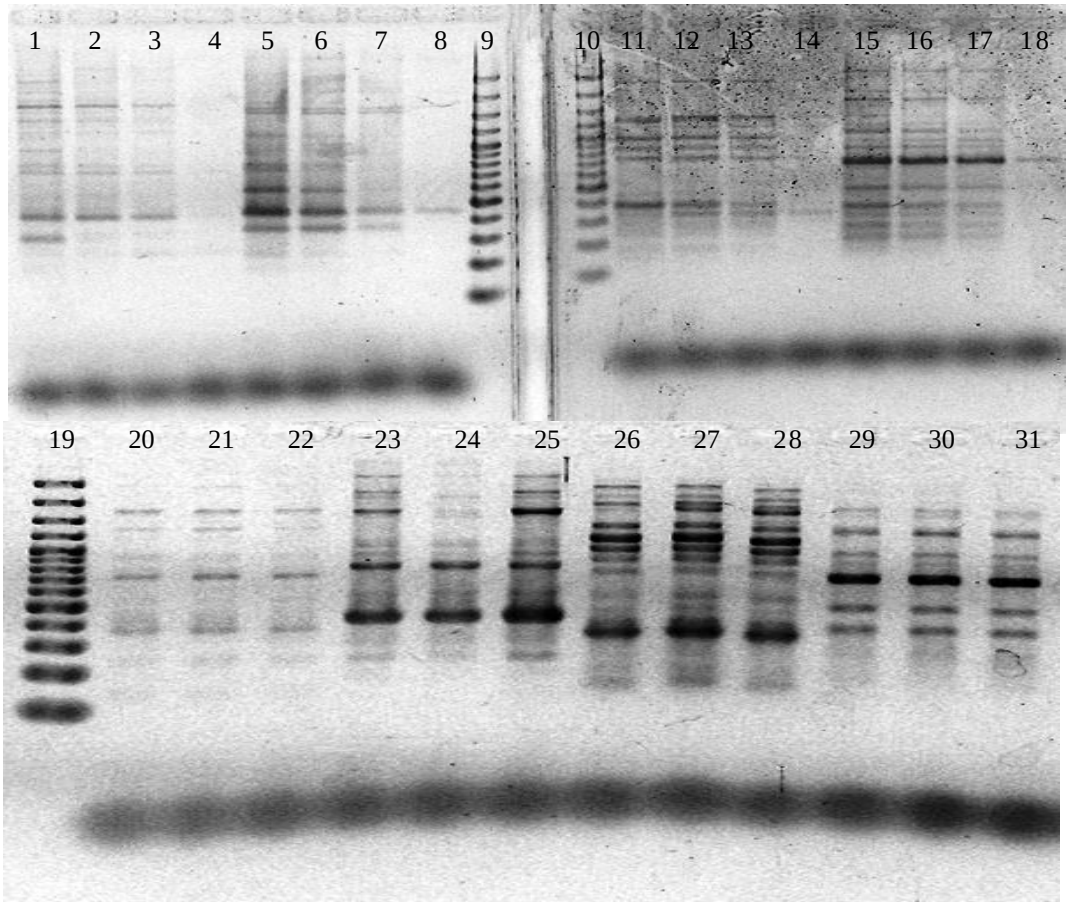


Figure 4.9: Repetitive sequence-derived amplification using the BOXA1R primer was used to generate genomic fingerprints. Amplification profiles with different annealing temperature (55ºC, 58ºC, 60ºC and 65ºC) to select for the optimum temperature that generate clear banding patterns (Lane 1-18). Amplifications were then repeated in triplicates with single annealing temperature of 55ºC (Lane 19-31). Selected samples were previously identified by 16S rRNA gene sequence.

Lane				Samples	16S rRNA gene sequence identification to family
Annealing temperature:					
55ºC	**58ºC**	**60ºC**	**65ºC**		
1	2	3	4	SE 2	*Streptomycetaceae*
5	6	7	8	PE 31	*Pseudonocardiaceae*
11	12	13	14	PE 25	*Streptomycetaceae*
15	16	17	18	SE 31	*Pseudonocardiaceae*

Lane			Samples	16S rRNA gene sequence identification to family
Replicates:				
1	**2**	**3**		
20	21	22	SE 2	*Streptomycetaceae*
23	24	25	PE 31	*Pseudonocardiaceae*
26	27	28	PE 25	*Streptomycetaceae*
29	30	31	SE 31	*Pseudonocardiaceae*

Lane 9, lane 10 and Lane 19: Gene Ruler 100bp Plus DNA Ladder, ready-to-use (Thermo Fisher Scientific Inc., United States of America)

Table 4.3: Clustering of isolates based on fingerprinting patterns obtained from repetitive sequence-derived amplification.

Cluster	Fingerprinting patterns	Isolates[a]
Streptomycetaceae	1	SE43[b], SE44, SE45, SE47
(Rescaled Distance: <10)	2	SE5[b], SE7
	3	PE25, PE30[b]
	4	SE18, SE19[b]
	5	SE8, SE9[b]
	6	SE6[b]
	7	SE40[b], SE48[b]
	8	PE28[b]
	9	SE1[b], SE2
	10	SE27, SE30[b]
	11	SE42[b]
	12	SE46[b]
	13	SE10, SE11[b], SE12, SE13, SE16, SE17
Micromonosporaceae	1	SE22, SE23[b]
(Rescaled Distance: <10)	2	PE2, PE4[b]
	3	PE24, PE29[b]
	4	PE8, PE9, PE12, PE20[b], PE21
	5	PE15, PE17[b]
	6	PE13, PE14[b]
	7	PE23[b]
	8	PE38[b], SE24[b]
	9	PE35[b]
Non-*Streptomycetaceae* and	1	SE25, SE37[b]
Non-*Micromonosporaceae*	2	SE31[b]
(Rescaled Distance: <5)	3	PE6[b], PE7
	4	PE5[b]
	5	PE10[b], PE11
	6	PE31[b]
	7	PE32[b]
	8	PE22[b]
	9	PE36[b]
	10	PE33, PE34, PE37[b]

[a] Prefix PE denotes isolates from *Padina antillarum*; SE denotes isolates from soil sediments

[b] Selected isolates identified with 16S rRNA gene sequences

Table 4.4: Identification of the representative strains based on phenotypic colour and genotypic fingerprinting pattern prior to 16S rRNA sequence based identification.

Cluster	Isolates[a,b]	GenBank Accession number	Closest 16S rRNA identification (Pairwise similarity, %)
Streptomycetaceae	SE43	KJ174478	*Streptomyces griseoincarnatus* group[c] (99.45)
	SE46	KJ174479	*S. wuyuanensis* (99.83)
	SE48	KJ174486	*S. wuyuanensis (99.83)*
	SE40	KJ174480	*S. qinglanensis* (99.65)
	PE30	KJ174482	*S. qinglanensis* (99.40)
	SE9	KJ174481	*S. qinglanensis* (99.73)
	SE1	KJ174483	*S. qinglanensis* (99.86)
	SE11	KJ174477	*S. qinglanensis* (99.72)
	SE19	KJ174484	*S. qinglanensis* (99.78)
	SE42	KJ174485	*S. rochei* group[c] (100.0)
	PE28	KJ174476	*S. rochei* group[c] (100.0)
	SE5	KJ174473	*S. wuyuanensis* (99.89)
	SE6	KJ174475	*S. matensis* (100.0)
	SE30	KJ174474	*S. iranensis* (97.34)
Micromonosporaceae	PE4	KJ174487	*Micromonospora tulbaghiae* (100.0)
	PE29	KJ174488	*M. aurantiaca* (99.90)
	PE35	KJ174489	*M. aurantiaca* (100.0)
	SE23	KJ174490	*M. aurantiaca* (100.0)
	PE20	KJ174491	*M. aurantiaca* (100.0)
	PE23	KJ174492	*M. aurantiaca* (100.0)
	PE14	KJ174493	*M. chalcea* (99.80)

Table 4.4, continued

Cluster	Isolates[a,b]	GenBank Accession number	Closest 16S rRNA identification (Pairwise similarity, %)
Micromonosporaceae	PE38	KJ174494	*M. maritima* (100.0)
	SE24	KJ174495	*M. maritima* (100.0)
	PE17	KJ174496	*Verrucosispora gifhornensis* (99.62)
Non-Streptomycetaceae and Non-Micromonosporaceae	SE37	KJ191451	*Tsukamurella inchonensis* (99.81)
	PE32	KJ191452	*Williamsia muralis* (99.61)
	SE31	KJ136015	*Sciscionella marina* (98.79)
	PE6	KJ191453	*S. marina* (99.15)
	PE5	KJ191454	*S. marina* (99.66)
	PE10	KJ191455	*S. marina* (98.29)
	PE31	KJ191456	*Pseudonocardia kunmingensis* (98.97)
	PE37	KJ191457	*Nocardia nova* (99.85)
	PE36	KJ136014	*Prauserella marina* (96.14)

[a] PE denotes isolates from *Padina antillarum*; SE denotes isolates from soil sediments.

[b] Selected isolates identified with 16S rRNA gene sequences. GenBank accession number given in the following column.

[c] A taxonomic group includes species that are not distinguishable by 16S rRNA gene sequence.

4.2 Salinity test

All isolates have salinity tolerance to MBA2 supplemented with 2% NaCl, and does not require the presence of sodium for growth (i.e. all grow on MBA2), though better growth rate were observed for isolates on MBA2 supplemented with 2% NaCl as growth of isolates was observed by the third day of incubation, but growth of isolates on MBA2 (0% NaCl) was only observed after the fifth day.

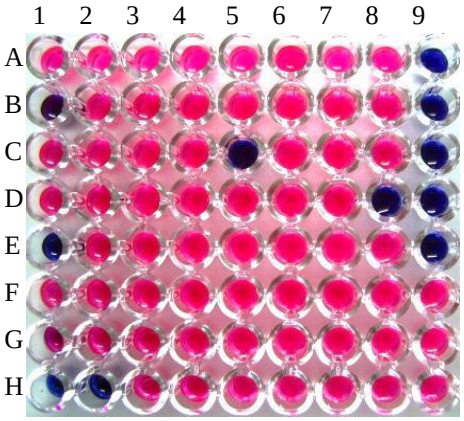
Only eleven isolates (three from seaweed and eight from sediment samples) that can tolerate 10% NaCl, and only two isolates (one from each seaweed and sediment samples) that can tolerate up to 15% of NaCl after 14 days of incubation (Table 4.5).

Table 4.5: Salinity tolerance of isolates from specimens of seaweed, *Padina antillarum* (total: 30 isolates) and its surrounding soil sediments (total: 31 isolates) when grown on MBA2 at 28ºC.

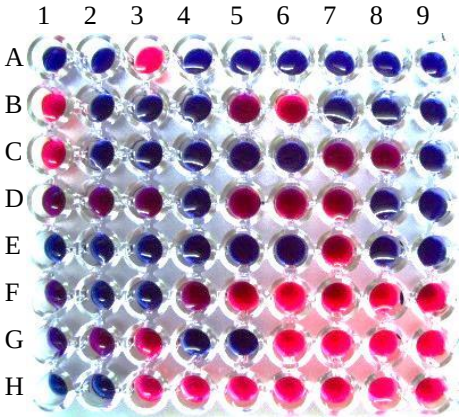
No. of isolates	**Salinity test (% NaCl)**			
	0	**2**	**10**	**15**
Isolated from *Padina antillarum*	30	30	3	1
Isolated from soil sediments	31	31	8	1
Total number of isolates	61	61	11	2

4.3 Presence of PKSI and NRPS gene fragment and antibacterial activity

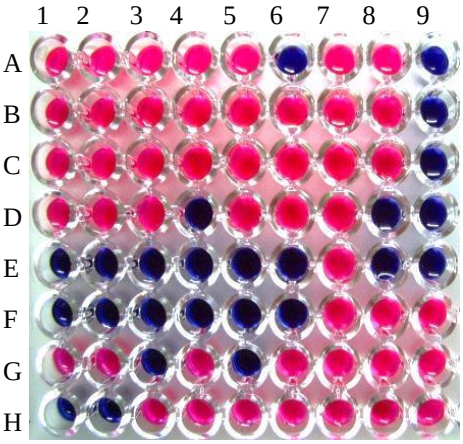
Sixty-one isolates were initially tested against *E. coli* M2, *S. aureus* M33 and *B. subtilis* M57 using REMA (Figure 4.10). A subsequent screening was carried to determine the minimum inhibitory concentration against the respective bacterial strains.



Set A



Set B



Set C

Figure 4.10: Primary screening employing REMA to test the concentrated (final concentration: 100mg/ml) and sterilized supernatant for bioactivity against either *E. coli* M2 (Set A), *S. aureus* M33 (Set B) or *B. subtilis* M57 (Set C). Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Solutions that show bioactivity in two of the three replicates will be confirmed with secondary screening.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
A	PE2	PE4	PE5	PE6	PE7	PE8	PE9	PE10	Nalidixic acid
B	PE11	PE12	PE13	PE14	PE15	PE17	PE20	PE21	Neomycin
C	PE22	PE23	PE24	PE25	PE28	PE29	PE30	PE31	Streptomycin
D	PE32	PE33	PE34	PE35	PE36	PE37	PE38	SE1	Penicillin G
E	SE2	SE5	SE6	SE7	SE8	SE9	SE10	SE11	Ampicillin
F	SE12	SE13	SE16	SE17	SE18	SE19	SE22	SE23	Ultrapure water
G	SE24	SE25	SE27	SE30	SE31	SE37	SE40	SE42	Ultrapure water
H	SE43	SE44	SE45	SE46	SE47	SE48	Ultrapure water	Ultrapure water	Ultrapure water

Thirty-nine isolates showed activities against one or more of the test bacteria when screened (Table 4.6). Thirty-two isolates were active against *S. aureus* M33 (Appendix B), twenty-two isolates showed activity against *B. subtilis* M57 (Appendix C), and ten isolates were active against *E. coli* M2 (Appendix D). The MIC ranged from 100mg/ml to 1mg/ml. Incidentally, though ten isolates showed broad spectrum bioactivity and twenty-nine isolates were antagonistic against *S. aureus* M33 and *B. subtilis* M57 only, however, no isolates were antagonistic against *E. coli* M2 only.

Table 4.6: Distribution of isolates from different samples antagonistic against test bacteria

No. of isolates	**Test bacteria**		
	***S. aureus* M33**	***B. subtilis* M57**	***E. coli* M2**
Isolated from *Padina antillarum*	18	2	1
Isolated from soil sediments	14	20	9
Total number of isolates	32	22	10

All isolates were also screened with degenerate primers for PKSI ketoacyl synthase and methyl-malonyl transferase domains and NRPS adenylation domain. PKSI gene was detected in 37 isolates (21 of seaweed isolates and 16 of sediment isolates) and NRPS gene was detected in 47 isolates (21 isolates of seaweed and 26 of sediment isolation) (Table 4.7). Eleven isolates yielded negative for either PKS1 or NRPS gene.

Table 4.7: Distribution of isolates with PKSI and NRPS gene fragment

Source of isolates	**PKSI only**	**NRPS only**	**PKSI and NRPS**	**Not detected**	**Total isolates**
Padina antillarum	1	6	20	3	30
Soil sediments	2	7	14	8	31
Total isolates	3	13	34	11	61

Furthermore, distribution of the presence of gene cluster and antibacterial activity of isolates were also compared (Table 4.8). Generally, the presence of gene cluster and antibacterial activity showed no association ($n = 61$; *phi* co-efficient, $\varphi = 0.09$).

Table 4.8: Presence of gene cluster and antibacterial activity

Gene cluster (PKSI and/or NRPS)	**Antibacterial activity**	**No. of isolates**		
		Padina antillarum	**Soil sediments**	**Total isolates**
Present	Present	18	15	33
Present	Absent	9	8	17
Absent	Present	0	6	6
Absent	Absent	3	2	5

Nonetheless, six *Streptomyces* isolates exhibited detectable antibacterial activity but were tested negative for either PKS1 or NRPS gene. Additionally, 4 isolates each from the genera *Streptomyces* and *Micromonospora*, 2 isolates each from the genera *Verrucosispora* and *Sciscionella*, and one isolate each from the genera *Tsukamurella*, *Williamsia*, *Prauserella*, *Pseudonocardia* and *Nocardia*, that were positive for either PKS1 or NRPS gene do not exhibit detectable antibacterial activity (Table 4.9).

Table 4.9: Distribution of bioactive isolates with gene cluster

Cluster	**Genus**	**Isolates with bioactivity highlighted in bold**	**Isolates with biosynthetic gene cluster (PKSI or NRPS) highlighted in bold**
Streptomycetaceae	*Streptomyces*	**PE25**, **PE28**, PE30, **SE1**, **SE2**, **SE5**, **SE6**, **SE7**, **SE8**, **SE9**, SE10, **SE11**, **SE12**, **SE13**, **SE16**, **SE17**, **SE18**, **SE19**, **SE27**, **SE30**, SE40, SE42, **SE43**, **SE44**, SE45, **SE46**, SE47, **SE48**	**PE25**, **PE28**, PE30, **SE1**, SE2, **SE5**, **SE6**, SE7, **SE8**, SE9, **SE10**, **SE11**, SE12, SE13, **SE16**, **SE17**, **SE18**, **SE19**, SE27, **SE30**, **SE40**, **SE42**, **SE43**, **SE44**, SE45, **SE46**, **SE47**, **SE48**
Micromonosporaceae	*Micromonospora*	**PE2**, **PE4**, **PE8**, **PE9**, **PE12**, **PE13**, **PE14**, **PE20**, **PE21**, **PE23**, **PE24**, **PE29**, **PE35**, PE38, SE22, SE23, SE24	**PE2**, **PE4**, **PE8**, **PE9**, **PE12**, **PE13**, **PE14**, **PE20**, **PE21**, **PE23**, **PE24**, **PE29**, **PE35**, **PE38**, **SE22**, **SE23**, **SE24**
	Verrucosispora	PE15, PE17	**PE15**, **PE17**
Non-*Streptomycetaceae* and Non-*Micromonosporaceae*	*Tsukamurella*	SE25, SE37	**SE25**, SE37
	Williamsia	PE32	**PE32**
	Prauserella	PE36	**PE36**
	Sciscionella	PE5, **PE6**, **PE7**, **PE10**, PE11, **SE31**	**PE5**, **PE6**, **PE7**, **PE10**, **PE11**, **SE31**
	Pseudonocardia	PE31	**PE31**
	Nocardia	PE33, PE34, PE37	PE33, PE34, **PE37**

CHAPTER 5

5.1 Characterization and identification of actinobacterial isolates

In this study, sixty one actinobacterial isolates was isolated from intertidal sediment samples and *Padina antillarum* (Dictyotales, *Phaeophyceae*) from Tanjung Tuan (Cape Rachado), Port Dickson, Malaysia. However, marine actinobacterial isolates *sensu stricto* should only include members of the taxonomic group indigenous to the marine environment (Ward & Bora, 2006). However, it is difficult to establish that they are indigenous to the marine environment (Tsueng & Lam, 2008; Imada, *et al.*, 2010; Khan, *et al.*, 2010). Nonetheless, the salinity test was conducted to determine the effect of sodium chloride on the isolates to facilitate the subsequent culturing and maintenance of the isolates. All isolates have sodium tolerance up to 2%. Though growth was observed in media without additional NaCl, better growth rates were observed on media supplemented with 2% NaCl. Although the specific requirement of sodium salt has been identified as a primary characteristic of marine actinobacteria, however, the quantity, stability, and uniqueness of the sodium requirement are not established (Khan, *et al.*, 2010). Our observations have shown that the isolates have adapted to have higher growth rate in higher salinity environment. Furthermore, there are reports that the production of secondary metabolites by marine actinobacteria are only triggered under halophilic conditions (Okami, *et al.*, 1976; Imada, *et al.*, 2007; Tsueng & Lam, 2008; Imada, *et al.*, 2010).

Nonetheless, the characteristically diverse Gram-positive actinobacteria is characterized mainly with its high guanine and cytosine content in its DNA (Stackebrandt, *et al.*, 1997), therefore, it is important to be able to putatively group the isolates into smaller working taxonomic units prior to phenotypic characterisation and genotyping. Hence, isolates were first screened with primers sets specific for

Streptomycetaceae and *Micromonosporaceae* as members of these families are often isolated in abundance from the marine environment (Ward & Bora, 2006; Yoshida, *et al.*, 2008; Maldonado, *et al.*, 2009). This approach allows for 47 of 61 isolates to be clustered as either *Streptomycetaceae* (28 isolates) or *Micromonosporaceae* (19 isolates. Most of the isolates belonging to the family *Streptomycetaceae* derived from the sediments whereas, most of the non-*Streptomycetaceae* isolates were derived from *Padina antillarum*. The ecological implication of this observation, however, had not been thoroughly explored.

Subsequently, each cluster ('*Streptomycetaceae*', '*Micromonosporaceae*', or 'Non-*Streptomycetaceae* and Non-*Micromonosporaceae*') were subjected to simultaneous characterization through colony phenotyping and molecular genotyping. Thirty four representative member selected from the grouping were identified through 16S rRNA gene sequence (Table 4.4).

The identified isolates revealed that genotyping based on fingerprinting patterns obtained from repetitive sequence-derived amplification showed higher resolution than colony phenotyping based on colour-grouping (colour of aerial mycelium, substrate mycelium and pigmentation colour) of the isolates grown on different media. Identified isolates belonging to the same species were assembled into many different single-member or two-members grouping when clustering were based on fingerprinting patterns. Molecular-typing had been known to have higher resolution than phenotypic approach, whereby, molecular-typing, in some cases could distinguish synonymous species or differentiate isolates within the same species (Lanoot *et al.*, 2004; Guo *et al.*, 2008). Therefore, genomic variation within the same species could yield different banding patterns among the isolates of the same species.

On the other hand, some of the identified isolates belonging to different species were assembled into the same grouping when clustering was based on colour of the isolates grown on different media. This may due to the morphological similarity between closely related species when characterised solely on colour of the isolates grown on different media. However, phenotyping especially through the numerical taxonomy approaches for the Actinobacteria had been used for systematic and classification studies, in which species and isolates were differentiated and delineated (Austin *et al.*, 1977; Goodfellow *et al.*, 1979; Goodfellow *et al.*, 1990; Grund & Kroppenstedt, 1990). This phenotyping approach, especially, for the *Streptomyces* had been helpful in species identification and now is the minimal standard of characterization of the member of the genus (Shirling & Gottlieb, 1966; Kämpfer *et al.*, 1991; Kämpfer, 2012). Commercial kits such as API® identification kits (bioMérieux Inc., United States of America), Biolog Microbial ID System (Biolog, Inc., United States of America) and BBL™ Crystal™ Identification System (Becton, Dickinson and Company, United States of America) have also utilises phenotypic approach for microbial identification and characterization. Thus, the heart of phenotyping approach relies heavily on the total number of test and the amount of character useful to differentiate and describe the isolates.

Consequently, phenotypic and genotypic approaches were used simultaneously for the delineation of the isolates enabling the identification of all species isolated in this study as both phenotypic and genotypic approaches revealed positive correlation (correlation co-efficient: $r = 0.989$; $p < 0.0001$). Molecular identification by 16S rRNA gene revealed five families, nine genera, and 17 species namely *Streptomycetaceae*

(*Streptomyces griseoincarnatus* group[1], *S. iranensis*, *S. matensis*, *S. qinglanensis*, *S. rochei* group[2] and *S. wuyuanensis*), *Micromonosporaceae* (*Micromonospora aurantiaca*, *M. chalcea*, *M. maritima*, *M. tulbaghiae* and *Verrucosispora gifhornensis*), *Pseudonocardiaceae* (*Prauserella marina*, *Pseudonocardia kunmingensis*, and *Sciscionella marina*), *Nocardiaceae* (*Nocardia nova* and *Williamsia muralis*) and *Tsukamurellaceae* (*Tsukamurella ichonensis*).

Furthermore, strain PE36 may possibly be a novel species within the genus *Prauserella*, with 16S rRNA gene sequence showed highest pairwise similarity of with *Prauserella marina* MS498^T (96.73%) and pairwise similarity of 96.30% with both *Prauserella rugosa* DSM 43194^T and *Saccharomonospora azurea* NA-128^T. The 16S rRNA gene sequence similarities between members of the genus *Prauserella* range from 95.8% to 100%. The phylogenetic analysis also revealed that the genus *Prauserella* is most closely related to the genus *Saccharomonospora* and forms a distinctive branch within the family *Pseudonocardiaceae* (Labeda *et al.*, 2011; Kim & Goodfellow, 2013) (Figure 5.1).

[1] A taxonomic group which includes *S. griseoincarnatus* (Pridham *et al.*, 1958), *S. variabilis* (Pridham, *et al.*, 1958), *S. labedae* (Lacey, 1987) and *S. erythrogriseus* (Falcão de Morais & Maia, 1959) that are not distinguishable by 16S rRNA sequence.

[2] A taxonomic group which includes *S. rochei* (Berger *et al.*, 1953), *S. enissocaesilis* (Gause *et al.*, 1983), *S. plicatus* (Pridham, *et al.*, 1958), *S. geysiriensis* (Wallhausser *et al.*, 1965), *S. ghanaensis* (Wallhausser, *et al.*, 1965) and *S. vinaceusdrappus* (Pridham, *et al.*, 1958) that are not distinguishable by 16S rRNA sequence.

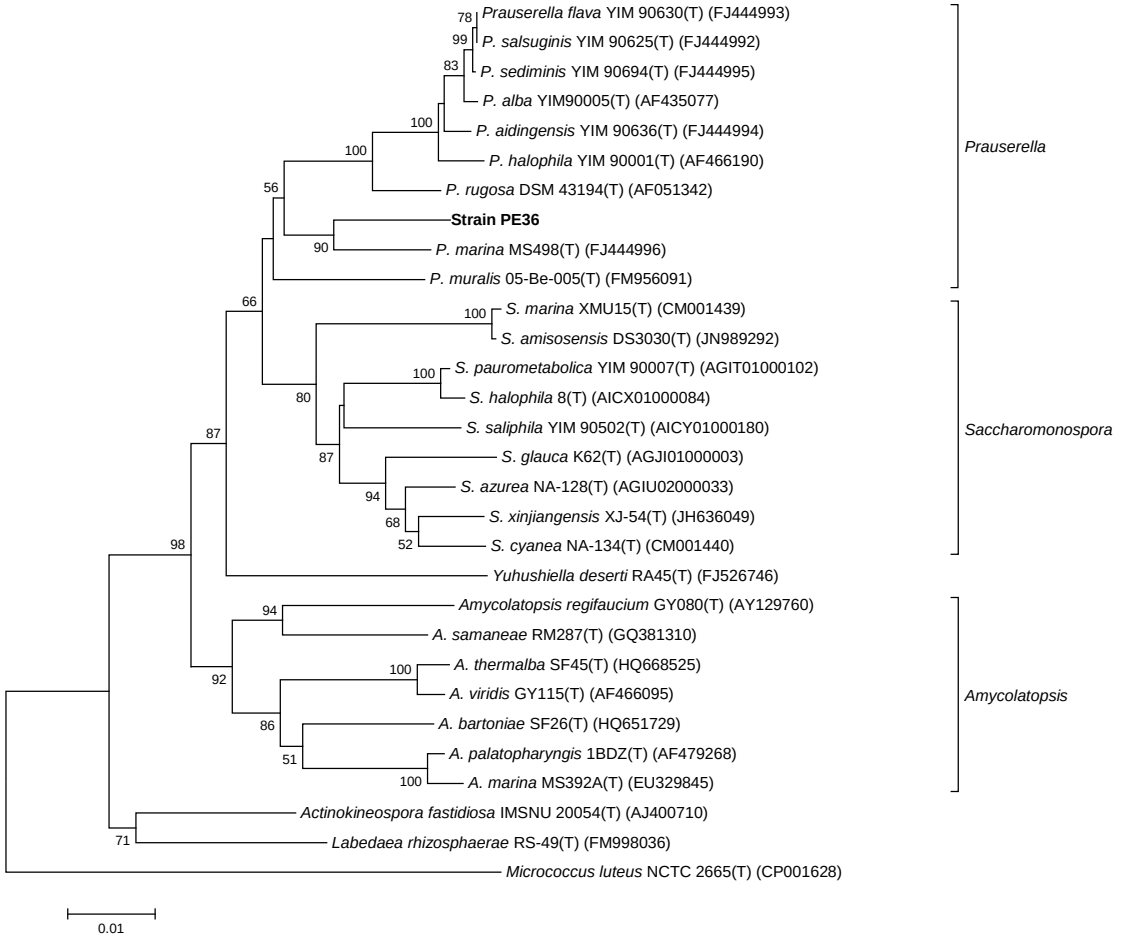


Figure 5.1: Consensus neighbour-joining tree (Kimura 2-parameter method) was constructed for phylogenetic analysis of strain PE36 and 28 representative members of its closest relative within the family *Pseudonocardiaceae*, based on 1344bp of the 16S rRNA gene sequence. The bootstrap consensus tree was inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (bar, 1% sequence divergence). *Micrococcus luteus* NCTC 2665(Type-strain) (GenBank accession number: CP001628) was used as outgroup.

Additionally, strain SE31 could also possibly be a new member of the genus *Sciscionella* on the basis of 16S rRNA gene sequence analysis (Figure 5.2). Moreover, two genome-based species delineation approaches were then employed to infer whole-genome distances between the genome of *Sciscionella* strain SE31 (Appendix E) and the genome of *S. marina* DSM 45152^{T} for the purpose of estimating the relatedness between both strains (Richter & Rosselló-Móra, 2009). These two different approaches revealed that strain SE31 could possibly be a novel species based on estimated of 54.4% DNA-DNA hybridization (Meier-Kolthoff *et al.*, 2013) and 92.6% average nucleotide identity (Goris *et al.*, 2007). The genus *Sciscionella* currently contains Gram-positive aerobic marine bacterium, and since the initial description of the genus in 2009, with the type-species *S. marina*, no additional new species have been described (Tian *et al.*, 2009; Labeda & Goodfellow, 2012). The type species *S. marina* was isolated from a grey sand sediment at a depth about 500 m of South China Sea (Tian, *et al.*, 2009). Recently, a bacterial strain isolated from the blood culture of a lymphoma patient was reported to this species (Sinha *et al.*, 2013).

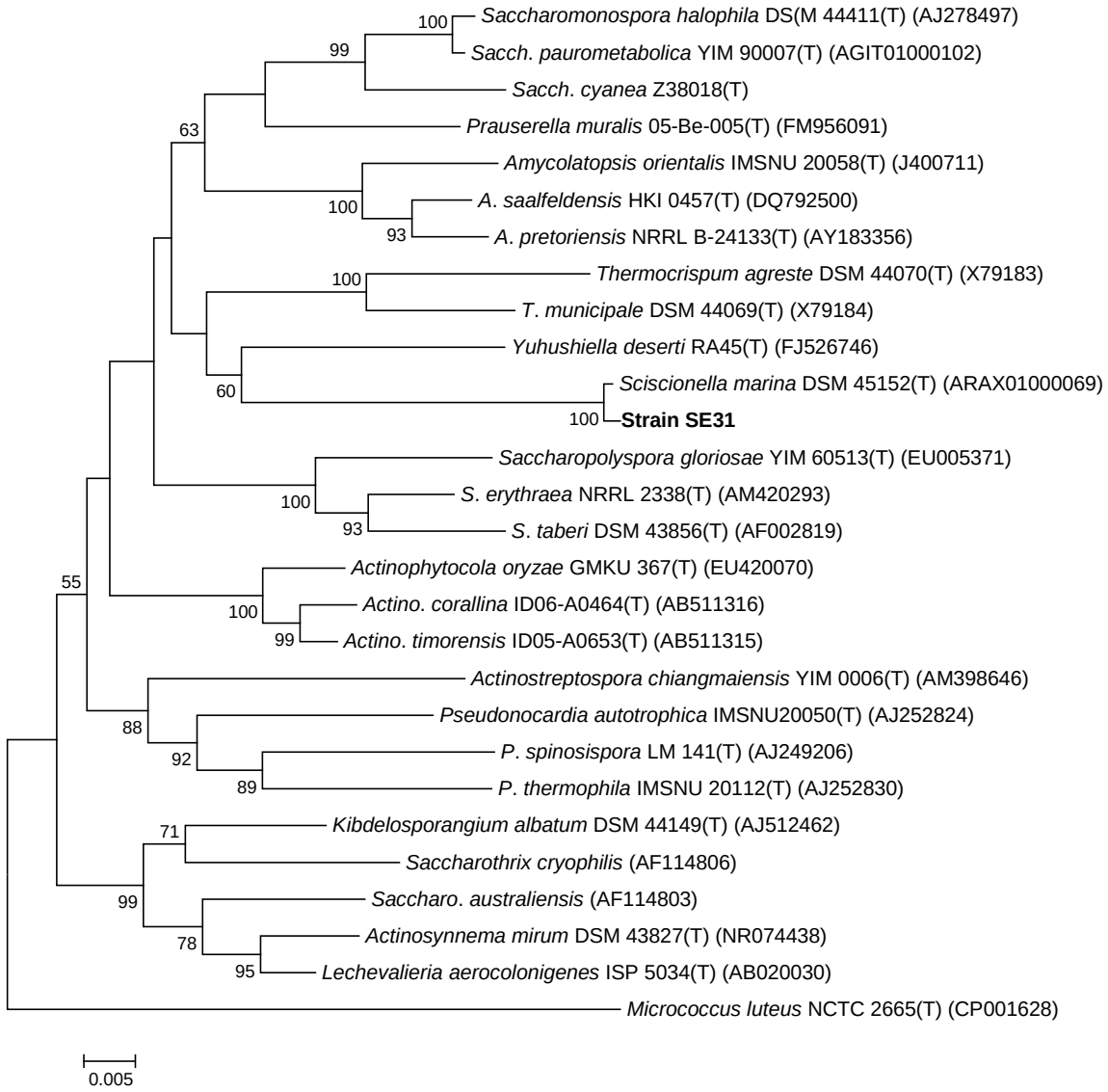


Figure 5.2: Consensus neighbour-joining tree (Kimura 2-parameter method) was constructed for phylogenetic analysis of strain SE31 and 27 representative members of its closest relative within the family *Pseudonocardiaceae*, based on 1217bp of the 16S rRNA gene sequence. The bootstrap consensus tree was inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (bar, 0.5% sequence divergence). *Micrococcus luteus* NCTC 2665(Type-strain) (GenBank accession number: CP001628) was used as outgroup.

5.2 Antibacterial activity and potential of actinobacterial isolates

Bioactive metabolites that are analogous of a bioactive compound may not necessarily be indicative of similar bioactivity (Han *et al.*, 2012). Therefore, bioassay guided profiling of isolates for the selection of bioactive compound is still widely used for the target of specific function (Strobel & Daisy, 2003; Sacramento *et al.*, 2004; Bull & Stach, 2007; Saurav *et al.*, 2013; Schulze, *et al.*, 2013). Therefore, this study is focused solely on antibacterial activity against *Escherichia coli*, *Staphlylococcus aureus* and *Bacillus subitilis*.

Moreover, it would suffice that with a genome that has a higher number of biosynthetic gene clusters, molecular screening approach is more likely to result in a positive hit. Hence, simply surveying culturable isolates for genes encoding for polyketides and non-ribosomal peptides can be helpful for determining a possible potential of the isolate (Ayuso, *et al.*, 2005; Ostash, *et al.*, 2005; Schirmer, *et al.*, 2005; Savic & Vasiljevic, 2006; Baltz, 2007). In this study, all but four of the isolates belonging to the family *Streptomycetaceae* that tested positive for the biosynthetic gene cluster, also exhibited bioactivity. Furthermore, out of 19 *Micromonosporaceae* isolates, only six of the isolates do not exhibit any detectable antibacterial activity although tested positive for the biosynthetic gene cluster. Additionally, of the 11 isolates belonging neither to *Streptomycetaceae* nor *Micromonosporaceae* which tested positive for the biosynthetic gene cluster, only four exhibited bioactivity. This showed that although a positive hit in molecular gene screening provide evidence of the production of a corresponding metabolites, however, it may also indicate the existence of further metabolic pathways of secondary metabolite synthesis not detected in the current bioassay screening (Pimentel Elardo, 2008; Schneemann, *et al.*, 2010).

But on the other hand, the 17 isolates that do not inhibit any of the test bacteria but possess PKS1 and/or NRPS gene clusters may not be expressing these genes. In another study, Laureti *et al.* (2011) were able to isolate polyketide, stambomycins, that are not usually expressed under laboratory growth condition by activation of a silent gene in *Streptomyces ambofaciens*. Moreover, many of these genes appeared cryptic and the functions are yet to be elucidated (Komaki & Harayama, 2006), therefore the isolates maybe producing novel polyketides and/or peptides that could not be detected from the antibacterial assay screening.

Contrarily, the lack of detectable gene fragments in six *Streptomycetaceae* isolates does not definitely prove the absence of a biosynthetic gene clusters as there are also other metabolites and other biosynthetic pathways that may exist in the genomes but not picked up during PCR screening (Nett *et al.*, 2009; Jiménez, *et al.*, 2010; Schneemann, *et al.*, 2010).

In comparison with other studies, this study that survey the actinobacteria isolated from marine environment has shown that PKSI and NRPS were detected in 61% and 77% of the isolates respectively. Study done by Qin *et al.* (2009) showed that PKSI and NRPS detected in endophytic actinomycetes isolated from medicinal plants collected from tropical rainforest were 11% and 26%, respectively. This is in contrast with the survey done by Ayuso, *et al.* (2005) on terrestrial actinomycetes isolated from tropical soils collected on Martinique, Central America that showed 62% detection of PKSI and 66% detection of NRPS. Furthermore, survey of the presence of PKS1 and NRPS in the actinomycetes type-isolates available at Merck Culture Collection had shown 57% and 80% respectively (Ayuso-Sacido & Genilloud, 2005).

Since majority of actinobacteria-derived compounds are usually shown to be complex polyketides and non-ribosomal peptides (Donadio, *et al.*, 2007; Minowa, *et al.*, 2007; Lin, *et al.*, 2009; Donadio, *et al.*, 2010; Jiménez, *et al.*, 2010; Schneemann, *et al.*, 2010; Han, *et al.*, 2012; Jang *et al.*, 2013; Palaniappan *et al.*, 2013) and no other taxonomic groups have devoted such high percentages of the coding capacity to polyketide and non-ribosomal peptide functions as in actinobacteria (Baltz, 2008), thus this further emphasizes the rationale to focus compound discovery efforts on actinobacteria through bioactivity guided assay and molecular gene screening.

CHAPTER 6

A total of 61 isolates of actinobacteria were isolated and characterized from marine sediments and *Padina antillarum*. All isolates do not require the presence of NaCl for growth but better growth rate was observed when cultured with 2% NaCl. The combined phenotypic and genotypic approaches used simultaneously for the delineation of the isolates enabled the identification of all species isolated in this study. Molecular identification by 16S rRNA gene sequence analysis revealed the presence of members of *Streptomycetaceae*, *Micromonosporaceae*, *Pseudonocardiaceae*, *Nocardiaceae* and *Tsukamurellaceae* from the samples. The actinobacteria isolates were tested for antibacterial activity and 39 isolates showed activity against one or more of the test bacteria. Additionally, the actinobacteria isolated from marine environment has shown that PKSI and NRPS were detected in 61% and 77% of the isolates respectively. The higher number of positive hit in molecular gene screening had indicated the possible existence of further metabolic pathways of secondary metabolite synthesis not detected in the antibacterial bioassay. Furthermore, the genome analysis of isolate SE31 revealed potentially new source of antibiotics and the possibility of pathogenicity of environmental isolates. This study had shown that the actinobacteria isolated from the marine environment remain to be of interest for the discovery of new species and potential new source of bioactive compounds.

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PRESENTATIONS IN CONFERENCES

1. Authors: Teo Wee Fei Aaron, Khoo Kong Soo & Geok Yuan Annie Tan

Title of poster presentation: Isolation of Actinobacteria from *Padina* sp

Title of conferences: International Congress of Malaysian Society for Microbiology 2009 (ICMSM 2009)

Date & venue: 1st – 4th December 2009, Park Royal Hotel, Penang

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Category: Microbial Diversity/Microbial Systematics & Taxonomy/General Microbiology

2. Authors: Teo Wee Fei Aaron & Geok Yuan Annie Tan

Title of poster presentation: Bioactive Actinomycetes Isolated from *Padina antillarum*

Title of conferences: My1Bio Conference 2010

Date & venue: 30th – 31st October 2010, Berjaya Times Square Hotel and Convention Centre, Kuala Lumpur

Published in Book of Abstract, page 60

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Title of oral presentation: Presence of Biosynthetic Genes in Actinomycetes Isolated from Intertidal Sediments and *Padina antillarum*

Title of conferences: 15th Biological Science Graduate Congress (BSGC 2010)

Date & venue: 15th – 17th December 2010, University of Malaya, Kuala Lumpur

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APPENDIX A

Table 1: Enumeration (CFU/ml) of total bacteria and putative actinobacteria in different media from *Padina antillarum* and its surrounding soil sediments.

Media	***Padina antillarum***		**Soil**	
	Average CFU/ml of total bacteria	**Average CFU/ml of putative Actinobacteria**	**Average CFU/ml of total bacteria**	**Average CFU/ml of putative Actinobacteria**
MBA	2.0×10^5	1.0×10^2	8.6×10^7	7.0×10^3
$\frac{1}{10}$MBA	3.0×10^7	1.0×10^3	8.5×10^8	1.0×10^3
MBG	NA [#]	NA [#]	3.4×10^4	2.0×10^2
MOA	4.4×10^6	6.0×10^5	8.6×10^7	7.0×10^3
MSC	2.4×10^3	1.0×10^2	5.0×10^4	2.0×10^3
PEA	1.8×10^9	6.0×10^3	1.1×10^8	2.0×10^6
M1	7.0×10^7	2.0×10^3	1.7×10^9	NA [#]
M2	1.0×10^8	1.0×10^4	1.0×10^6	NA [#]
M4	8.0×10^7	8.0×10^3	2.5×10^9	NA [#]
M7	5.0×10^7	1.0×10^3	1.0×10^6	NA [#]
M8	9.0×10^7	5.0×10^5	3.1×10^9	NA [#]

[#]No colony growth was observed.

APPENDIX B

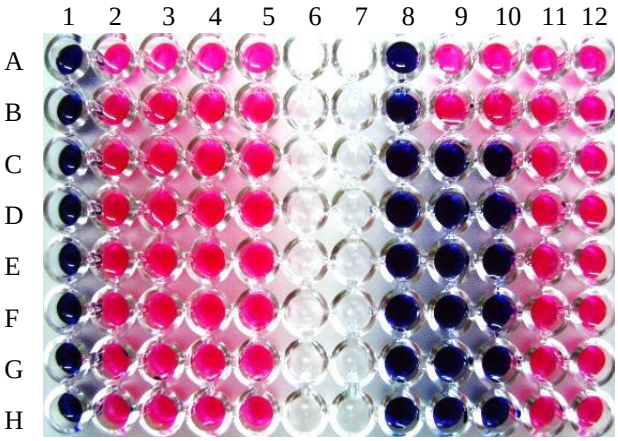

Figure 1: Secondary screening employing REMA to test the supernatant for bioactivity against *S. aureus* M33. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	PE2	100mg/ml
B1-B4	PE4	100mg/ml
C1-C4	PE6	100mg/ml
D1-D4	PE7	100mg/ml
E1-E4	PE8	100mg/ml
F1-F4	PE9	100mg/ml
G1-G4	PE10	100mg/ml
H1-H4	PE12	100mg/ml

Wells	Solution	MIC
A8-A11	PE13	100mg/ml
B8-B11	PE14	100mg/ml
C8-C11	PE20	1mg/ml
D8-D11	PE21	1mg/ml
E8-E11	PE23	1mg/ml
F8-F11	PE24	1mg/ml
G8-G11	PE25	1mg/ml
H8-H11	PE28	1mg/ml

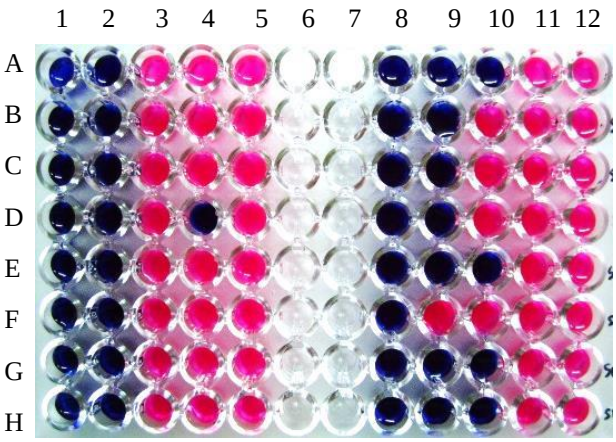

Figure 2: Secondary screening employing REMA to test the supernatant for bioactivity against *S. aureus* M33. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	PE29	10mg/ml
B1-B4	PE35	10mg/ml
C1-C4	SE1	10mg/ml
D1-D4	SE2	10mg/ml
E1-E4	SE5	10mg/ml
F1-F4	SE6	10mg/ml
G1-G4	SE7	10mg/ml
H1-H4	SE8	10mg/ml

Wells	Solution	MIC
A8-A11	SE9	1mg/ml
B8-B11	SE11	10mg/ml
C8-C11	SE13	10mg/ml
D8-D11	SE16	10mg/ml
E8-E11	SE30	1mg/ml
F8-F11	SE31	100mg/ml
G8-G11	SE43	1mg/ml
H8-H11	SE44	1mg/ml

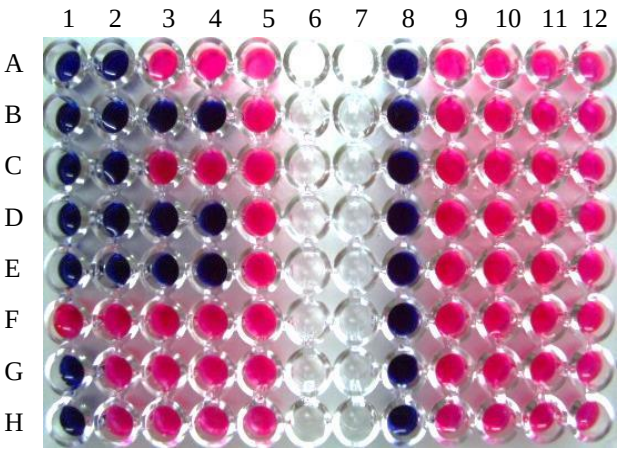

Figure 3: Secondary screening employing REMA to test the supernatant for bioactivity against *S. aureus* M33. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	Nalidixic acid	10mg/ml
B1-B4	Neomycin	0.1mg/ml
C1-C4	Streptomycin	10mg/ml
D1-D4	Penicillin G	0.1mg/ml
E1-E4	Ampicillin	0.1mg/ml
F1-F4	Ultrapure water	Not detected
G1-G4	PE2	100mg/ml
H1-H4	PE4	100mg/ml

Wells	Solution	MIC
A8-A11	PE6	100mg/ml
B8-B11	PE7	100mg/ml
C8-C11	PE8	100mg/ml
D8-D11	PE9	100mg/ml
E8-E11	PE10	100mg/ml
F8-F11	PE12	100mg/ml
G8-G11	PE13	100mg/ml
H8-H11	PE14	100mg/ml

APPENDIX C

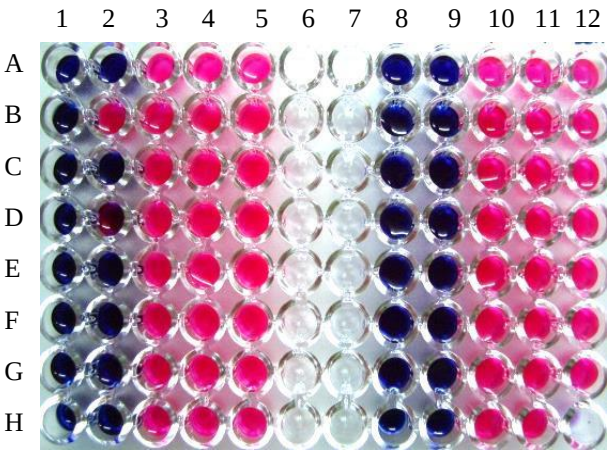

Figure 1: Secondary screening employing REMA to test the supernatant for bioactivity against *B. subtilis* M57. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	PE8	10mg/ml
B1-B4	PE35	100mg/ml
C1-C4	SE1	10mg/ml
D1-D4	SE2	10mg/ml
E1-E4	SE5	10mg/ml
F1-F4	SE6	10mg/ml
G1-G4	SE7	10mg/ml
H1-H4	SE8	10mg/ml

Wells	Solution	MIC
A8-A11	SE9	10mg/ml
B8-B11	SE11	10mg/ml
C8-C11	SE12	10mg/ml
D8-D11	SE13	10mg/ml
E8-E11	SE16	10mg/ml
F8-F11	SE17	10mg/ml
G8-G11	SE18	10mg/ml
H8-H11	SE19	10mg/ml

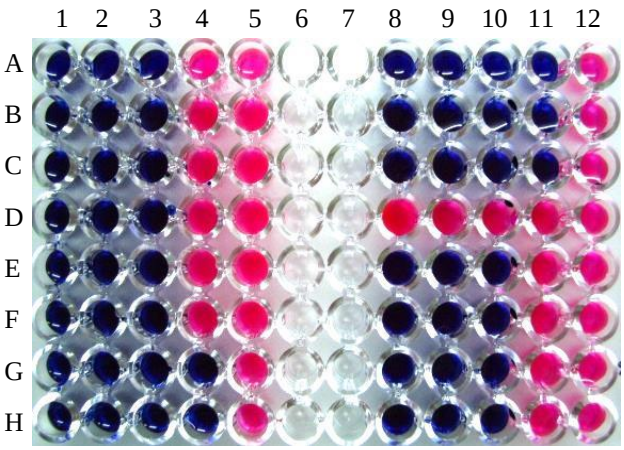

Figure 2: Secondary screening employing REMA to test the supernatant for bioactivity against *B. subtilis* M57. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	SE27	1mg/ml
B1-B4	SE31	1mg/ml
C1-C4	SE43	1mg/ml
D1-D4	SE44	1mg/ml
E1-E4	SE46	1mg/ml
F1-F4	SE48	1mg/ml
G1-G4	Nalidixic acid	0.1mg/ml
H1-H4	Neomycin	0.1mg/ml

Wells	Solution	MIC
A8-A11	Streptomycin	0.1mg/ml
B8-B11	Penicillin G	0.1mg/ml
C8-C11	Ampicillin	0.1mg/ml
D8-D11	Ultrapure water	Not detected
E8-E11	SE44	1mg/ml
F8-F11	SE43	1mg/ml
G8-G11	SE31	1mg/ml
H8-H11	SE27	1mg/ml

APPENDIX D

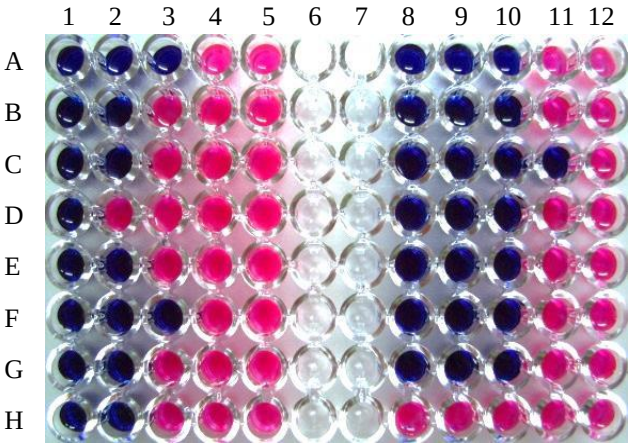

Figure 1: Secondary screening employing REMA to test the supernatant for bioactivity against *E. coli* M2. Bioactivity is determined by the colour changes from blue to purple or pink. Placements of each solution were as table below. Initial concentration of 100mg/ml (column 1 and column 8) was serially diluted to 10mg/ml, 1mg/ml and 0.1mg/ml. Column 5 and column 12 as negative control (Ultrapure water was used).

Wells	Solution	MIC
A1-A4	PE28	1mg/ml
B1-B4	SE1	10mg/ml
C1-C4	SE2	10mg/ml
D1-D4	SE6	100mg/ml
E1-E4	SE7	10mg/ml
F1-F4	SE8	1mg/ml
G1-G4	SE9	10mg/ml
H1-H4	SE13	10mg/ml

Wells	Solution	MIC
A8-A11	SE43	1mg/ml
B8-B11	SE44	1mg/ml
C8-C11	Nalidixic acid	0.1mg/ml
D8-D11	Neomycin	1mg/ml
E8-E11	Streptomycin	1mg/ml
F8-F11	Penicillin G	1mg/ml
G8-G11	Ampicillin	1mg/ml
H8-H11	Ultrapure water	Not detected

APPENDIX E

Whole genome sequence of isolate SE31 (accepted for publication in Marine Genomics; ISSN: 1874-7787)

The genome of *Sciscionella* strain SE31 was shotgun-sequenced using the Illumina HiSeq 2000 pair-end sequencing platform. Approximately 108 million raw reads in this study. The sequencing reads were pre-processed using PRINSEQ-lite version 0.20.1 to remove duplicated reads and poor quality reads. The pre-processed reads were *de novo* assembled using the CLC Genomics Workbench version 5.1 (CLC bio, Denmark).

Assembled raw reads resulted in 217 contigs, with a N50 contig size of 83,637bp, N75 size of 43,978 and N25 size of 173,814. The genomic size of this assembly is 7,425,729bp with a G+C content of 68.7%. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JALM00000000.

The sequenced genome was also annotated using the Rapid Annotation Subsystem Technology (RAST) pipeline (Aziz *et al*., 2008). RAST predicted 7,039 protein coding genes, 46 tRNAs and 3 rRNAs. RAST functional annotation of the predicted protein-coding genes showed that 669 genes are involved with amino acids and derivatives synthesis, 371 genes are involved with cofactors, vitamins, prosthetic groups, pigments, and 429 are linked to fatty acids, lipids and isoprenoid. Interestingly, 113 putative genes are associated with the virulence, disease and defense, suggesting that these genes may play important roles in the virulence or resistance of antibiotics. Though the pathogenicity of this bacteria is still not known, further analysis of the genome sequence may suggest better understanding of this potential pathogen.

Furthermore, the potential of this strain to produce secondary metabolites was also tested in vitro and has shown antibacterial activity (Ayuso *et al*., 2005). Therefore, the assembled genome were analyzed through secondary metabolites search tool antiSMASH (Medema *et al*., 2011). Preliminary data revealed the presence of 46 putative gene clusters involved in the biosynthesis of various natural products. In comparison, the genome of type-strain *S. marina* DSM 45152^{T} contains only 33 secondary metabolite clusters.

Among the clusters found in both *Sciscionella* strains, a biosynthetic gene cluster which shared a high homology with calicheamicins, an enediyne antitumor antibiotics initially isolated from Micromonospora echinospora (Ahlert *et al*., 2002), was predicted.

References:

Ahlert, J., Shepard, E., Lomovskaya, N., Zazopoulos, E., Staffa, A., Bachmann, B. O., . . . Thorson, J. S. (2002). The calicheamicin gene cluster and its iterative type I enediyne PKS. *Science*, 297(5584), 1173-1176. doi: 10.1126/science.1072105

Ayuso, A., Clark, D., González, I., Salazar, O., Anderson, A., & Genilloud, O. (2005). A novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. *Applied Microbiology and Biotechnology*, 67, 795–806.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Kubal, M. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics*, 9(1), 75.

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