Induction of secondary metabolism across actinobacterial genera

A thesis submitted for the award
Doctor of Philosophy
at Flinders University of South Australia

Rio Risandiansyah

Department of Medical Biotechnology
Faculty of Medicine, Nursing and Health Sciences
Flinders University

2016

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

SUMMARY

Current research in drug discovery suggests that co-culture of actinobacteria would result in the activation of “cryptic” genes in the production of novel secondary metabolites. In an effort to study co-culture, 388 unique combinations of actinobacteria were screened for interactivity (defined as changes in morphology, pigment production, sporulation and antibiotic activity), in which 45 possible interactions were discovered. Among these interactions, *Pseudonocardia* sp. PIP 161 was found to interact with at least 17 other *Streptomyces* species, in which an increase in antibiotic activity and sporulation was observed.

HPLC-DAD analysis, however, revealed that co-culture with *Pseudonocardia* sp. PIP 161 had varying effects on the induction of secondary metabolism in different *Streptomyces* species. While both co-cultured *Streptomyces* spp. showed an increase in antibiotic production, *Streptomyces* sp. EUC 63 showed an increase in the production of most, if not all, compounds, with up to a 7 fold increase in the production of an enterobactin-like compound. Furthermore, the interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* spp. were found to occur via a signal from a secreted chemical, which was producible in axenic conditions of *Pseudonocardia* sp. PIP 161 in both liquid and solid medium.

As enterobactin was known as a potent siderophore, co-culture studies were undertaken in conditions of varying iron levels, and whether an increase in iron levels would mimic the changes observed during co-culture with *Pseudonocardia* sp. PIP 161. However, while iron was observed to play an essential role in the formation of aerial mycelia and sporulation, the production of antibiotics and pigments were found, in this case, to be more dependent on chemical signals from *Pseudonocardia* sp. PIP 161 compared to iron levels. Therefore, efforts were made to isolate the signal producing chemical, in order to be used as an inducer for secondary metabolite production.

Pseudonocardia sp. PIP 161 was then subcultured and tested for inducing activity in several *Streptomyces* spp from broth eluates. The extraction of these compounds followed by tandem MS/MS reveals a major chemical compound from *Pseudonocardia* sp. PIP 161 to be a diketopiperazine, cyclo (His-Pro), as well as at least one other diketopiperazine. Although previously detected from mammals and plant pathogens, this is the first report of cyclo (His-Pro) being detected in endophytic actinobacteria. Induction with cyclo (His-Pro) significantly increased antibiotic activity in *Streptomyces* sp. EUC 63 and increased blue pigment production from *Streptomyces* sp. SC 36. However, cyclo (His-Pro) induction alone fails to

completely mimic the morphological and metabolic changes observed in co-culture with *Pseudonocardia* sp. PIP 161.

Therefore, based on these findings, it was concluded that the interactivity of *Pseudonocardia* sp. PIP 161 was in part mediated by diketopiperazines, and provides preliminary evidence of the role of diketopiperazines as cell signalling compounds in actinobacteria. Continuation of this study may show the potential of diketopiperazine induction in awakening previously unexpressed genes from actinobacteria, and thus be used as inducers in drug discovery projects.

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.

Signed.....

Date.....

ACKNOWLEDGEMENTS

Bismillahirrahmanirrahiim. Alhamdulillahi robbil 'alamin. Arrahman-Arrahiim.

I thank Universitas Islam Malang, in which through the funding from Indonesian Directorate General of Higher Education (DIKTI) from the Health Profesionnal Education Quality scheme, has entrusted me with the opportunity and the funding to complete my education in Flinders University.

I sincerely thank my supervisor, Prof. Chris M.M Franco, for his guidance in this project, and giving me valuable lessons that I would hopefully be able to carry with me in conduct of my profession. I also extend my gratitude to my co-supervisor, A/Prof. Mike Perkins, for his truthful advice and support, encouragement and interest in this project. Thank you both for the time and patience that you provided in an effort to complete this project.

I am grateful for the lecturers in the department: Prof. Wei Zhang, Dr. Barbara Sanderson, and Dr. Fiona Young for their example of excellence. The passes in the hallway sometimes makes the day. Life at the lab would not be possible if not for the efforts of Mrs. Barbara Kupke and Dr. Hanna Krysinska.

I am thankful for the lessons and knowledge passed through Ernest Lacey, Alistair Lacey, and Andrew Crombie for the time spent on me in Microbial Screening Technologies, in Sydney. Thank you for the rich and priceless knowledge you have provided, and for the lessons in work ethics and passion for science.

To my friends and brothers, Mousa Al-ghazwi, whom I have known the longest, and Dr. Yadollah Bahrami, whom I have talked to the most, I extend much gratitude for the friendship and kindness you have shown me throughout my years during my studies.

To my PC-2 lab partners: Evita, Xuyen, Eric, Steve, Elmo, Qi, Sophia, and Onuma, who have kept me company and helped me greatly in my studies, by providing me with ideas and methods, and encouragements, I couldn't have completed this thesis without your help.

Lastly, to my family, my mother and father, for the constant barrage of reminders and sincere prayers. To my wife Hanggia Primadita, for her constant patience and believe in me. It would be impossible for me to finish this project without their support. For this, I am indebted.

CHAPTER 1. LITERATURE REVIEW

1.1 Actinobacteria as a source of novel bioactive compounds

1.1.1 Natural product discovery from actinobacteria

Natural products are known to be a rich source for novel bioactive compounds. In a recently updated report, 67% of all the new drugs approved by the FDA in 1981 to 2014 either originated or were derived from natural products, whereas only 27% were purely synthetic compounds (with 6% vaccines), as shown in figure 1.1 (Newman & Cragg 2016). A comprehensive list of natural products discovered up to today can be readily viewed in two different databases, whereas approximately 200,000 compounds are listed in the 'dictionary of natural products' and approximately 370,000 compounds listed in ZINC database (Sterling & Irwin 2015). From this, it is approximated that around 65-80% of natural bioactive compounds were found to conform to 'Lipinski's rule of five' (a rule commonly used to assess the viability of bioactive compounds to be leads/drugs), showing more promise for a successful 'hit' when compared to synthetic compounds (Harvey, Edrada-Ebel & Quinn 2015; Quinn *et al*. 2008).

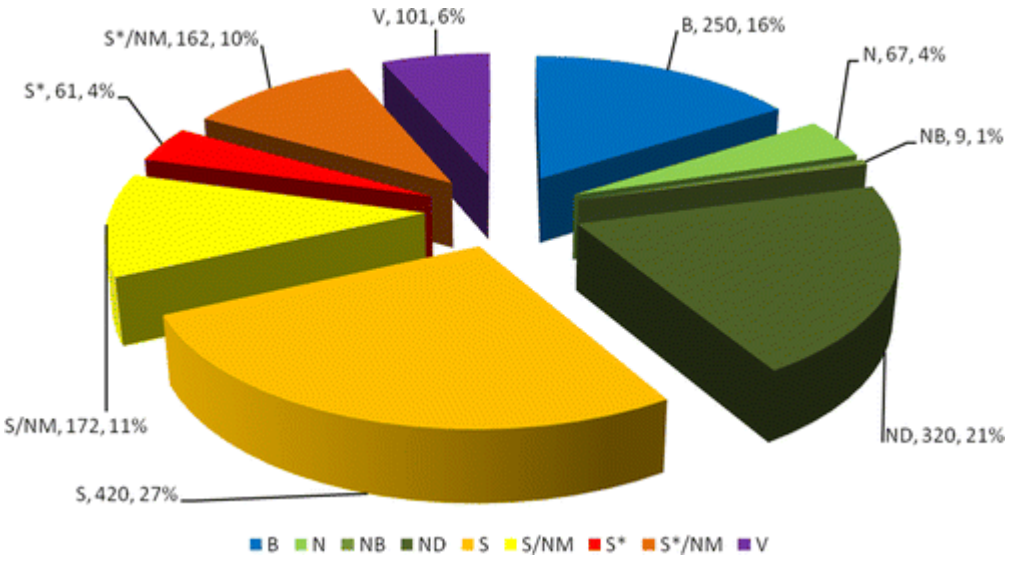


Figure 1.1 All new approved drugs 1981 – 2014; n 1562, as displayed in Newman and Cragg (2016) based on the source, whereas B= biological macromolecule, N= unaltered natural product, NB= botanical drug (defined mixture), ND= natural product derivative, S= synthetic drugs, S*= synthetic drugs with an NP pharmacophore, V= vaccine, and /NM=mimic of natural products.

Natural products are used in a wide range of therapeutic bioactive compounds, including treatments for type 1 and type 2 diabetes, anti-inflammatory medications,

anti-cancer, anti-Alzheimers, anticoagulants and anti-ulcers. A large portion of drugs approved from 1981 to 2014, however, were found to be anti-infective agents (16%, or 221 drugs from 1328 drugs), with 63.4% directly from natural sources, derived from natural products, or synthetics with a natural product pharmacophore (Newman & Cragg 2016). The 'golden era' of antibiotics between the periods of 1940s – 1960s discovered most of the antibiotic classes used today, followed by an 'innovation gap' for 40 years (Fischbach & Walsh 2009). During this period, several antibiotic sub-classes were being discovered and clinically tested (Bush 2012), but new classes of antibiotics only were discovered in the 2000s (Fair & Tor 2014; Fischbach & Walsh 2009), as shown in figure 1.2.

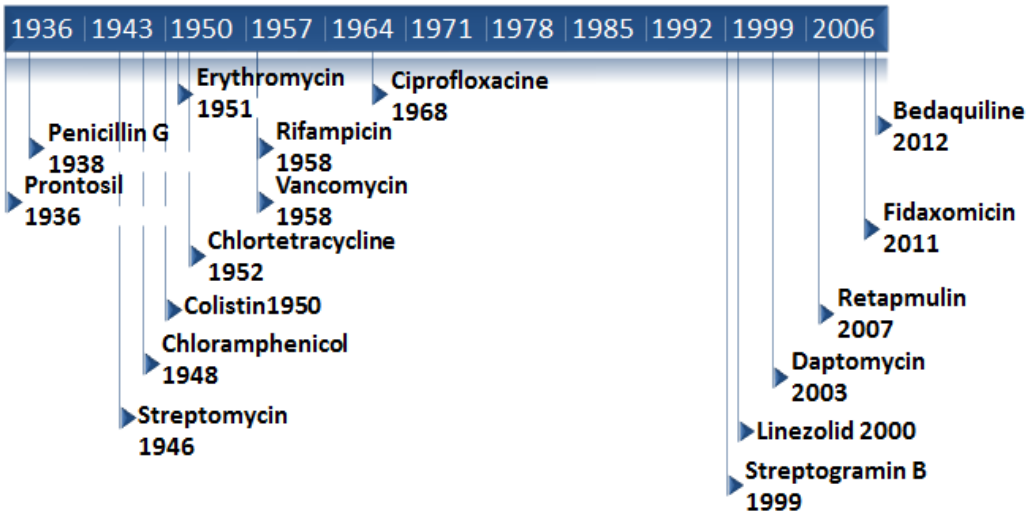


Figure 1.2 Timeline of first clinical introduction of antibiotics (Fair & Tor 2014)

Among the new classes of antibiotics discovered, oxazolidinones and diarylquinolines are completely synthetic, although the leads for oxazolidinones were discovered in the 1970s. Comparison of the chemical structure between synthetic antibiotics and natural product derived antibiotic compounds shows the relative simplicity of the synthetic antibiotics, as shown in figure 1.3 (Fair & Tor 2014). Furthermore, the bioactivity and selectivity of natural products are attributes that are desirable within the medical field, in which investigation of the mechanism of action is information that can be used in the creation of a semi-synthetic or a synthetic drug (Cragg & Newman 2013).

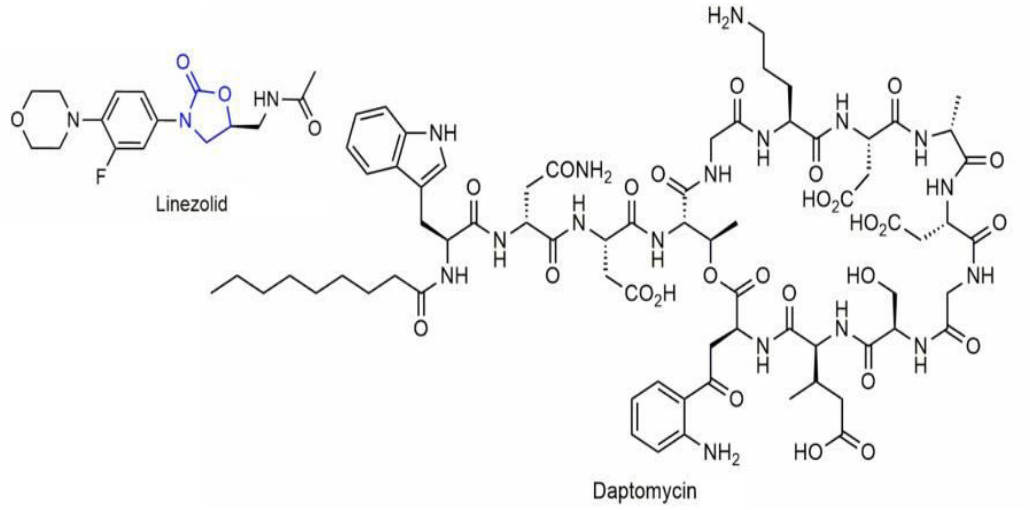


Figure 1.3 The complexity of Daptomycin (natural product) compared to Linezolid (synthetic antibiotic) (2014), as shown in Fair and Tor (2014).

The search for natural products is still relevant today and is an emerging area of interest recently. Efforts to isolate novel compounds is today includes by isolating different rare actinobacteria from various sources (Azman *et al*. 2015; Jafari *et al*. 2014; Lazzarini *et al*. 2000; Tanvir *et al*. 2016; Wang, X *et al*. 2013), awakening 'silent genes' by using various elicitors, such as rare elements (Kawai *et al*. 2007; Tanaka, Y, Hosaka & Ochi 2010), stress or chemicals (Yoon & Nodwell 2013) or co-culture (Bertrand *et al*. ; Chen, H *et al*. 2015; Netzker *et al*. 2015), and others. As this research is mainly focused co-culture, this subject would be discussed in further paragraphs.

1.1.2 The need for new antibiotics

The discovery of antibiotics reduced the mortality rate of infectious and communicable diseases in a time when it was one of the greater threats faced by mankind worldwide. Although some researchers were confident that antibiotics would be the solution to end infectious diseases, after more than a decade later,infectious diseases are still a threat both in developed and developing countries. The fact is, in the early 2000s, infectious diseases still rank in the top ten highest cause of death worldwide, consisting of lower respiratory tract infection (such as Pneumonia) in the higher, middle and lower income nations, and diarrhoeal diseases, tuberculosis, and HIV/AIDS in lower income countries. Although the rate of infectious diseases is projected to decline by the 2030s, lower respiratory tract infection is projected to remain in the top four causes of death, and other diseases such as tuberculosis and HIV/AIDS rate to fall out of the top ten at that time (WHO 2008).

However, this projection underestimates other contributing factors, among them the increasing rise of antibiotic resistance due to the misuse or overuse of antibiotic treatment prescription (Alvan, Edlund & Heddini 2011). This over-exposure of antibiotics is thought to lead to a selection process of resistant strains over susceptible strains in the host, and directly contributes to the growth of resistant strains communicable to the surrounding population (Alanis 2005; Chan *et al*. 2012). To further complicate matters, investments in antibiotic research and development by the pharmaceutical industry has declined since 1985, resulting in fewer antibacterial agent discoveries (Spellberg *et al*. 2004; Wenzel 2004). Antibiotic investment is a risky undertaking: the transition of novel antibiotic compounds from preclinical phase to the clinical phase was rarely successful; regulations are in place which would ensure first generation of antibiotics to be used instead of a newer one; and as antibiotics work by curing the disease instead of mitigating its symptoms, it would mean to gain lesser profit compared to some drugs against non-communicable, degenerative diseases (Morel & Mossialos 2010; Spellberg *et al*. 2004).

Although the solution for the emergence of antibiotic resistance mostly required the adjustment of factors including improved screening of disease, the discovery and application of vaccines, and adjustments in health regulation to optimize rational antibiotic administration, antibiotic research and development would still play a pivoting role in management antibiotic resistance (Alvan, Edlund & Heddini 2011). The development of antibiotic has lately been focused on improving existing antibiotics, increasing efficacy or improving its spectrum, or mitigating side effects (Alanis 2005; Spellberg *et al*. 2004), however, reducing selective pressure posed by antibiotic usage would rely on novel antibiotics having a different mechanism of action and/or different target (Alvan, Edlund & Heddini 2011).

1.1.3 Secondary metabolite biosynthetic pathways in actinobacteria

Understanding the biosynthetic pathways responsible for the production of secondary metabolites are essential in discovering factors influencing the production of the compound of interest. Several studies have been focused in determining the genomic properties of certain uncommon actinobacteria as a means to comprehend the biosynthetic pathways involved in the production of several different metabolites and the factors influencing production. It is a known fact that genomic data would encode enzymes influencing both primary and secondary metabolism, directly linked to the production of useful compounds or even certain enzymes. A comparative study of the genomic properties in several genera of actinobacteria has revealed that common

enzymes, and therefore, common biosynthetic pathways, exist despite a significant variety of the genome size between the members of the genus (Alam *et al*. 2011).

A common biosynthetic pathway known to exist in different actinobacteria which are known to produce useful secondary metabolites in the polyketide synthase (PKS) pathway and the non-ribosomal peptide synthase (NRPS) pathway (Fischbach & Walsh 2006). The biosynthesis of several metabolites through the PKS and NRPS pathways are shown to go through a series of reactions catalysed by similar classes of enzymes having similar functions, in which its metabolic pathway is similar to fatty acid biosynthesis, only having a wider range of acyl thioesters precursors, including malonyl coenzyme A (CoA), propionyl-CoA, methylmalonyl-CoA, ethylmalonyl-coA and methoxymalonyl-CoA (Ridley & Khosla 2009). The precursors are then subjected to a series of elongation reactions not unlike fatty acid synthesis, in which precursors are transformed into a ketone form by ketosynthase (KS), acyltransferase (AT) and an acyl carrier protein (ACP), followed by, when required, a conversion into a B-hydroxyl or an alkane group by ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) (Schirmer *et al*. 2005). These enzymes are grouped together into modules, and in some cases also contain active and inactive enzymes within these modules (such as shown in the production of amphotericin B (Caffrey *et al*. 2001)). An example of this is the production of an erythromycin precursor 6-deoxyerythronolide B synthase (DEBS), as shown in figure.

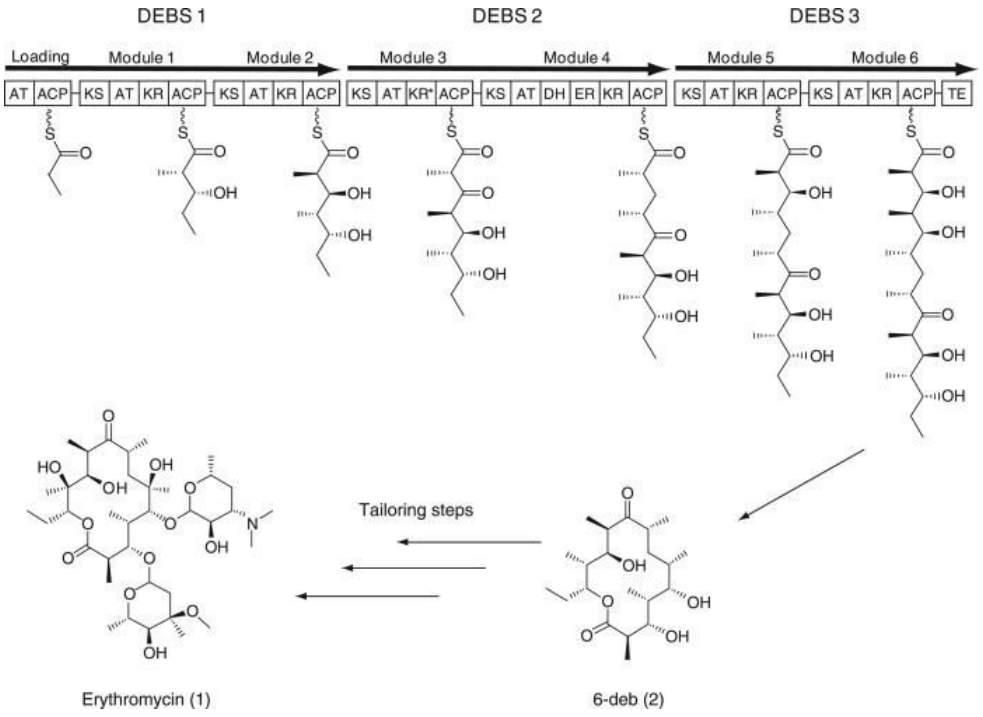


Figure 1.4 Biosynthesis of erythromycin precursor 6-deoxyerythronolide B synthase (DEBS) (Ridley & Khosla, 2009).

The conformation of these enzymes determines the type of polyketide synthase system that is possessed by an actinobacteria. Type I PKS systems consists of megasynthases containing multiple modules, each responsible for elongation and reduction reactions, with the potential of creating a diverse range of bioactive metabolites, depending on the modules present. In type II PKS systems, these modules are dissociated multicomplex enzymes with one or two active sites, and iteratively produce compounds with a defined length. Type III PKS systems iterative homodimeric condensing enzymes, in which CoA thioesters are condensed directly, and therefore negates the use of an acyl carrier protein, but may only produce small aromatic metabolites (Ridley & Khosla 2009; Shen 2003). However, some instances of PKS systems which deviate from this classification also exist. In the PKS type I system biosynthesis of leinamycin, for instance, several modules contains an inactive AT and instead its activity was provided by an iterative protein. The biosynthesis of macrotetrolide relies on a type II PKS system which uses acyl CoAs directly, similar to a type III PKS system (Shen 2003).

The genetic organization of the modules for PKS systems commonly exist in clusters, in which several modules of minimal polyketide units exist and work iterative, or non-iteratively, to synthesize molecules having complex, and diverse, structures (Schirmer *et al*. 2005). This allows detection of the potential for polyketide production, by specifically screening for the genes which encodes minimal polyketide units, with a classical polymerase chain reaction approach. This is achieved with reported success using degenerate primers corresponding to the minimal polyketide units (ketosynthase, acetyltransferase, and acyl carrier protein) conserved regions (Ayuso-Sacido & Genilloud 2005). This method is used to accurately select potential producers from living source or its associated microorganisms which potentially may contain hundreds of different species, reducing the otherwise required for laborious and time consuming screening (Schirmer *et al*. 2005).

1.1.4 *Streptomyces* genetic potential: cryptic/silent genes

A majority of bioactive natural products are mostly derived from actinobacteria. As *Streptomyces*, a major constituent of the *Streptomycetaceae* family, are the predominant actinobacteria in soil communities and the most explored and researched (Labeda *et al*. 2011), it would therefore be reasonably likely that most of these compounds were discovered from *Streptomyces*. A database focusing in

cataloguing compounds isolated from *Streptomyces* showed that more than >1900 *Streptomyces* have produced >2400 novel and unique compounds, with at least half found with bioactivity (Lucas *et al*. 2013). This number was thought to be an underestimation, however, as current work was only based on abstracts of published work, and thus only showing those most important in that research.

Despite this high number, a concern of 'diminishing returns' is currently voiced by several researchers, whereas despite screening efforts of novel antibiotics in actinobacteria that were conducted in the 1990s, few novel compounds were acquired, and instead more compounds that were already previously discovered was obtained (Watve *et al*. 2001). This was also supported historically, with no novel antibiotic classes discovered after 1980 until the early 2000s (Fischbach & Walsh 2009). While this would certainly be a factor in the lack of interest in industrial investments in antibiotic discovery from natural sources, researchers also cite the innovation gap to be exacerbated by a perceived low profit margin of antibiotics. This was mentioned to be due to the low lifespan of antibiotics in general (to be worsen by antibiotic resistance), governmental regulation that aims to further reduce the number of antibiotics used (to curb antibiotic resistance emergence) and the recommendation to use pre-existing antibiotics as first line defence (Morel & Mossialos 2010; Wenzel 2004).

Despite this, with proper regulation and new approaches aimed to increase the efficiency of discovery, natural products remain the most promising source of novel bioactive compounds (Peláez 2006). While the metabolite produced from *Streptomyces* would definitely be limited, Watve *et al*. (2001) predicted another 150,000 compounds to be undiscovered. Recent genome sequencing studies that have discovered a large number of gene clusters, however, many of these genes are silent or inactive in normal conditions (Challis & Hopwood 2003; Clardy, Fischbach & Currie 2009; Seyedsayamdost *et al*. 2012), or expressed in very little concentrations that it would not be detectable (Olano *et al*. 2008).Genomic studies have revealed actinobacteria species sequenced up to this day have an average genome size of 2 – 10 MB, with 2000 to 9000 protein encoding genes. *Streptomyces* species have a genomic size of 8 to 10 Mb with 20 to 30 secondary metabolite gene clusters (as shown in the following figure) (Nett, Ikeda & Moore 2009), but only 3 – 5 metabolite per species are currently known (Tanaka, Y *et al*. 2013).

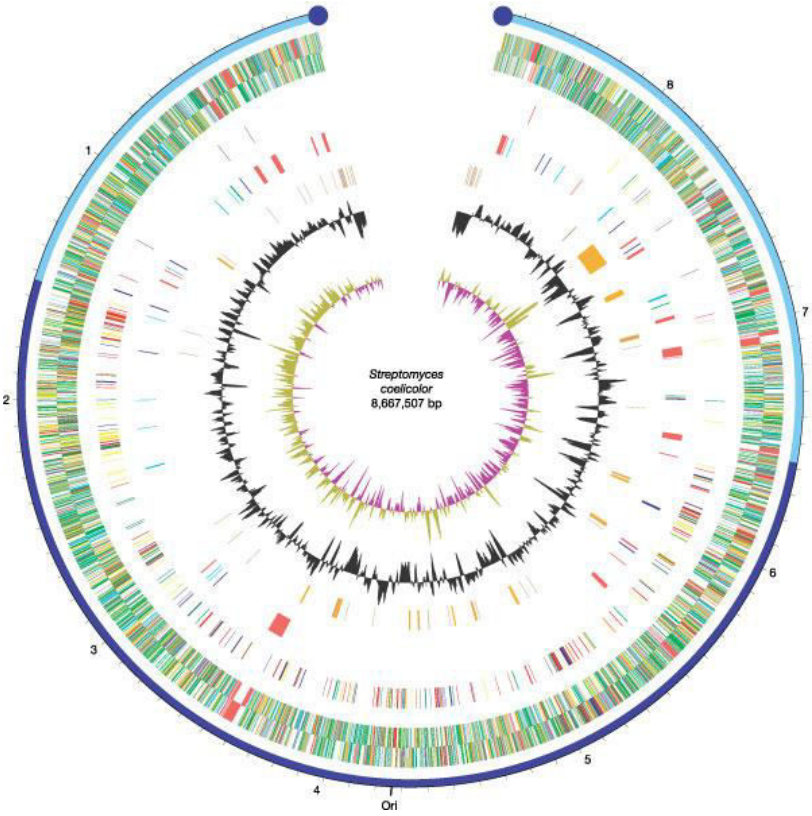


Figure 1.5 Circular representation of the *Streptomyces* coelicolor chromosome, as shown in Bentley *et al*. (2002). The outer scale is numbered anticlockwise to indicate megabase size. Circles from 1, 2, 3, 4 and 5 (from outside in) show genes with predicted function (1 and 2 represent all genes in reverse and forward strand, 3 represents 'essential' genes, 4, represents 'contingency' genes, 5, shows mobile elements), circle 6 represents G+C content, and circle 7 shows GC bias ((G-C/G+C), khaki indicates values >1, purple <1) (Bentley, Chater, Cerdeno-Tarraga, *et al*. 2002).

From previous research, the role of the supply of nutrients in an axenic actinobacteria growth condition in secondary metabolite production is (1) the activation of certain gene clusters, (2) the provision of precursors (Li, L, Qiao & Yuan 2007), and (3) the adjustment of the metabolic flux in favour of secondary metabolism, (Li, L, Zheng & Yuan 2007; Naeimpoor & Mavituna 2000). The supply of different carbon and/or nitrogen sources is found to alter the production metabolites; not only changing the concentration of the compounds produced (Jonsbu, McIntyre & Nielsen 2002), but also changing the metabolic profile or the types of compounds produced (Gesheva, Ivanova & Gesheva 2005). This is observed in metabolic studies of 971 metabolic pathways in different media conditions, in which certain metabolite pathways were active in different media conditions, as shown the following figure (Borodina, Krabben & Nielsen 2005).

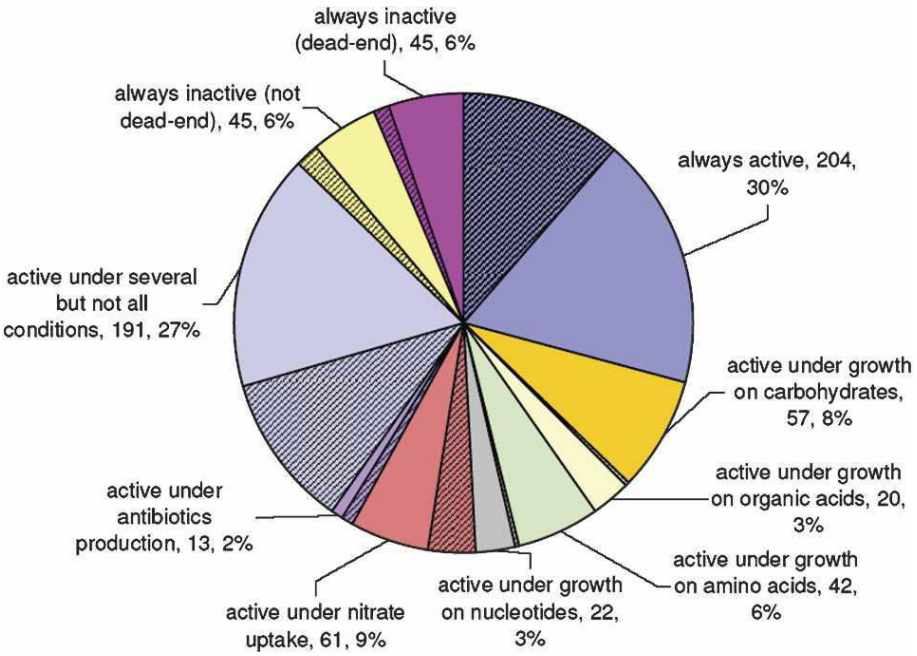


Figure 1.6 Percentage of metabolic pathways active in different conditions. Gene expression in metabolism were controlled by nutrient composition, yet some metabolites were expressed only if casamino acid was added (Borodina, Krabben & Nielsen 2005).

Optimisation studies has shown that adjusting carbon and nitrogen sources in a certain species may increase the production of interesting compounds, as shown for example in the production of poly-epsilon-lysine from *Streptomyces noursei* (Bankar & Singhal 2010), clavulanic acid from *Streptomyces clavuligerus* (Wang, Y-H *et al*. 2005), and laccase from *Streptomyces psammoticus* (Niladevi *et al*. 2009). This approach would greatly benefit in the production of a known useful compound, and could result in an increase of production of up to 23 times (such as the case in the production of oligomycin A from *Streptomyces avermitilis* using fatty acid precursors) (Olano *et al*. 2008), enabling feasible mass production of the compound. Other methods to 'awaken' these genes have been discussed in other papers (Scherlach & Hertweck 2009; Zarins-Tutt *et al*. 2016). Among these was by co-culturing actinomycetes with different organisms, which would be discussed in further detail in the following paragraphs.

1.2 Actinobacteria life cycle and secondary metabolite production

1.2.1 Actinobacteria taxonomy, morphology and life cycle

Actinobacteria can be defined as Gram positive filamentous spore-forming bacteria, having a high G-C content, found most commonly in soil. Taxonomically, based on 16S and 23S rRNA gene profile, Actinobacteria can be divided into six classes: *Actinobacteria*, *Acidimicrobidiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia*, and elevated the status of suborders to orders (Ludwig *et al*. 2012). Based on this taxonomy, 8 orders (previously sub-orders), namely, *Actinomycitales*, *Streptomycetales*, *Corynebacteriales*, *Micrococcales*, *Micromonosporales*, *Propionibacteriales*, *Streptosporangiales*, and *Frankinales*, with approximately more than 20 families, are known to be antibiotic producing (Adegboye & Babalola 2012; Ludwig *et al*. 2012). *Streptomyces* are the dominant and morphologically diverse genus in the family *Streptomycetaceae,* order *Streptomycetales*, and divided into 130 clades based on its 16S rRNA sequences (Labeda *et al*. 2011). Furthermore, *Streptomyces* has a high frequency for discovery, and therefore is the most studied genus.

The life cycle of the *Streptomyces* has been described in several literatures (Barka *et al*. 2016; Dyson 2009). As *Streptomyces*, and most actinobacteria, are known to be non-motile, dispersion relies on the formation of spores from aerial hyphae, which was also a response to nutrient starvation and/or other unfavourable conditions. The spores are transported to another location with favourable conditions, and the spores begin to germinate. Following this is the formation of vegetative or substrate hyphae, which was primarily used to absorb nutrients in its surrounding. After a certain biomass is achieved, secondary metabolism (such as antibiotic production) is initiated and reproductive aerial hyphae are formed (Beppu 1992). A schematic representation of the life cycle of actinobacteria is shown in the following figure 1.7.

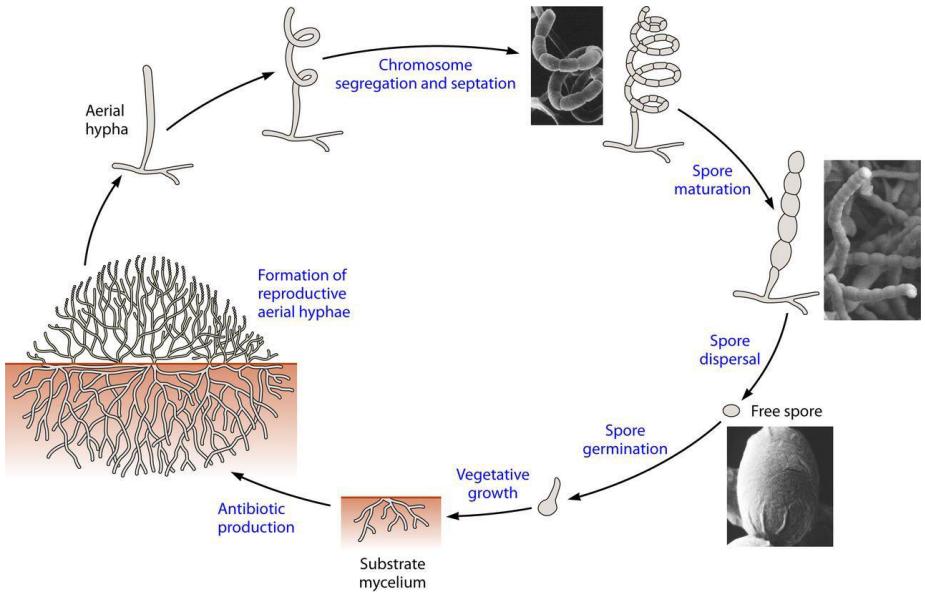


Figure 1.7 Schematic representation of an actinobacterial life cycle (Barka *et al*. 2016).

While primary metabolism can be defined as metabolism occurring during the period of growth, and thus concerned growth and biomass accumulation by the utilization of carbon catabolism pathways (glycolytic, pentose phosphate and TCA cycle pathways) (Dyson 2009), secondary metabolism occurs after growth reduction or growth cessation (Bibb 2005). Thus, most secondary metabolites are often produced approaching stationary phase, with certain exceptions. Secondary metabolites are typically bioactive compound with a certain role that would increase the survivability of the organism in a biological setting, including antibiotic compounds (Atta 2015), metal transporters (Liu, N *et al*. 2013), auto-regulatory hormones (Beppu 1992) or having an unknown function. The regulation of secondary metabolite production is further discussed below.

1.2.2 A-factor regulates sporulation and secondary metabolite production

Based on a pioneering work in the 1960s, a regulatory factor known as γ-butyrolactone, also called the autoregulatory factor or A-factor, which was found to induce sporulation and the production of Streptomycin in *Streptomyces griseus* (Ohnishi *et al*. 1999; Takano 2006). From several *Streptomyces* species, a number of A-factors are identified today, each having varying structures, as collated in a review by Takano (2006). *S. griseus* mutants deficient in A-factors has been shown to have lost their ability to produce certain streptomycin as well as form spores (Hara & Beppu

1982), indicating a link between sporulation and production of secondary metabolites. This is supported by future research which shows the presence of a regulatory factor which controls both sporulation and production of secondary metabolites (Du *et al*. 2011; Horinouchi & Beppu 1992b; Li, W *et al*. 2006).

Several researchers have attempted to elucidate the regulatory cascade of A-factors to sporulation and production. The A-factor is speculated to be synthesized from a glycerol derivative and a beta-keto acid by the AfsA protein and binds to a repressor regulatory protein (AfsA binding protein) resulting in the expression of a positive A-signal signal transducer (AdpA) which would influence both morphological development in form of sporulation and the production of secondary metabolites (Horinouchi & Beppu 1992b). The nature of AfsA binding proteins (ArpA) is supported by other research, in which a strains deficient in this protein result in a normal to slightly increased sporulation and not a loss in production compared to the wild type bacteria (Miyake *et al*. 1990). In the case of the production of Streptomycin by *S. griseus*, AdpA binds to upstream regions and activates the transcription of *strR* (Ohnishi *et al*. 1999), which would then bind to multiple sites in the streptomycin biosynthetic gene cluster (Retzlaff & Distler 1995).

In regards to its regulatory function, it is suggested that A-factors are microbial hormones (Horinouchi 2002), and has a key function in unlocking secondary metabolite cryptic genes. As mentioned previously, addition of external A-factors in *S. griseus* bald mutants is able to restore secondary metabolite and sporulation (Hara & Beppu 1982). However, A-factor signalling is found to be time and concentration sensitive, in which the exogenous addition of A-factor outside the natural time frame caused the inhibition of both sporulation and secondary metabolite production (Ando, Ueda & Horinouchi 1997). In *S. griseus*, A-factors are produced 12-36 h after inoculation, slightly before the stationary phase of the bacteria, and is only detected for a short period of time, due to *afsA* not being expressed during the stationary phase and A-factor having a low stability (Horinouchi 2002; Neumann, Piepersberg & Distler 1996), as shown in figure 1.8. Neumann *et al*. (1996) described this phase as the “decision phase” in which the presence of A-factors would determine whether sporulation occurs and secondary metabolites are produced.

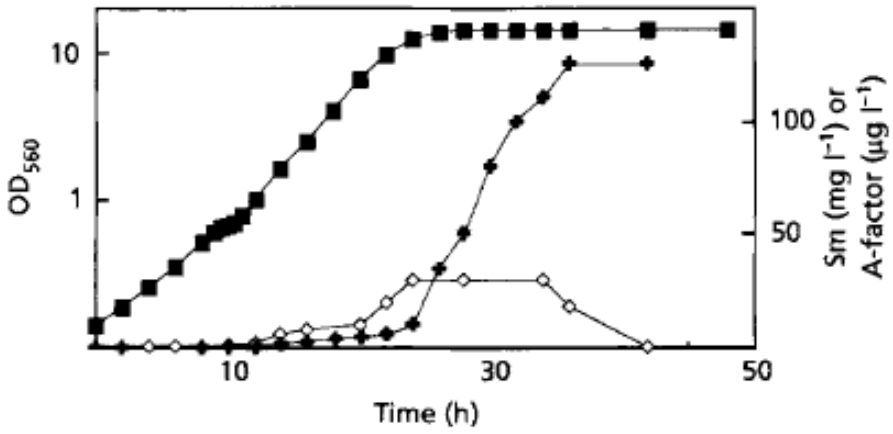


Figure 1.8 Growth of S. griseus in defined medium showing level of biomass (■), Streptomycin (+) (Sm) and A-factor (◊) production. A-factor levels increases and decreases shortly entering stationary phase, followed by an increase in Streptomycin production (Neumann, Piepersberg & Distler 1996).

Other findings suggest that A-factors or other regulatory signals also would determine nutrient uptake and shifts in primary metabolism enzyme expression. This is described in a research by Du *et al*. (2011) using *Streptomyces chattanoogensis* in which genes expressing two gamma-butyrolacones (GBL), scgA and scgX, and a GBL receptor protein, scgR are deleted. Concurrent with previous studies, this resulted in a reduction in sporulation and secondary metabolite production in ΔscgA and ΔscgX strains, while ΔscgR had identical characteristics with the wild type. However, ΔscgA strains also had different expression of key proteins in the primary metabolic pathways. This finding is supported by previous researchers in which differential sporulation pattern is observed when afsA deleted mutants were inoculated in medium with different carbon sources which may indicate that AfsA proteins might play a role in nutrient uptake (Ando, Ueda & Horinouchi 1997; Neumann, Piepersberg & Distler 1996).

Currently, several research studies with actinobacteria has discovered and characterized GBL from mainly *Streptomyces* sources, with little research on non-*streptomycetes* (Takano 2006). In a review by Takano (2006) several GBL discovered from *Amycolatopsis mediterranei* and *Actinoplanes teichomyceticus*, with GBL receptor proteins were also found in both bacteria and *Micromonospora echinospora* (Choi *et al*. 2003). However, little progress has been made in the discovery of A-factors or other gamma-butyrolactones from non-*Streptomyces*.

1.3 Actinobacteria in microorganism communities

1.3.1 Actinobcteria isolation from microorganism communities

Organisms targeted for bioactive compound discovery are firstly isolated and characterized from the natural environment with an emphasis on the isolation of novel species to increase the chances of the discovery of a unique bioactive compounds. The search for novel actinobacteria, for instance, may be expanded to include regions not yet explored for actinobacteria isolation (such as conducted in the Himalayas (Duraipandiyan *et al*. 2010) or a racecourse in South Africa (Everest & Meyers 2008)). It is also well known that actinobacteria may also originate from plant sources (Li, W-J *et al*. 2004), or marine sources such as seaweeds and sea-cucumber. In the 2000s, novel species from uncommon genera are still being discovered from soil collected from forests (Wang, Y-X *et al*. 2008; Wang, YM *et al*. 2001), plant litter (Sakiyama *et al*. 2010), mines (Carlsohn *et al*. 2007) and the roots of specific plant surrounding mines (Trujillo *et al*. 2006).

Plants are known to host a population of actinobacteria inside the leaves, stems and roots of plants, thus called 'endophytic actinobacteria' (Kaewkla, O & Franco 2013), as well as inside its surrounding soil. Most actinobacteria are considered to be mostly saprophytic, residing inside the plants without causing any adverse effects and eliciting a systemic immune response from the plant host (Crawford *et al*. 1993; Hasegawa *et al*. 2006). Previous isolation efforts using cultivation based methods in this laboratory by Kaewkla and Franco (2013) have shown that a majority of the actinobacteria from native Australian trees are isolated from the roots of the plants, compared to the stems and leaves (409 cultures compared to 55 and 56 cultures, respectively). Other isolation studies seem to follow this trend: 317 actinobacteria were discovered from legume plant roots (Mingma *et al*. 2013), while only 47 successfully isolated from legume plant leaves.

However, visualization of endophytic actinobacteria using current technology is currently limited, with some confirmation of actinobacteria colonisation in the roots (Franco *et al*. 2007) but little to none in any other organs. Researchers have used other technologies to deduce the localization of actinobacteria inside the plants. Common methods employ molecular based strategies to determine the number of actinobacteria in organs of the plant structure, as well as monitor the changes of endophytic actinobacteria population due alteration in the plants environment (Conn & Franco 2004). A pyrosequencing based method has been used to determine the endophytic bacterial communities in *Arabidopsis thaliana* and compare the population of bacteria in the roots and the leaves of the plant. The results of this study report that

a higher number of actinobacteria can be discovered from the root systems compared to the leaves (Bodenhausen, Horton & Bergelson 2013). This laboratory has used T-RFLP and PCR based screening in wheat and discovered that not only that a large population of root endophytic actinobacteria exists, but also different communities of root endophytic ecosystem differs when the host is planted in different soils (Conn & Franco 2004).

As the majority of actinobacteria are initially discovered from soil sources, it seems natural that the root systems are the highest in actinobacteria populations. Soil characteristics play a role in nutrient availability as well as providing physico-chemical and spatial characteristics which would support the growth of a microbial community (Marschner *et al*. 2001). However, through control of nutrient availability in the surrounding soil and even more in the root systems in particular the rhizospheres, plant type are also known to be able to influence its microbial population (Grayston, Vaughan & Jones 1997; Ladygina & Hedlund 2010). The correlation of root exudates in the alteration the bacterial density is confirmed by addition of artificial exudates in maize, and comparing the bacterial density in the dry roots and the surrounding soil seems to imply a that plants play a greater function in determining the microbial population (Baudoin, Benizri & Guckert 2003). Similarly, in the case of actinobacteria, rhizospheres actinobacteria are shown to have a higher diversity and number compared to the root endophyte actinobacteria (Mingma *et al*. 2013).

1.3.2 *Pseudonocardia*: a case study on interactivity

Pseudonocardia is a rare actinobacteria isolated from soil (Park, SW *et al*. 2008; Prabahar *et al*. 2004; Qin *et al*. 2008), plant material (Duangmal *et al*. 2009; Gu *et al*. 2006; Kaewkla, O & Franco 2012; Sakiyama *et al*. 2010; Zhao *et al*. 2012), marine sediments (Jafari *et al*. 2014; Tian *et al*. 2013; Zhang, D-F *et al*. 2014) and attine ants (Barke, Jorg *et al*. 2010; Carr *et al*. 2012; Meirelles *et al*. 2014). Based on its 16S rRNA sequences, the family *Pseudonocardiaceae* was divided into 14 genera and separated further based on its cell wall type, whereas 8 genera had cell wall type IV, and 6 genera had cell wall type III (Lee, SD, Kim & Hah 2000; Stackebrandt, Rainey & Ward-Rainey 1997). The genus *Pseudonocardia* contains a type IV cell wall meso-diaminopimelic acid, arabinose and galactose in the peptidoglycan (Lee, SD, Kim & Hah 2000), and, at the time of writing, include 54 species (http://www.bacterio.net/*Pseudonocardia*.html), in which is at least partially categorized into 10 clades (Mueller *et al*. 2010).

The relationship between *Pseudonocardia* and attine ants in particular, is a subject of interest in this research. Attine ants, also known as leaf-cutting ants, live off a basidiomycete fungus as a main carbon food source, and in exchange provide protection of that fungus from other fungal parasites (Poulsen, Michael *et al*. 2007; Rodrigues *et al*. 2008). This was mainly achieved by microbial communities which reside in the attine-ant which produced antimicrobial and antifungal agents, which was composed of a dominant *Pseudonocardia* species and actinobacteria species, including *Streptomyces* and *Amycolatopsis* species (Barke, Jorg *et al*. 2010; Haeder *et al*. 2009; Meirelles *et al*. 2014; Poulsen, M. *et al*. 2005; Seipke *et al*. 2011; Sen *et al*. 2009).

Furthermore, a specific *Pseudonocardia* species in each ant population is maintained by vertical transmission from the queen to its progeny (Andersen, Sandra B. *et al*. 2015; Cafaro *et al*. 2011; Poulsen, Michael *et al*. 2011). Thi*s* was suggested to be the evidence that *Pseudonocardia* co-evolved with attine ants (Andersen, S. B. *et al*. 2013; Andersen, Sandra B. *et al*. 2015), and may indicate that *Pseudonocardia* would be more equipped for interaction or induction of other species in its immediate vicinity to allow the production of useful secondary metabolism, including antibiotic and antifungal agents.

Ant-associated *Pseudonocardia* were found to have bioactivity when cultured singularly, such as in the production of pseudonocardons A-C, and produce other non-specific antibacterial and antifungal agents in live cultures (Carr *et al*. 2012; Meirelles *et al*. 2014; Sen *et al*. 2009). However, several effective antifungal agents were also produced by ant-associated *Streptomyces* species (with potentially more compounds undiscovered in laboratory conditions), and a more diverse compounds were isolated from *Streptomyces* species, including actinomycin, antimycins, valinomycin, and candicidin (Haeder *et al*. 2009; Schoenian *et al*. 2011; Seipke *et al*. 2011). Ant-associated microbial communities were found to utilize more antifungals compared to the compounds isolated from *Pseudonocardia*, or even *Streptomyces*, alone (Barke, Jorg *et al*. 2010), and antibiotics were found to be distributed differently throughout the ant body (Schoenian *et al*. 2011).

The potential of *Pseudonocardia* to act as symbiotes beyond leaf-cutting ants was described in the study of the phylogenetics of *Pseudonocardiaceae*, in which multiple species were isolated from locations having high micro-communities (Mueller *et al*. 2010). *Pseudonocardia* beyond attine ants has been discovered in productive microbial communities in other studies (Guo *et al*. 2015). Previously, *Pseudonocardia* has been found to also increased the production of artemisinin in *Artemisia annua*

(also known as ‘sweet wormwood’) by upregulating genes which contribute to the conversion of amorpha-4,11-diene to artemisinic acid (Li, J *et al*. 2012). Therefore, concurring with the idea presented by Mueller *et al*. (2010), this may show the potential for *Pseudonocardia* to induce the production of compounds from other species.

1.4 Co-culture for secondary metabolite production

1.4.1 Co-culture increases unique metabolite production

Although it is well known that an interaction between the plant, pathogen (bacterial, fungi, or insect), and its residential microorganism (e.g. rhizobacteria, actinobacteria) is ever present in the ecosystem, these interactions in relation to its secondary metabolite production has only recently gained momentum. It is already well known that a mixed culture of bacteria would produce different compounds compared to when grown individually. An example of this is the production of Biphenomycin A from the co-cultivation of *Streptomyces griseorubiginosus* 43608 and *Pseudomonas maltophila* 1928, whereas one species produces the precursor and the other converts that precursor into the compound (Ezaki *et al*. 1992). Recently, mixed cultures has been used in various biotechnological applications: the production of biofuels (Shu *et al*. 2013), the production of various compounds, such as fatty acids (Zhang, F *et al*. 2013), acetic acid (Wang, Z *et al*. 2013) and bioremediation efforts (Chen, S *et al*. 2012; Ibn Abubakar *et al*. 2012; Saez, Benimeli & Amoroso 2012).

Actinobacteria are known as a rewarding source of novel compounds. Despite the high number of potential novel compounds produced, however, it is thought that a majority of these compounds are not expressed in normal conditions (Challis & Hopwood 2003; Clardy, Fischbach & Currie 2009). Challis and Hopwood (2003) has anticipated that different compounds can act synergistically in biological competition. Somewhat in support of this idea, previous studies have already shown that interspecies interaction may produce different metabolites and alter the morphological development, particularly sporulation, of the *Streptomyces* involved (Ueda *et al*. 2000). In this case, Ueda *et al*. (2000) suspected that a diffusible signalling molecule(s) were shared between each *Streptomyces* species which would trigger sporulation or antibiotic production. Surprisingly, A-factors producing *Streptomyces griseus* showed no stimulatory effects to the receiver strains, despite prior knowledge that A-factors (and γ-butyrolactones) are important signalling molecules for sporulation (Takano 2006).

Further evidence was provided by Traxler *et al*. (2013), in which a similar approach with Ueda *et al*. (2000) was used, but with a more advanced technique in the detection of secondary metabolite production. Traxler *et al*. (2013) co-cultivated *Streptomyces coelicolor* with 20 other different actinobacteria, and discovered 5 interactions which show temporal and phenotypic responses in *S. coelicolor*, either stimulating, partially stimulating, or not stimulating pigmentation or development. An earlier research using a slightly different and automated method by Vetsigian *et al*. (2011) discovered 42 different interaction profiles from 64 isolates, in which 45% were of growth or sporulation inhibition and only 19% were growth promotion. Vetsigian *et al*. (2011) employed a method in which the actinobacteria, designated as “senders”, were incubated on filters on a nutrient medium, in order to allow produced molecules to diffuse in the medium. After a certain number of days, the filter, along with the actinobacteria colony, was removed, and another set of actinobacteria, designated as “receivers”, were point-inoculated on the “conditioned” medium and observed using a camera in 4 hour intervals resulting in a time-lapse image (Vetsigian, Jajoo & Kishony 2011). Both images obtain from Traxler *et al*. (2013) and Vetsigian *et al*. (2011) are shown in figure 1.9.

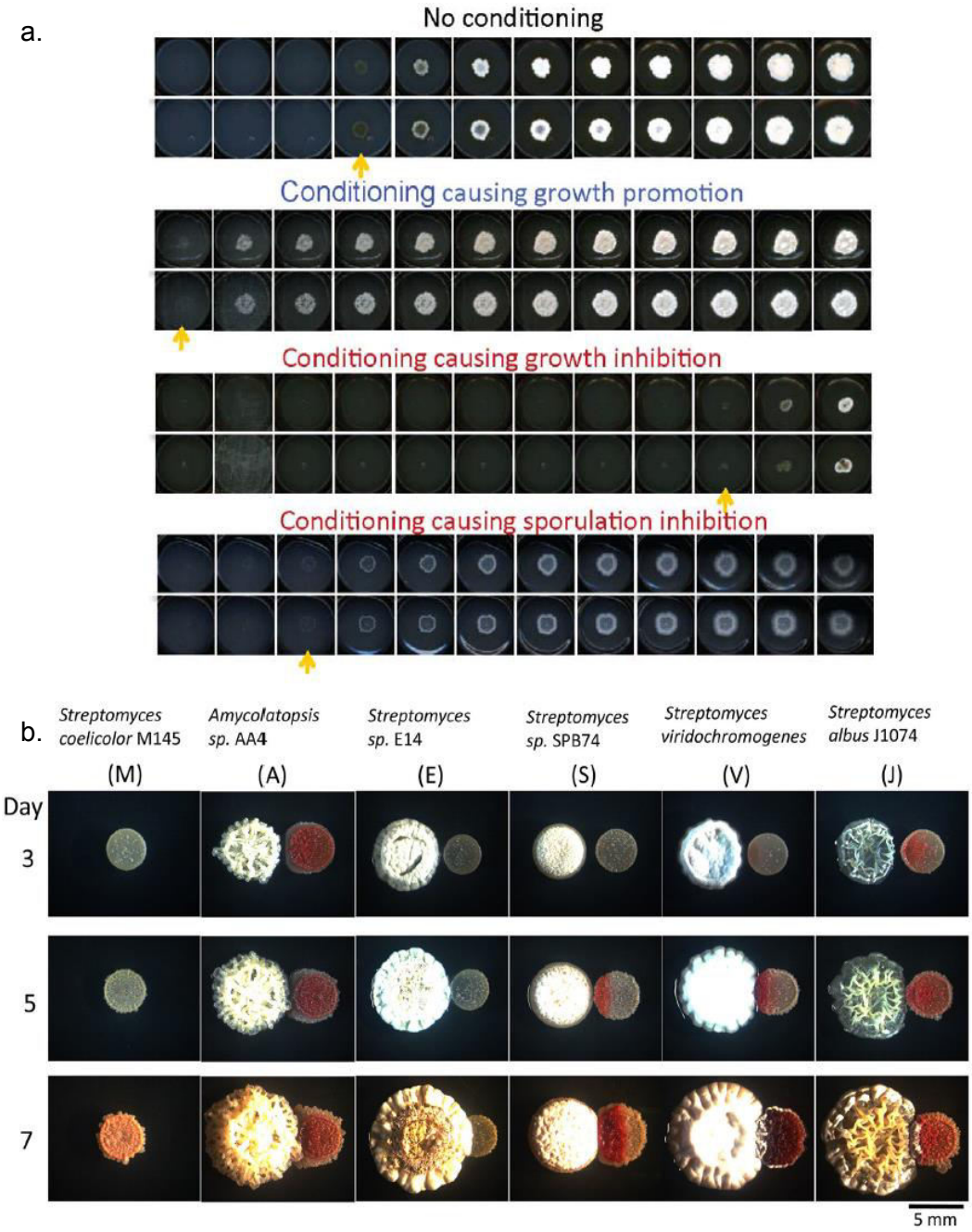


Figure 1.9 (a.) Vestigian *et al*. (2011) and (b.) Traxler *et al*. (2013) images depicting interspecies interaction causing morphological and temporal alterations to growth, pigmentation and sporulation. The time-lapse images from displayed in (a.) were able to pinpoint growth and sporulation times, as well as notice exact acceleration and delays in growth and sporulation pattern.

There are clear advantages and disadvantages of using the co-cultivation methods employed by Vetsigian *et al*. (2011) compared to Ueda *et al*. (2000) and Traxler *et al*. (2013). Firstly, the removal of the "sender" actinobacteria colonies and administering a nutrient resupply of the spent medium after a certain period of time negated the possibility of sporulation in the "receiver" actinobacteria to merely be triggered by nutrient deficiency due to competition. Secondly, the time-lapse images obtained

would not only show eventual alterations in growth and sporulation, but would also be able to show delays and acceleration of growth and pin-point the exact time of sporulation when with and without conditioning. However, as the “sender” actinobacteria are grown in axenic conditions, the interactions that observed would mostly be a chemical interaction.

As shown in figure 1.9, time-lapse images of growth promoted actinobacteria shows an initial acceleration of bacterial density, but sporulation on those bacteria occurred at the same rate or slower as the unconditioned bacteria, as well as actinobacteria which seems to only inhibit sporulation and not growth of the actinobacteria (Vetsigian, Jajoo & Kishony 2011). Interestingly, the network which was observed by Vetsigian *et al*. (2011) showed an asymmetry of sender-receiver interaction, whereas more sender actinobacteria are able to inhibit the growth of almost all receivers, and one actinobacteria inhibited itself. Similarly, two actinobacteria were stimulated by almost all actinobacteria, except when the conditioned by the effective inhibitors. The mechanistic explanation of why this occurred is beyond the scope of the paper, however, Vestigian *et al*. (2011) attributed the inhibition phenomenon to antibiotic interactions, whereas some actinobacteria would be susceptible to antibiotic and others, particularly those in close phylogenetic relationship with the antibiotic producers, would be resistant. Whether this resistance was only active when the antibiotic was present in the medium or would always be active (hence alteration in secondary metabolite production) as well as growth promoted receiver actinobacteria were left unanswered in this paper.

Findings that actinobacteria interaction does alter secondary metabolite production is shown using two metabolomics approaches, nanospray desoption electrospray ionization (NanoDESI) and microbial matrix-assisted laser desoption ionization time of flight (MALDI-TOF) imaging mass spectrometry (IMS) (Traxler *et al*. 2013a). Within the five interactions (*S. coelicolor* M145 against five other ‘initiator’ actinobacteria) studied in this paper, a large number of individual compounds were observed whereas a majority were interaction specific, and were unique depending on the interacting strain and the time of sampling. Sub-networks of at least four major compound families (antibiotics actinorhodin and prodiginine, and siderophores coelichelin and desferrioxamines B and E) were discovered across all 5 interactions at any time point.

Closer examination of the production of recognized compounds resulted in several interesting observations. The production of coelichelin in the interaction between *S. viridochromogenes* and *S. coelicolor* was produced by either or both of the corresponding strains, as both possess the genes encoding its production.

1.4.2 General theories in the mechanism of co-culture

Several paper explain the general theories in co-culture, depicted in figure 1.10 (Scherlach & Hertweck 2009; Zarins-Tutt *et al*. 2016).

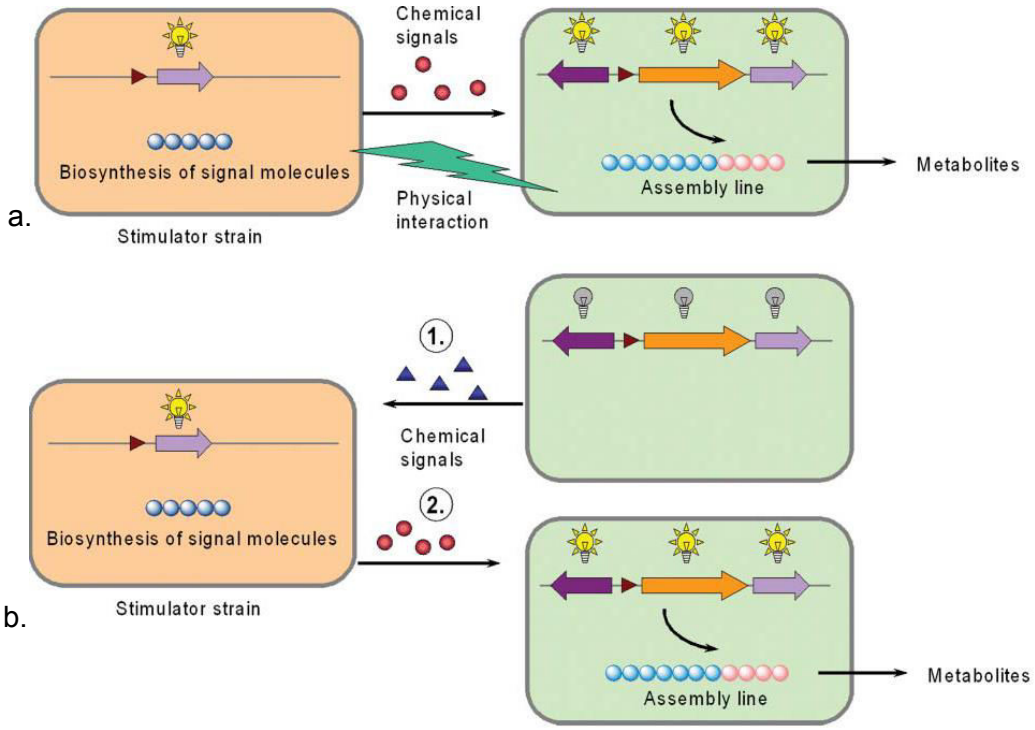


Figure 1.10 Co-culture mechanism from Schelrlach and Herweck (2009), in which (a) inducer cell activates secondary metabolite pathways by sending chemical signals or by cell to cell components or physical interaction, and (b) producer cell induce the biosynthesis of chemical signals from the inducer cell, resulting in secondary metabolite pathway activation in the producer cell.

The induction in co-culture in the activation of the pathway-specific genetic clusters can be a result of (a) cell to cell physical interaction, (b) the activation of a global regulatory system due to small chemical signals produced by the inducer strain, or an exchange between the producer and the inducer culture, in which it would result in secondary metabolism pathway in the producer culture. These points are further discussed as follows.

1.4.3 Physical contact in induction of secondary metabolites

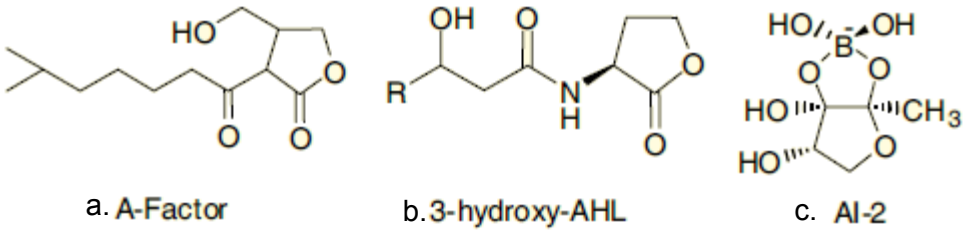
Some successful interaction between microorganisms was discovered to be induced by cell wall components. It was discovered that the co-culture between *Streptomyces lividans* TK23 and mycolic acid containing actinobacteria, *Tsukamurella pulmonis* TP-

B0596 resulted in the formation of red pigments produced by the *Streptomyces* species (Onaka *et al*. 2011). In this study, *Tsukamurella* butanol extracts were not found to be able to result in the same induction, nor was the effect seen when the cultures were separated into two compartments separated by a dialysis membrane. Thus, it was concluded that physical cell to cell interaction was required for the induction. Lastly, the induction was also not stimulated when using mycolic acid deficient *Corynebacterium glutanicum* mutants, therefore concluding that the interaction was due to the presence of mycolic acid.

This method led to the discovery of novel and unique compound. A novel butanolide, chojalactones A-C, was produced from *Streptomyces* sp. CJ-5 in co-culture with *Tsukamurella pulmonis* TP-B0596, and resulted in the enhancement of multiple other secondary metabolites (Hoshino, Wakimoto, *et al*. 2015). Arcyriaflavin E, was also isolated by co-culture of *Streptomyces* cinnamoneus NBRC 13823 and other mycolic acid containing bacteria, and from marine sources, 65 *Micromonosporaceae* was co-cultured with 5 mycolic acid bacteria, resulting in the increased production of antibiotic activity in several of those strains (Adnani *et al*. 2015; Hoshino, Zhang, *et al*. 2015).

1.4.4 Small molecules in induction of secondary metabolite

The production or exchange of small molecules or chemical signals is known to be one method of interaction in both Gram positive and Gram negative bacteria. One notable example of this is seen in the phenomenon called ‘quorum sensing’, where a chemical signal exchange between microorganisms in a community eventually resulting in the expression of a gene, forming a coordinated response within the population (Diggle, Crusz & Cámara 2007). As described in the previous papers, quorum sensing results in the many observable changes - including the production of biofilm, secondary metabolite production, sporulation, increased virulence, motility, clumping and pigmentation. Several known quorum sensing molecules are homoserine lactones (HSL), A-factors, and autoinducers, as shown in figure 1.11 (Diggle, Crusz & Cámara 2007; March & Bentley 2004).



a. A-Factor b. 3-hydroxy-AHL c. AI-2

Figure 1.11 Structure of known quorum sensing molecules as depicted in Diggle *et al*. (2007), whereas (a) A-factor, (b) 3-hydroxy-AHL, and (c) Autoinducer-2 (AI-2).

Homoserine lactones (HSL) are exclusively found in Gram negative bacteria (and is the equivalent of the A-factors from Gram positive bacteria), and operate by acting on the LuxR receptor proteins to regulate multiple genetic pathways (Cooley, Chhabra & Williams 2008; March & Bentley 2004; Williams 2007). These pathways would be responsible for increased virulence, or survivability of bacteria by producing biofilm, or antibiotic resistance (Diggle, Crusz & Cámara 2007). Endophytic actinobacteria is a source for HSL degrading enzyme, reducing the survivability of pathogenic bacteria (Chankhamhaengdecha *et al*. 2013; Park, S-Y *et al*. 2005), and can be speculated to be a result of bacterial competition.

In Actinobacteria, A-factors or butyrolactones are found to be responsible for both sporulation and secondary metabolite production (Horinouchi & Beppu 1992b). Addition of external butyrolactones or butyrolactone mimics restored pimaricin production on impaired mutant *Streptomyes natalensis npi287* (Recio *et al*. 2004), and landomycin E and streptomycin from *Streptomyces globisporus* 1912-B2 and *Streptomyces griseus* 1439, respectively (Matselyukh *et al*. 2015). A-factors are suggested to be produced by most *Streptomyces*, and responsible for the activation or repression of more than 500 genes (Higo *et al*. 2012), including daptomycin from *Streptomyces roseosporus* (Mao *et al*. 2015).

Unlike HSL and butyrolactones, autoinducer-2 (AI-2) was found in both Gram positive and Gram negative bacteria (Cooley, Chhabra & Williams 2008). AI-2 is produced in more than 500 bacterial species, and is responsible for the formation of biofilm as well as chemotaxis and motility (Pereira, Thompson & Xavier 2013; Sun, J *et al*. 2004). The activity of AI-2 is highly dependent on cell density, as high amounts of AI-2 which entered the cells via the phosphorylation and activation of multiple AI-2 ABC transporters was required for the expression of LuxR, followed by other gene expression (Pereira, Thompson & Xavier 2013). Research of AI-2 in *Streptomyces* and other actinobacteria is currently lacking, with AI-2 (in Actinomycetes) known to be produced by *Propionibacterium acnes*, *Bifidobacteria* sp. and *Mycobacterium avium*

(Geier *et al*. 2008; Polkade *et al*. 2016; Sun, Z *et al*. 2014). The relationship between AI-2 and secondary metabolism in actinobacteria is currently unexplored.

Quorum sensing manipulation has resulted in several successful production of secondary metabolites. For instance, addition of 100 nM butyrolactone I in liquid cultures of *Aspergillus terreus* has resulted in an induction of *lovB* and *lovF* genes, leading to the increased production of lovastatin (Raina *et al*. 2012). Similarly, addition of spent medium containing butyrolactone molecules was also found to result sclerotiorin from *Penicillium sclerotiorum*, although in this case, other compounds might also be responsible for this effect (Raina, Odell & Keshavarz 2010). However, this area, particularly in *Streptomyces* or uncommon actinobacteria, is vastly under-explored.

1.4.5 Conclusion

The study of actinobacteria interaction seems to be a promising research to unlock the potential of novel compound production from actinobacteria. The current state of research indicates that chemical crosstalk between species would result in an increased discovery of secondary metabolites, either by the activation of silent genes or the induction of compound production by chemical responses driven by interspecies interaction. Isolation studies conducted in this faculty has resulted in a library of more than 1500 cultures. The potential of interactivity of these cultures, in particular involving non-*Streptomyces*, has not been explored. Therefore, the aims of this project is to study the interaction between multiple actinobacteria species in the secondary metabolite production.

CHAPTER 2. PUTATIVE SPECIES IDENTIFICATION USING 16S RRNA SEQUENCING OF ACTINOBACTERIA USED IN THIS STUDY

2.1 Introduction

Actinobacteria have been a source of novel compounds, yet many more compounds remain unexpressed, requiring induction from external sources (Scherlach & Hertweck 2009; Yoon & Nodwell 2013). The aim of this research project was to screen for interactions between actinobacteria that would lead to changes in morphology, sporulation, pigment production, and/or antibiotic production. There was a selection of *Streptomyces* and non-*Streptomyces* isolated in this laboratory that consisted of more than 500 endophytic actinobacteria from native Australian trees, which were then identified based on morphology, T-RFLP, or 16S rRNA gene sequencing (Kaewkla, O & Franco 2012).

The initial aim was to induce metabolite production in non-streptomycetes as this group contained a higher proportion of non-antibiotic producers among the cultures tested. Researchers has already began to look into rare or uncommon actinobacteria (and therefore non-streptomycetes) for the production of novel compounds (Lazzarini *et al*. 2000). However, the research later shifted to also include *Streptomyces* species that were isolated in later weeks (the late emerging *Streptomyces*) from native Australian plants (Kaewkla, O & Franco 2012), and several *Streptomyces* isolated from wheat root and soils. From those, a randomly selected number of non-*Streptomyces* was used for the screening study, with the results shown in later chapters.

In this section the morphological characteristics of several actinobacteria that were found to be of interest is described. Furthermore, identification of the actinobacteria (both non-*Streptomyces* and *Streptomyces*) based on comparison of 16S rRNA gene sequence was conducted. This allows the identification of cultures of interest, as well as the detection of potential novel species.

2.2 Method

2.2.1 Spore suspension

Cultures were maintained using stocks obtained from glycerol stocks stored at -80°C prepared previously (Kaewkla, O & Franco 2012). The cultures were inoculated onto different media (HPDA, MS, and/or ISP-2) and incubated at 27°C for 7 – 14 days, until good sporulation was achieved. A spore suspension was made by first inoculating working cultures onto plates containing ISP-2 medium, incubated in 27°C for 7 days, until a good sporulation was achieved. Using a sterile loop, the spores were gently scraped off the surface of the plates and inserted into a sterile cryotube containing 1 ml sterile 50% glycerol, and stored in -20°C until further use.

2.2.2 Comparison of morphology in different medium

From the prepared glycerol stocks, 50 µl was inoculated on to ISP-2, ISP-3, and ISP-5 media, and spread on the plate in a grid pattern. These plates were then incubated at 27°C for 28 days. The vegetative and aerial mycelium colour noted, as well as the presence of spore colour and diffusible pigment for each culture in the different medium.

2.2.3 DNA extraction

For DNA extraction, the cultures were first grown on medium which would best support good culture growth, layered with sterile cellophane, and incubated until good growth is observed. In this case, cultures were inoculated on ISP-2 with cellophane and incubated at 27°C for 7 – 14 days. DNA extraction was conducted using a CTAB method. Up to two loops of mycelia were transferred into a loop containing 600 µl of TRIS-EDTA and 10 µl of lysozyme, vortexed and spun using a mini centrifuge. These were then incubated at 37°C for 1 hour before adding 10 µl of Proteinase K and 32.5 µl of SDS. Samples were mixed gently and incubated at 55°C for 1 hour. Preheated in the same temperature, 65 µl of CTAB/NaCl mixture (10 g CTAB, 4.1 g NaCl in 100 ml of RO water) was transferred into the tube, and added with 100 µl of 5 M NaCl, incubated at 55°C for 10 minutes. DNA extraction was achieved by adding 600 µl phenol:chloroform:isoamyl alcohol (25:24:1), left for 30 minutes with intermittent shaking every 10 minutes, followed by two time chloroform (500 µl) wash for 15 minutes. Between each wash, the samples were centrifuged for 12,000 g for 15 minutes, and the supernatant was transferred to a new tube. After the final wash, 20 µl of RNAse was added and incubated for 60 minutes. DNA precipitation was

accomplished by adding 50 µl 3 M Na Acetate and 1 ml of pure ethanol. The samples were left at -20°C overnight, and the supernatant removed by centrifugation at 16,000 g for 15 minutes. The pellet was washed twice with 70% ethanol and dried by placing in the heating block at 55°C until dry. Finally, the DNA was eluted with 50 µl nuclease free water.

2.2.4 16S rRNA gene sequencing

Samples showing good DNA purity (λ 260 nm / 280 nm within 1.6 – 2.0) and concentration (>30 ng/µl DNA sample) were amplified for 16S rRNA gene for identification purposes. PCR amplification of the gene was using a universal 16S rRNA primer. The first portion (R1) of the gene was amplified using primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 765R (5'-GTAGCGGTGAAATGCGTAGA-3'), and the second portion (R2) was amplified using primers 704F (5'-CTGTTTGCTCCCCACGCTTTC-3') and 1492R (5'-CACGGATCCTACGGGTACCTTGTTACGACTT-3'), with the estimated band size to be 739 bp and 789 bp, respectively. For sequencing, a 50 µl PCR reaction was prepared, with the following composition: 10x Taq buffer (1x Taq buffer: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), dNTP (5 mM), Taq polymerase (5 units/µl), water to 37 µl, and DNA template 2 µl. The PCR conditions used for the amplification was 94°C initial denaturation for 2 minutes, followed by 40 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, and an final extension of 72°C for 10 minutes. For sequencing, the PCR product was purified from its remaining PCR reagents using an Ultra-clean® PCR product cleaning kit (Mobio Laboratories, Inc, CA, USA).

2.2.5 Phylogenetic analysis

DNA sequence quality was checked using CHROMAS v.2.5.0 (www.technelysium.com.au/), in which peaks with low quality (unclear or double) were deleted using the software for the forward and reverse primers. Both sequence was then combined by reverse-complementing the reverse primer sequence using the same program, and saved in a FASTA format. The 16S DNA gene sequence was compared online through the EZTAXON (www.ezbiocloud.net/eztaxon) database, and compared to the closest annotated species. The phylogenetic tree of several species was made by firstly making multiple sequence alignment (MSA) using Mega

5.0 software. The phylogenetic relationship of each species was analysed with a neighbour-joining algorithm bootstrap with 1000 replicates using the same software.

2.3 Results and Discussion

2.3.1 *Pseudonocardia* species show two or more different variants

Actinobacteria grown on different medium would be expected to have different morphological characteristics on each medium (Shirling & Gottlieb 1966). These differences can be used to classify actinomycetes within genera. As our study had a selected a large number of *Pseudonocardia* species, the data obtained from this genus would be more meaningful. For the *Pseudonocardia* species used in this study, we noted four main types based on the morphological characteristics on ISP-2, ISP-3 and ISP-5, as shown in table 2.1.

Based on their morphology, we conclude that *Pseudonocardia sp*. EUM 208, EUM 212, EUM 224, EUM 227, CAP 122, PIP 161, and PIP 197 were closely related to each other or belong to the same clade or even species, while *Pseudonocardia* sp. CAP 111, CAP 47 and CAP 60, showed differences when subcultured on ISP-5 medium. We therefore confirmed this using 16S rRNA sequencing.

Table 2.1 Morphology of several *Pseudonocardia* species used in this study

Variant	Culture Code	ISP-2					ISP-3					ISP-5				
		Aerial hyphae colour	Veg. hyphae colour	Growth	Sporulation	Spore Colour	Aerial hyphae colour	Veg. hyphae colour	Growth	Sporulation	Spore Colour	Aerial hyphae colour	Veg. hyphae colour	Growth	Sporulation	Spore Colour
I	EUM 208	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	EUM 212	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	EUM 224	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	EUM 227	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	CAP 122	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	PIP 161	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
	PIP 197	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	-	-
II	CAP 47	Br	Br	+	+	Wh	Br	Br	+	+	Wh	Br	Br	+	+	Wh
III	CAP 60	Yw	Yw	+	+	Yw	Br	Br	+	+	Wh	Yw	Yw	±	-	-
IV	CAP 111	Yw	Yw	+	+	Wh	Br	Br	±	±	Wh	Br	Br	±	-	-

†Br = Brown, Yw = Yellow, Wh = White; Growth and sporulation, - = no growth/sporulation, ± little growth/sporulation, + good growth/sporulation

‡All observations were made after 28 days of incubation in 27°C. In ISP-2 medium, actinomycetes were subcultured on top of cellophane, as the plates were further used for DNA isolation

2.3.2 Putative species identification using 16S rRNA gene sequencing

2.3.2.1 Pseudonocardia species

DNA isolation and 16S rRNA gene sequencing was conducted on Pseudonocardia species with varying success. The DNA from *Pseudonocardia* species variant I and IV was successfully sequenced, however, after multiple attempts, sufficient DNA concentration and purity for variant II and III (CAP 47 and CAP 60) was not achieved. The identification of *Pseudonocardia* species sequenced was summarized in the table 2.2. Most of the *Pseudonocardia* species sequenced were found to be closely related to *Pseudonocardia carboxydivorans* Y8(T), which was the closest type strain, as denoted by the (T) symbol. A phylogenetic tree was then constructed using *Amycolatopsis alba* DSM 44262(T) species as an outlier, as shown in figure 2.1.

Table 2.2 *Pseudonocardia* species identification based on 16S rRNA gene sequences

Culture	Closest Similarity	Accession	%	Diff/Total nucleotide†
CAP 122	*Pseudonocardia carboxydivorans* Y8(T)	EF114314	100.0	0/1341
EUM 208				0/1331
EUM 212				0/1341
EUM 224				0/1331
EUM 227				0/1333
PIP 161				0/1335
PIP 197				0/1326
CAP 111	*Pseudonocardia xishanensis* YIM 63638(T)	FJ817397	99.55	6/1325

†Number of different nucleotides from total nucleotides

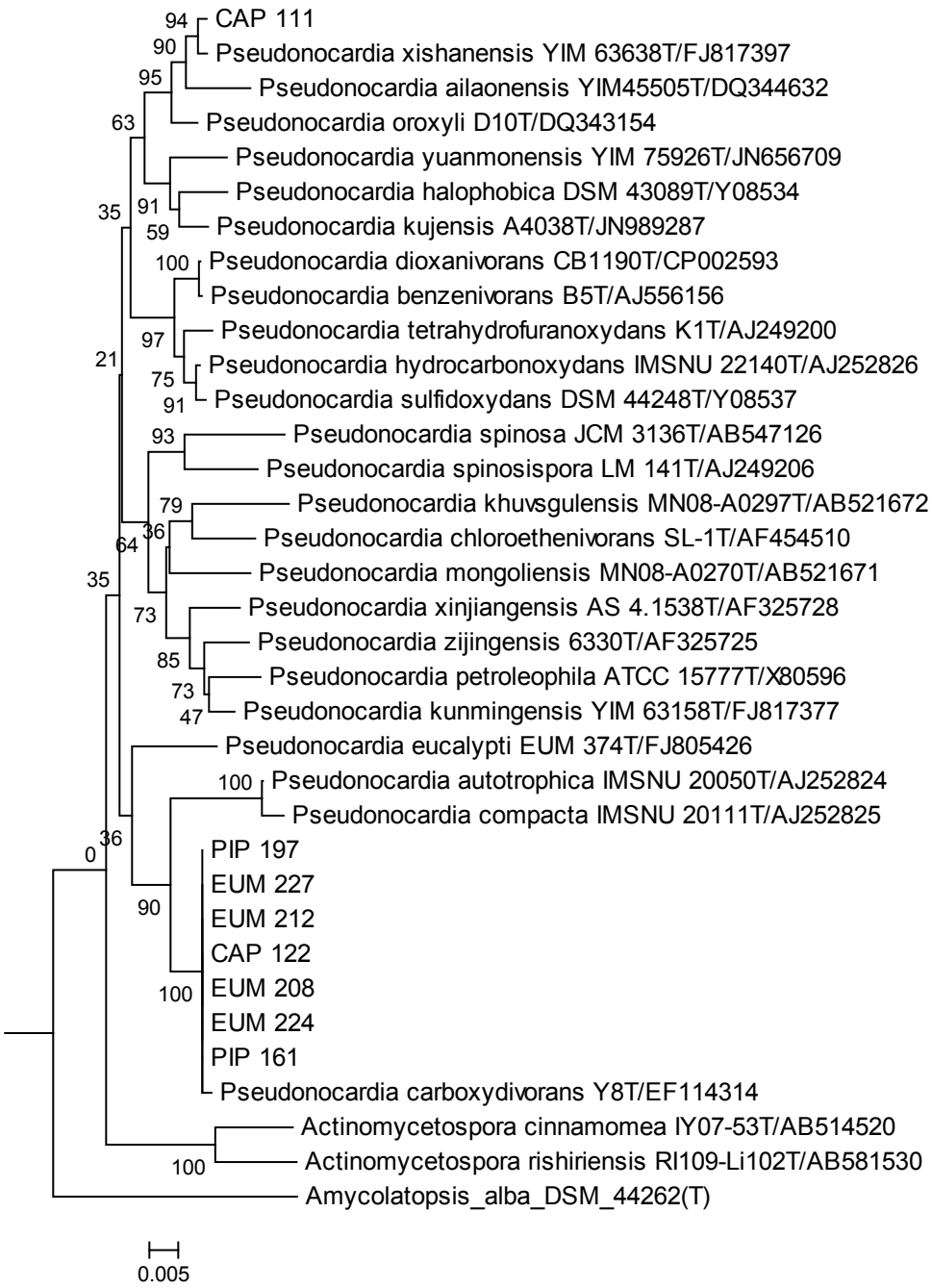


Figure 2.1 Neighbour-joining tree showing the relationship between *Pseudonocardia* strains used in this study and related species of the genus *Pseudonocardia* species using Amycolatopsis alba DSM 44262(T) as the outlier. Bootstrap percentages based on 1000 replicates. Bar, 0.005 substitutions per nucleotide positions.

2.3.2.2 Streptomyces species

Several *Streptomyces* species were morphologically similar with each other when subcultured in ISP-2 medium. EUM 356 and EUM 359 showed similar 'bald-like' appearance early in the development, followed by the formation of dark-brown coloured aerial hyphae and white sporulation. CAP 168 was morphologically similar with EUC 63, in which both has a pink-red hyphae colour with white spores. Of those

that were isolated from soil samples, SC 78 and SC 36 had similar characteristics, having cream coloured vegetative hyphae, red violet coloured aerial hyphae and late forming dark grey spores as well as blue or purple pigment formation in ISP-2. These cultures were identified using 16S rRNA gene sequencing followed by a BLAST analysis comparing it to other type strains in the EZTAXON database, as shown in table 2.3.

Table 2.3 *Streptomyces* species identification based on 16S rRNA gene sequences

Culture	Closest Similarity	Accession	%	Diff/Total nucleotide†
EUM 356	*Streptomyces ederensis* NBRC 15410	AB184658	99.70	4/1326
EUM 359	*Streptomyces cellulosae* NBRC 13027	AB184265	100	0/1341
CAP 168	*Streptomyces galilaeus* JCM 4757	AB045878	100	0/1328
EUC 63	*Streptomyces canus* NRRL B-1989	AY999775	99.04	13/1351
SC 78	*Streptomyces rubrogriseus* LMG 20318	AJ781373	100	0/1327
SC 36	*Streptomyces coelescens* DSM 40421	AF503496	100	0/1327
PIP 201	*Streptomyces badius* NRRL B-2567	AY999783	100	0/1339
PIP 156	*Streptomyces olivaceus* NRRL B-3009	JOFH01000101	99.93	1/1338
CAP 288	*Streptomyces anulatus* NRRL B-2000	DQ026637	100	0/1337
CAP 103	*Streptomyces ambofaciens* ATCC 23877	M27245	99.70	4/1336

†Number of different nucleotides from total nucleotides

Based on the table above, most *Streptomyces* were identified as having a 100% sequence similarity with a type species, except for *Streptomyces* sp. EUC 63, *Streptomyces* sp. EUM 356, and *Streptomyces* sp. CAP 103. Therefore, a phylogenetic tree was made with these species, as shown in figure 2.2, 2.3, and 2.4, respectively.

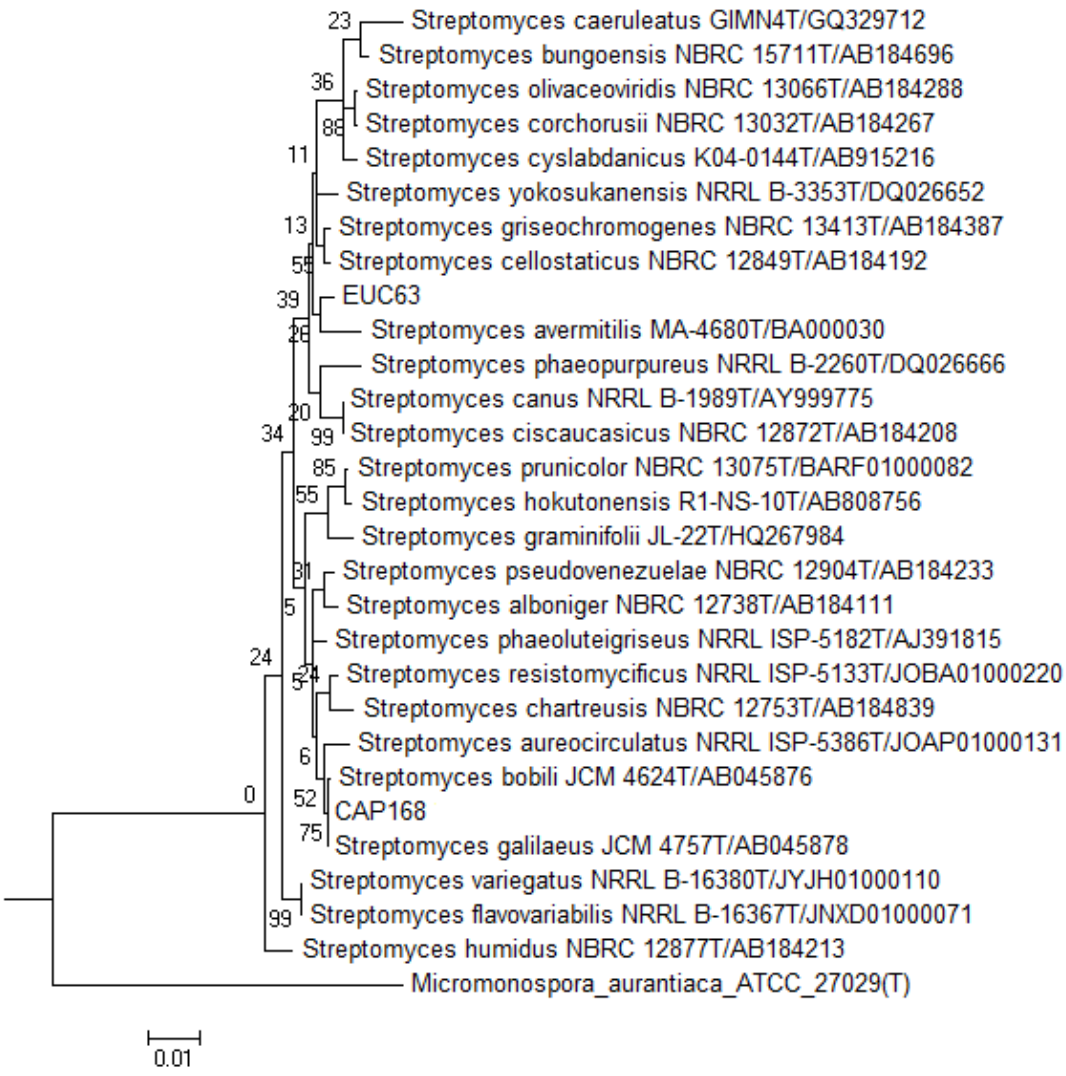


Figure 2.2 Neighbour-joining tree showing the relationship between *Streptomyces* sp. EUC 63 and *Streptomyces* sp. CAP 168 used in this study and related species of the genus *Streptomyces* species using *Micromonospora* aurantiaca ATCC 27029 as the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.01 substitutions per nucleotide positions.

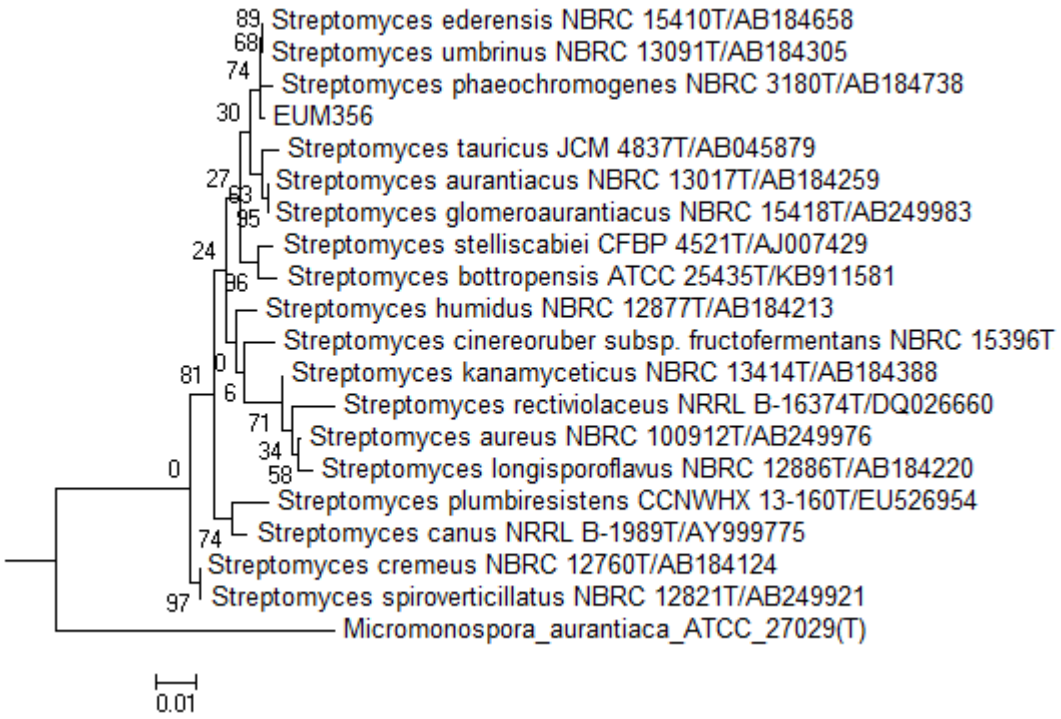


Figure 2.3 Neighbour-joining tree showing the relationship between *Streptomyces* sp. EUM 356 used in this study and related species of the genus *Streptomyces* species using *Micromonospora* aurantiaca ATCC 27029 as the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.01 substitutions per nucleotide positions.

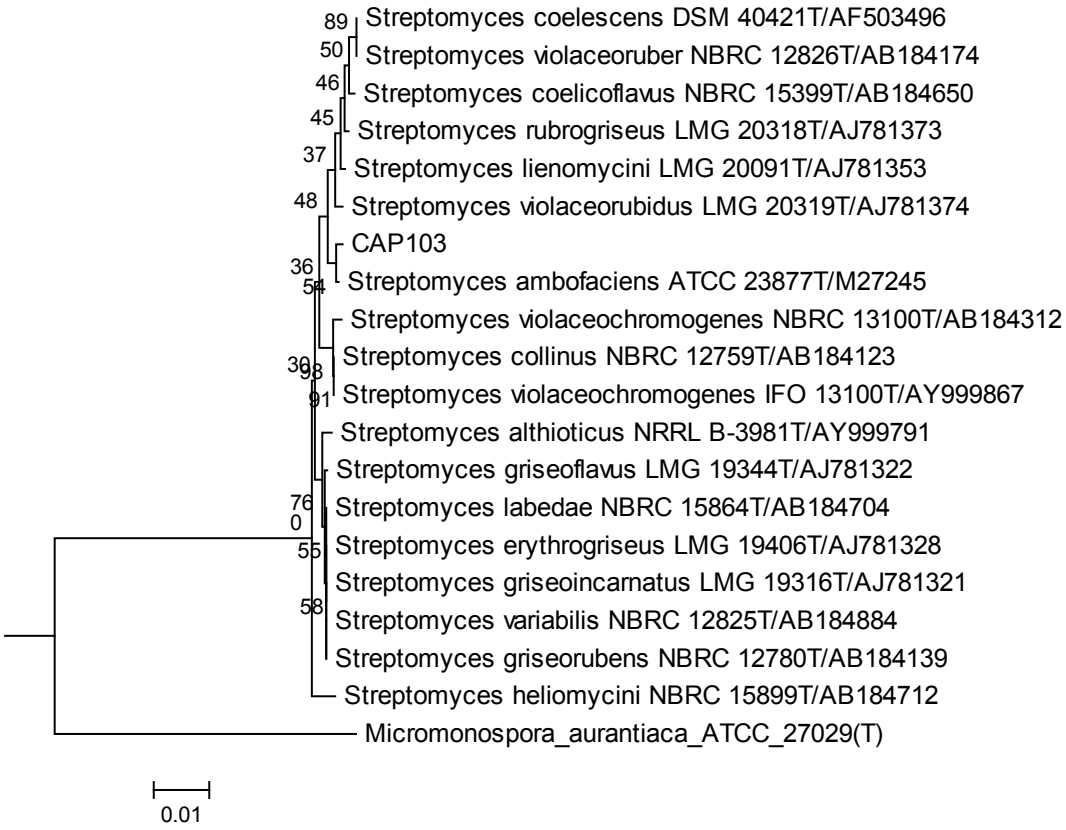


Figure 2.4 Neighbour-joining tree showing the relationship between *Streptomyces* sp. CAP 103 used in this study and related species of the genus *Streptomyces* species using *Micromonospora* aurantiaca ATCC 27029 as the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.01 substitutions per nucleotide positions.

2.3.2.3 Other species

2.3.2.3.1 *Amycolatopsis* sp. PIP 207

PIP 207 (sequence length 1336 bp, 92.9% completeness) had closest similarity with *Amycolatopsis keratiniphila* subsp. keratiniphila DSM 44409 and *Amycolatopsis lurida* DSM 43134 at 99.6257% and 99.6251% (Different nucleotides/total nucleotides was 5/1336 and 5/1334, respectively), and *Amycolatopsis keratiniphila* subsp. *nogabecina* DSM 44586 at 99.40% (8/1335), which was supported with phylogenetic tree analysis, as shown in figure 2.5. PIP 207 media showed yellow to brown substrate mycelia and aerial mycelia with white spores and dry and flaky colonies.

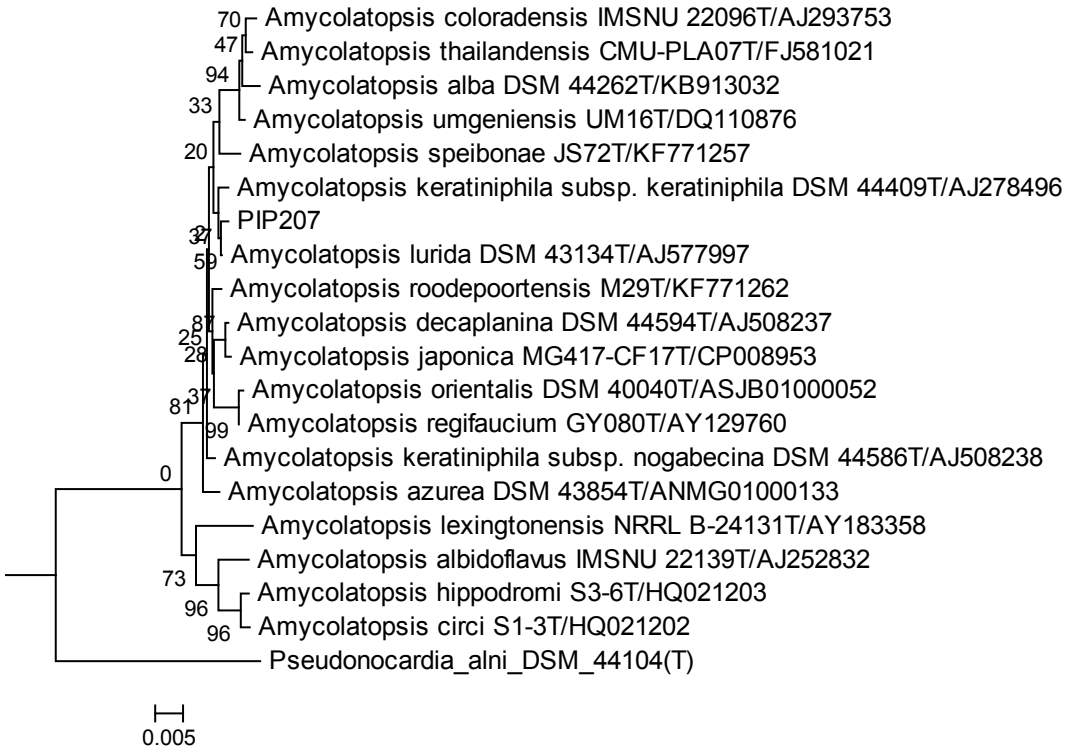


Figure 2.5 Neighbour-joining tree showing the relationship between Amycolatopsis sp. PIP 207 used in this study and related species of the genus Amycolatopsis species using *Pseudonocardia* alni DSM 44104 as the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.01 substitutions per nucleotide positions

2.3.2.3.2 *Nocardia* sp. CAP 40

CAP 40 (sequence length 1308 bp, 90.7% completeness) had closest similarity with *Nocardia soli* DSM 44488 and *Nocardia cummidelens* R89 at 99.39% similarity (8/1307 for both species). Based on its phylogenetic tree (as shown in figure 2.6), CAP 40 was most similar to *Nocardia fluminea* S1T/AF277204 (99.23%).

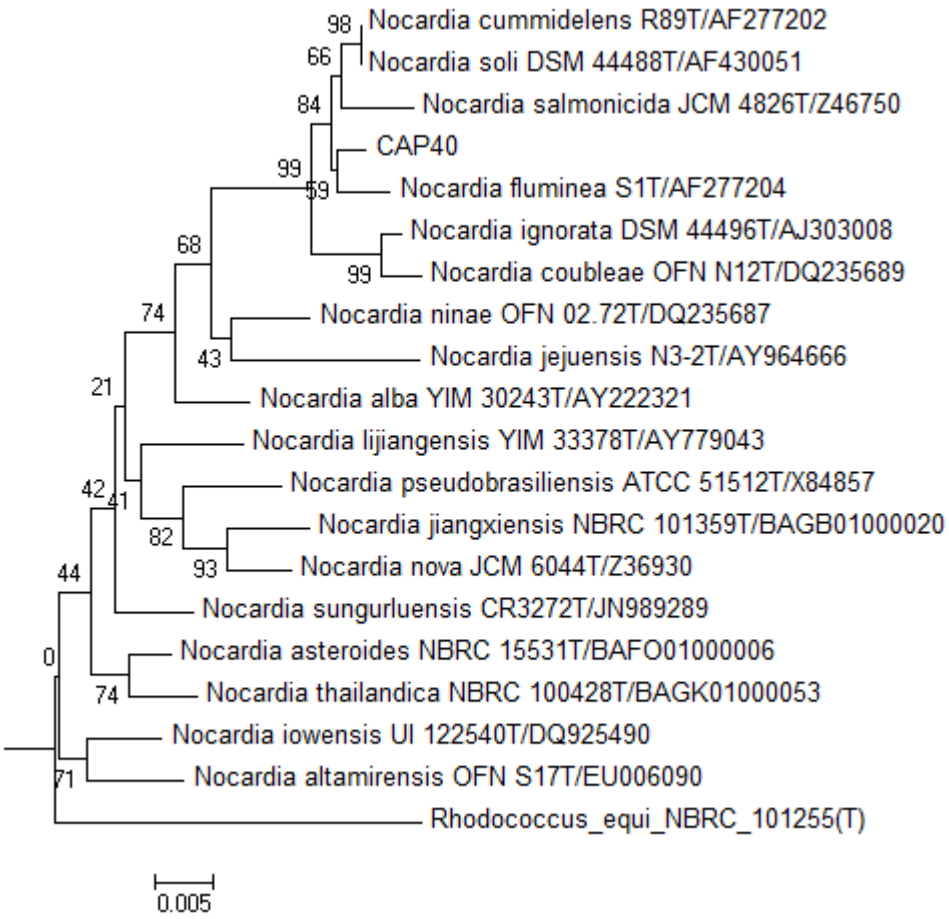


Figure 2.6 Neighbour-joining tree showing the relationship between Nocardia sp. CAP 40 used in this study and related species of the genus Nocardia species using Rhodococcus equi NBRC 101255 the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.005 substitutions per nucleotide positions.

2.3.2.3.3 *Kribbella* species

PIP 158 (sequence length 1333 bp, 92.4% completeness) had closest similarity with *Kribbella albertanoniae* BC640, *Kribbella hippodromi* S1.4 at 99.25%, and *Kribbella sandramycini* ATCC 39419 at 99.17%. Similarly, PIP 166 (sequence length 1324 bp, 91.8% completeness) was have closest similarity with *Kribbella hippodromi* S1.4 at 99.32%, followed with *Kribbela albertanoniae* BC640 and *Kribbella sandramycini* ATCC 39419 at 99.24%. The phylogenetic tree was shown in figure 2.7. It has recently been accepted as a new species of *Kribbella, Kribbella pittospori* (Kaewkla, O- & Franco 2016).

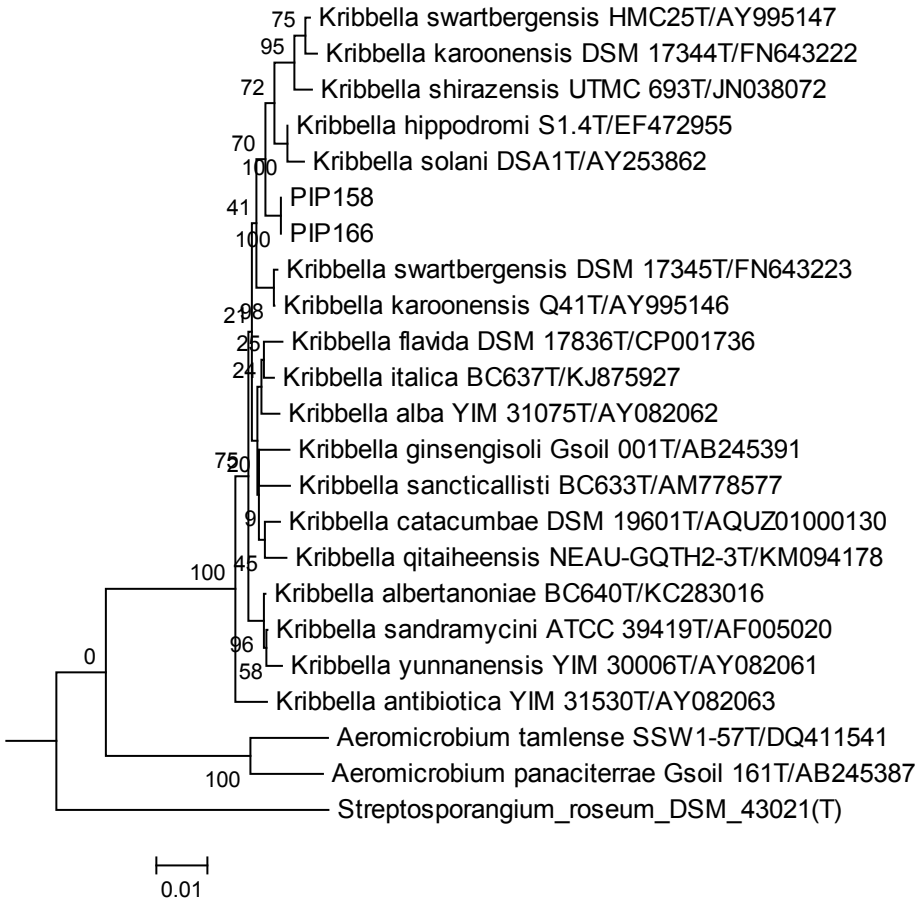


Figure 2.7 Neighbour-joining tree showing the relationship between *Kribbella* sp. PIP 158 and *Kribbella* sp. PIP 166 used in this study and related species of the genus *Kribbella* species using Streptosporangium roseum DSM 43021 the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.01 substitutions per nucleotide positions

2.3.2.3.4 *Micromonospora* species

CAP 181 (sequence length 1328 bp, 92.4% completeness) had closest similarity with *Micromonospora coerulea* DSM 43143 at 99.62%, *Micromonospora chaiyaphumensis* MC5-1 at 98.95% and *Micromonospora auratinigra* TT1-11 at 98.87%. EUC 38 (sequence length 1327 bp, 92.3% completeness) was identified to have closest similarity with *Micromonospora aurantiaca* ATCC 27029 at 100%, *Micromonospora sediminicola* SH2-13 at 99.7%, and *Micromonospora marina* JSM1-1 at 99.62%. The constructed phylogenetic tree was shown in figure 2.8.

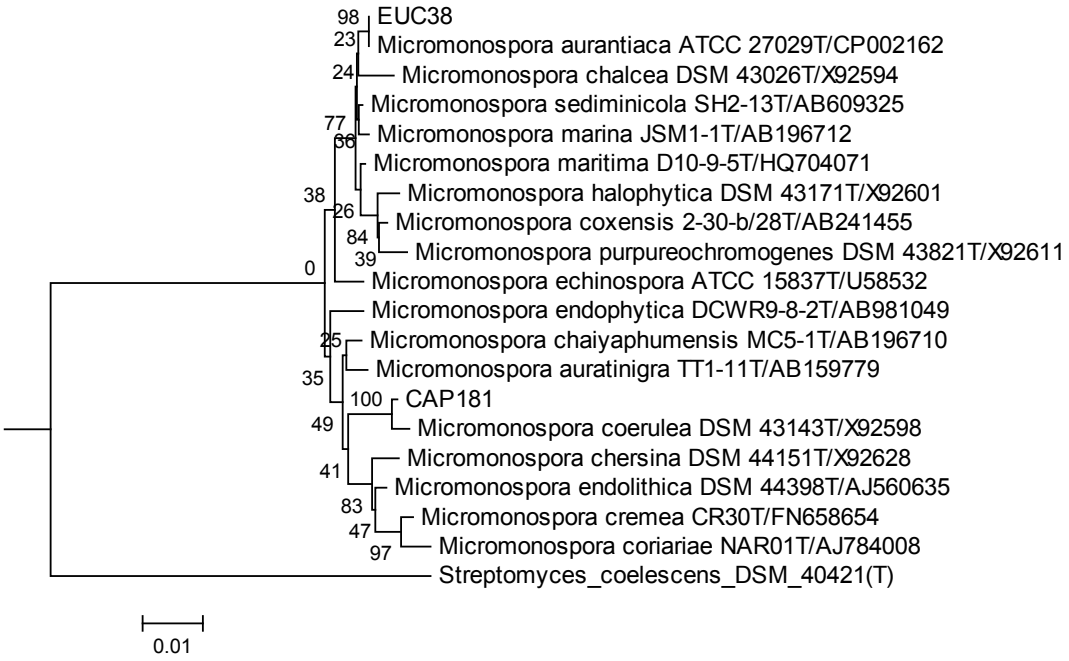


Figure 2.8 Neighbour-joining tree showing the relationship between *Micromonospora* sp. CAP 181 and *Micromonospora* sp. EUC 38 used in this study and related species of the genus *Micromonospora* species using *Streptomyces* coelescens DSM 40421 as the outlier group. Bootstrap percentages based on 1000 replicates. Bar, 0.005 substitutions per nucleotide positions

2.3.3 Potential symbiosis capability of *Pseudonocardia*

We discovered that a majority of the *Pseudonocardia* species used in this study was closely related to *Pseudonocardia carboxydivorans* Y8. *Pseudonocardia carboxydivorans* Y8 was isolated from a roadside soil samples in Seoul, Korea, and published in 2008. *Pseudonocardia carboxydivorans* Y8 was described as follows:

"Aerobic, Gram-positive, non-motile actinomycete. Forms brown substrate mycelium and white aerial mycelium. The aerial mycelium fragments into rod-shaped spores. Grows optimally at 25 °C and tolerates up to 4 % (w/v) NaCl. Positive for catalase and urease, but negative for oxidase. Utilizes N-acetyl- d-glucosamine, inulin and mannan but not cellobiose, d-fructose, d-galactose, myo-inositol, maltose, d-mannitol, d-mannose, melezitose, raffinose, l-rhamnose, d-ribose, sorbitol, sucrose, trehalose, xylitol or d-xylose as sole carbon sources. meso-Diaminopimelic acid is present in the cell wall and MK-9 is the major menaquinone. Predominant fatty acids are 16:0 iso and 16:1 iso. The G+C content of the type strain is 77 mol%." (Park, SW *et al*. 2008)

Pseudonocardia isolation studies have showed that these species were ubiquitous in nature, being found in soil samples (Park, SW *et al*. 2008; Prabahar *et al*. 2004), as endophytes (Kaewkla, O & Franco 2012; Zhao *et al*. 2012), from marine sources (Tian *et al*. 2013; Zhang, D-F *et al*. 2014), and, most famously, from attine ants (Andersen, S. B. *et al*. 2013; Meirelles *et al*. 2014). Furthermore, although it is common for microorganisms in general to interact with other species in its microbiome, attine ants studies have indicated that *Pseudonocardia* plays an integral role in the attine ant survival, often by production of anti-fungal (Meirelles *et al*. 2014) and antibacterial (Jafari *et al*. 2014) or anti-parasitic agents (Poulsen, Michael *et al*. 2010). Other findings show that multiple antifungal agents were also found to be produced by other attine ant-associated *Streptomyces* species (Barke, Jörg *et al*. 2010; Haeder *et al*. 2009; Seipke *et al*. 2011), suggesting the possibility of mutualistic interaction between species determined by a central *Pseudonocardia* species (Andersen, S. B. *et al*. 2013).

Researchers have suggested that *Pseudonocardia* species co-evolved or co-adapted with its attine ant hosts (Andersen, S. B. *et al*. 2013; Andersen, Sandra B. *et al*. 2015). While the microbiome in the ant cuticle may change, some findings suggested that a dominant single *Pseudonocardia* species was maintained (Barke, Jörg *et al*. 2010; Cafaro *et al*. 2011), resulting in a genetic stability and uniformity among *Pseudonocardia* species (Poulsen, M. *et al*. 2005). Concurring with this, phylogenetic analysis reveals a pattern of ant-*Pseudonocardia* association, whereas *Pseudonocardia* of a different clades would be more likely to be found with specific attine ant species (Cafaro *et al*. 2011). In this case, clade 3 (out of a total 10 clades) was found to have the highest number of *Pseudonocardia* isolated from attine ants and was previously thought to be mostly attine ant specific – however, recently, multiple environmental counterparts has been discovered, including *Pseudonocardia carboxydivorans* Y8 (Mueller *et al*. 2010).

Mueller *et al*. (2010) discovered that *Pseudonocardia* was also found in association in the environment with a wide diversity of other species, “including plants, marine sponges, anemones and mammals”. The capability of *Pseudonocardia* to interact with other species beyond fungal ants communities were shown in recent finding, where *Pseudonocardia sp.* YIM 63111 was found to induce production of artemisinin in *Artemisia annua* (Li, J *et al*. 2012). Thus, concurring with Mueller *et al*. (2010), *Pseudonocardia* may be equipped for symbiotic interactions with other species. Therefore, we attempt to screen *Pseudonocardia,* in particular, and other non-*Streptomyces* in interaction with other species.

2.3.4 Conclusion

We identified several actinomycetes which were found to be of interest. *Pseudonocardia* species used in this study can be clustered into two or more groups based on its morphology, and 16S rRNA sequence analysis reveals that a majority of the samples tested were *Pseudonocardia carboxydivorans* Y8. Other non-*Streptomyces* sequenced reveal several species closely related to *Amycolatopsis keratiniphila* subsp. keratiniphila DSM 44409, *Nocardia soli* DSM 44488, *Micromonospora coerulea* DSM 43143, *Micromonospora aurantiaca* ATCC 27029 and *Kribbella pittospori* DSM23717. *Streptomyces* species showed similarity to various species despite similar morphology, including *Streptomyces ederensis* NBRC 15410, *Streptomyces cellulosae* NBRC 13027, *Streptomyces galilaeus* JCM 4757, *Streptomyces anulatus* NRRL B-2000, *Streptomyces olivaceus* NRRL B-3009, *Streptomyces* badius NRRL B-2567, *Streptomyces ambofaciens* ATCC 23877, and soil isolated *Streptomyces* species, including *Streptomyces rubrogriseus* LMG 20318 and *Streptomyces coelescens* DSM 40421.

Based on the literature, we postulate that the interaction between *Pseudonocardia* and other species has potential. This will be reported in the following chapters.

CHAPTER 3. SCREENING FOR INTERACTION BETWEEN VARIOUS *STREPTOMYCES* AND UNCOMMON ACTINOBACTERIA

3.1 Introduction

A number of active compounds applicable in many fields of biology and medicine are discovered from actinobacteria. *Streptomyces*, which is the largest genus, is also the most studied, and has been a source of more than 2400 unique bioactive compounds from more than 1900 species (Lucas *et al*. 2013), indicating 1 or 2 compounds per *Streptomyces* species. Genomic analysis of the *Streptomyces coelicolor* alone, however, shows 20 – 30 potential metabolic pathways (Bentley, Chater, Cerdeño-Tárraga, *et al*. 2002; Nett, Ikeda & Moore 2009). This indicates that pathways to synthesize many compounds are left unexpressed when using common laboratory media and culture techniques (Nett, Ikeda & Moore 2009), and some researchers even went so far to suggest a potential of 100,000 compounds to be produced by *Streptomyces* (Watve *et al*. 2001). The few examples to manipulate the production of these 'cryptic compounds' have met with varying success, and include ribosome engineering (Ochi & Hosaka 2013), resistance gene mutation (Tanaka, Y *et al*. 2013), use of synthetic inducers or chemical probes (Ahmed *et al*. 2013; Yoon & Nodwell 2013), and the addition of rare earth elements (Tanaka, Y, Hosaka & Ochi 2010).

A different approach used by other researchers to elicit natural products is by exploiting cell-to-cell communication which is likely to occur in the large diverse microbial communities. In natural product discovery research, different compounds are produced in a mixture of different bacteria. The earliest suggested example of co-culture to produce a bioactive compound is the discovery of Penicillin in 1929 (Abdelmohsen *et al*. 2015). Since then, co-culture was utilized or implicated in different research throughout the years, for example, in *Clostridium* toxin production in 1950s (Sakaguchi & Tohyama 1955), lactic acid stimulation in the 1970s (Nath & Wagner 1973), the production of ethanol in 1980s (Kurosawa, Nomura & Tanaka 1989; Ng, Ben-Bassat & Zeikus 1981; Tanaka, H, Kurosawa & Murakami 1986), and others.

Research in *Streptomyces* and other actinobacteria probably first advanced the advent of advanced molecular techniques in the late 1990s, when regulatory and communication pathways of secondary metabolism were discovered (Horinouchi & Beppu 1992a), pioneering a path to understand the phenomenon observed in the last

40 years. Recent evidence of these interactions in actinobacteria is made available by metabolic analysis using advanced chemical techniques, suggesting that an array, up to 629 chemical compounds were produced during co-culture, with specific compounds produced in different interactions (Traxler *et al*. 2013b; Watrous *et al*. 2013).

While it is clear that research in understanding the interaction in actinobacteria is still in its infancy, fewer studies have attempted to branch out and attempt to discover the interaction among uncommon actinobacteria. Recent studies report an interesting and successful interaction between *Actinokineospora* and *Nocardiopsis* (Dashti *et al*. 2014) and between *Rhodococcus* sp. and *Micromonospora* sp., *Solwaraspora* sp., and *Verrucosispora* sp. (Adnani *et al*. 2015), mostly from marine sources.

We currently have a culture library consisting of around 1,500 endophytic actinobacteria isolated from previous studies (Kaewkla, O & Franco 2012), consisting of a large number of uncommon actinobacteria as well as early and late (after 12 weeks in the isolation process) emerging *Streptomyces* species. Among these cultures are a several *Pseudonocardia* species, which, with other microorganisms, are also often found to form large communities and known to produce anti-fungal compounds on the surface of attine ants (Sen *et al*. 2009). However, to this date, the potential for *Pseudonocardia* in co-culture studies are largely unexplored.

Therefore, in this study, we attempt to screen for various interactions among several different uncommon actinobacteria and Streptomycetes. As described in previous chapter, we attempt to observe signs of interactions such as changes in sporulation, growth, and antibiotic or pigment production (Ueda *et al*. 2000). The mechanism and metabolic changes of the interaction would be further explored in other chapters.

3.2 Method

3.2.1 Glycerol stock and spore suspension of cultures

Original cultures from Kaewkla and Franco (2012) were stored in 50% glycerol stocks. The cultures used in the following experiments were 23 non-*Streptomyces*, consisting of *Pseudonocardia* sp., *Kribbella* sp., *Micromonospora* sp*., Nocardia* sp., and *Amycolatopsis* sp*.,* and 20 *Streptomyces* species which were obtained after 12 – 16 weeks in the isolation period. Cultures were maintained using stocks obtained from glycerol stocks stored in -80°C. A working plate was first made by subculturing these culture in ISP-2 and incubating at 27°C for 7 – 14 days. If pure cultures are visible,

single spores were then collected and transferred in to a new glycerol stock and stored in -20°C.

For experiments, a spore suspension was used. This was made by first inoculating and spreading 50 µl of cultures from prepared glycerol stocks on to ISP-2 plates using a sterile metallic loop. The plates were incubated for 7 days in 27°C, or until good sporulation was observed. Using a sterile loop, the surface of the plates were gently scraped off from the surface of the plates and inserted in to a sterile cryotube containing 1 ml of sterile 50% glycerol, and stored in -20°C until further use.

3.2.2 Preliminary screening for interaction

The preliminary screening for interaction between actinobacteria was first conducted among 24 actinobacteria, consisting of 12 .*Pseudonocardia* sp. 4 *Micromonospora* sp., 3 *Kribbella* sp., 1 *Amycolatopsis* sp., 1 *Nonomurae*, 2 *Nocardia* sp., and 1 *Streptomyces* species. The screening was conducted on ISP-2 as a production medium, and interaction was defined as changes of morphology, pigmentation, sporulation and antibiotic production in co-culture which differed from monocultures.

First, the order of the cultures were randomized and given a numerical code of 1 to 24. Screening methodology was modified from Seyedsayamdost *et al*. (2012), and other researchers. Instead of two cultures, preliminary screening was conducted on two group of two cultures (e.g., 1, 2 and 3, 4), forming one 'cluster' (e.g., 1, 2, 3, and 4). To ensure that each culture would interact with another culture at least once, the second group of cultures were alternated (e.g., 1, 2, 3, 4, and 1, 2, 5, 6, and so on). This was conducted for all 24 cultures in the preliminary screening.

A 'cluster' was made by spotting 10 µl from prepared spore suspension using a cut tip in an ISP-2 plate, whereas a 0.3 to 0.5 cm gap was left between each culture. Each plate consisted of 6 clusters, placed in the edges of the plates at a 60° angle with each other. A control plate consisting of 6 spots of cultures placed at similar areas as treatment. The plates were incubated at 27°C to up to 10 days, and observed at day 3, 5, 7 and 10 for interaction. These were done in triplicates, where a change in one out of three interactions were considered a positive result and rechecked in the secondary screening (as described below).

Increase or decrease of growth was measured based on whether vegetative mycelia were present or absent in the co-culture compared to monoculture, or if a clear zone of inhibition was observed. Increase or decrease in sporulation was measured based

on whether spores were present or absent in the co-culture compared to monoculture, and also if a clear difference was observed in the distribution of spores in the cell surface. Lastly, increase or decrease of pigment formation was measured based on the presence or absence of the pigments in the media, whether the pigments were observed in the surrounding cultures, or a clear difference in the hue of the pigment colour were observed.

3.2.3 Secondary screening for interaction

Secondary screening was based on Seyedsayamdost *et al* (2012) and Traxler *et al* (2012). The samples used in the secondary screening was based on the results of preliminary screening, with similar methodology, except that a one on one co-culture was conducted. In this case, an ISP-2 plate was divided into 6 regions, in which each co-culture was placed in the edges of the plates at a 60° angle with each other. From the spore suspension, 10 µl were spotted using a cut tip, with a 0.3 – 0.5 cm distance with each culture in an interaction. A control was prepared consisting of 6 monocultures in an ISP-2 plate of all cultures which were passed through this screening. Each plate was prepared in triplicates. The plates were then incubated at 27°C for 10 days, with observation every two days. Similar marking criteria was used, as stated in 3.2.2.

3.2.4 *Pseudonocardia* sp. and non-*Streptomyces* with a two to one interaction testing

The effect of several *Pseudonocardia* sp. in its interaction with non-*Streptomyces* were also tested in a method that was similar to secondary screening with some modification. In this case, a single *Pseudonocardia* sp. was tested with two other cultures spotted on either side. The methods for the spotting was as described in 3.2.3, with marking criteria used as stated in 3.2.2.

3.2.5 *Streptomyces* and non-*Streptomyces* screening for interaction

Ten *Streptomyces* species were tested for interaction against different actinobacteria used in the preliminary screening, with a similar method as described in Seyedsayamdost *et al* (2012) with few modifications. A 6 well plate was used in which each well was filled with 5 ml ISP-2 medium using a 5 ml pipette. Each well was then spotted with two cultures with a 0.3 – 0.5 cm gap, whereas each culture was spotted

with 10 µl from its corresponding prepared spore suspension. Each 6 well plate was then incubated at 27°C for 10 days, with observation for interaction conducted every 2 days. Similar marking criteria was used, as described in 3.2.2.

3.2.6 Screening of *Pseudonocardia* sp. PIP 161 and other *Streptomyces* species with the 'Ueda' configuration

Screening for interaction between *Pseudonocardia* sp. PIP 161 and several late-emerging *Streptomyces* species and 4 cultures isolated from wheat or wheat soil. In this case, a method was used based on Ueda *et al*. (2000), followed by a flip plate antibiotic activity assay. Each plate measures the interaction between *Pseudonocardia* sp. PIP 161 and one *Streptomyces* species. Firstly, 10 µl of one culture were spotted on one edge of the plate, in which the other culture was spotted adjacently, with a 0.3 – 0.5 cm gap between the two cultures. On the same plate, each culture were spotted apart (with 3 – 5 cm gap between each culture) as a control. This configuration in this study was stated as the 'Ueda configuration', based on its source. The plates were then incubated at 27°C for 10 days, with observation for interaction conducted every 2 days. Each interaction were tested in triplicates. Similar marking criteria was used, as described in 3.2.2.

3.2.7 Medium preparation for antibiotic activity measurement

To measure antibiotic activity, a modified agar diffusion method was used. Laboratory grown *Staphylococcus aureus* was prepared previously which was stored in 50% glycerol in a -80°C freezer. A working plate was made by streaking 50 – 100 µl of cultures from the glycerol stock on to a tryptone soy agar (TSB, Oxoid added with agar 15 g/l) plate and incubating at 37°C overnight to obtain single colonies. The plates were made into a working plate and stored in a 4°C fridge until use and refreshed on a monthly basis.

For antibiotic assay, a bacterial suspension was prepared by transferring one colony of *S. aureus* in 1 ml of tryptone soy broth (TSB) in a 1.5 Eppendorf tube using a flame-sterilized loop. A control was prepared consisting of an un-inoculated Eppendorf containing 1 ml of TSB. The Eppendorf tubes were incubated in 37°C for 18 – 20 hours in order to achieve log phase. The cultures were then measured for OD_{600nm} using a spectrophotometer, and diluted to OD_{600nm} 0.2 using TSB medium.

S. aureus cultures were equally mixed into agar for antibiotic testing. The medium used for testing was antibiotic medium no. 1 (Oxoid) was prepared and sterilized according to manufacturer direction. Diluted *S. aureus* cultures suspension were then transferred into the medium (at 10 ml/L of medium) when the medium temperature was approximately 40 – 50°C. This medium was then used in following antibiotic assay measurement procedures.

3.2.8 "Flip-plate" assay for antibiotic activity measurement

Treatment plates intended to be tested for antibiotic activity (for example, interaction plates after the 10th day) was first prepared using the following procedure. Using a sterile scalpel or a heat sterilized metallic spatula, the treatment plates were dislodged from the 90 mm petri dish by running through the spatula the inside edges of the petri dish. Then, the medium was 'flipped', either by releasing the medium upside down into a new sterile petri dish, or by 'flipping' using the spatula in the same 90 mm petri dish. Thus, the sterile plate would contain a "flipped" medium with the cultures at the bottom of the plate. To the upper side of the plate was then poured with 5 – 10 ml of prepared antibiotic medium no. 1 containing *S. aureus* bacterial suspension, as described in 3.2.7, and left to solidify for 10 minutes. These plates were then incubated at 37°C overnight. A zone of inhibition was observed from above and the diameter of the zone measured in millimetres.

3.2.9 Statistical analysis

All statistical analysis was done using student t-test (one tailed, equal variance) in Excel, in which values were considered significant if the p value is <0.05.

3.3 Results and Discussion

3.3.1 Preliminary screening method used reduced plate number

We first attempted to screen for interactions with 24 different non-streptomycetes belonging to the genera *Pseudonocardia*, *Kribbella*, *Nocardia*, *Promicromonospora*, and *Micromonospora*, which were identified by the previous researchers based on morphology and T-RFLP (Kaewkla, O & Franco 2012). However, a 16S rRNA identification which was conducted later (after the screening process) on the species

indicated that the single *Promicromonospora* culture was instead closely related to *Streptomyces* (as previously showed in chapter 2).

The equation to calculate the number of unique combinations from a large number of interactions has been described elsewhere (Seyedsayamdost *et al*. 2012). Therefore, the number of combinations required for screening of our samples and thus indicative of spots required was calculated using the formulae described as follows:

$$Number\ of\ combinations = \frac{n!}{(n-r)! \times (r!)}$$

where n = total number of cultures tested and r = number of culture used for each combination. As the intention was to test the interaction of two actinobacteria, the resulting number of tests to cover all possible unique interactions was 276, which would mean the same number of plates required, or 46 plates if each plate contained six unique combinations. In triplicates, this would amount to 150 plates including triplicate control plates of monocultures for each of the 24 cultures screened. Using this method, the total number of 552 spots of cultures would be required for the interaction and another 24 for the controls, which would amount to 1,728 spots when screened in triplicates.

To reduce the number of plates and work needed to a more manageable level, the cultures were spotted in four items per combination, i.e in quadruplets, with 6 'clusters' of quadruplet cultures, per plate. Each cluster would therefore mainly assess 6 unique binary combinations, although it would unintentionally assess combinations of up to four cultures, although not all possible combinations.

The clusters were configured in a way that each culture would interact at least once, displayed in figure 3.1. With this configuration, the 276 possible combinations of the 24 cultures were screened using 11 plates. Therefore, the number of spots required for 24 cultures would be 264, and another 24 for monoculture controls. This allows for three replicates to be done for each interaction screening, whereas the positive (or negative) interactions were considered when even 1 out of the 3 replicates indicate a result, whereas conventional binary screening was used to confirm the results in a secondary screening. Thus, using this method, we have reduced the work required by half, and the plates required by 2/3rd.

		Actinobacteria cultures																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Actinobacteria cultures	1																								
	2	1.1																							
	3	1.1	1.1																						
	4	1.1	1.1	1.1																					
	5	2.1	2.1	2.2	2.2																				
	6	2.1	2.1	2.2	2.2	1.2																			
	7	2.3	2.3	2.4	2.4	1.2	1.2																		
	8	2.3	2.3	2.4	2.4	1.2	1.2	1.2																	
	9	2.5	2.5	2.6	2.6	3.1	3.1	3.2	3.2																
	10	2.5	2.5	2.6	2.6	3.1	3.1	3.2	3.2	1.3															
	11	3.3	3.3	3.4	3.4	3.5	3.5	3.6	3.6	1.3	1.3														
	12	3.3	3.3	3.4	3.4	3.5	3.5	3.6	3.6	1.3	1.3	1.3													
	13	4.1	4.1	4.2	4.2	4.3	4.3	4.4	4.4	4.5	4.5	4.6	4.6												
	14	4.1	4.1	4.2	4.2	4.3	4.3	4.4	4.4	4.5	4.5	4.6	4.6	1.4											
	15	5.1	5.1	5.2	5.2	5.3	5.3	5.4	5.4	5.5	5.5	5.6	5.6	1.4	1.4										
	16	5.1	5.1	5.2	5.2	5.3	5.3	5.4	5.4	5.5	5.5	5.6	5.6	1.4	1.4	1.4									
	17	6.1	6.1	6.2	6.2	6.3	6.3	6.4	6.4	6.5	6.5	6.6	6.6	7.1	7.1	7.2	7.2								
	18	6.1	6.1	6.2	6.2	6.3	6.3	6.4	6.4	6.5	6.5	6.6	6.6	7.1	7.1	7.2	7.2	1.5							
	19	7.3	7.3	7.4	7.4	7.5	7.5	7.6	7.6	8.1	8.1	8.2	8.2	8.3	8.3	8.4	8.4	1.5	1.5						
	20	7.3	7.3	7.4	7.4	7.5	7.5	7.6	7.6	8.1	8.1	8.2	8.2	8.3	8.3	8.4	8.4	1.5	1.5	1.5					
	21	8.5	8.5	8.6	8.6	9.1	9.1	9.2	9.2	9.2	9.2	9.4	9.4	9.5	9.5	9.6	9.6	10.1	10.1	10.2	10.2				
	22	8.5	8.5	8.6	8.6	9.1	9.1	9.2	9.2	9.2	9.2	9.4	9.4	9.5	9.5	9.6	9.6	10.1	10.1	10.2	10.2	1.6			
	23	10.3	10.3	10.4	10.4	10.5	10.5	10.6	10.6	11.1	11.1	11.2	11.2	11.3	11.3	11.4	11.4	11.5	11.5	11.6	11.6	1.6	1.6		
	24	10.3	10.3	10.4	10.4	10.5	10.5	10.6	10.6	11.1	11.1	11.2	11.2	11.3	11.3	11.4	11.4	11.5	11.5	11.6	11.6	1.6	1.6	1.6	

Figure 3.1 A modification of the checkerboard assay used in this study, in which quadruple interactions were used instead of the binomial interaction, wherein the first number represents the plate number and the second number represents the position within that plate, forming different ‘clusters’ of quadruplet cultures. This design allows for each culture to interact with another culture at least one time, and cutting down the number of tests required (in triplicates, including controls).

3.3.2 Preliminary screening suggests that *Micromonospora* sp. and *Streptomyces* sp. were most susceptible to induction

An interaction was scored by comparison with a control monoculture as a baseline (as shown in table 3.1). Most cultures showed good growth in ISP-2 plates; however, some cultures had minimal growth only after the 5th day or even the 10th day, as observed on *Pseudonocardia* sp. CAP 111, CAP 60, and CAP 47 (referring to table 3.1.; 10, 15 and 18, respectively), *Micromonospora sp*. 181r and 181g (13 and 24, respectively), Nonomurae sp. CAP 329 (17), and *Kribbella* sp. PIP 166 (11). Therefore, any growth of these cultures in proximity with other cultures was considered to be a positive interaction. The result of the initial interaction is displayed in figure 3.2.

Based on the initial screening, interaction noticeably occurred in only a few species; most noticeable was on the *Micromonospora* sp. and the single *Streptomyces sp*. screened. *Micromonospora* sp. EUM 48, CAP 181r and CAP 181g (4, 13 and 24, respectively) was induced in several clusters, which manifested in increased growth or increased pigmentation compared to monocultures. *Micromonospora* sp. EUM 48 had increased surface black pigmentation in cluster 1.1, 2.4, 6.2 and 7.4. In three of these clusters, a *Pseudonocardia* sp. of a similar morphology was present, namely, *Pseudonocardia* sp. 208, 212, 224, in cluster 1.1, *Pseudonocardia* sp. 197 in cluster 2.4, and *Pseudonocardia* sp. PIP 161 in cluster 7.4. Other cultures in the clu2sters include *Pseudonocardia* sp. CAP 47 (which had a different morphology with aforementioned *Pseudonocardia* sp.), *Nonomurae* sp. CAP 329, and *Nocardia* sp. CAP 40.

Micromonospora sp. CAP 181r and CAP 181g (13 and 24, respectively) (an dark orange and green variance of the same source), on the other hand, did not visibly show an increase of pigmentation, but mainly showed increased growth compared to monocultures in several clusters. *Micromonospora* sp. CAP 181r had increased growth in clusters 4.2 to 4.6 and 5.6., while *Micromonospora* sp. CAP 181g had increased growth in cluster 1.6, 10.3, 10.5, 10.6, 11.5, and 11.6. Both cultures were stimulated in the presence of *Pseudonocardia sp*. EUM 227, *Streptomyces* sp. EUM 76, *Micromonospora* sp. EUC 38 and *Pseudonocardia sp*. PIP 197 (5, 6, 7, and 8, respectively). However, antibiotic production, at least in this early screening, was only from *Micromonospora* sp. CAP 181g in cluster 1.6, in conjunction with *Pseudonocardia sp*. CAP 335, *Pseudonocardia* sp. CAP 122 and *Nocardia* sp. CAP 290 (21, 22, and 23, respectively).

Contrary to previous trends, *Micromonospora* sp. EUC 38 (7) had growth inhibition in cluster 2.4 and 4.4. In this case, *Micromonospora* sp. EUC 38 was in contact with *Pseudonocardia* sp. EUM 224 (3), *Micromonospora* sp. EUM 48 (4), *Pseudonocardia* sp. PIP 197 (8), *Micromonospora* sp. 181r (13), and *Amycolatopsis* sp. PIP 207 (14). While the two *Pseudonocardia* in this case was morphologically similar, *Micromonospora* sp. EUC 38 was not inhibited by the other morphologically similar *Pseudonocardia*. This led us to speculate that the inhibition of *Micromonospora* sp. EUC 38 was due to contact with other *Micromonospora* sp. and/or *Amycolatopsis* sp. PIP 207, instead of *Pseudonocardia* sp.

Increased sporulation was observed in *Streptomyces* sp. EUM 76 (6) in clusters 1.2, 7.5, and 9.1. *Streptomyces* sp. EUM 76 was in contact with four morphologically similar *Pseudonocardia* sp. sp. in these clusters, namely *Pseduonocardia* sp. EUM 227, *Pseudonocardia* sp. PIP 197, *Pseudonocardia* sp. PIP 161, and *Pseudonocardia* sp. CAP 122 (5, 8, 20, and 22, respectively). Other species in contact with *Streptomyces* were *Micromonospora* sp. EUC 38, *Nocardia* sp. CAP 40, *Nocardia* sp. CAP 290, and *Pseudonocardia* sp. CAP 335 (7, 19, 23, and 21, respectively) which had a different morphology with the aforementioned *Pseudonocardia* sp. While at this stage, the inducer of *Streptomyces* cannot be determined, whereas *Streptomyces* sp. EUM 76 can be either induced by *Nocardia* sp. and/or *Pseudonocardia* sp., it shows increasing evidence that morphologically similar *Pseudonocardia* sp. had influence on multiple species.

Supporting this was increased growth and sporulation observed in *Pseudonocardia* sp. CAP 111 (10), which had morphological similarities with *Pseudonocardia* sp. CAP 60 and *Pseudonocardia* sp. CAP 335, and *Kribbella* sp. PIP 158 and PIP 166 (11 and 12, respectively) in several different clusters. In those clusters, induction with the usual *Pseudonocardia* sp. was more apparent, in which induction, as with the previous interactions, often occurred when in conjunction with *Pseudonocardia* sp. with orange aerial mycelia (referring to table 3.1).

The initial screening allows quick macroscopic assessment on the presence of interaction between different species, but it does so with a risk of false positives, as many interactions may only be governed by two species. Furthermore, there was a possibility that the response observed may the conjunction of multiple species (non-binary) (Ahring & Westermann 1987; Laube & Martin 1981; Wu, Jain & Zeikus 1994). Therefore, in order to re-confirm the positive interactions, existing clusters were then broken down into binary co-cultures, with focus to those exhibiting changes.

Table 3.1 Growth, sporulation, and day 10 morphological characteristics of actinobacteria used in the initial screening study

No	Culture	Day 3		Day 5		Day 7		Day 10						
		g	s	g	s	g	s	g	s	Aerial color	Vegetative color	Shape	Margin	Elevation
1	*Pseudonocardia* sp. EUM 208	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
2	*Pseudonocardia* sp. EUM 212	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
3	*Pseudonocardia* sp. EUM 224	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
4	*Micromonospora* sp. EUM 48	+	0	+	0	+	0	+	+	Orange-black	Orange	Circular	Smooth	Flat
5	*Pseudonocardia* sp. EUM 227	+	+	+	+	+	+	+	+	White	Orange	Irregular	Lobate	Raised
6	*Streptomyces* sp. EUM 76	+	0	+	0	+	0	+	+	White	Cream	Circular	Smooth	Flat
7	*Micromonospora* sp. EUC 38	+	+	+	+	+	+	+	+	Orange	Orange	Circular	Smooth	Flat
8	*Pseudonocardia* sp. PIP 197	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
9	*Pseudonocardia* sp. PIP 148	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
10	*Pseudonocardia* sp. CAP 111	0	0	-	+	-	+	-	+	Yellow	Yellow	Punctiform	Lobate	Pulvinate
11	*Kribbella* sp. PIP 166	0	0	-	+	-	+	-	+	White	White	Irregular	Lobate	Pulvinate
12	*Kribbella* sp. PIP 158	-	+	-	+	+	+	+	+	White	White	Irregular	Lobate	Pulvinate
13	*Micromonospora* sp. CAP 181r	0	0	0	0	0	0	0	0	-	-	-	-	-
14	*Amycolatopsis* sp. PIP 207	+	+	+	+	+	+	+	+	Yellow	Yellow	Irregular	Lobate	Raised
15	*Pseudonocardia* sp. CAP 60	0	0	0	0	0	0	0	0	-	-	-	-	-
16	*Kribbella* sp. PIP 118	+	0	+	0	+	0	+	0	White	White	Circular	Smooth	Flat
17	*Nonomurae* sp. CAP 329	0	0	0	0	0	0	0	0	-	-	-	-	-
18	*Pseudonocardia* sp. CAP 47	0	0	0	0	0	0	0	0	-	-	-	-	-
19	*Nocardia* sp. CAP 40	+	+	+	+	+	+	+	+	White	Pink	Irregular	Smooth	Flat
20	*Pseudonocardia* sp. PIP 161	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
21	*Pseudonocardia* sp. CAP 335	0	0	0	0	0	0	0	0	-	-	-	-	-
22	*Pseudonocardia* sp. CAP 122	+	+	+	+	+	+	+	+	White	Brown	Irregular	Lobate	Raised
23	*Nocardia* sp. CAP 290	-	+	+	+	+	+	+	+	White	White	Circular	Smooth	Flat
24	*Micromonospora* sp. CAP 181g	0	0	-	-	-	-	-	+	Green-Black	Black	Punctiform	Smooth	Pulvinate

Score for growth (g) and sporulation (s) (0 = none, - = little, + =good);

		Cultures in the proximity of cultures exhibiting differences																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Cultures where differences were exhibited	1																								
	2	g-				g-	g-																		
	3				g-											g-	g-								
	4	p+	p+	p+				p+	p+									p+	p+	p+	p+				
	5																								
	6					as+		as+	as+											s+	s+	s+	s+		
	7			g-	g-				g-					g-	g-										
	8																								
	9																								
	10	gs+	gs+					gs+	gs+	gs+		gs+	gs+									gs+	gs+		
	11	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+		gs+												
	12	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+	gs+													
	13			g+	g+	g+	g+	g+	g+	g+	g+	g+	g+		g+	g+	g+								
	14																								
	15																								
	16																								
	17																								
	18																								
	19																								
	20																								
	21																								
	22																								
	23																								
	24	g+	g+			g+	g+	g+	g+									g+	g+	g+	g+	ag+	ag+	ag+	

Figure 3.2 Screening for interaction between 24 actinobacteria for differences in growth (g+/g-), sporulation (s+/s-), and pigment production (p+/p-) in any time up to 10 days compared to controls grown as monocultures. At the 10th day, the cultures were tested for antibiotic activity (a) against S. aureus. The cultures tested were **(1)** *Pseudonocardia* sp. EUM 208, **(2)** EUM 212, **(3)** EUM 224, **(4)** *Micromonospora* sp. EUM 48, **(5)** *Pseudonocardia* sp. EUM 227, **(6)** *Streptomyces* sp. (formerly *Proomicromonospora* sp.) EUM 76, **(7)** *Micromonospora* sp. EUC 38, **(8)** *Pseudonocardia* sp. PIP 197, **(9)** PIP 148, **(10)** CAP 111, **(11)** *Kribbella* sp. PIP 166, **(12)** PIP 158, **(13)** *Micromonospora* sp. CAP 181r, **(14)** Amycolatopsis sp. PIP 207, **(15)** *Pseudonocardia* sp. CAP 60, **(16)** *Kribbella* sp. PIP 118, **(17)** *Nonomurae* sp. CAP 329, **(18)** *Pseudonocardia* sp. CAP 47, **(19)** *Nocardia* sp. CAP 40, **(20)** *Pseudonocardia* sp. PIP 161, **(21)** CAP 335, **(22)** CAP 122, **(23)** *Nocardia* sp. CAP 290, and **(24)** *Micromonospora* sp. CAP 181g. The interactions were measured in triplicates, in a modified checkerboard assay, in which one plate were spotted with 6 groups of four cultures in a square.

3.3.3 Co-culture screening shows that most interaction in clusters can be replicated in binary interactions

The previous screening identified cultures which exhibited changes when subcultured in the proximity of other cultures; most notably *Micromonospora* sp. EUM 48, *Micromonospora* sp. CAP 181g, *Micromonospora* sp. EUC 38, and *Streptomyces* sp. EUM 76. However, cultures in several cluster in which the induction occur also were tested, in order to further confirm the validity of the finding. The result of the secondary screening is displayed in figure 3.3. Due to time limitations, not all interactions were measured.

Most interactions tested in previous screening can be split into interactions consisting of only two actinobacteria that were directly tested. We observed that increased pigment production in *Micromonospora* sp. EUM 48 which was found in one cluster (1.1) in previous screening, for instance, was inducible by three *Pseudonocardia* species separately. Similarly, this was replicated in the sporulation of *Streptomyces* sp. EUM 76, which was induced by cultures of *Pseudonocardia* species, *Micromonospora* sp. EUM 48 and *Nocardia* sp. CAP 40, separately.

Low levels of antibiotic production were observed in several binary interactions. Although sporulation was induced in *Streptomyces* sp. EUM 76, low levels of antibiosis were only seen in co-cultures with *Pseudonocardia* sp. This was also observed in *Micromonospora* sp. CAP 181g when co-cultured with *Pseudonocardia* sp. CAP 122. On the other hand, we observe antibiotic produced in *Amycolatopsis* sp. PIP 207; however, as the monocultures also produced antibiotics, no differences were observed in any interaction.

Several interactions found in initial screening were not replicated by these results, suggesting either that multiple species were required for interaction or components of the interaction were not sufficiently tested. However, we could safely conclude that most of the interaction found in this case, at least based on visible macroscopic changes, was binary. We then attempt to replicate the results in order to characterise the interaction which occurred.

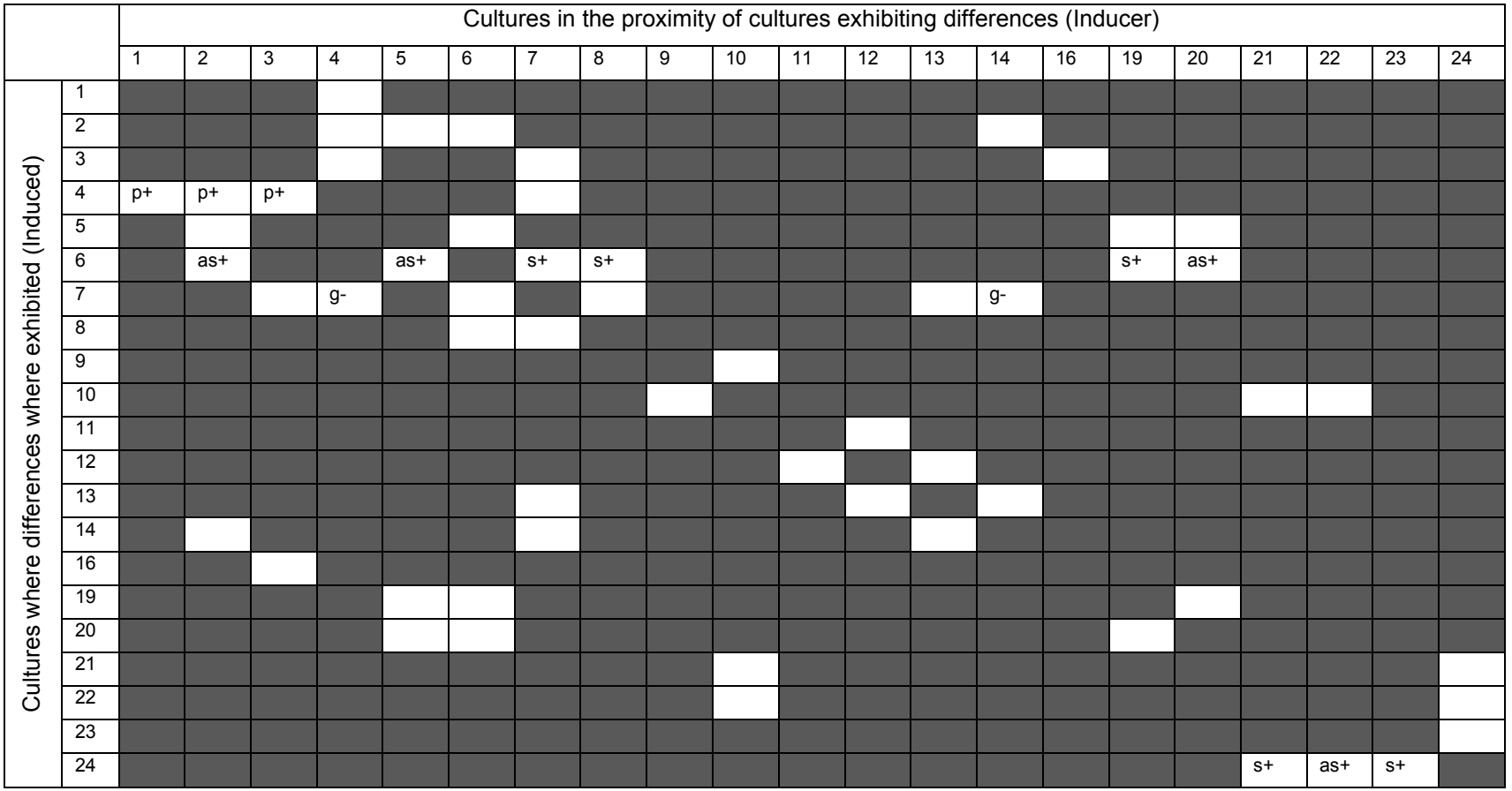


Figure 3.3 Confirmation of interaction by binary interaction screening for cultures showing differences in growth (g+/g-), sporulation (s+/s-), pigment production (p+/p-) and antibiotic production (a) against S. aureus based on the results from initial screening assay. The cultures tested were **(1)** *Pseudonocardia* sp. EUM 208, **(2)** EUM 212, **(3)** EUM 224, **(4)** *Micromonospora* sp. EUM 48, **(5)** *Pseudonocardia* sp.EUM 227, **(6)** *Streptomyces* sp. (formerly *Proomicromonospora* sp.) EUM 76, **(7)** *Micromonospora* sp. EUM 38, **(8)** Pseudoncoardia sp. PIP 197, **(9)** PIP 148, **(10)** CAP 111, **(11)** *Kribbella* sp. PIP 166, **(12)** PIP 158, **(13)** *Micromonospora* sp. CAP181r, **(14)** Amycolatopsis sp. PIP 207, **(16)** *Kribbella* sp. PIP 118, **(19)** Nocardia sp. CAP 40, **(20)** *Pseudonocardia* sp. PIP 161, **(21)** CAP 335, **(22)** CAP 122, **(23)** Nocardia sp. CAP 290, and **(24)** *Micromonospora* sp. CAP 181g. The interactions were tested in triplicates, in which one plate were spotted with 6 groups of two cultures in a side-by-side configuration, and tested for antibiotic activity against S. aureus on the 10th day.

3.3.4 Morphologically similar *Pseudonocardia* spp. induce morphological changes in different species of actinobacteria

Based on previous results, a subtype of *Pseudonocardia* sp. was able to induce growth and antibiotic production in *Micromonospora* and *Streptomyces*. Based on its morphological characteristics as well as the aforementioned 16S rRNA sequencing, several *Pseudonocardia* sp. samples that were used in the study had 100% sequence similarity with each other, as well as to a type culture *Pseudonocardia carboxydivorans* Y8(T). The *Pseudonocardia* sp. were mainly those having orange aerial mycelia and white spores, namely *Pseudonocardia* sp. EUM 208, EUM 212, EUM 224, EUM 227, PIP 197, PIP 148, PIP 161 and CAP 122.

While the limitations of 16S rRNA sequencing in differentiating interspecies has been documented (Clarridge 2004; Janda & Abbott 2007; Woo *et al*. 2008), there are some reports of *Pseudonocardia* sp. being phylogenetically closely related (Lee, SD, Kim & Hah 2000), with one interspecies difference of merely 0.3%-0.4% (Park, SW *et al*. 2008; Prabahar *et al*. 2004) yet showing enough phenotype differences to warrant speciation. Therefore, it could be possible, that *Pseudonocardia* sp. used in this study could also be poorly differentiated using 16S rRNA sequencing alone, and requires significant phenotypic analysis to determine differences. Thus, several *Pseudonocardia* sp. with the same morphology were tested for differences in inducing abilities against previously used actinobacteria, as displayed in table 3.2.

Table 3.2 Co-culture results of morphologically similar *Pseudonocardia* sp. against various actinobacteria.

Actinobacteria exhibiting changes	*Pseudonocardia sp*.					
	EUM 212	EUM 227	PIP 197	PIP 148	PIP 161	CAP 122
Micromonospora sp. EUM 48	p+	p+	p+			p+
Streptomyces sp. EUM 76	s+	s+	s+		s+	
Micromonospora sp. EUM 48						
Kribbella sp. PIP 166						
Kribbella sp. PIP 158						
Amycolatopsis sp. PIP 207						
Nonomurae sp. CAP 329						
Nocardia sp. CAP 40						
Nocardia sp. CAP 290						
Micromonospora sp. CAP 181g	g+	g+	g+	g+	g+	g+

†Observed up to day 10

Our previous results show that the production of antibiotics from *Streptomyces sp*. EUM 76 occurred when co-cultured with *Pseudonocardia* sp. PIP 161, but did not show any antibiotic activity when co-cultured with *Pseudonocardia* sp. PIP 197, although in both cases, sporulation occurred. It should be noted, however, that the

antibiotic produced in this stage was inconsistent between replications, whereas only one out of three replicates showed antibiotic activity.

According to table 3.2, however, differences in inducing abilities between *Pseudonocardia* sp. subtypes were discovered. *Pseudonocardia* sp. EUM 212, EUM 227 and PIP 197, for instance, were able to both induce sporulation in *Streptomyces* sp. EUM 76 and pigment production in *Micromonospora* sp. EUM 48, yet *Pseudonocardia* sp. PIP 148 did neither. *Pseudonocardia* sp. CAP 122 induced pigment production in *Micromonospora* sp. EUM 48, but did not induce sporulation in *Streptomyces* sp. EUM 76, whilst the reverse happened with *Pseudonocardia* sp. PIP 161. All *Pseudonocardia* sp. however, was able to induce growth in *Micromonospora* sp. CAP 181g.

Therefore, it was concluded that differences exist between the *Pseudonocardia* sp. tested, in terms of its inducing ability. However, proper differentiation between these subtypes would require a set of experiment and analysis which was beyond the scope of this study, as the main concern was with the interaction between actinobacteria. At this point, the mechanism to induce sporulation and alteration in secondary metabolism of *Pseudonocardia* sp. was still unknown, except that it may be shared between different *Pseudonocardia* sp. within the same subtype. In order to further study the induction, *Pseudonocardia* sp. PIP 161 was selected as the inducer species, considering that the mechanism to be less non-specific (and therefore be more determinable) compared to other *Pseudonocardia* sp., and results in antibiotic production on *Streptomyces* sp. EUM 76.

3.3.5 Sporulation was induced in *Streptomyces* sp. EUM 76 when co-cultured with other *Streptomyces* species

The next step was to screen previous positive results with an array of late growing *Streptomyces* species to observe interaction between uncommon actinobacteria with *Streptomyces* species. While not all actinobacteria were tested, *Micromonospora* sp. EUC 38 and CAP 181g, *Amycolatopsis* sp. PIP 207, and several *Pseudonocardia* species from different subtypes was screened. *Streptomyces* sp. EUM 76 interactions were displayed in figure 3.4.

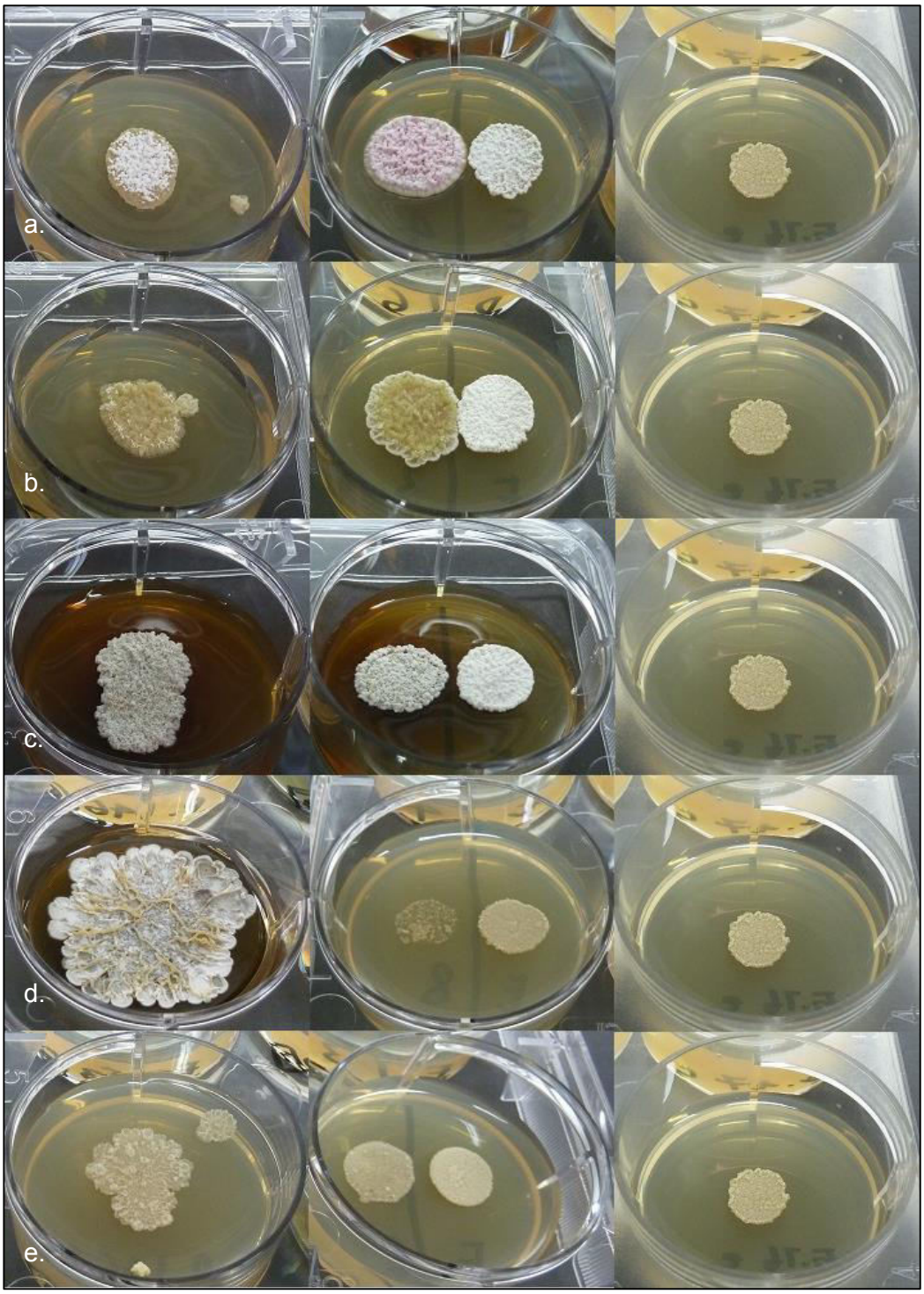


Figure 3.4 *Streptomyces* sp. EUM 76 (right column) interacts with different *Streptomyces* species (left column). (a) Co-culture with *Streptomyces* sp. EUC 63, (b) with *Streptomyces* sp. EUC 20, (b) with *Streptomyces* sp. CAP 288, (d) with *Streptomyces* sp. CAP 230, and (e) with *Streptomyces* sp. PIP 146.

Previous screening efforts discovered that sporulation in *Streptomyces* sp. EUM 76 was induced by four *Pseudonocardia* species, *Nocardia* sp. CAP 40, and *Micromonospora* sp. EUC 38. Furthermore, three *Streptomyces* species also interacted with *Streptomyces* sp. EUM 76. Sporulation was induced in both

Streptomyces species between *Streptomyces* sp. EUM 76 and *Streptomyces* sp. EUC 63, while *Streptomyces* sp. EUC 63 had also increased pigment production in co-culture. Sporulation was also induced between *Streptomyces* sp. EUM 76 with *Streptomyces* sp. EUC 20 and *Streptomyces* sp. CAP 288, while *Streptomyces* sp. CAP 230 was inhibited when co-cultured. Not all *Streptomyces* species tested induced sporulation, as shown in the co-culture with *Streptomyces* sp. PIP 146.

3.3.6 *Amycolatopsis* sp. PIP 207 also inhibits several *Streptomyces* species

Amycolatopsis sp. PIP 207 was seen to also interact with several of the *Streptomyces* species in co-culture, shown in figure 3.5.

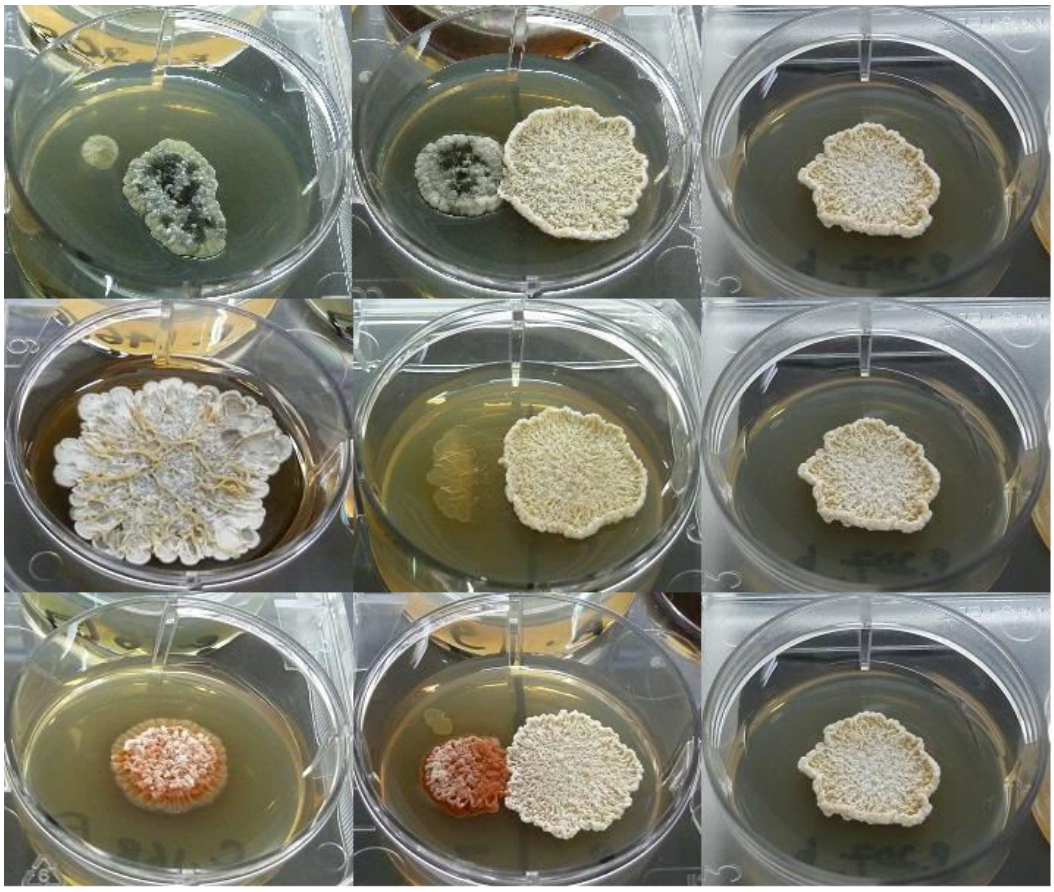


Figure 3.5 Amycolatopsis sp. PIP 207 (right column) interacts with three different *Streptomyces* species (left column). (Top) Co-culture with *Streptomyces* sp. CAP 76, (Middle) with *Streptomyces* sp. CAP 230, and (bottom) with *Streptomyces* sp. CAP 168.

Streptomyces sp. CAP 76 was uninhibited in the presence of *Amycolatopsis* sp. PIP 207; however, it causes a slight decolourisation on the surface of *Amycolatopsis sp*.

to be slight greyish. This was most likely to be the influence of pigments produced by *Streptomyces* sp. CAP 76. *Amycolatopsis* sp. PIP 207 also inhibited the growth of *Streptomyces* sp. CAP 230, with a clear inhibition zone around 0.3 cm around the edges. The inhibition zone was also observed on *Streptomyces* sp. CAP 168, where it prevented sporulation of *Streptomyces* sp. CAP 168, indicating some development retardation. Furthermore, in this interaction, *Amycolatopsis* species was less lobate in co-culture compared to mono-culture, indicating a bidirectional interaction.

3.3.7 *Micromonospora* sp. EUC 38 was also inhibited by *Streptomyces* sp. and other genera

Previous screening showed that several actinobacteria, i.e. *Micromonospora* sp. EUM 48, *Amycolatopsis* sp. PIP 207, inhibited the growth of *Micromonospora* sp. EUC 38. When the interaction was further screened against 9 *Streptomyces*, growth inhibition was observed to occur when co-cultured with *Streptomyces* sp. EUM 244, as shown in figure 3.6.

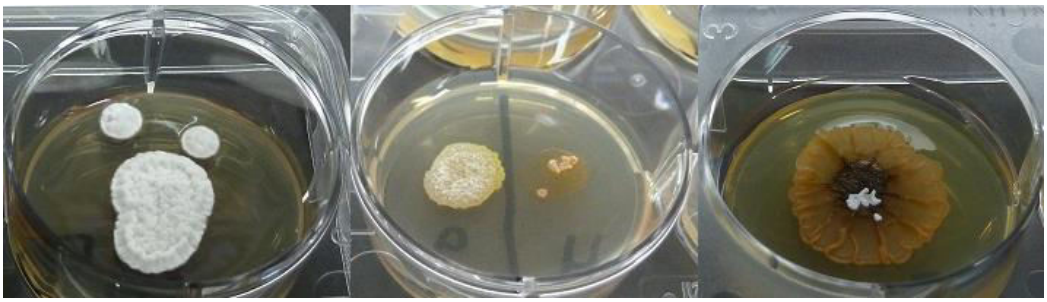


Figure 3.6 *Micromonospora* sp. EUC 38 (right column) interacts with *Streptomyces* sp. EUM 244 (left column). Co-culture between *Micromonospora* sp. EUC 38 and *Streptomyces* sp. EUM 244 was shown in the middle column.

Co-culture of *Micromonospora* sp. EUC 38 and *Streptomyces* sp. EUM 244 shows inhibition on both cultures when compared to monocultures. While the interaction between *Amycolatopsis* sp. PIP 207 can be explained by the production of siderophores or antibiotics (which both were found to be produced by *Amycolatopsis* sp. PIP 207 in monocultures), interaction between *Micromonospora* sp. EUM 48 and *Streptomyces* sp. EUM 244 would be harder to explain. However, although at this point we cannot determine the mechanism of interaction, we speculate that specific interactions occur between these species.

3.3.8 *Pseudonocardia* sp. PIP 161 was able to induce changes in multiple *Streptomyces* species

Previous screening shows that a subtype of *Pseudonocardia* species was able to induce sporulation of multiple non-*Streptomyces* as well as *Streptomyces* sp. EUM 76. This screening process was expanded to observe changes on 18 *Streptomyces* species and 4 unknown actinobacteria isolated from soil and wheat roots. Using method previous used, we discovered four different interactions with *Pseudonocardia* sp. PIP 161 and *Streptomyces* species, as shown figure 3.7.

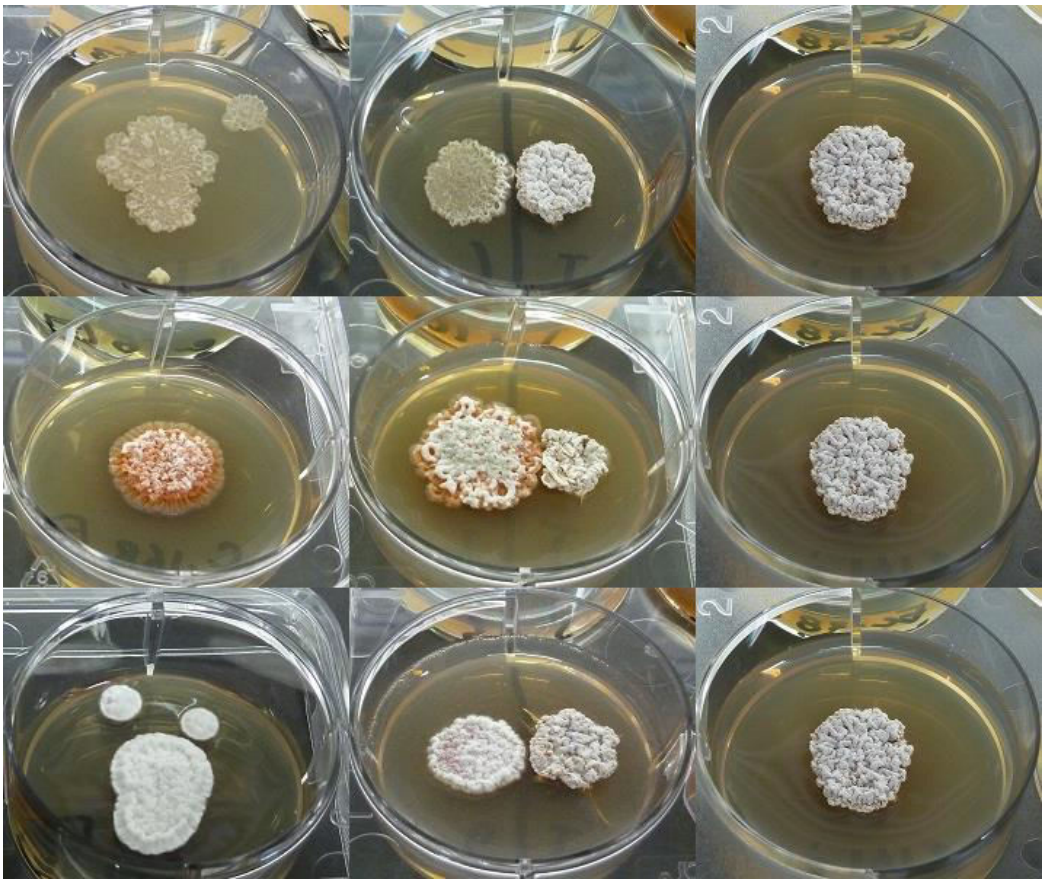


Figure 3.7 *Pseudonocardia* sp. PIP 161 (right column) interacts with three different *Streptomyces* species (left column). (Top) Co-culture with *Streptomyces* sp. PIP 146, (Middle) with *Streptomyces* sp. CAP 168, and (Bottom) *Streptomyces* sp. EUM 244.

Further studies use a methodology based on Ueda *et al*. (2000), which would enable the viewing of an interaction and non-interaction on a single plate. Several of these interactions were shown in figure 3.8.

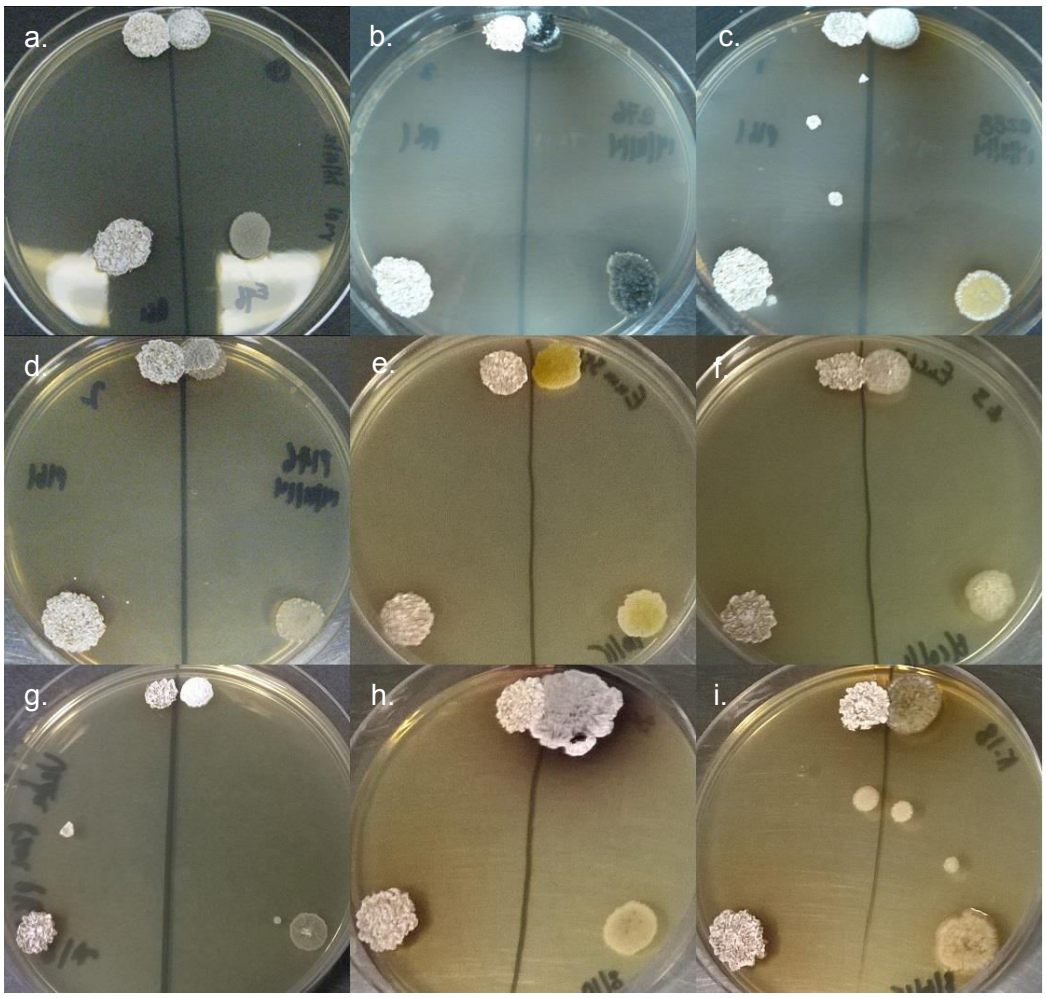


Figure 3.8 Interaction between *Pseudonocardia* sp. PIP 161 and several different *Streptomyces* sp. (a.) EUM 76, (b.) CAP 76, (c.) CAP 288, (d.) PIP 146, (e.) EUM 356, (f.) EUM 63, (g.) PIP 201, (h.) SC 36, (i.) K 18, showing generally increased sporulation.

As shown in above figure, *Pseudonocardia* sp. PIP 161 was seen to be able to interact with a great number of *Streptomyces* species, in which increased sporulation was observed in most cases. Some interactions were visible since day 4, such as the interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* sp. EUM 356, EUC 63 (as observed in figure 3.8), and SC 36, while others were visible after day 7.

3.3.9 *Pseudonocardia* sp. PIP 161 alters antibiotic production in various *Streptomyces* species.

Previous screening established that *Pseudonocardia* sp. PIP 161 was able to induce sporulation in three different *Streptomyces* species. We expanded this to include the other *Streptomyces* and measured antibiotic production of the interaction (table 3.3). As described in the methodology, a modified agar diffusion method was used to

measure the zone of inhibition against *S. aureus*, as it was readily available in this laboratory. The main interest of this study was to compare the antibiotic production of the cultures when in co-culture compared to when in mono-culture.

Table 3.3 Antibiotic production in co-culture with mono-culture between *Pseudonocardia* sp. PIP 161 and various *Streptomyces* species

Streptomyces sp.	Average zone of inhibition against *S. aureus*	
	Streptomyces species in mono-culture	*Streptomyces* species in co-culture
CAP 214[a]	clear plate	clear plate
PIP 245[a]	7.3±2.12	11±4.95
CAP 230[a]	clear plate	clear plate
EUC 209[a]	18.3±2.89	21.6±2.89
PIP 156[a]	**6.7±11.55**	**34.3±9.81**
EUC 7[a]	21±3.60	18.3±2.89
EUC 6[a]	20±0.00	20±0.00
CAP 28[a]	20±0.00	21.6±10.4
EUC 66[a]	**23.3±2.88**	**13.3±2.88**
PIP 196[a]	45±0.00	53.3±5.77
CAP 208[a]	51.6±2.88	46.6±5.77
PIP 201[a,b]	**0**	**12.3±2.51**
CAP 103[a]	**Contaminated**	**clear plate**
EUM 242[a]	25±0.00	25±0.00
EUM 359[a]	**46.6±5.77**	**clear plate**
EUM 76[b]	**None detected**	**13.67±1.52**
PIP 146[b]	**None detected**	**28.00±3.46**
CAP 288[b]	32.00±11.13	56.67±11.54
EUC 63[b]	**27.33±3.05**	**36.67±3.05**
CAP 168[b]	26.67±5.77	33.33±11.5
EUM 244[b]	**46.67±5.77**	**Clear Plate**
SC 36[c]	**None detected**	**35.67±1.15**
CAP 76[b]	Clear plate	Clear Plate

(a)Co-cultures and mono-cultures were in different plates,(b)Co-culture were in the same plate with mono-culture, overlaid with 5 ml of agar infused with *S.aureus* OD_{600nm} of 0.02,(c) same as b, with 10 ml of *S.aureus* agar.

Most of the *Streptomyces* sp. tested produced some amount of antibiotics, with some producing at lower levels than others. In several cases, co-culture was enabled higher activity of *Streptomyces* species against *S. aureus*, indicating higher antibiotic production, ranging from 1.5 to 2x the activity in monoculture. It should be noted that a 'clear plate' (when the no *S. aureus* was able to grow in the overlaid plate) would cloud the activity of monocultures when monocultures and co-cultures were on the same plate (such as when using the method by Ueda *et al* (2000)), therefore changes in activity would be undetected.

3.3.10 Interaction map showing interactions between actinobacteria

Based on our findings so far, we can summarize the interaction that occurs between several actinobacteria tested in this study. The number of actinobacteria screened for interactions was 12 *Pseudonocardia* sp., 3 *Kribbella* sp., 2 *Nocardia* sp., 1 *Nonomurae* sp., 1 *Amycolatopsis* sp., and 29 *Streptomyces* species. The interaction screening covered interaction between 22 different species (and 1 variant) of non-*Streptomyces* and one *Streptomyces* with each other, between 8 non-*Streptomyces* and 1 *Streptomyces* with 10 different *Streptomyces*, and 1 *Pseudonocardia* with 18 *Streptomyces* and 4 unknown actinobacteria isolated from wheat root and soil samples. Thus, 388 unique combinations of actinobacteria were screened in this study. The summary of the positive results, based on binomial screening, was displayed in figure 3.9.

From the screening, more than 45 interactions were discovered, including unidirectional or bidirectional increases or decreases in growth, sporulation, pigment production and antibiotic production. Based on this screening effort, it was discovered that the interactions were not solely one on one, and some species can interact with more than one species in different manners. It was discovered that one *Streptomyces* was responsive or compatible to signals from various *Streptomyces* and non-*Streptomyces* species, although at this point, whether the signals or triggers were similar or dissimilar was unknown. More importantly, it was also discovered that *Pseudonocardia* spp., with PIP 161 being the most extensively tested, was able to induce changes in a range of different *Streptomyces*. As most research in co-culture of actinobacteria interactions rarely use an approach to observe the interaction in more than one species, this is most likely to be among the first studies to discover the multiple interactions within a single species.

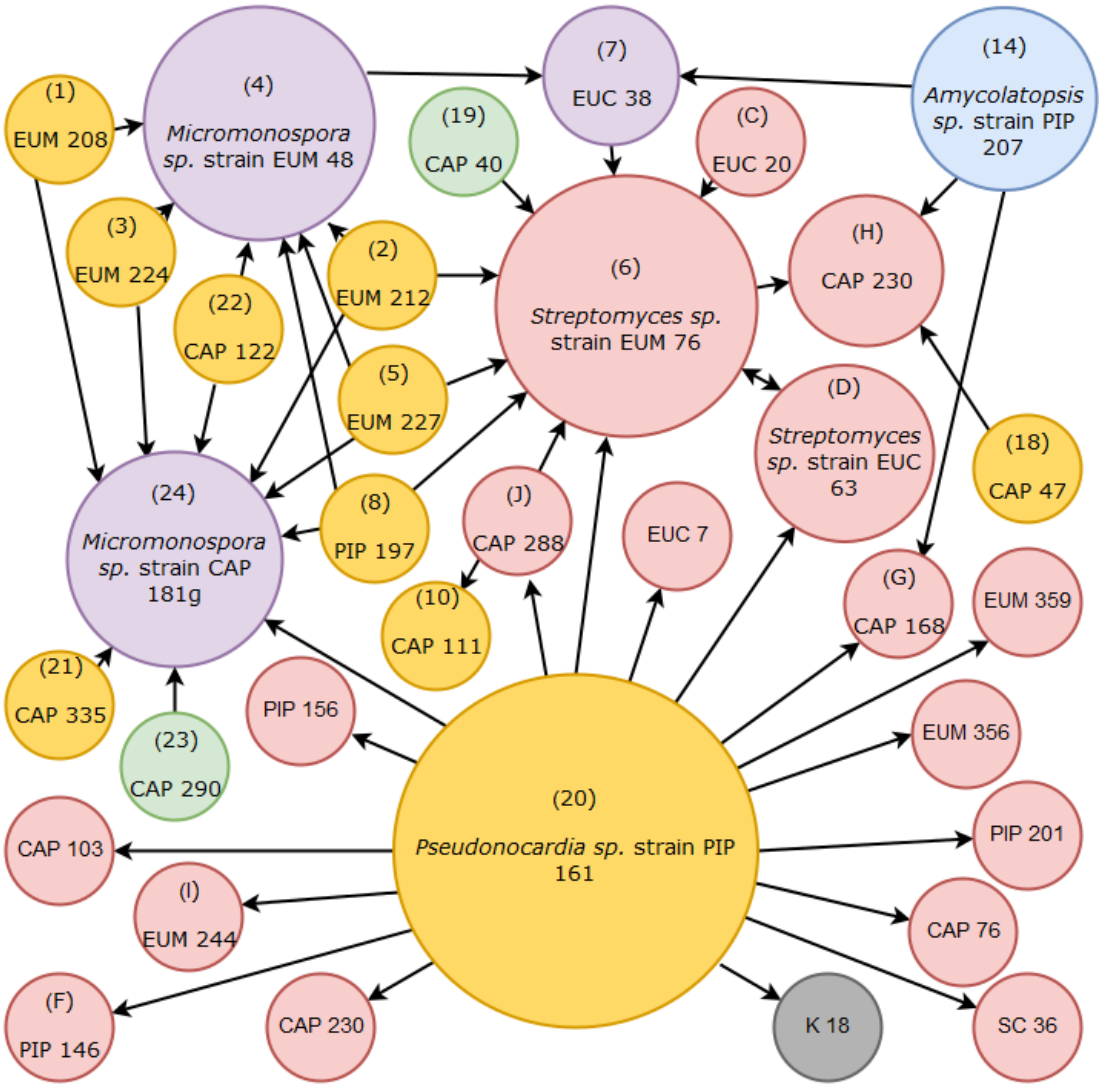


Figure 3.9 A map of most interactions observed this study. Each circle represents a different species, with numbers or letters in brackets to refer to previous tables, and were colour coded based on the genus of each species: red denotes *Streptomyces*, orange denotes *Pseudonocardia*, purple denotes *Micromonospora*, blue denotes Amycolatopsis, green denotes Nocardia and grey denotes actinobacteria with a currently unknown genus. Larger circles are made to accommodate a higher number of interactions.

Based on the sample distribution of this study, it would be most likely that the number of interactions that were discovered to be between species from *Streptomyces* and *Pseudonocardia* genera were due to the higher number of samples within those genera compared to other non-*Streptomyces* screened. Furthermore, whilst the *Streptomyces* screened were relatively morphologically varied, there were only possibly two or three phenotypes of the *Pseudonocardia* tested, and whether each member of the phenotype was from the same or were different species was still undetermined.

A majority of the inducing *Pseudonocardia* spp. were morphologically similar, while the remaining *Pseudonocardia* sp., namely CAP 335, CAP 111 and CAP 47, had very different morphology. From the major phenotype, only *Pseudonocardia* sp. PIP 161 was more extensively screened against other *Streptomyces*. Therefore, while it may be possible that other *Pseudonocardia* spp. with the same phenotype as PIP 161 may also interact with various *Streptomyces*, only the interaction of PIP 161 could be confirmed with any confidence. It should be noted that many of the interactions between *Streptomyces* were not tested, nor were the tests done with the full repertoire of the non-*Streptomyces* available. Thus, the possible interactions should not be considered to be limited to what was depicted in the interaction map above.

3.3.11 Current concepts on the mechanism of interactions between actinobacteria

Several main interactions observed in this study seem to agree with findings from Vetsigian *et al*. (2011). The finding that morphologically and phylogenetically similar species to influence different species, as observed in *Pseudonocardia* species, was explainable as there was low correlation between 16S rRNA gene similarity and interactivity profile (Davelos Baines, Xiao & Kinkel 2007; Vetsigian, Jajoo & Kishony 2011). The principle of reciprocity, as explained in the same journal, was also observed in our findings, where *Amycolatopsis sp*. PIP 207 seems to inhibit the growth of many different actinobacteria, and was inhibited by *Streptomyces* sp. PIP 168, which also produced antibiotics – i.e., mutual inhibition. Finally, we discovered both a 'sender'-centric actinobacteria able to induce the growth and aerial mycelia formation and sporulation of many *Streptomyces* species, and a 'receiver'-centric actinobacteria which were influenced by different actinobacteria. These extremes were also present in the soil community network in the aforementioned study.

Advances in understanding interactions in microorganism communities were only made available due to the pioneering work of researchers on which this work was based upon. As this was a screening for interaction, future studies would be focused on metabolic analysis to increase the capability of analysing and understanding the interaction which occurs between the species, as also conducted in other published work in this area. Therefore, the hypothesis of this study was answered based on the theoretical mode of interactions observable from macroscopic changes which occurred during the microorganism interaction.

Most research in interaction often describes microbial induction to be caused by (1) cell to cell contact, (2) small chemical signalling, (3) enzymatic activation of precursors

from a producer species, and/or (4) horizontal genetic transfer between species (Abdelmohsen *et al*. 2015; Bertrand *et al*. 2014; Ochi & Hosaka 2013). Evidence of these, in actinobacteria, is not lacking (Dashti *et al*. 2014; Hoshino, Wakimoto, *et al*. 2015) – however, the reality is evidently more complex, and intriguing, than what is described. For instance, the co-culture between *Streptomyces* sp. and *Tsukumurella pulmonis* in the production of alchivemycin A was concluded to be a result of interaction with mycolic acid, which was a component of the cell wall, by conducting interaction with mycolic acid deficient mutant cells (Onaka *et al*. 2011). In *Streptomyces lividans* with several mycolic acid bacteria, however, the killing of the bacterial cell prevented the aggregation of *Streptomyces sp*. to the cell wall, and nullifying the induction (Asamizu *et al*. 2015). As the cell wall was not damaged in the process, it can be speculated that chemical signal may be directing the co-aggregation of the two co-cultures. Thus, the interaction process can be a combination between both cell to cell contact and chemical signalling (Schroeckh *et al*. 2009), making the isolation and elucidation of a responsible component difficult.

Thus, this description, while accurate, is a simplification of the complex concepts which occur in an interaction. However, these points are useful as a guideline in categorizing and understanding the interactions which occur in our case, and should be considered in further studies to elucidate the interaction discovered in this study. The interaction between *Streptomyces sp*. EUM 76, for instance, can be observed to occur even without a direct cell to cell contact, as seen in the interaction with *Streptomyces* sp. EUC 63 and CAP 288 (figure 3.4 a and c, respectively), suggesting that a chemical signal might be responsible for the interaction. However, as it cannot be assumed that each interaction would have the same mechanism, concurring with studies by Traxler *et al*. (2012), it could therefore also be interpreted that the induction in the form of increased sporulation and antibiotic production, in this case, is an indirect result of nutrient limitation (Ohnishi, Seo & Horinouchi 2002; Rigali *et al*. 2008).

Our main interest, however, is the ‘sender’-centric inducer, *Pseudonocardia sp*. PIP 161, which is able increase sporulation of 17 actinobacteria, as well as increase antibiotic activity on 9 *Streptomyces* species. However, at this point, it is unclear whether the interaction requires direct cell to cell contact or a result of chemical signalling. Clearly, more research to determine the inducing mechanism of *Pseudonocardia* sp. in an effort to understand methods to ‘awaken sleeping genes’ is required.

3.4 Conclusion

This screening process indicates a wide range of interaction occurs between actinobacterial species, whereas 45 of both promotion and inhibitory interactions were detected out of 388 combinations of actinobacteria. A ‘receiver’-centric actinobacteria, *Streptomyces* sp. EUM 76, was seen to produce aerial mycelia and sporulation in co-culture with 9 different actinobacteria from three different genera. Interestingly, a ‘sender’-centric actinobacteria, *Pseudonocardia* sp. PIP 161, was seen to be able to induce sporulation and antibiotic production in at least 17 different actinobacteria. This study did not explore the metabolic changes that occur in an interaction, and therefore limiting analysis to determine the mechanism of the interaction that was observed. Therefore, future studies which discuss the molecular or metabolic changes in co-culture are required.

CHAPTER 4. THE INTERACTION BETWEEN *STREPTOMYCES* SP. EUC 63 WITH *PSEUDONOCARDIA* SP. PIP 161 IN THE INDUCTION OF ANTIBIOTICS

4.1 Introduction

In the previous chapter, co-culture of non-*Streptomyces* and *Streptomyces* uncovered 45 interactions out of 388 possible combinations of pairs of actinobacteria cultures. *Pseudonocardia* sp. PIP 161, which was more extensively tested, was able to induce the formation of aerial hyphae and sporulation, and/or increased antibiotic production in at least 17 different actinobacteria in solid medium. While any change in morphology is considered to be an interaction, the detection of increase in antibiotic activity indicate that an alteration in the metabolic profile occurred. This was in support of previous research, in which co-culture was found to be able to 'awaken cryptic genes' in search of unique novel compounds with high bioactivity (Abdelmohsen *et al*. 2015; Bertrand *et al*. 2014).

In *Streptomyces* species, several global regulators had been identified in previous literature, including AdpA, regulated by γ-butyrolactones (Ohnishi *et al*. 2005), AfsR, a member of the *Streptomyces* antibiotic regulatory protein (SARP) (Horinouchi *et al*. 1990; Lee, P-C, Umeyama & Horinouchi 2002; Tanaka, A *et al*. 2007), cyclic AMP receptor protein (Crp) (Gao, C *et al*. 2012), DasR (Rigali *et al*. 2008), AbsA1/A2 two component system regulators (Anderson, Brian & Champness 2001; Santos-Beneit, Rodríguez-García & Martín 2012) and others, as reviewed previously (van Wezel & McDowall 2011). Literature studies has shown that the activation of these receptors by specific external signals either activates biosynthetic cluster regulators (as in the case of AfsR and Crp, for instance (Gao, C *et al*. 2012; Tanaka, A *et al*. 2007)), or by releasing its repressing activity on the biosynthetic clusters that it regulates (as was the case in DasR, and AbsA2 (Craig *et al*. 2012; Rigali *et al*. 2008; Santos-Beneit, Rodríguez-García & Martín 2012)), resulting in production of multiple secondary metabolites.

The link between morphogenesis and secondary metabolite production have been explained in other research, in which multiple regulatory cascades have been found to regulate development of *Streptomyces* as well as activate secondary metabolism. For example, A-factors, which are γ-butyrolactones produced by many *Streptomyces* species, accumulate in a growth-dependent fashion, and activate AdpA, a

transcriptional activator which controls streptomycin production (Horinouchi & Beppu 1992b; Ohnishi, Seo & Horinouchi 2002), and daptomycin production in *Streptomyces roseosporus*, via *atrA* expression (Mao *et al*. 2015). AdpA, however, was found to also regulate morphological development, including aerial mycelium formation, septum formation, and sporulation (Higo *et al*. 2012; Ohnishi *et al*. 2005).

The activation of secondary metabolite biosynthetic clusters, regulated by AdpA, for instance, or other global regulatory systems, was often controlled by cluster-situated regulators (van Wezel & McDowall 2011). Studies in *Streptomyces coelicolor* reveal at least five known cluster-situated regulators for five known antibiotics produced by this species (actinorhodin, undecylprodigiosins, calcium-dependent ionophore antibiotic (CDA), methylenomycin (MM), and the partially characterized cryptic polyketide (CPK)) (Liu, G *et al*. 2013; van Wezel & McDowall 2011). The complexity of the regulation of these antibiotics, in particular CPK, was found to involve a specific γ-butryolactone (SCB) and the interplay of other global regulatory system, such as DasR (a negative repressor which was activated by N-acetyl-glucosamine) (Rigali *et al*. 2008), as well as the products of ACT and RED regulation (Liu, G *et al*. 2013).

The *in silico* assessment of AdpA binding sites (Guyet *et al*. 2014; Higo *et al*. 2012), showed the possibility of regulatory cross-links in the cluster-situated regulatory system for antibiotics. As with CPK activation, however, a certain combination of chemical signals, as would potentially be provided by co-culture, could theoretically activate previously unexpressed genes. Observation of metabolic changes after co-culture would be the first step in understanding the mechanism of the interaction. However, while antibiotic activity was found to be increased in co-culture with *Pseudonocardia* sp. PIP 161 (suggesting alteration in secondary metabolite production), metabolic profiles between interactions were not explored. Therefore, in this study, the metabolic changes in several *Streptomyces* species due to the interaction of *Pseudonocardia* sp. PIP 161 were analysed in more detail.

4.2 Methodology

4.2.1 Spore suspension

From prepared glycerol stocks, 50 µl of *Streptomyces* sp. EUC 63, *Streptomyces* sp. PIP 146, *Streptomyces* sp. PIP 201 and *Pseudonocardia* sp. PIP 161 was inoculated and spread using a sterile metallic loop on to ISP-2 plate. This plate was incubated for 3 to 7 days until good sporulation was observed. Using a sterile loop, the surface

of the plate were gently scraped off and inserted into a sterile cryotube containing 1 ml of sterile 50% glycerol, and stored at -20°C until further use.

4.2.2 Liquid cultivation of monocultures and co-cultures

From the prepared spore suspensions, 50 µl each of *Streptomyces* sp. EUC 63, *Streptomyces* sp. PIP 146 and *Pseudonocardia* sp. PIP 161 was inoculated and spread separately using a sterile metallic loop onto an ISP-2 plate and incubated for 5 days. Four 1 x 1 cm squares were cut and used to inoculate a 250 ml flask containing 50 ml IM-22 liquid growth medium (composed of glucose (anhydrous) 15 g/l, soya meal 15 g/l, Corn steep liquor 5 g/l, NaCl 5 g/l, $CaCO_3$ 2 g/l, pH adjusted to 7.2±0.2 prior to autoclaving), and incubated on a rotary shaker at 150 rpm at 27°C for 5 days.

On the 5^{th} day, 2 ml was transferred into a new 250 ml Ehrlenmeyer flask containing 50 ml of liquid ISP-2 medium. For co-culture, the same volume was transferred from IM-22 medium into 50 ml of ISP-2 broth medium after *Streptomyces* species cultures were inoculated. These were placed on a rotary shaker at 150 rpm for 5 days for *Streptomyces* sp. EUC 63, and 7 days for *Streptomyces* sp. PIP 146 monocultures. For scale up production followed by HPLC analysis, 5 flask for each treatment (monoculture and co-culture) were used, which were pooled for extraction.

4.2.3 Metabolite extraction from liquid cultures

At the day of the harvest, each flask was transferred into a sterile 50 ml Falcon tube, and centrifuged at 2500 rpm for 20 minutes. Each tube was then passed through a Whatman no. 1 filter paper into a clean flask. The pellet was then removed from each tube using 10 – 14 ml methanol pooled into one flask. The volume was then adjusted to 70 ml using methanol. The broth was placed in a 250 ml flask and 70 ml ethyl acetate added, Flasks were then shaken overnight (120 – 150 rpm).Ethyl acetate extracts were separated from the broth using a separating funnel. Mycelial methanol extracts were separated into two falcon tubes (at 35 ml each) and centrifuged at 3000 rpm for 10 minutes, and the pellets discarded. One Falcon tube was transferred to 2 ml Eppendorf tubes at 1.8 ml/ tube. These tubes were then concentrated and evaporated for 6 hours. Each 2 eppendorf tubes were then pooled into one, and these tubes were stored in -20°C until further use. The un-concentrated samples were also stored in -20°C until further use.

4.2.4 Thin layer chromatography (TLC) and Scale-up TLC

Thin layer chromatography was performed using a silica gel 60 F_{254} aluminium sheet (Merck) cut into a 10 x 10 cm square. The TLC plates were activated in a 70°C incubator for 15 – 30 minutes before use. Samples were spotted onto the TLC plate, and after drying the plate, it was eluted with ethyl acetate:methanol (4:6 and 7:3). The solvents were run 1 cm from the top of the plate, and the retention factor (R_f) (distance of band divided by distance of solvent) of each band was measured, and observed under UV-light, at 254 nm and 365 nm wavelengths, in which each band was marked using a pencil. A bioautogram was used to test for antibiotic activity, as stated below.

Preparative TLC applies the same basic methodology. However, the plate was cut in to a 20 x 10 cm rectangle, and 200 µl of the concentrated samples was spotted per silica plate. After running in the appropriate solvent and marking the bands at 254 nm and 365 nm wavelength, a 1 x 10 cm strip was cut off and tested for activity against *S. aureus* using bioautogram. The corresponding active bands within the active R_f range were scraped off and extracted using 1 ml of methanol, by vigorous shaking for 10 minutes. After centrifugation at 10,000 g for 30 seconds, the supernatant was transferred into a fresh tube and the solvent evaporated for 2 – 3 hours. The sample were then re-tested for antibiotic activity.

4.2.5 Bioautogram

The TLC plate as prepared above was cut to fit on a 90 mm petri dish containing *S. aureus*, as described in Chapter 3. Areas where no samples (below the area where the samples were spotted and the area after the solvent front) was cut, and the plate placed with the aluminium sheet facing upwards and the silica gel in direct contact with the medium. At the back of the plate, each visible band and direction of the plate was marked. The plates were left in a sterile compartment to allow sample diffusion for at least 30 minutes. On the same plate, a well containing the 40 µl of the original samples in the TLC plate was added as a control. The TLC plates were then carefully removed using flame sterilized tweezers, and the plates placed in 37°C overnight. Zones of inhibition would be matched with initial retention times to observe which bands with bioactivity.

4.2.6 High Performance Liquid Chromatography (HPLC) conditions

HPLC of mycelial extracts of *Streptomyces* sp. EUC 63 in monoculture and co-cultured with *Pseudonocardia* sp. PIP 161, as prepared in 4.2.3, was conducted in Microbial Screening Technologies, Sydney. In this case, each sample contained in the Eppendorf tubes were freeze dried and sent for analysis. These were reconstituted in Methanol and run through analytical HPLC (column used was Alltima, 53 mm x 7 mm C-18 column, with a 3 µm pore size), followed with a photodiode array analyser. The solvent system used was acetonitrile-0.01% TFA and MilliQ water-0.01% TFA, with a gradient system of 0 – 7 minutes, 0 to 100% acetonitrile-0.01% TFA, followed by a 2 minute holding time and a 3 minute re-equilibration to 10% Acetonitrile-0.01% TFA. The peaks were measured at 205 nm, 210 nm, and 230 nm.

4.2.7 "Preconditioning" of media

4.2.7.1 Preconditioning in solid medium

This method for preconditioning was modified from Vetsigian *et al*. (2011). For solid medium "preconditioning", a sterile rectangle cellophane was placed on the surface on the medium. Co-culture of *Streptomyces* sp. 201 and *Streptomyces* sp. EUC 63 with *Pseudonocardia* sp. PIP 161 was subcultured on the left and right area of the cellophane, by transferring 50 µl of the respective cultures from glycerol stocks, and spreading using a loop. The plates were then incubated for 7 days in 27°C. After 10 days, the cellophane was carefully removed, and 10 µl of *Streptomyces* species cultures were spotted onto different areas of the plate: in the area where *Pseudonocardia* sp. PIP 161 was spread, the area where *Streptomyces* species was spread, the juncture of *Pseudonocardia* sp. PIP 161 and *Streptomyces* species, and outside the cellophane area. After 30 minutes air-drying, the plates were then incubated in 27°C and observed daily for changes in sporulation and/or pigmentation. The test was conducted in triplicates.

4.2.7.2 Preconditioning in liquid medium

This method was similar to liquid cultivation as described in 4.2.2, with several modification. Firstly, the inducer species, *Pseudonocardia* sp. PIP 161 was subcultured in IM-22 for 5 days, as described previously, and 4 ml transferred in three 500 ml flasks containing 100 ml ISP-2. At this time, four cubes of *Streptomyces* sp. EUC 63 was transferred into IM-22 from plates and all flasks were incubated in 27°C, and shaken at 150 rpm. At the fifth day, broth and mycelia of *Pseudonocardia* sp. PIP

161 was separated using a Whatman no. 1 filter paper, and the broth was transferred into a new sterile 500 ml flask by filter sterilization (with a 0.4 µm injection filter). The nutrient was reconstituted by adding 10 ml of a 10x concentrated ISP-2, and the volume returned to 100 ml by adding 40 ml of sterile RO water. Lastly, 4 ml of *Streptomyces* sp. EUC 63 was added from IM-22 flasks and the flasks incubated at 27°C for 5 days. This was followed by extraction protocol as described in 4.2.3.

4.2.8 Colony selection methodology

From *Streptomyces* sp. EUC 63 glycerol stocks, 50 µl was streaked on an ISP-2 plate in obtain single colonies. After 5 day incubation at 27°C, based on its sporulation (well sporulated or not), colony colour (red or white), and colony size, 22 colonies were selected and streaked on a new ISP-2 plate and re-incubated in the same conditions, in which the back colour and sporulation was noted, and made into a spore suspension, as described in 4.2.1.

To assess the interactivity, six 10 µl spore suspension of *Streptomyces* sp. EUC 63 were spotted on the left and right side of an ISP-2 plate at approximately 0.5 cm from the edge, and 1 cm distance between spots in one column, and approximately 3 cm distance between spots in one row. Adjacent (with a 0.1 – 0.3 cm gap) with the *Streptomyces* sp. EUC 63 spots in the right column, 10 µl spore suspension of *Pseudonocardia* sp. PIP 161 were spotted. This was done for all selected colonies, except colony 20 due to contamination. The plates were incubated at 27°C for 5 days, and graded for sporulation (0 = no spores, 1 = sparse spores (<25% of colony covered), 2 = >25% and <80% colony covered, 3 = >80% colony covered, but < 100%, and 4 = 100% colony covered and fully developed) and pigmentation scores viewed from the back (0 = no red pigments, 1 = <25% had red pigment, 2 = >25% and <80% colony had red pigments, and 3 = >80% had red pigments), and checked for back colour. After the 5th day, the plates were measured for antibiotic activity using the flip plate assay.

4.2.9 Flip plate antibiotic assay

Refer to chapter 3.

4.2.10 Statistical analysis

All statistical analyses were done using the student t-test (one tailed, equal variance) in Excel, in which values were considered significant if the p value is <0.05.

4.3 Results and Discussion

4.3.1 Co-culture increased antibiotic activity of *Streptomyces* sp. EUC 63 or *Streptomyces* sp. PIP 146 with *Pseudonocardia* sp. PIP 161 in liquid cultivation

Preliminary and secondary screening from solid medium shows several positive interactions between several *Streptomyces* species and *Pseudonocardia* sp. PIP 161, the latter as the inducer. Considering that (a) larger amounts of sample for analysis could be more feasibly acquired in liquid cultivation compared to solid medium (Bertrand *et al*. 2014) and (b) most of the interaction phenomenon observed in solid medium did not necessarily reflect processes in liquid medium, liquid cultivation was tested for several of the interactions. Therefore, antibiotic compounds against *S. aureus* produced extracellularly were obtained from the broth culture and compared between monocultures and co-culture as a gauge for successful interaction in liquid medium.

Varying results were obtained, as noted in several interactions where extracellular antibiotics against *S. aureus* were produced in equally low or high levels between monocultures and co-cultures. *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146, however, showed increased antibiotic activity in co-culture compared to monocultures, and was therefore further scaled up and extracted in order to also determine intracellular levels of antibiotic production, as shown in figure 4.1.

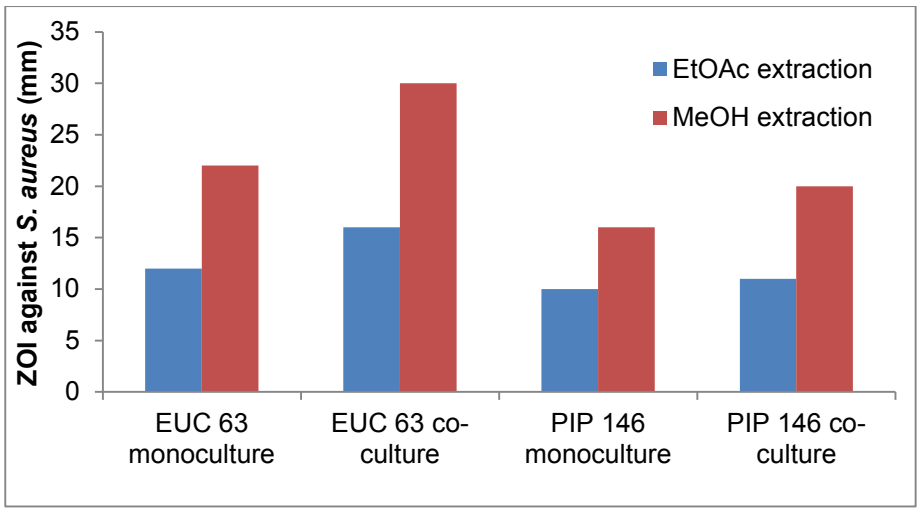


Figure 4.1 Antibiotic activity from ethylacetate and methanol extracts of *Streptomyces* sp. EUC 63 and PIP 146 liquid cultures in mono-cultures and co-cultured with *Pseudonocardia* sp. PIP 161, extracted at day 5 and day 7, respectively.

Comparison of concentrated broth ethyl acetate extracts and methanol mycelial extract between monoculture and co-culture showed a 1.5 and 2-fold increase in antibiotic activity against *S. aureus* by *Streptomyces* sp. PIP 146 and *Streptomyces* sp. EUC 63, respectively, indicating that most of the antibiotic was located intracellularly. As equal volumetric ratio and treatment applied in the extraction protocol between monoculture and co-cultures were identical, it can be concluded that the increase of antibiotic production between monocultures and co-cultures was due to the interaction with *Pseudonocardia* sp. PIP 161. Furthermore, as these interactions were also obtained in solid medium, it was assumed that the interaction between these two species may indicate that similar genes were expressed in both solid and liquid medium.

4.3.2 Co-culture increases multiple antibiotic compounds produced by *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146

Preliminary efforts to detect and eventually isolate the antibiotic compound were first conducted using thin layer chromatography (TLC) followed by a bioautogram against *S. aureus*. TLC comparisons between EtOAc and MeOH extracts show similar bands in an ethylacetate:methanol (7:3) solvent system, both from *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146 in monoculture and co-culture. As antibiotic activity was higher in mycelial extracts, the efforts to isolate the antibiotic compound were mainly focused on mycelial extracts.

Interestingly, TLC of intracellular metabolites showed different metabolite profile between monocultures and co-cultures in both *Streptomyces* species, as shown in table 4.1, indicating that the possibility that *Pseudonocardia* sp. PIP 161 interaction influenced secondary metabolite production pathways. Although only a slight difference in the number of bands between co-culture and monocultures in both *Streptomyces* species tested was observed, this consisted of bands with different R_f values. With *Streptomyces* sp. EUC 63, for instance, the co-culture with *Pseudonocardia* sp. PIP 161 resulted in the 7 different bands compared to the monocultures visible at 254 nm, and 3 new bands at 365 nm. However, several bands visible in monocultures (such as 0.09 and 0.12 in 254 nm and 0.42 and 0.47 in 365 nm) were not found in co-culture.

Furthermore, few bands were observed differently between monocultures and co-cultures which was hypothesized to be a result of changes in the metabolite profile. An example was band 0.91 in Streptomyces sp. EUC 63 which was visible in monoculture at 254 nm and co-cultures in both wavelength, but not in monocultures at 365 nm wavelength. A possible explanation was that there were two different compounds having the same Rf value of 0.91. One was the band that was visible at 254 nm in both monocultures and co-cultures, and the other band that emerged only in co-culture which was visible at 365 nm.

Table 4.1 Thin layer chromatography (TLC) using an Ethylacetate:Methanol (7:3) elution system of monocultures and co-cultures of *Streptomyces* species and *Pseudonocardia* sp. PIP 161

Species	Treatment	Σ of bands	R_f at 254 nm	R_f at 365 nm
Streptomyces sp. EUC 63	Monoculture	11	0.09, 0.12, 0.45, 0.68, 0.91	0.42 – 0.47, 0.68, 0.71, 0.77, 0.83
	Co-culture	14	0.38 – 0.45, 0.60, 0.68, 0.73, 0.77, 0.82, 0.83, 0.87, 0.91	0.25 – 0.32, 0.77, 0.91
Streptomyces sp. PIP 146	Monoculture	9	0.17, 0.51, 0.74, 0.77, 0.83	0.51, 0.71, 0.88, 0.94
	Co-culture	11	0.10, 0.17, 0.53, 0.60, 0.71, 0.77, 0.87	0.10, 0.51, 0.83, 0.94
Pseudonocardia sp. PIP 161	Monoculture	2	0.77	0.88

†*Pseudonocardia* sp. PIP 161 monocultures thin layer chromatography was conducted in a previous sample
‡R_f values in red showed difference in co-culture with *Pseudonocardia* sp. PIP 161 compared to monoculture

Comparison between *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146 in co-culture with *Pseudonocardia* sp. PIP 161, shows reoccurring bands with an R_f of 0.60, 0.77, and 0.87 in 254 nm. As these bands were not detected in *Pseudonocardia* sp. PIP 161 monoculture bands, the detection of these bands in the co-culture extracts of both species may be coincidental.

Another possibility that can be explored in future experiments was that a two-way interaction occurred between *Pseudonocardia* sp. PIP 161 and other *Streptomyces* species. In typical co-culture experiments, the origin of the compounds produced in the co-culture was difficult to ascertain; co-culture of *Nocardiopsis* sp. RV163 and *Actinokineospora* sp. EG49, for example, resulted in the production of new compounds that were not visible in monocultures of both species (Dashti *et al*. 2014), as was the case in several other examples (Adnani *et al*. 2015; Taniguchi *et al*. 1998; Wang, Y *et al*. 2014; Zuck, Shipley & Newman 2011). While this information has been acquired in other studies by analysing gene expression in co-culture conditions (such as using microarrays), chemical analysis might suggest metabolic changes in both cultures occurs despite having a single producing culture (Schroeckh *et al*. 2009; Traxler *et al*. 2013b; Watrous *et al*. 2013).

Previous findings in this study had discovered that a lower level of antibiotic production was observed in monocultures of *Streptomyces* species, while no antibiotic activity was seen in *Pseudonocardia* sp. PIP 161. Therefore, it can be assumed that antibiotic production mostly originated from *Streptomyces* species, despite possible metabolic changes in the inducer species. To measure changes in antibiotic production, a bioautogram against *S. aureus* was conducted on the same TLC plate, as shown in the figure 4.2.

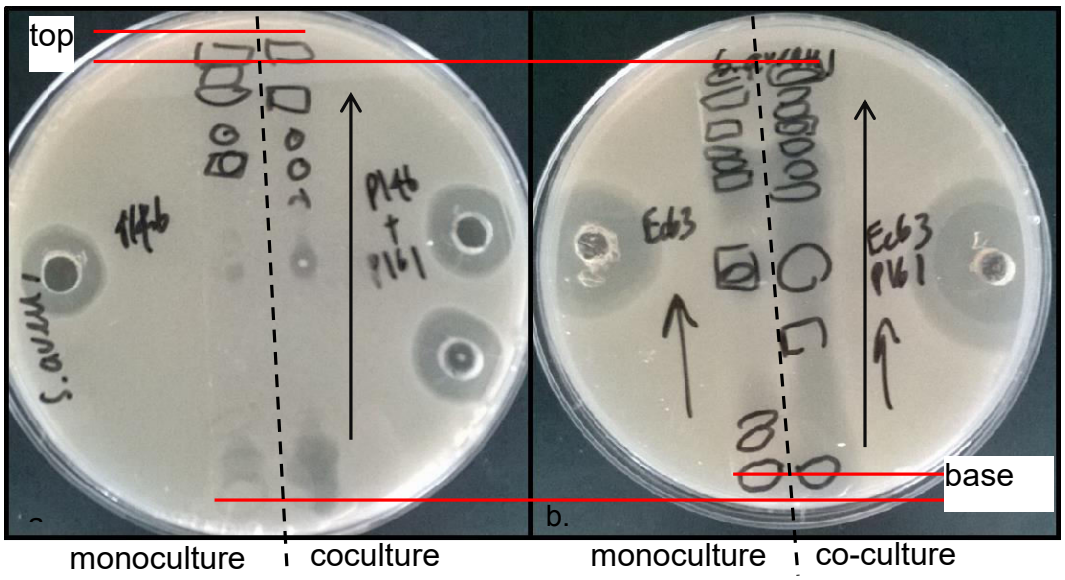


Figure 4.2 Bioautogram of mycelial methanolic extracts (a) *Streptomyces* sp. PIP 146 and (b) *Streptomyces* sp. EUC 63 in monoculture and co-cultured with *Pseudonocardia* sp. PIP 161 (left and right bands, respectively).

It was found that a different response to co-culture was observed between co-culture of *Pseudonocardia* sp. PIP 161 and the two tested *Streptomyces* species. Multiple bands of compounds with antibiotic activity were observed from both *Streptomyces* sp. PIP 146 and *Streptomyces* sp. EUC 63, in monoculture and co-culture. Interestingly, co-culture resulted in the increase of antibiotic production in multiple bands, indicating multiple compounds were upregulated in the presence of *Pseudonocardia* sp. PIP 161. This was further supported by the results of antibiotic testing on individual TLC fractions, as is shown in Table 4.2.

Table 4.2 Antibiotic activity of TLC fractions of *Streptomyces* species in monocultures and co-cultures with *Pseudonocardia* sp. PIP 161

Sample	Fraction number	R_f range of fraction	Antibiotic activity against *S. aureus* after extraction
Streptomyces sp. EUC 63 monoculture (E:M 7:3)	1	0 – 0.41	0
	2	0.78 – 0.81	10
	3	0.81 – 1.0	11
Streptomyces sp. EUC 63 co-cultured with *Pseudonocardia* sp. PIP 161 (E:M 7:3)	1	0 – 0.15	10
	2	0.18 – 0.60	n.t ‡
	3	0.65 – 0.76	11
	4	0.80 – 0.83	0
	5	0.87 – 0.95	14

Streptomyces sp. PIP 146 monoculture (E:M 4:6)	1	0 – 0.13	0
	2	0.13 – 0.26	6.5
	3	0.26 – 0.39	11
	4	0.39 – 0.52	7
Streptomyces sp. PIP 146 co-cultured with *Pseudonocardia* sp. PIP 161 (E:M 4:6)	1	0 – 0.13	8
	2	0.13 – 0.25	11
	3	0.25 – 0.38	13
	4	0.38 – 0.51	12

†A scaled up TLC was used for to obtain fractions, resulting in slightly different bands to appear.
‡n.t = not tested, fractions were not extracted due to lack of activity in scaled up bioautogram experiment

While, as aforementioned, co-culture was observed to increase antibiotic production in all bands in both *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146, antibiotic activity was detected in a specific R_f range. For *Streptomyces* sp. EUC 63, the highest antibiotic activity was observed in fraction 3 and fraction 5, in monoculture and co-culture, respectively, within the R_f range of 0.81 – 0.95, thus indicating that the antibiotic compounds were relatively non-polar. On the other hand, the highest antibiotic activity in *Streptomyces* sp. PIP 146 was observed in fraction 3 for both monoculture and co-culture, therefore showing the compound to be relatively polar with an R_f of 0.25 – 0.39 in the solvent system used.

4.3.3 HPLC photodiode array analysis shows co-culture with *Pseudonocardia* sp. PIP 161 alters metabolism levels in *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146

Previous TLC results observed the increase of the production of multiple metabolites in co-culture. In order to analyse the interaction in more detail, a more sensitive technique was required. Thus, the extracts, which were treated to have the same concentration of compounds as the original extract, were further analysed in using HPLC-DAD in order to understand the extent of the induction, as was observed in figure 4.3 (a) and (b).

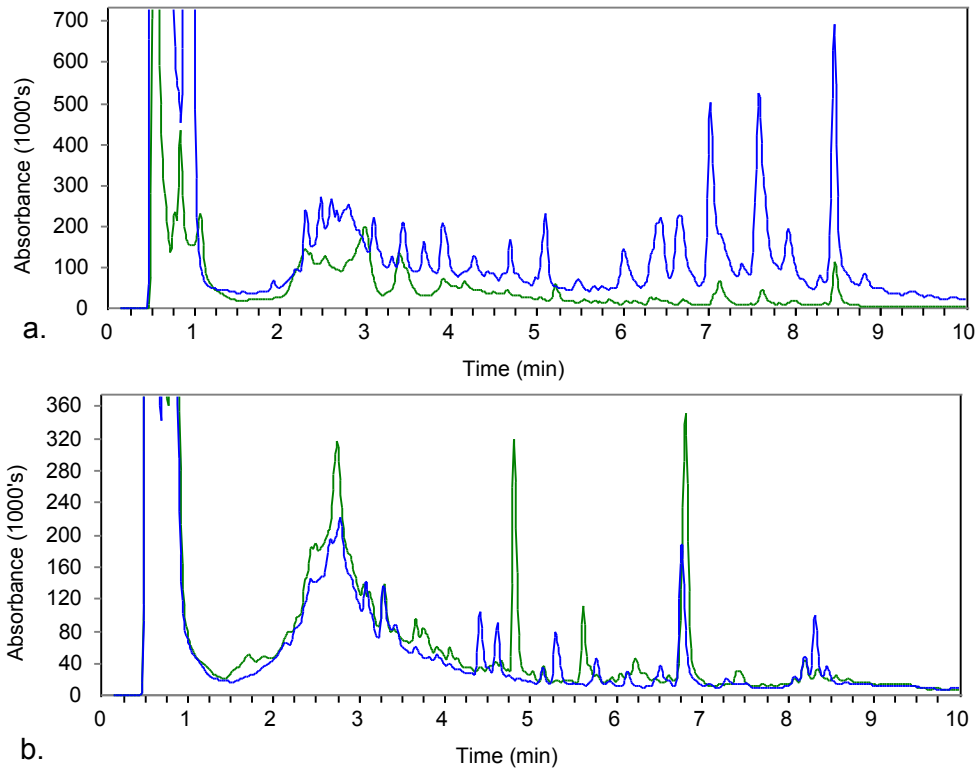


Figure 4.3 HPLC-DAD analysis overlay of mycelial methanolic extracts of (a) *Streptomyces* sp. EUC 63 monocultures (green) and co-cultured with *Pseudonocardia* sp. PIP 161 (blue) at 205 nm, showing increased metabolite production, and (b) *Streptomyces* sp. PIP 146 monocultures (green) and co-cultured (blue) with *Pseudonocardia* sp. PIP 161 at 205 nm, showing altered metabolite production

An analysis of the compounds produced in *Streptomyces* sp. EUC 63 reveals that the levels of several compounds were increased such as compounds with an R_t of 7.01, 7.56 and 8.46 minutes. Interestingly, these three compounds had similar UV-spectra (as shown in figure 4.4 a), indicating the possibility of a similar basic structure between these compounds. Enterobactins, a catecholate siderophore commonly produced from enteric bacteria, was found to have a UV spectrum within the range of these three compounds, with visible differences after 400 nm (as shown in figure 4.4 b), and similar retention time (around 7.4 minutes using HPLC with similar conditions) (Fiedler *et al*. 2001).

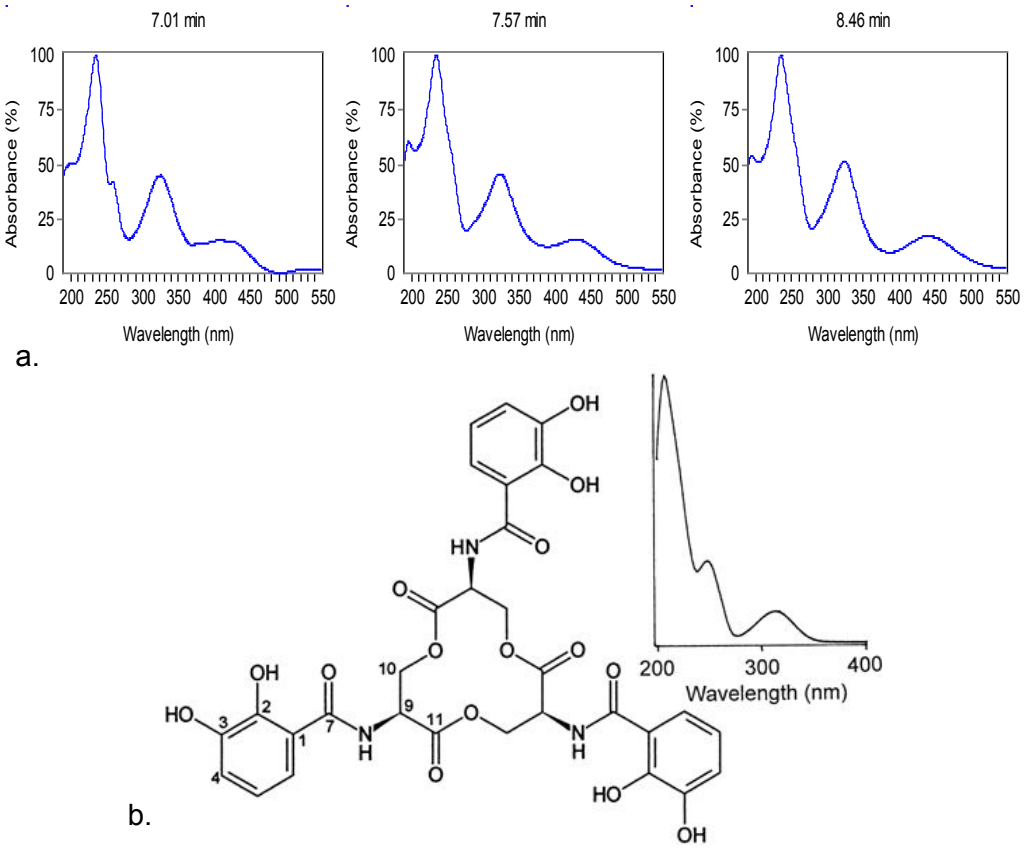


Figure 4.4 (a) UV spectrum of three major compounds found be increased in *Streptomyces* sp. EUC 63 and *Pseudonocardia* sp. PIP 161 co-cultures, at 7.01, 7.57, and 8.46 minutes, (b) enterobactin UV-spectrum and structure based on other literature (Fiedler *et al*. 2001)

In *Streptomyces* sp. EUC 63, co-culture was seen to increase the metabolite production ranging from a 2-fold increase (in compound at 2 minutes, for instance) to a 7-fold increase (in compound at 8.5 minutes). In *Streptomyces* sp. PIP 146, however, several compounds were either upregulated or downregulated. Furthermore, HPLC analysis of mycelial methanolic extracts of *Pseudonocardia* sp. PIP 161 (as displayed in Chapter 6) showed compounds that were mostly polar which did not correspond to any compounds in both *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146. Therefore, it was concluded that the changes of the profile of the peaks were due to induction.

These findings indicate that different species have different responses to co-culture with *Pseudonocardia* sp. PIP 161, suggesting that different metabolic pathways were affected in each species. This conclusion was supported by findings from other research which tested the interactivity of a single inducer species. For instance, in the study of the interaction between *Amycolatopsis* sp. AA4 and five different *Streptomyces* species resulted in different sporulation and morphogenesis

responses, and different metabolite pathways being activated (Traxler *et al*. 2013b). Unique and unequal bioactivity or secondary metabolite production was also observed in the interaction between *Mycobacterium* sp. with different marine *Micromonospora* spp., *Solwaraspora* spp., and *Verrucosispora* spp., also supporting this conclusion (Adnani *et al*. 2015).

The co-culture of *Pseudonocardia* sp. PIP 161 with *Streptomyces* sp. EUC 63, as mentioned previously, showed an increase of most of the compounds produced compared to monocultures. While each peak in co-culture seems to correspond with a peak in monocultures, albeit at a lower concentration, it appears that no novel compounds were produced in co-culture when compared to monocultures. However, as an increase in metabolism was observed, the increased levels allowed for better assessment for secondary metabolites produced as well as increased antibiotic activity.

Secondary metabolite synthesis, in particularly antibiotics, are known to be arranged in biosynthetic clusters, whereas its expression would be regulated by a specific regulators acting on these clusters (Bibb 2005). Secondary metabolite production typically occurs at the late stage of the *Streptomyces* life cycle or in conditions of environmental stress, indicating the presence of regulators which controls the expression of multiple secondary metabolite clusters, or so called global regulators (Gao, C *et al*. 2012; van Wezel & McDowall 2011). The activation of these global regulators would activate pathway-specific regulators (or release the repression of these regulators), and result in the production of secondary metabolites (such as pigments, antibiotics, or siderophores).

Enterobactin, a catecholic siderophore commonly produced by enteric bacteria, is biosynthetically dependent on NRPS activation, and required for the transport and utilization of iron from the environment (Fiedler *et al*. 2001; Raymond, Dertz & Kim 2003). The production of siderophores, such desferrioxamine and enterobactins, were often found to be tied to iron availability (Günter, Toupet & Schupp 1993; Moelling *et al*. 2007). Interestingly, desferrioxamine was found to also be controlled under DasR global regulators, in which the release of DasR increases *dmdR1* which encodes iron uptake repressors, and therefore lowering siderophore production (Craig *et al*. 2012).

While recent studies discovered that a metabolic cross-link exists between enterobactin production and other chorismate-derived secondary metabolites, (Cano-Prieto *et al*. 2015), currently no studies have explored the catecholic siderophore global regulation in *Streptomyces*. However, based on this information, it can be speculated that the increase in multiple metabolite production in co-culture of

Streptomyces sp. EUC 63 may indicate that *Pseudonocardia* sp. PIP 161 induces secondary metabolite production by acting on an global regulatory system.

4.3.4 Antibiotic production from *Streptomyces* sp. EUC 63 can be induced by *Pseudonocardia* sp. PIP 161 in cell-free interaction

In an effort to understand the mechanism of the interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* sp. EUC 63, the identification of the 'inducer' produced by *Pseudonocardia* sp. PIP 161 must be achieved. In regards with previous findings (refer to Chapter 3), whereas sporulation in *Streptomyces* sp. PIP 201 was increased in co-culture with *Pseudonocardia* sp. PIP 161 without direct cell to cell contact, it was hypothesized that a diffusible compound from *Pseudonocardia* sp. PIP 161 acted as an inducer. In order to prove this hypothesis a *Pseudonocardia* sp. PIP 161 cell-free interaction was carried out.

Firstly, to replicate and observe the previous interaction more clearly, a preconditioned medium was prepared as per previously described methodology (Vetsigian, Jajoo & Kishony 2011). In this method, the medium was 'preconditioned' with metabolites from the inducer species, *Pseudonocardia* sp. PIP 161, on cellophane to prevent the presence of vegetative mycelia. To account for the presence of autoregulators, the medium was also preconditioned with the test cultures, e.g, *Streptomyces* sp. PIP 201 or *Streptomyces* sp. EUC 63. Areas were then outlined using a marker at the back of the plate upon cellophane removal, to mark areas where each culture was dominant.

The results show that sporulation of *Streptomyces* sp. PIP 201 was achieved when spotted in areas preconditioned with *Pseudonocardia* sp. PIP 161 or in areas where both *Pseudonocardia* sp. PIP 161 and *Streptomyces* sp. PIP 201 were co-cultured. On the other hand, no sporulation was observed when *Streptomyces* sp. PIP 201 was spotted on areas preconditioned with *Streptomyces* sp. PIP 201, nor in areas outside the preconditioning, as a control, as shown in figure 4.5a.

Similarly, in *Streptomyces* sp. EUC 63, increased pigmentation was achieved when spotted in areas preconditioned with *Pseudonocardia* sp. PIP 161 or in areas where *Pseudonocardia* sp. PIP 161 and *Streptomyces* sp. EUC 63 mixed, as shown in figure 4.5b. Although sporulation occurred in all spots, increased pigment production was only observed in cultures spotted those aforementioned areas. Therefore, based on this result, it can be concluded that the inducer from *Pseudonocardia* sp. PIP 161 was indeed a producible compound, at least in solid medium.

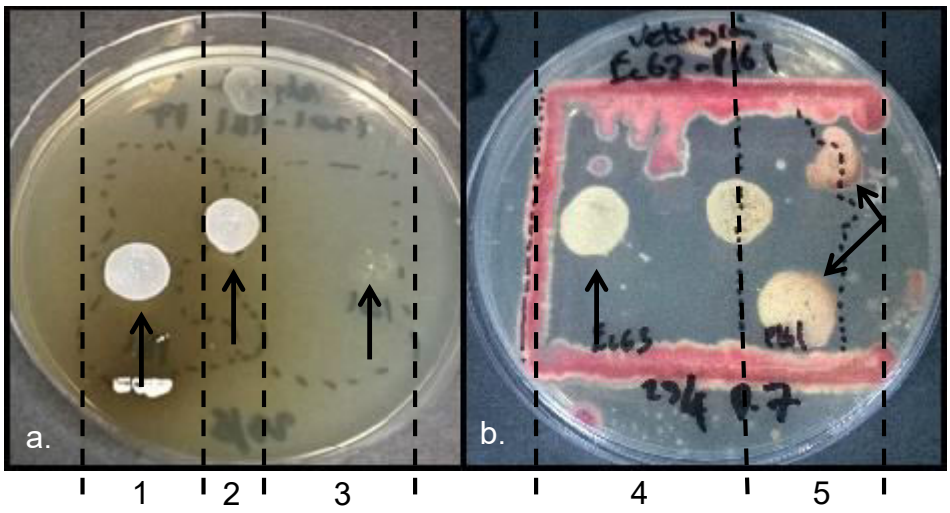


Figure 4.5 “Preconditioned” solid media induces sporulation in (a) *Streptomyces* sp. PIP 201, viewed from the top, with (1) areas preconditioned with *Pseudonocardia* sp. PIP 161, (2) areas preconditioned with both *Streptomyces* sp. PIP 201 and *Pseudonocardia* sp. PIP 161, and (3) areas preconditioned with *Streptomyces* sp. PIP 201. , ; in (b) *Streptomyces* sp. EUC 63, viewed from the bottom of the plate, with (4) areas preconditioned with *Pseudonocardia* sp. PIP 161 and (5) preconditioned with *Streptomyces* sp. EUC 63. Arrows depict point of interest, with sporulation observed in *Streptomyces* sp. PIP 201 in (1) and not in (3), and pigment production

To determine whether the inducer compounds were produced in liquid medium, a similar test was carried out, as described in methodology. The results show similar production of antibiotic in preconditioned medium to co-culture with *Pseudonocardia* sp. PIP 161, further corroborating previous results, as shown in figure 4.6. These results were similar to initial results, wherein co-culture (and in this case, preconditioned medium) significantly increased antibiotic production compared to monocultures in *Streptomyces* sp. EUC 63.

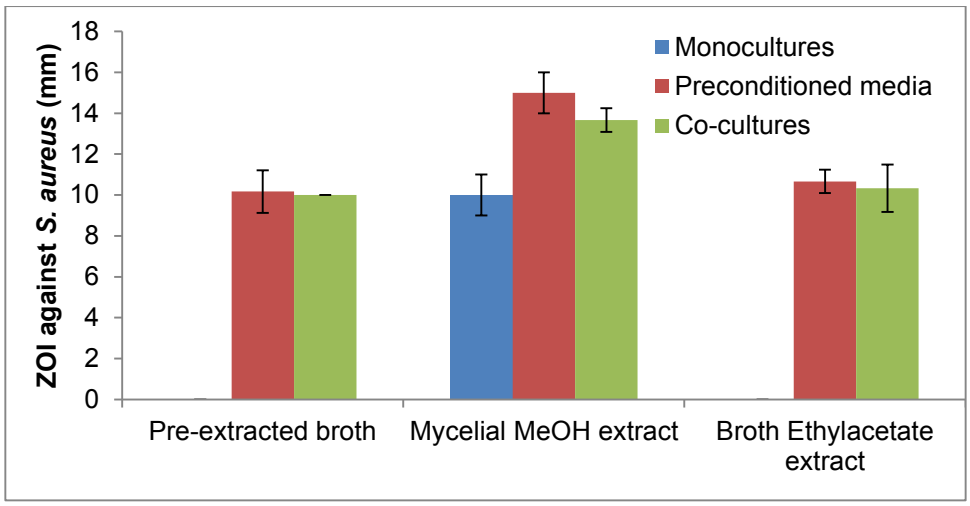


Figure 4.6 Comparison of antibiotic production from *Streptomyces* sp. EUC 63 monocultures, subcultured in *Pseudonocardia* sp. PIP 161 preconditioned liquid medium, and co-culture with *Pseudonocardia* sp. PIP 161.

Comparison of visual characteristics of *Streptomyces* sp. EUC 63 in monoculture, co-culture and in preconditioned liquid medium also shows that successful induction, at least at some level, was observed in "preconditioned" medium, as shown in table 4.3. In preconditioned medium, it was observed here that while mycelial colour was more similar to the monoculture compared to co-culture, the colour of the extracts were more similar to the co-culture. This also suggests that the "preconditioned" medium was able to induce multiple compounds, aside from antibiotic activity. The darker hue that was observed in broth, however, can be attributed to the re-nutrition, which was done by adding a sufficient amount of concentrated ISP-2 medium ingredients.

Table 4.3 Observable characteristics of *Streptomyces* sp. EUC 63 morphogenesis and extract between treatments

Sample or treatment	Mycelial colour	Broth colour	Broth EtOAc extract colour	Mycelial MeOH extract colour
Streptomyces sp. EUC 63 monoculture	Cream	Light orange	Colourless	Light orange
Streptomyces sp. EUC 63 in preconditioned medium	Cream	Dark red (due to re-nutrition)	Pink-red/ orange	Dark orange
Streptomyces sp. EUC 63 co-cultures	Red	Dark orange	Light orange	Dark red-orange

Based on these results, it was concluded that the induction from *Pseudonocardia* sp. PIP 161 was in the form of a secreted compound(s), thus supporting multiple studies in this area (Abdelmohsen *et al*. 2015; Bertrand *et al*. 2014; Scherlach & Hertweck

2009). Differing from several researchers (Onaka *et al*. 2011), these findings show that, at least in the production of antibiotics in *Streptomyces* sp. EUC 63, physical cell to cell interaction was not required in this interaction. For example, Scherlach and Herweck (2009), suggested that elicitation of cryptic biosynthetic pathways could be a result of chemical signals sent by the producer compound to the inducer compound, resulting in a different set of chemical signals inducing biosynthetic pathways in the producer. Furthermore, other research indicates that this kind of exchange was present in various interacting species, as shown by metabolic analysis (Traxler *et al*. 2013b; Watrous *et al*. 2013).

In this study, however, it was observed that monocultures of *Pseudonocardia* sp. PIP 161 was able to induce antibiotic production in *Streptomyces* sp. EUC 63, as well as sporulation and morphogenesis in *Streptomyces* sp. PIP 201, without the presence of the producer species. This enables the compound to be isolated and applied to other cultures in order to observe the extent of the interaction, as well as understand the mechanism of the interaction itself. Another added benefit, was that it could lessen the complexity of co-cultivation in the production of antibiotics in a larger scale, as it would bypass several hurdles when dealing with co-culture, such as culture compatibility and culture condition optimisation (Bertrand *et al*. 2014; Scherlach & Hertweck 2009). Efforts to isolate the inducing compounds from *Pseudonocardia* sp. PIP 161 are described in chapter 6.

4.3.5 Colony selection reveals varying degrees of morphogenesis, antibiotic production and induction responses

To achieve the best results in subsequent experiments, a colony selection step was introduced for *Streptomyces* sp. EUC 63, by testing single colonies for observable phenotypic characteristics. The selection criteria were based primarily on pigment production, sporulation level, and the morphological variation (such as, for instance, the roughness or smoothness of the culture surface on the day of observation). While the same criteria were applied in *Pseudonocardia* sp. PIP 161, no observable differences were seen between each colony. Therefore, this process was mainly focused on *Streptomyces* sp. EUC 63.

Colony selection resulted in 22 single colonies, showing various levels of pigment production, as observed in figure 4.7. Large variation in pigmentation was observed in these different colonies, ranging to very low production of pigments (colony 5, 10, 18, and 19), to very high pigment production (colony 8 and 16). Colony 18 showed a

low level of growth, but was still included in further tests. Colony 20 resulted in heavily contaminated by bacteria and was not used in further tests.

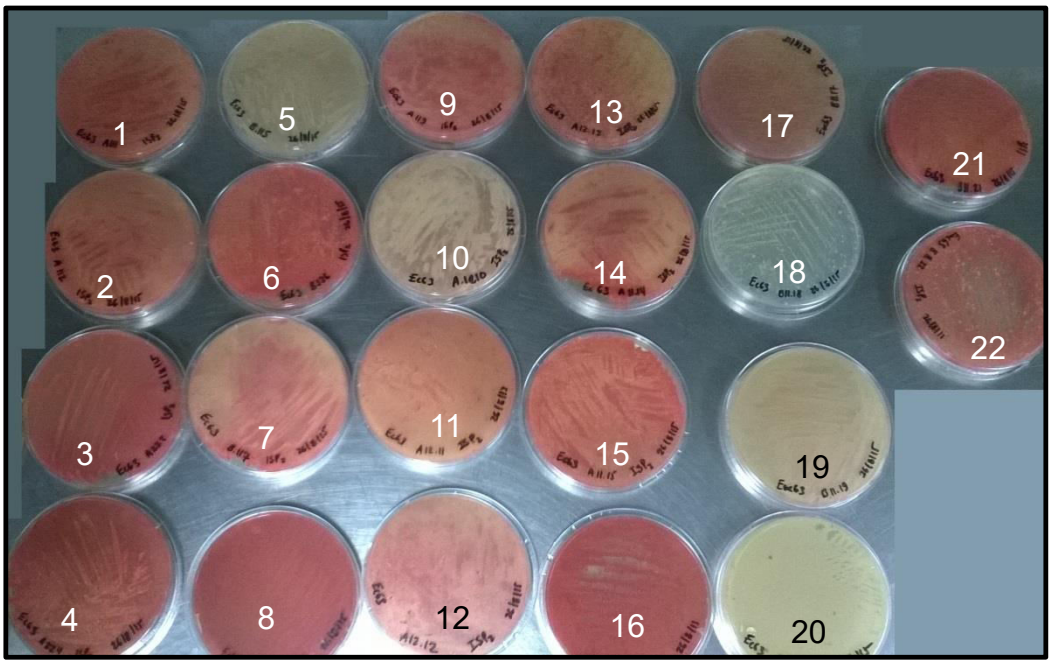


Figure 4.7 Colony selection shows varying degrees of morphogenesis and pigment production levels. Each plate represents a single colony. Colony numbering was in vertical order starting from the top left corner.

These colonies were then tested for individual response to interaction, by spotting each colony with *Pseudonocardia* sp. PIP 161 and observing changes in pigmentation and sporulation, as shown in figure 4.8, with results stated in table 4.4. Again, various responses to *Pseudonocardia* were observed, based on these criteria. Several cultures show increased sporulation when in co-culture, however, several colonies show opposite or no response to induction. Colony 3, 10, 11, 13, 15, and 19, for instance formed spores in both monocultures and when in co-culture. On the other hand, colony 5 and 17 formed spores in monocultures which were inhibited by co-culture. While colony 18 showed low growth in monoculture and co-culture, a slight increase of growth can be observed when in conjunction with *Pseudonocardia* sp. PIP 161.

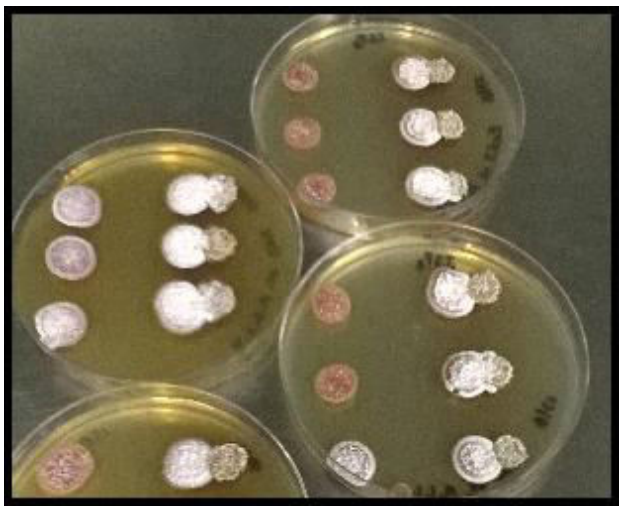


Figure 4.8 An example of colony selection shows varying degrees of morphological changes and responses to interaction with *Pseudonocardia* sp. PIP 161. Each plate represents individual colony interactivity response, whereas monocultures were placed in the left-hand side of the plate, and co-cultures were placed in the right-hand side.

Table 4.4 Characteristics of *Streptomyces* sp. EUC 63 colonies in co-culture with *Pseudonocardia* sp. PIP 161 compared to monoculture at day 6

Colony #	Mono-culture			Co-culture		
	Aerial spores	Colour (back)	Pigment score	Aerial spores	Colour (back)	Pigment score
1	2 – 1[†]	Cream	2 – 1	3	Red	3
2	1	Cream	1	3	Red	3
3	3	Cream	1	3	Dark red	3
4	2	Cream	1 – 3	3	Dark red	3
5	3	Red	3	2	Cream	1 – 2
6	1 – 3 (v)[‡]	Red	1 – 3 (v)	3	Red	3
7	1	Cream	1	3	Red	3
8	0 – 3 (v)	Cream	0 – 2 (v)	3	Red	3
9	3 – 2	Cream	1	3 – 2	Red	3 – 2
10	4	Cream	1	4	Red	3
11	4	Cream	1 – 2	4	Red	3
12	2	Cream	1	3	Red	3
13	3	Cream	1	3	Red	3
14	3	Cream	1	2 – 3	Red	3 – 2
15	3	Red	3	3	Red	3
16	2	Red	3 – 1	2	Red	2
17	4	Red	2	3	Red	3
18	0	Cream	0	0	Cream	0
19	4	Red	3	4	Red	3
21	1	Cream	1	3	Red	3
22	1 – 2	Cream	1 – 3	2	Red	3

Scores for sporulation was as follows: 0 = no spores, 1 = little/ sparse spores (<25% of colony covered), 2 = >25% and <80% colony covered, 3 = >80% colony covered, but < 100%, and 4 = 100% colony covered and fully developed. Pigmentation scores (from the back) was as follows, 0 = no red pigments, 1 = <25% had red pigment, 2 = >25% and <80% colony had red pigments, and 3 = >80% had red pigments.

†The dominant of three replicates was mentioned first, with the second score showing the single variance (for example, 1 – 3 would mean 1, 1 and 3).
‡(v) denotes high variability, whereas each showing different score within that range (for example, 1 – 3(v) would mean a 1, 2, and 3 score, while 1 – 3 would mean 1, 1 and 3).

Finally, using the flip plate method, antibiotic activity was tested and compared between monocultures and co-culture with *Pseudonocardia* sp. PIP 161, as shown in figure 4.9. In most colonies, regardle ss of the level of sporulation and pigmentation, a significant ($p<0.05$) increase of antibiotic production was observed in co-culture compared to monocultures. However, colony 4 and colony 19 did not show significant increase of antibiotic activity in co-culture compared to monoculture, while colony 5 showed a decrease of antibiotic activity in co-culture; however, this difference was not significant. The five highest average of antibiotic production was observed in colony 4 (24±3.4 mm), colony 6 (23.3±0.6 mm), colony 1 (22.3±2.5 mm), colony 3 (22 mm) and colony 11 (21.7±1.2). The largest difference between monoculture and co-culture was observed in colony 10, as monocultures did not show any antibiotic activity, followed by colony 11 and colony 8, while the lowest was colony 21 and colony 6. Colony 18 had low production of antibiotics, reflective of the low growth, which was only slightly improved by co-culture.

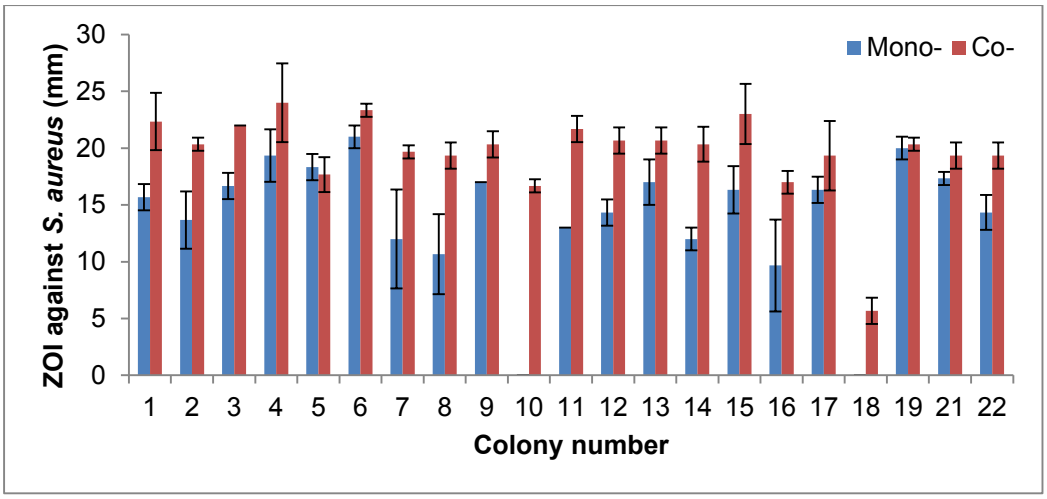


Figure 4.9 Antibiotic production produced in different selected colonies between monoculture and co-culture with *Pseudonocardia* sp. PIP 161.

Based on this, the colony with the best interactivity, at least when viewed in its antibiotic producing capacity, was used in future experiments. Colony 10 although showing high interactivity, lost red pigment formation (as seen in figure 4.7), and had different characteristics compared to the original culture. Colony 11 had the second largest difference between monoculture and co-culture antibiotic production,

indicating good interactivity with *Pseudonocardia* sp. PIP 161, as well as showing higher activity after co-culture compared to colony 10. Therefore, colony 11 was chosen for further tests.

4.4 Conclusion

Pseudonocardia sp. PIP 161 produces a diffusible chemical signal which influences multiple *Streptomyces* species differently. Co-culture with *Pseudonocardia* sp. PIP 161 results alterations in the metabolic profile, which in some cases increases the production of antibiotics and siderophores, and increase sporulation in *Streptomyces* sp. EUC 63 and *Streptomyces* sp. PIP 146. This allows increased visibility of compounds that were previously produced in undetectable concentrations in monocultures, although it is unlikely that novel compounds were produced. Lastly, it was hypothesized that *Pseudonocardia* sp. PIP 161 produces an extractable compound which caused the activation of a pleiotropic global regulatory protein in *Streptomyces* species.

4.5 Acknowledgements

We would like to extend our gratitude to Dr Ern Lacey at Microbial Screening Technologies for running HPLC-DAD experiments, as well as his help in identifying several compounds produced in this study.

CHAPTER 5. THE EFFECTS OF IRON ON THE PRODUCTION OF SECONDARY METABOLITES IN MONOCULTURE AND CO-CULTURES OF *PSEUDONOCARDIA* SP. PIP 161 WITH TWO *STREPTOMYCES* SPECIES

5.1 Introduction

Co-culture in microorganisms has previously been shown to be able to stimulate or induce secondary metabolite gene clusters, resulting in the production of compounds which are uniquely produced (Bertrand *et al*. 2014; Hopwood 2013; Traxler *et al*. 2013b; Watrous *et al*. 2013). While the majority of the studies focus on the product of the interaction rather than the inducers, some advances in discovering and isolating the inducers has been accomplished. For example, some studies found the interaction to be contributed by cell wall components, i.e, mycolic acid or its associated components (Asamizu *et al*. 2015; Hoshino, Wakimoto, *et al*. 2015; Onaka *et al*. 2011), or quorum sensing molecules, i.e, acyl-homoserine lactones (Kitani, Hoshika & Nihira 2008; Recio *et al*. 2004). However, further studies on factors influencing the interaction are clearly needed.

In a natural setting, the interaction between different microorganisms is mostly a bid for dominance or co-existence, which may manifest in efforts to secure various growth limiting nutrients (Hibbing *et al*. 2010). Iron is considered an essential requirement in normal housekeeping functions of microorganisms, as well as having a role in sporulation (Purohit, Sassi-Gaha & Rest 2010; Traxler *et al*. 2012), biofilm formation (Modarresi *et al*. 2015), and even secondary metabolite or antibiotic production (Béchet & Blondeau 1998; Coisne, Béchet & Blondeau 1999). An efficient uptake of iron, therefore, not only would be necessary for the survival of a microorganism, but can also provide an advantage in co-existing in a microbial community.

In the environment, however, iron resides in its Fe (III) state, which is insoluble, and thus inaccessible by microorganisms. Microorganisms bypass this by producing siderophores: iron carriers capable of binding to the iron in its insoluble state, transferring the iron into the cytosol, which would then be converted into a soluble Fe (II) state (Neilands 1995; Saha *et al*. 2015). Thus, under conditions of iron deficiency, a natural response would be to increase siderophore production (Nakouti, Sihanonth & Hobbs 2012). Instead, recent research has revealed microorganism favour up-regulation of siderophore uptake proteins (Moelling *et al*. 2007; Mulé *et al*. 2015).

Therefore, in a bacterial community, the possibility of one microorganism taking up siderophores produced by another microorganism would be quite high. Hibbing *et al* (2010) described this phenomenon as 'social cheaters' – microbes which would be able to benefit from the availability of a required nutrient, without expending the energy to acquire the nutrients. While inter-kingdom examples of siderophore 'piracy' have been reported (Johnson 2008), other studies highlight the involvement of actinobacteria (Arias *et al*. 2015; Galet *et al*. 2015), thus suggesting that the phenomenon is common.

Previous studies suggest that the genetic clusters responsible for the production of siderophores also contain ferric-siderophore uptake systems which were siderophore-specific (Barona-Gómez *et al*. 2006; Bunet *et al*. 2006; Schalk, Mislin & Brillet 2012). Furthermore, multiple siderophore uptake systems may exists in a single microorganism, supporting the uptake of different siderophores from different sources, whereas specific siderophores were more likely to result in the increase of growth and morphogenesis (Tierrafría *et al*. 2011). Therefore, the absorption of specific siderophores which enables iron acquisition might be from different sources determined by the compatibility of each species. As some studies discovered that the lowering of the iron content would up-regulate siderophore uptake receptors (Moelling *et al*. 2007; Mulé *et al*. 2015; Traxler *et al*. 2012), the likelihood of xeno-siderophore uptake in interacting species would be high.

The addition of iron and/or the production of siderophores were found to be correlated with the appearance of morphogenesis: the increased growth and sporulation rate, morphology differences, and/or pigment production (Lambert *et al*. 2014; Traxler *et al*. 2012). As these changes were also observed in the co-culture of *Pseudonocardia* sp. with other *Streptomyces* species in this study, it can be hypothesized that iron levels would stimulate these changes. Inspired by Traxler *et al* (2012), this chapter attempts to discover how monocultures and co-cultures would respond to various iron levels, and thus provide a possible explanation for the interaction mechanism which was previously discovered. In order to accomplish this, the effects of iron content in the interaction between *Pseudonocardia* sp. PIP 161 and two *Streptomyces* species were studied. In this case, *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36, were selected as both species show pigment production as well as antibiotic activity.

5.2 Method

5.2.1 Spore suspension

From prepared glycerol stocks, 50 µl of *Streptomyces* sp. EUC 63 colony 11 (refer to chapter 4), *Streptomyces* sp. SC 36, and *Pseudonocardia* sp. PIP 161 was inoculated and spread using a sterile metallic loop on to ISP-2 plate. This plate was incubated for 7 days until good sporulation was observed. Using a sterile loop, the surface of the plate were gently scraped off and inserted into a sterile cryotubes containing 1 ml of sterile 50% glycerol, and stored in -20°C until further use.

5.2.2 Siderophore production using CAS assay

Siderophore production of actinobacteria isolated was measured using chrome azurol S (CAS) assay (Alexander & Zuberer 1991). A dark blue indicator solution (solution 1) was first made by adding 10 ml of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl), 50 ml of CAS at 1.21 mg/ml and 40 ml of Hexadecyl-trimethylammonium bromide (CTAB) at 1.82 mg/ml. A buffer solution (solution 2) consisting of 30.24 g PIPES (piperazine-N,N'-bis 2-ethanesulfonic acid) in 750 ml salt solution (0.3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl) was prepared and its pH adjusted to 6.8 using 50% KOH. This was added with 15 g of agar and added with 50 ml of RO water. A nutrient solution (solution 3), consisting of 70 ml water, 2 g gluose, 2 g mannitol, 493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.4H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 1.2 mg $ZnSO_4.7H_2O$ and 1.0 mg $Na_2MoO_4.2H_2O$ was then made, and all prepared solution was then autoclaved and cooled to 50°C. Solution 3 was then added into the buffer solution, mixed and added with solution 4 (solution 4 consists of 30 ml of 10% (w/v) casamino acids) via filter sterilisation. This was mixed properly and added with solution 1. The solution was mixed again and poured on to the plates. After solidifying, the isolates were spotted (10 µl) on the four corners of the plate at from prepared spore suspension. Positive reaction was indicated by an orange halo surrounding the colony. The diameter of this halo was then measured in millimetres.

5.2.3 Subculture in different iron levels

5.2.3.1 Media preparation

The media used is ISP-2 with different iron levels. This was prepared by making ISP-2 medium as described previously, autoclaved and cooled down to 50°C. A stock solution of 100 mM, 10 mM, and 1 mM of $FeCl_3$ was then made by mixing 162.2 mg of $FeCl_3$ or 270.3 mg of $FeCl_3.6H_2O$ in 1 ml water (to make 1 M), which was

consecutively diluted to prepare the stock solution. These stocks were then filter sterilized in a new sterile tube. To make ISP-2 media supplemented with 0.1, 1, 10, and 200 µM of $FeCl_3$, from the stock solution 100 µl, 1 ml (from 1 mM stock solution), 1 ml from (from 10 mM stock solution), and 200 µl (from 100 mM stock solution), respectively, were each transferred per litre of medium into tubes containing ISP-2 medium and mixed prior to pouring onto plates. In this experiment, ISP-2 was added with 10 µM and 200 µM $FeCl_3.6H_2O$, while the other concentrations were made with $FeCl_3$ (anhydrous).

Similarly, a stock solution was made using the same method on 2,2-dipyridyl, in which 150.3 mg was diluted in 1 ml water, and was diluted into similar stock solutions (100 mM, 10 mM and 1 mM). From the proper stock solutions the same method was used to supply ISP-medium with 2,2-dipyridyl with a concentration of 1, 5, 20, 50, 200 and 400 µM prior to pouring in to plates. Using this method, the insertion of small volumes (10 µl or less) in making $FeCl_3$ and 2,2-dipyridyl dilutions in ISP-2 medium was avoided.

5.2.3.2 Primary screening with a 'Ueda configuration'

To test for colony diameter in different iron levels, 10 µl of cultures were spotted from prepared stock solutions on the medium using the Ueda configuration, as explained in chapter 3 (refer to 3.2.6). This resulted in a spot of 10 mm, which would serve as a baseline for all cultures used. Each treatment was done in triplicates. Observations were made on day 3, 5 and 7, in which the colony diameter (in mm) of the *Streptomyces* species were measured in monocultures and co-cultures. Morphological changes were also observed in which the edges of the colony were noted for serration or smoothness, colony pigment colour on the reverse plate, and roughness or smoothness of the colony surface. Finally, sporulation was scored based on the spore distribution on the colony, whereas (-) was used for no sporulation, (±) for sporulation in less than 50% of the colony, and (+) for more than 50% of the colony.

5.2.3.3 Co-inoculation of cultures in solid medium

Co-inoculation method was used to scale up production in solid medium, and was based on a method used in Microbial Screening Technologies, Sydney. From the spore suspension (refer to 5.2.1), 50 µl transferred using a cut tip and was spread on a single ISP-2 plates to form a lawn of growth, and incubated for 27°C for 7 days.

Using a sterile metallic triangle or scalpel, 1 x 1 cm blocks were cut from each *Streptomyces* culture and transferred into an autoclaved 250 ml flask containing 50 ml of water in sterile conditions. The flasks were then shaken vigorously until spores appeared to be dislodged from the agar blocks. Using a cut tip, transfer 1 ml of the spores in water suspension into an ISP-2 plate, in which the plates were gently stirred to spread the inoculum. For co-culture, this was overlaid with 1 ml of *Pseudonocardia* sp. PIP 161 culture. The plates were air dried for 0.5 to 1 hour under a laminar flow. These were done in triplicates. These plates were then incubated in 27°C for 10 days, followed by methanol extractions for antibiotic assay.

5.2.4 Metabolite extraction from solid medium

After 10 days, the scale up plates (refer to 5.2.3.3) were extracted with methanol by first cutting the three plates into small blocks, using a scalpel or a metallic triangle, and transferred into a single flask containing 150 ml of methanol. The flasks were then covered with aluminium foil and shaken for 4 hours at 150 rpm on a rotary shaker. The extracts were then filtered using a Whatman no. 1 under vacuum conditions to remove agar blocks. The extracts were evaporated until all the methanol was evaporated (approximately one-sixth of the volume remain). The extracts were then equilibrated to the same volume (30 ml) using 60% methanol in water and tested for antibiotic activity. The extracts were temporary stored in -4°C cold room until further analysis.

5.2.5 Antibiotic assay of methanol extracts

Agar plates containing *S. aureus* was prepared as previously described (refer to 3.2.7), and poured. After the plates were solidified, a heat sterilized 6 mm cork-borer was used to make 10 wells per bacteria plates. Each well was filled with 40 µl of the extracts (refer to 5.2.4), incubated at 37°C overnight, in which the zone of inhibition was measured.

5.3 Results and Discussion

5.3.1 Siderophore levels in several actinobacteria

The induction of antibiotic production in several actinobacteria was found in previous screening studies, some of which suggest that these interactions between

actinobacteria were mediated by siderophores. Therefore, several actinobacteria which previously showed an interaction (refer to chapter 3) were then screened for siderophore production, using the CAS assay (Alexander & Zuberer 1991; Shin *et al*. 2001). The zone of the siderophore produced was measured to gauge the quantity and relative strength of the siderophore produced, as shown in table 5.1. As a positive control, we used *Streptomyces* sp. Lup 30, which was previously found to produce siderophores (Le 2015).

Table 5.1 Detection of siderophore production using CAS assay after 10 days of incubation

Culture	Orange halo diameter
Streptomyces sp. Lup 30 (positive control)	18 mm
Pseudonocardia sp. PIP 161	31.5 mm
Pseudonocardia sp. EUM 212	29.5 mm
Pseudonocardia sp. CAP 111	Not detected
Amycolatopsis sp. PIP 207	25.5 mm
Micromonospora sp. EUM 48	Not detected
Micromonospora sp. CAP 181	Not detected
Micromonospora sp. EUC 38	Not detected

Siderophore production was detected for *Pseudonocardia* sp. PIP 161 and *Pseudonocardia* sp. EUM 212. *Amycolatopsis* sp. PIP 207 was also found to produce siderophores and inhibition was observed in previous screening with Streptomyces sp. CAP 230 (refer to Chapter 3), similar to the conclusions drawn in Traxler *et al*. (2012). Thus, as *Pseudonocardia* sp. PIP 161 was found to induce production of secondary metabolites in various *Streptomyces* species (refer to Chapter 3), it was possible that the mechanism of the interaction would be mediated by siderophores, as described by previous researchers (Traxler *et al*. 2012). Therefore, it was interesting to observe whether changes in *Streptomyces* species can be stimulated with alteration in iron levels. This part of the study follows previous research by Traxler *et al*. (2012), to determine whether different iron levels were able to stimulate the changes observed in co-culture.

5.3.2 The influence of co-culture in colony diameter and morphology of *Streptomyces* species

It was discovered that the co-culture of *Streptomyces* sp. SC 36 and *Streptomyces* sp. EUC 63 with *Pseudonocardia* sp. PIP 161 increased antibiotic production and sporulation in *Streptomyces* species. While increased changes in colony morphology was observed in *Streptomyces sp*. EUC 63 and *Streptomyces sp*. SC 36 in co-culture compared to monoculture, these differences were not always observed in colony diameter measurements, as shown in figure 5.1. As described in methodology, spotting of cultures resulted in a colony diameter baseline of 10 mm.

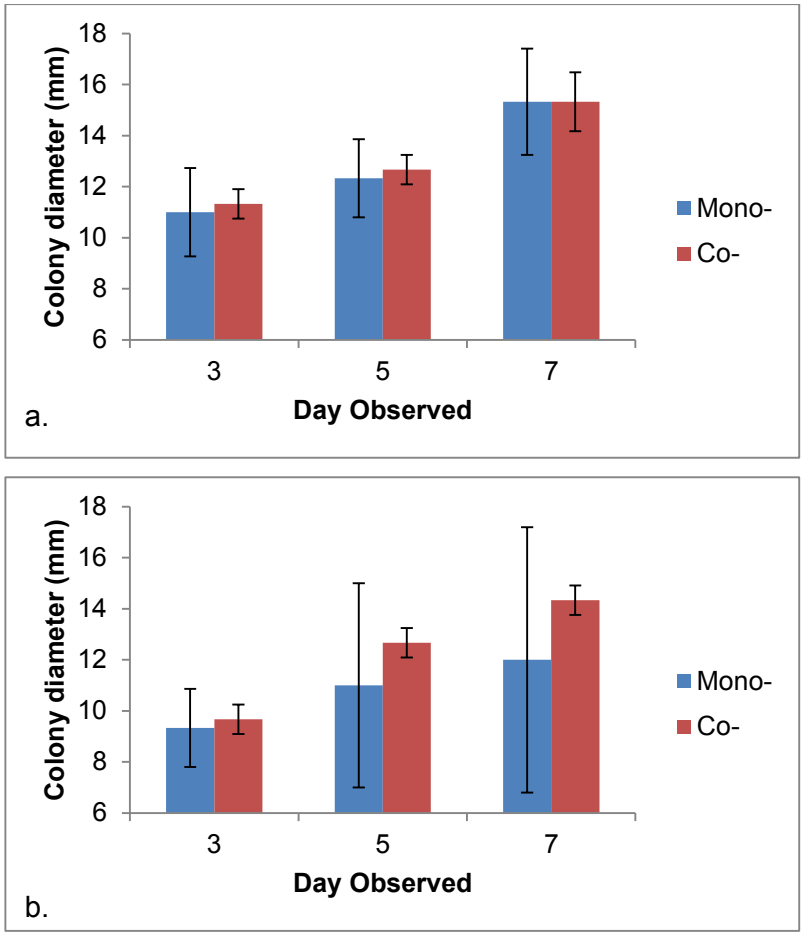


Figure 5.1 Colony diameter measurement of (a) *Streptomyces* sp. EUC 63 and (b) *Streptomyces* sp. SC 36, observed in day 3, 5, and 7, in monocultures and co-cultivated with *Pseudonocardia* sp. PIP 161. No difference was observed in *Streptomyces* sp. EUC 63 in co-culture compared to monocultures, but an increase in colony size was observed in co-culture of *Streptomyces* sp. SC 36.

In this case, colony diameter measurements show no significant difference between monocultures and co-cultures of *Streptomyces* sp. EUC 63 in day 3, 5 and 7, while *Streptomyces* sp. SC 36 showed a larger colony diameter in co-culture compared to monocultures (although these changes were not significant). Morphological

observations showed increased morphogenesis in co-cultures compared to monocultures. In *Streptomyces* sp. EUC 63, colony surface "roughness" (or rugose structures) differences were noticeable on co-cultures compared to monocultures on day 3 (as shown in table 5.2), while in *Streptomyces* sp. SC 36, these differences were observed on day 5 (as shown in table 5.3). A slight difference in the formation of aerial hyphae was observed in *Streptomyces* sp. EUC 63 co-culture compared to monoculture, wherein the difference was more noticeable in *Streptomyces* sp. SC 36. Furthermore, colony pigment formation was more pronounced in the co-culture of *Streptomyces* sp. SC 36 compared to monoculture.

In previous studies, early colony development and aerial hyphae or sporulation production was shown to be one characteristic that was indicative of a positive interaction (Seyedsayamdost *et al*. 2012; Traxler *et al*. 2013b; Vetsigian, Jajoo & Kishony 2011). Complex structures in colony development has been found to be a result of multiple regulation and differentiation signals, in which iron has been shown to play some part (Pelchovich, Omer-Bendori & Gophna 2013). In *Streptomyces*, the formation of wrinkles or a rugose surface was found to be related with iron concentration, and was hypothesized to provide a higher surface to volume ratio, allowing more oxygen intake (Dietrich *et al*. 2008; Traxler *et al*. 2012). Furthermore, pigmented compounds which were redox-active were also found in some way to influence the formation of wrinkles although this has not been elaborated (Dietrich *et al*. 2008), which may also contribute in iron metabolism. Therefore, we explore the role of iron in the interaction between these species.

5.3.3 *Streptomyces* sp. EUC 63 colony changes in co-culture and altered iron levels

Iron levels were adjusted either by supplementing $FeCl_3$ or $FeCl_3.6H_2O$ in equal molarities, or by adding 2, 2-dipyridyl, an iron chelator often used to simulate iron limitation. These were added to ISP-2, a complex medium that was mainly used in the production of the secondary metabolites from *Streptomyces* species. While this medium was not iron free, addition of external iron, or binding of available iron, would, to some extent, influence the morphogenesis and growth of the cultures tested. A range of iron concentrations (0.1, 1, 10 and 200 μM) was tested to stimulate iron excess, with a similar range for 2, 2-dipyridyl.

In *Streptomyces* sp. EUC 63, supplementation of iron had little effect on the colony diameter (figure 5.2) and morphogenesis (table 5.2). In conditions of iron excess, planar growth, determined by colony diameter, was lower compared to control, with

similar scores for monocultures and co-cultures. One explanation for this was iron-mediated oxidative stress as a result of free iron potentiating the conversion of reactive oxygen species (ROS) and hydroxyl radicals from hydrogen peroxides via the Fenton reaction (Cornelis *et al*. 2011; Touati 2000). ROS and other reactive species were known to cause DNA damage, lipid peroxidation, and protein carbonylation (Meneghini 1997), and therefore could influence bacteria survivability and growth.

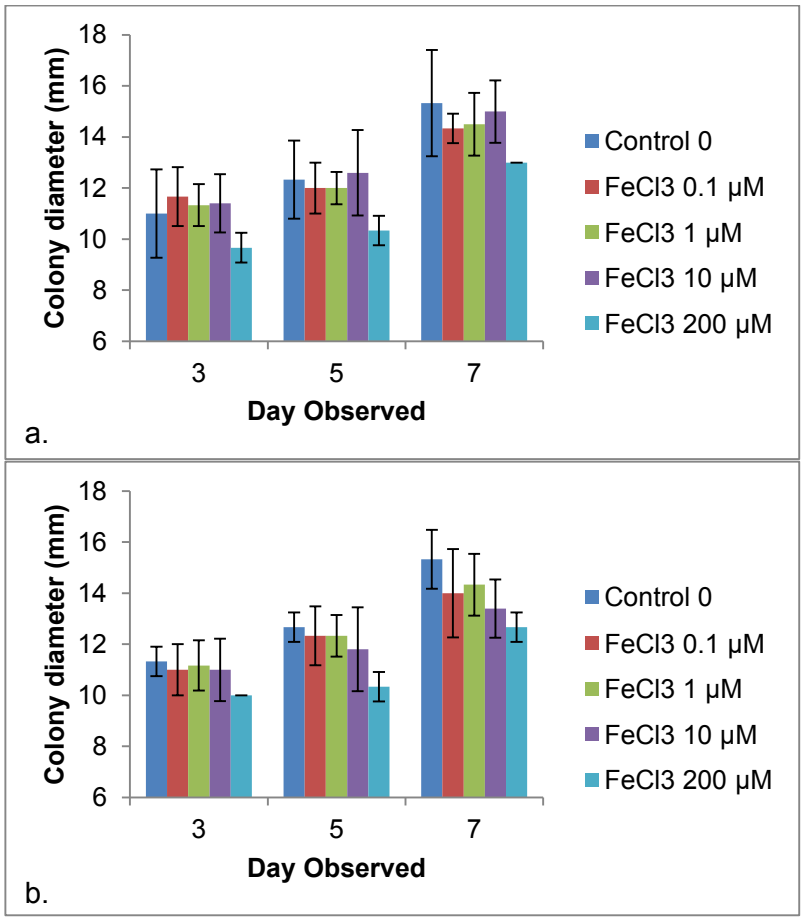


Figure 5.2 Colony diameter of *Streptomyces* sp. EUC 63 (a) monocultures and (b) co-cultured with *Pseudonocardia* sp. PIP 161 in high iron levels, observed in day 3, 5, and 7

A study of literature however, reveals that acquisition of iron was tightly controlled by microorganisms. As the Fenton reaction would often occur using free irons (Fe^{2+}), a tight regulation of iron would be necessary by microorganisms in order to maintain a concentration of iron intracellularly that would be beneficial for the growth. While siderophores were one way of regulating the iron intake and acquisition, most research in this area notice that iron excess would cause a down-regulation of siderophore uptake receptors, with many iron regulators having a DNA-binding component (Escolar, Pérez-Martín & de Lorenzo 1999; Kobayashi, Fujikawa &

Kozawa 2014; Yang *et al*. 2008). However, down-regulation of siderophores biosynthesis were found to have detrimental effects to growth and morphogenesis,

In low iron conditions, retardation of growth occurred after addition of 2,2-dipyridyl at concentrations as low as 5 μM, and complete inhibition occurred starting at concentration of 50 μM in *Streptomyces* sp. EUC 63 monocultures, which was not affected by co-culture (Fig 5.3). Morphogenesis of *Streptomyces* sp. EUC 63 in monocultures were mostly similar with co-culture under these conditions, with cultures retaining their smooth surface and edges in 2,2-dipyridyl, indicating that the formation of wrinkles and undulate/serrated edges requires iron, concurring with previous research (Dietrich *et al*. 2008; Traxler *et al*. 2012).

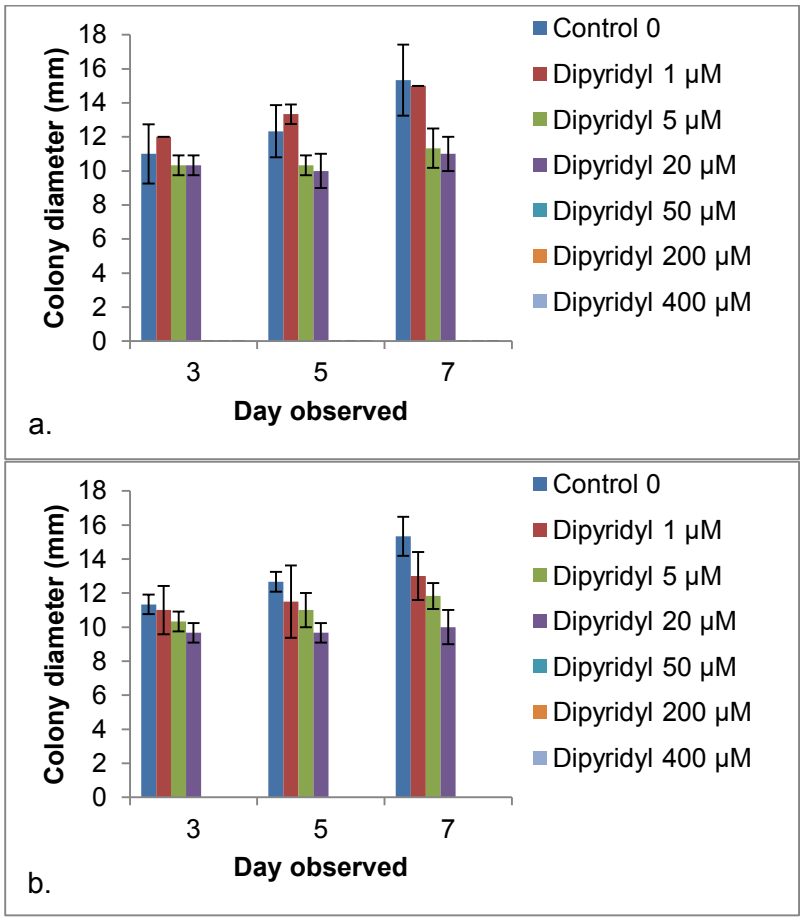


Figure 5.3 Colony diameter of *Streptomyces* sp. EUC 63 (a) monocultures and (b) co-cultured with *Pseudonocardia* sp. PIP 161 in low iron levels, observed in day 3, 5, and 7. Complete inhibition was observed after addition of 2,2-dipyridyl at 50 to 400 μM.

Finally, the formation of pink to red pigmentation which occurred between day 6 and 7 from *Streptomyces* sp. EUC 63 was seen to be produced both in monocultures and co-cultures in all conditions, with a co-cultivation resulting in pigments with a darker hue. Thus, the production of this pigment seems to be more reliant on co-culture

compared to iron regulation. Previous research (see chapter 4) revealed that the increase in production of pigments was followed by an increase in other secondary metabolites, therefore it can be concluded that iron manipulation alone cannot stimulate the production of unique compounds in *Streptomyces* sp. EUC 63 which were produced via co-culture.

Table 5.2 Observation of morphological changes in *Streptomyces* sp. EUC 63 in monocultures and co-cultures subcultured in ISP-2 media with iron surplus and limitation

Day	*Streptomyces* sp. EUC 63 in ISP-2 with the following treatments:	Monoculture				Co-culture with *Pseudonocardia* sp. PIP 161			
		Shape	Edges	Colour	Sporulation	Shape	Edges	Colour	Sporulation
5	Control	Rough	Smooth	None	±	Rough	Smooth	None	+
	+$FeCl_3$ 0.1 µM	Rough	Smooth	None	±	Rough	Smooth	None	+
	+$FeCl_3$ 1 µM	Rough	Smooth	None	±	Rough	Smooth	None	+
	+$FeCl_3$ 10 µM	Rough	Smooth	None	+	Rough	Smooth	None	+
	+$FeCl_3$ 200 µM	Rough	Smooth	None	-	Rough	Smooth	None	+
	+2,2-Dipyridyl 1 µM	Rough	Smooth	None	±	Rough	Smooth	None	+
	+2,2-Dipyridyl 5 µM	Rough	Smooth	None	±	Rough	Smooth	None	+
	+2,2-Dipyridyl 20 µM	Smooth	Smooth	None	-	Rough	Smooth	None	-
	+2,2-Dipyridyl 50 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-
	+2,2-Dipyridyl 200 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-
	+2,2-Dipyridyl 400 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-
7	Control	Rough	Serrated	None	+	Rough	Serrated	None	+
	+$FeCl_3$ 0.1 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+$FeCl_3$ 1 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+$FeCl_3$ 10 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+$FeCl_3$ 200 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+2,2-Dipyridyl 1 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+2,2-Dipyridyl 5 µM	Rough	Serrated	Pink	+	Rough	Serrated	Light red	+
	+2,2-Dipyridyl 20 µM	Smooth	Smooth	None	-	Smooth	Smooth	Light red	-
	+2,2-Dipyridyl 50 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-
	+2,2-Dipyridyl 200 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-
	+2,2-Dipyridyl 400 µM	No Growth	No Growth	No Growth	-	No Growth	No Growth	No Growth	-

†Sporulation scores used were (-) for no sporulation, (±) for low sporulation, less than 50% of the colony surface and (+) for more than 50% of the colony surface

5.3.4 *Streptomyces* sp. SC 36 colony changes in co-culture and altered iron levels

In *Streptomyces* sp. SC 36, similar to the results with *Streptomyces* sp. EUC 63, iron supplementation had little effect on growth rate in monocultures (figure 5.4). An increase, which was not significant, in colony diameter and growth rate was seen in monocultures with supplementation of 10 μM $FeCl_3$. Furthermore, unlike *Streptomyces* sp. EUC 63, excess iron did not significantly reduce the growth rate in monocultures, which we consider to be due to a better ability to regulate iron uptake, or a sufficient oxidative stress defence mechanism (Bogel, Schrempf & Ortiz de Orué Lucana 2008; Wedderhoff *et al*. 2013).

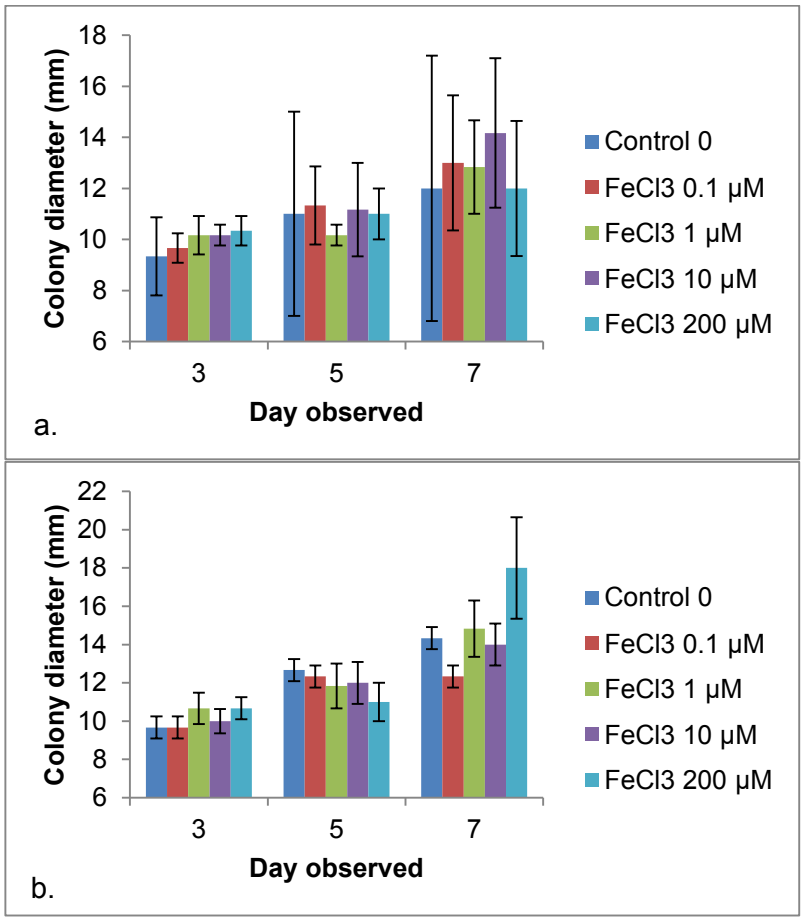


Figure 5.4 Colony diameter of *Streptomyces* sp. SC 36 (a) monocultures and (b) co-cultured with *Pseudonocardia* sp. PIP 161 in high iron levels.

Interestingly, co-cultivation with *Pseudonocardia* sp. PIP 161 with excess iron conditions increased colony diameter with a growth surge observed at day 7. Observation on colony characteristics in iron surplus showed that co-culture increased colony development (indicated by rough surfaces and serrated edges), as well as a pigment formation and sporulation, regardless of the concentration of iron

supplementation. However, an increase in iron levels was seen to also increase sporulation in monocultures, albeit at a slower rate (table 5.3).

Under iron starvation conditions, growth inhibition was seen to occur with the addition of 200, and 400 µM 2, 2-dipyridyl in monoculture and co-culture, which was in higher compared to *Streptomyces* sp. EUC 63 (figure 5.5). Furthermore, these concentrations also prevented the formation of wrinkles and undulation/serration on the edges of the colony, as well as sporulation and red pigment formation, both in monocultures and co-cultures. Sporulation was inhibited by 2, 2-dipyridyl, both in monocultures and co-cultures, in concentrations as low as 5 µM, although pigment formation in co-culture was only inhibited in 200 and 400 µM.

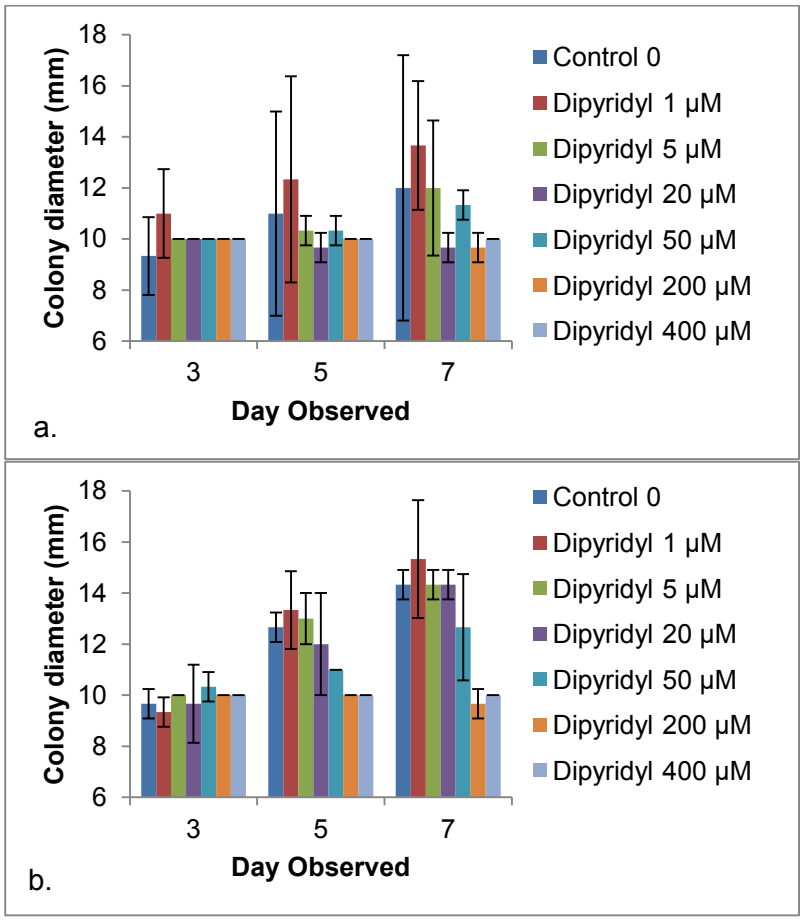


Figure 5.5 Colony diameter of *Streptomyces* sp. SC 36 (a) monocultures and (b) co-cultured with *Pseudonocardia* sp. PIP 161 in low iron levels.

Although it can be concluded based on this study that iron was necessary for colony development in *Streptomyces* sp. SC 36, which was also established by previous literature, whether *Pseudonocardia* sp. PIP 161 acts as an inducer by increasing iron uptake to its neighbouring colony is still uncertain. Furthermore, the metabolic change and antibiotic production due to the interaction, which was observed previously, in

different iron conditions in this case, was unknown. We elaborate this point in later paragraphs.

Table 5.3 Observation of morphological changes in *Streptomyces* sp. SC 36 in monocultures and co-cultures subcultured in ISP-2 media with iron surplus and limitation

Day	*Streptomyces* sp. SC 36 in ISP-2 with the following treatments:	Monoculture				Co-culture with *Pseudonocardia* sp. PIP 161			
		Shape	Edges	Colour	Sporulation	Shape	Edges	Colour	Sporulation
5	Control	Smooth	Serrated	None	-	Rough	Serrated	Red	+
	+$FeCl_3$ 0.1 µM	Smooth	Serrated	None	-	Rough	Serrated	Red	+
	+$FeCl_3$ 1 µM	Rough	Serrated	None	-	Rough	Serrated	Red	+
	+$FeCl_3$ 10 µM	Rough	Serrated	None	±	Rough	Serrated	Red	+
	+$FeCl_3$ 200 µM	Rough	Serrated	None	-	Rough	Serrated	Red	-
	+2,2-Dipyridyl 1 µM	Smooth	Serrated	None	-	Rough	Serrated	Red	±
	+2,2-Dipyridyl 5 µM	Smooth	Serrated	None	-	Rough	Serrated	Red	-
	+2,2-Dipyridyl 20 µM	Smooth	Serrated	None	-	Rough	Serrated	Red	-
	+2,2-Dipyridyl 50 µM	Smooth	Smooth	None	-	Smooth	Smooth	None	-
	+2,2-Dipyridyl 200 µM	Smooth	Smooth	None	-	Smooth	Smooth	None	-
	+2,2-Dipyridyl 400 µM	Smooth	Smooth	None	-	Smooth	Smooth	None	-
7	Control	Smooth	Serrated	None	-	Rough	Serrated	Red	+
	+$FeCl_3$ 0.1 µM	Rough	Serrated	None	+	Rough	Serrated	Red	+
	+$FeCl_3$ 1 µM	Smooth	Serrated	None	+	Rough	Serrated	Red	+
	+$FeCl_3$ 10 µM	Smooth	Serrated	Red	+	Rough	Serrated	Red	+
	+$FeCl_3$ 200 µM	Rough	Serrated	None	-	Rough	Serrated	Red	+
	+2,2-Dipyridyl 1 µM	Smooth	Serrated	None	±	Rough	Serrated	Red	+
	+2,2-Dipyridyl 5 µM	Smooth	Serrated	None	-	Rough	Serrated	Red	-
	+2,2-Dipyridyl 20 µM	Smooth	Serrated	None	-	Smooth	Smooth	Red	-
	+2,2-Dipyridyl 50 µM	Smooth	Smooth	None	-	Smooth	Serrated	Red	-
	+2,2-Dipyridyl 200 µM	Smooth	Smooth	None	-	Smooth	Smooth	None	-
	+2,2-Dipyridyl 400 µM	Smooth	Smooth	None	-	Smooth	Smooth	None	-

†Sporulation scores used were (-) for no sporulation, (±) for low sporulation, less than 50% of the colony surface and (+) for more than 50% of the colony surface.

5.3.5 Co-inoculated culture shows that the *Pseudonocardia* sp. PIP 161 interaction was complex and multifactorial on both *Streptomyces* species

In order to observe the interaction closer and observe whether co-cultivation under different iron concentrations alters antibiotic production, as was reported previously (Coisne, Béchet & Blondeau 1999), in our species, the interaction was scaled up in iron surplus with 10 µM $FeCl_3$ (for *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36) and 200 µM $FeCl_3$ (for *Streptomyces* sp. SC 36), and iron limitation conditions with 50 µM 2,2-dipyridyl. As it was known that the iron resistance was higher in *Streptomyces* sp. SC 36, and this culture was able to benefit from excess iron (with addition of 200 µM $FeCl_3$) in co-culture with *Pseudonocardia* sp. PIP 161, iron excess conditions was only tested with *Streptomyces* sp. SC 36.

Pseudonocardia sp. PIP 161 was observed to have robust growth in different iron levels (figure 5.6). Similar growth rates and morphogenesis occurred in all media, indicating that both iron surplus and limitation were neither detrimental nor beneficial, at least when viewed in terms of growth and development. In iron excess, we observe a darker hue of vegetative mycelia of *Pseudonocardia* sp. PIP 161.

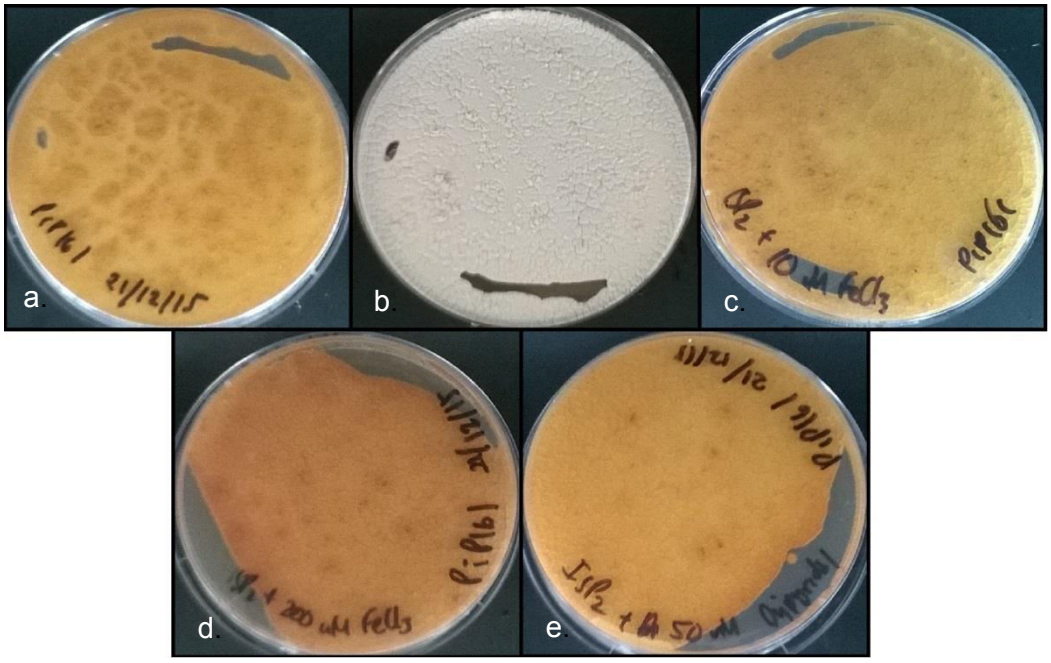


Figure 5.6 *Pseudonocardia* sp. PIP 161 in (a) untreated ISP-2 medium from the back, (b) from the top, (c) ISP-2 with 10 µM $FeCl_3$, (d) ISP-2 with 200 µM $FeCl_3$, and (e) ISP-2 with 50 µM 2, 2-dipyridyl.

Streptomyces sp. EUC 63, sporulation and pink pigmentation was observed in monocultures control plates and with iron supplementation, at day 7 of incubation

(figure 5.7). Consistent with previous testing, pigment produced during co-inoculated culture had a darker hue, in comparison to results from monoculture, suggests that secondary metabolism was enhanced with co-cultivation. When supplemented with 50 µM 2, 2-dipyridyl, *Streptomyces* sp. EUC 63 monocultures showed little growth. Interestingly, co-cultivation with *Pseudonocardia* sp. PIP 161 increased sporulation (not visible in picture) and pigment production by *Streptomyces* sp. EUC 63.

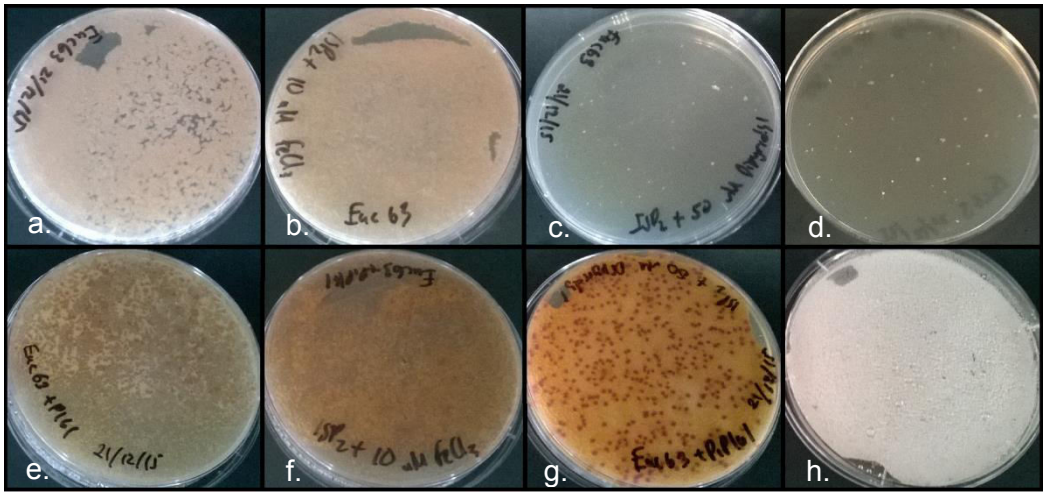


Figure 5.7 Co-inoculated culture of *Streptomyces* sp. EUC 63 and *Pseudonocardia* sp. PIP 161 after 7 days of incubation in media treated with various iron levels observed for growth, sporulation and pigment production. [Top row] *Streptomyces* sp. EUC 63 monocultures in (a) untreated ISP-2 media, (b) ISP-2 with 10 µM $FeCl_3$, (c) ISP-2 with 50 µM 2,2-dipyridyl bottom plate, and (d) ISP-2 with 50 µM 2,2-dipyridyl top plate, while [Bottom row] shows *Streptomyces* sp. EUC 63 mixed with *Pseudonocardia* sp. PIP 161 in (e), (f), (g), and (h) with the respective configuration.

Thus, these results correlate with previous results, as well as work from previous researchers (Yamanaka *et al*. 2005). Based on these results, iron was required for *Streptomyces* sp. EUC 63 in its culture development, particularly the formation of sporulation and pigmentation, as seen when iron limitation was introduced. However, increase in iron alone did not increase the pigment production (as observed in figure 5.7 a and b), indicating that the inducing compound which enabled the production of increased secondary metabolites were produced by *Pseudonocardia* sp. PIP 161.

It was therefore concluded that *Pseudonocardia* sp. PIP 161, in its interaction with *Streptomyces* sp. EUC 63, increased the culture development of the *Streptomyces* species in low iron conditions, and possibly also significantly enhancing secondary metabolite production. This was further supported when the interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* sp. SC 36 was examined under the same conditions, described as follows, as shown in figure 5.8.

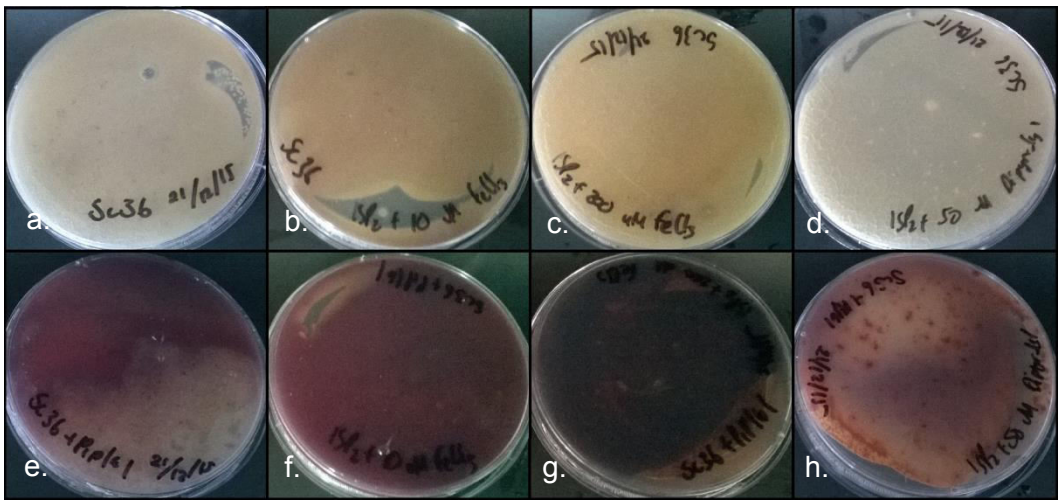


Figure 5.8 Co-inoculated culture of *Streptomyces* sp. SC 36 and *Pseudonocardia* sp. PIP 161 after 7 days of incubation in media treated with various iron levels observed for growth, sporulation and pigment production. [Top row] *Streptomyces* sp. SC 36 monocultures in (a) untreated ISP-2 media, (b) ISP-2 with 10 µM FeCl3, (c) ISP-2 with 200 µM FeCl3, and (d) ISP-2 with 50 µM 2,2-dipyridyl, while [Bottom row] shows *Streptomyces* sp. SC 36 mixed with *Pseudonocardia* sp. PIP 161 in (e), (f), (g), and (h) with the respective configuration.

Streptomyces sp. SC 36 monocultures had good growth on all media, however, sporulation was inhibited on ISP-2 medium added with 50 µM 2, 2-dipyridyl. While some pigmentation was observed in monocultures that seemed to increase when iron was supplemented, purple pigments were only produced when co-cultured with *Pseudonocardia* sp. PIP 161. Furthermore, sporulation was also observed in co-cultures on medium with 50 µM 2,2-dipyridyl, again, indicating that *Pseudonocardia* sp. PIP 161 supported the growth and development of *Streptomyces* species in low iron concentrations.

These findings concur with previous findings in this area. As also discovered in this experiments, iron was required for the formation of spores and colony morphological characteristics (Cabaj & Kosakowska 2009; Lambert *et al*. 2014; Pelchovich, Omer-Bendori & Gophna 2013; Tierrafría *et al*. 2011; Traxler *et al*. 2012), in particular the formation of rugose structures in the colony surface, and serrated edges. Colony baldness was observed in when iron limitation was introduced in varying degrees, which also inhibited the formation of sporulation of both *Streptomyces* species. Finally, iron limitation inhibited the formation of colony pigmentation on both *Streptomyces* species, which indicates retardation in secondary metabolism.

On the other hand, increase in levels of iron, which did result in some morphological changes in monocultures in *Streptomyces* sp. SC 36 when subcultured in initial tests (as observed in 5.3.4.1), appeared to have no effect on *Streptomyces* sp. EUC 63. Furthermore, changes due to increased iron levels were not visible in co-inoculation

of both cultures, in which both *Streptomyces* species showed no differences in morphology or sporulation compared to control, and particularly when compared to co-culture. Therefore, an increase in iron levels by itself had no added benefit to the *Streptomyces* species, either due to the inability of the *Streptomyces* to uptake and utilize the iron (Tierrafría *et al*. 2011), or that a homeostasis with the current iron level was already achieved (Condon *et al*. 2014).

Co-culture with *Pseudonocardia* sp. PIP 161 appears to have not only restored morphology and sporulation in co-culture, but also increase the formation of pigments in both *Streptomyces* species. In both *Streptomyces* species, co-culture with *Pseudonocardia* sp. PIP 161 resulted in increased colony pigment formation regardless of the iron level. Interestingly, this also applies where iron limitation inhibited the growth and sporulation in *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36. Therefore, it can be concluded that the inducer produced from *Pseudonocardia* sp. PIP 161 was essential for the increased activation of secondary metabolites for both *Streptomyces* species, in which the mechanism of the inducer may not be related to iron level.

5.3.6 Monoculture and co-culture antibiotic production in different iron levels

Antibiotic activity from co-inoculation plates methanol extracts were tested against *S. aureus* for *Streptomyces* sp. EUC 63, and *Streptomyces* sp. SC 36, as shown in figure 5.9. The extracts were tested for activity against *S. aureus*, as both cultures has previously shown activity (refer to chapter 3).

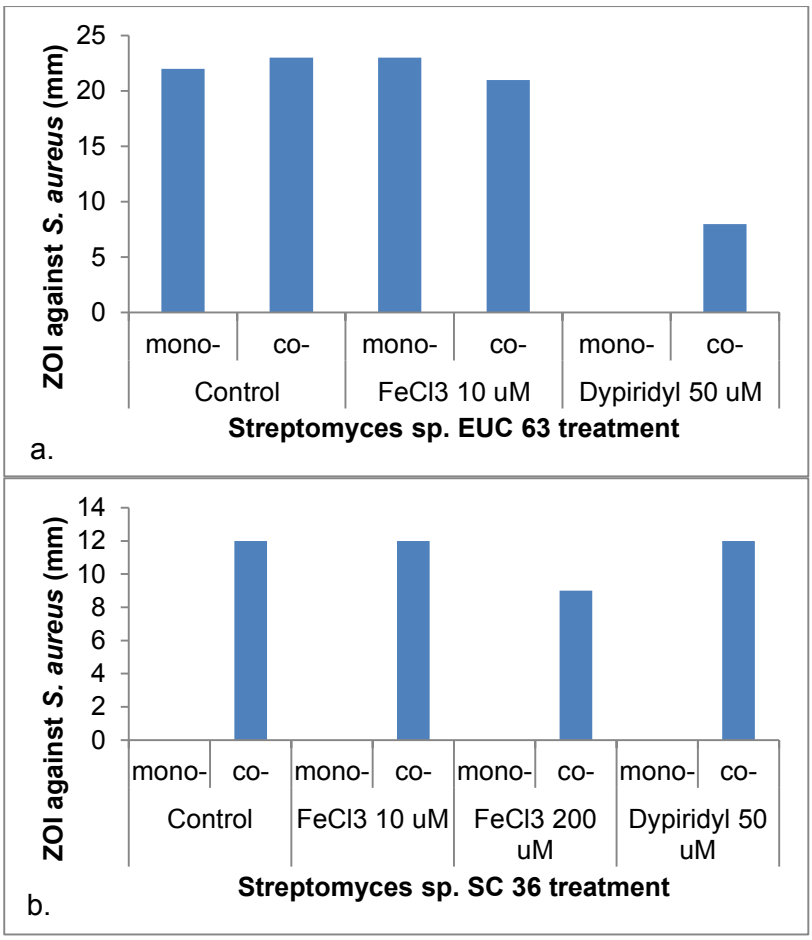


Figure 5.9 Antibiotic activity of (a) *Streptomyces* sp. EUC 63 and (b) *Streptomyces* sp. SC 36 in monocultures and co-inoculated with *Pseudonocardia* sp. PIP 161 in ISP-2 medium, and ISP-2 medium with altered iron levels.

In *Streptomyces* sp. EUC 63, similar antibiotic activity was observed in both monocultures and co- inoculated cultures in untreated ISP-2 and in ISP-2 with increased iron levels. This was contrary to previous findings, in which co-inoculated with *Pseudonocardia* sp. PIP 161 was seen to increase antibiotic activity in *Streptomyces* sp. EUC 63. However, as previous results has shown success mostly in liquid medium, it can be suggested that the interaction with *Pseudonocardia* sp. PIP 161, in particular with *Streptomyces* sp. EUC 63, influenced genes that were well expressed in liquid medium but not in solid medium (Yagüe *et al*. 2014). Another possible explanation was that the antibiotic compounds produced by *Streptomyces* sp. EUC 63 were less stable under prolonged storage, while awaiting evaporation, resulting in a loss of antibiotic activity. Nevertheless, an increase in co-inoculation was seen compared to monocultures in low iron content, signifying that co-inoculation with *Pseudonocardia* sp. PIP 161 increased the survivability *Streptomyces* sp. EUC 63, allowing some production of antibiotic compounds.

In *Streptomyces* sp. SC 36 however, an increase of antibiotic activity was observed in co-culture compared to monocultures, regardless of the iron level. In conditions of iron excess, a lowering of antibiotic activity was observed, although the significance of this was difficult to ascertain. It can be hypothesized that the production of antibiotics in *Streptomyces* sp. SC 36 was linked to the production of siderophores either by metabolic crosstalk, as was found in benzoxazole production in *Streptomyces* species (Cano-Prieto *et al*. 2015) or a genetic regulation (Flores & Martín 2004), as increasing iron levels in the medium was found to reduce siderophore production (Mulé *et al*. 2015).

5.4 Conclusion

It was hypothesized that the interaction that occurred between *Pseudonocardia* sp. PIP 161 and other *Streptomyces* sp. was mediated by iron via siderophore piracy. In these experiments, it was discovered that iron by itself was not able to replicate the changes that occur in co-culture, both in *Streptomyces* sp. EUC 63 and in *Streptomyces* sp. SC 36. The production of enterobactin-like compound (as hypothesized in previous results) from *Streptomyces* sp. EUC 63, was not observed in low iron concentration, as marked by the low growth and survivability of *Streptomyces* sp. EUC 63 with the addition of 2,2-dipyridyl. Therefore, it can be concluded that an inducer from *Pseudonocardia* sp. PIP 161 was essential for the increased production of pigment and antibiotics in *Streptomyces* species.

CHAPTER 6. DIKETOPIPERAZINES PRODUCED BY *PSEUDONOCARDIA* SP. PIP 161 INDUCE INCREASED SECONDARY METABOLIC PRODUCTION IN *STREPTOMYCES* SP. EUC 63 AND *STREPTOMYCES* SP. SC 36

6.1 Introduction

Our studies show that *Pseudonocardia* sp. PIP 161 was able to induce sporulation and changes in metabolism in various *Streptomyces* species. In our tests, we find that these interactions could occur without direct cell to cell contact, and was induced by incubation in spent medium of *Pseudonocardia* species monocultures (refer to chapter 4). This indicates the presence of a chemical signal produced by *Pseudonocardia* species which mediates physical and metabolic changes in our tested *Streptomyces* species.

We propose that the phenomenon observed in our study was mostly, or in part, mediated by diketopiperazine (DKP). DKPs are the smallest cyclic configuration of two amino acids, and therefore are found in inter-kingdom, with the isolation of DKPs from various plants, fungi, bacteria, and humans with metabolic disorders. DKPs also possess a large spectrum of bioactivities, with reported activities to include antibiotics, antitumor, antihypertension and antiarrythmia (McCleland *et al*. 2004), and antihyperglyceaemic (Song *et al*. 2003). Although abundantly found in nature, chemical synthesis of DKPs can be achieved with relative ease (Fischer 2003), and therefore various bioactivities in synthetic DKPs has also been reported (Martins & Carvalho 2007).

There has been efforts made to isolate DKPs from various sources, including endophytic and marine actinobacteria. In the last 15 years, different forms of DKPs were isolated from various microorganisms and elucidated using MS or NMR studies, either with or without modifications of the structural amino acids, forming enantiomers, having diastereomeric forms, or forming a different structure entirely. Marine organism are known to be a rich source of DKPs, with 90 novel 2,5-DKPs were discovered between 2009 and 2014, of which 14 DKPs were discovered from marine actinobacteria (Huang *et al*. 2014).

Cell signalling (as in quorum sensing) has been defined to require a “reaction of a population of cells which is different from individual cells” in the effect of a molecule

(March & Bentley 2004). Quorum sensing molecules are found to regulate phenotypic differences in bacteria including sporulation, biofilm maturation, virulence, bacterial cross talk, pigmentation, secondary metabolite production, and others (Diggle, Crusz & Cámara 2007).

The inclusion of DKPs as cell signalling molecules and/or quorum sensing molecules was based on the evidence that several DKPs could mimic acyl-homoserine lactones (AHLs), and act on their receptors (Degrassi *et al*. 2002; Holden *et al*. 1999). Lactones are already established as cell signalling molecules and quorum sensing molecules in bacteria, and analogues are discovered in fungi and actinobacteria. In *Streptomyces*, γ-butyrolactones and A-factors were found to be auto-regulators and initiate for sporulation and secondary metabolite production (Du *et al*. 2011; Horinouchi & Beppu 1992a).

Although no direct link between DKP and secondary metabolite induction has been established in actinobacteria, several interaction studies had discovered that various DKPs were either found to be present in the interaction or was present in monocultures (Dashti *et al*. 2014; Guimarães *et al*. 2010). Several researches have speculated that DKPs would play a role in interspecies interactions, but so far, this link has not been strongly established (De Rosa, Mitova & Tommonaro 2003; Gao, Y *et al*. 2010). On the contrary, some researchers dispute the hypothesis that DKPs plays a role as quorum sensing molecules (Huvenne *et al*. 2008).

In this study, we discovered that a major component found in an active fraction obtained from *Pseudonocardia* sp. PIP 161 monocultures was a DKP. Thus, we hypothesize that the interaction between *Pseudonocardia* sp. PIP 161 and several *Streptomyces* sp. was mediated by a DKP(s), and tested the hypothesis by adding pure DKP compounds to induce sporulation and antibiotic production in *Streptomyces* species. These studies provide further evidence of the role of DKP as cell signalling molecules in actinobacterial interactions.

6.2 Methods

6.2.1 Spore suspension

A spore suspension for cultures was prepared as described earlier (chapter 3). From prepared glycerol stocks, 50 µl of *Streptomyces* sp. EUC 63 colony 11 (refer to chapter 4), *Streptomyces* sp. SC 36, *Streptomyces* sp. EUM 356 and *Pseudonocardia* sp. PIP 161 was inoculated and spread onto ISP-2 agar. This plate was incubated for

7 days until good sporulation was observed. Using a sterile loop, the surface of the plate was scraped off gently and inserted into a sterile cryotube containing 1 ml of sterile 50% glycerol, and stored at -20°C until further use.

6.2.2 HPLC analysis

HPLC of mycelial extracts of *Pseudonocardia* sp. PIP 161, as prepared in 4.2.3, was conducted in Microbial Screening Technologies (MST), Sydney. In this case, each sample contained in the Eppendorf tubes were freeze dried and sent. The method for HPLC were as stated previously (refer to 4.2.6).

6.2.3 Inducer production from *Pseudonocardia* sp. PIP 161

Previous studies has shown that inducers were secreted in the medium. A scaled-up production of the inducer was conducted as follows. *Pseudonocardia* sp. PIP 161 was first subcultured as a lawn on ISP-2 medium by transferring and spreading 50 µl from prepared spore suspension, and incubating for 5 days in 27°C. After achieving sufficient sporulation, twelve cubes (1 x 1 cm) were cut out using a sterile scalpel and inserted into three 250 ml flasks containing IM-22 medium to have obtain biomass production. These were incubated for 5 days with continuous shaking at 150 rpm in a 27°C incubator room.

After 5 days, 24 ml from each flask was transferred into three baffled 3-L flasks, each containing 600 ml of ISP-2 broth as production medium. This was then incubated with continuous shaking at 150 rpm in a 27°C incubator room for 5 days. The broth and mycelia were separated using Whatman no 1 filter paper and a Buchner funnel under vacuum.

6.2.4 XAD-4 Fractionation

The broth supernatant of *Pseudonocardia* sp. PIP 161 was subjected to XAD-4 column chromatography. A cylindrical column (length 70 cm x 10 cm) was filled to a bed volume of 200 ml XAD-4 resin in MilliQ water. The sample was then introduced into the column, in which the flow-through (Spent broth) was collected. The column was then washed with MilliQ-water, methanol, and acetone at 200 ml each, and the solvent eluates were collected, at a maximum 20 ml per fraction, with each fraction following colour changes observed in the eluates. Fraction numbering was started

when methanol was added into the column. Around 20 fractions were collected, with eluates becoming colourless after the 10th fraction; the first two fractions were aqueous were pooled into one fraction, and screened for induction activity. Thus, fractions 3 to 9 was then tested in solid medium for activity in both diluted and undiluted form.

6.2.5 Methods to test fractions for induction

6.2.5.1 Disc diffusion assay

From the prepared spore suspension of *Streptomyces* sp. EUC 63 colony 11, six spots of 10 µl were made on an ISP-2 plate in two columns with equal spots per column at both sides of the plate, leaving a 1 cm distance from the edge of the plate. Each spot within one column was at least 10 mm apart. This left to air dry for 30 minutes. The plates were then incubated at 27°C for 3 days.

Sterilized paper discs (Whatman® antibiotic assay discs, Sigma-Aldrich) were placed on a sterile plate and 40 µl (2x20 µl) of the XAD-4 fractions were added to each disc and left to dry for 10 minutes. On day 3, the discs were placed on the side of one column of colonies, with a 0.1 – 0.3 distance from the spotted colony edges on alternating sides. The plates were incubated at 27°C for 4 more days. Observations were made every two days, and at the 7th day, the plates were assayed for antibiotic activity using flip plate assay (refer to 3.2.8).

6.2.5.2 Testing of active fractions by spread plate assay

For this method, each active fraction was spread on an ISP-2 plate using the following method. A poured ISP-2 plate at 25 ml was prepared as described previously. Fractions 4 and 5 were then filter sterilized and diluted 10 times with methanol. To the solidified plates, 250 µl of the original fractions and the 1/10 dilution was spread using a flamed metallic triangle (resulting in a 1/100th and 1/1000th of the original concentration, respectively). Triplicates plates were left to air dry for 30 minutes. A control plate containing untreated ISP-2 plate was also prepared. Three 10 µl spots of *Streptomyces* sp. EUC 63 and *Streptomyces* sp. EUM 356 were spotted onto separate plates in a triangular fashion. To avoid bias, the plates of the same species were randomized prior to spotting. After 10 days, an antibiotic assay was conducted using the flip plate method.

6.2.6 Electrospray Ionisation Mass Spectrometry (ESI-MS) and Tandem MS/MS-MS

The ESI mass spectra of the sample fraction #4 and #5 were conducted by Dr. Yadollah Bahrami at the Flinders Advanced Analytical Facility, Flinders University. The ESI mass spectra were attained with a Waters Synapt HDMS (Waters, Manchester, UK) and data acquisition was using a Waters Masslynx (V4.1, Waters Corporation, Milford, USA). Both ion modes were used to acquire the mass spectra, with a capillary voltage of 2.0 kV and a sampling cone voltage of 40 V. The other conditions were as follows: extraction cone voltage, 4.0 V; ion source temperature, -80°C; desolvation temperature, 350 °C; desolvation gas flow rate, 500 $L.h^{-1}$. Positive ion mass spectra was retrieved over a mass range of 600 -1600 m/z using continuum mode acquisition in the V resolution mode. Mass calibration was performed by infusing sodium formate solution (0.5 mM, 1:9 (v/v) water:isopropanol). Accurate mass analysis was performed in the positive ion mode. For MSMS data, Argon was used as the collision gas and a collision energy between 15 and 25 eV depending on the amount of fragmentation produced.

6.2.7 Diketopiperazine (DKP) induction method in liquid medium

Liquid cultivation for secondary metabolite production was conducted as previously described for mono-cultures of *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36, and *Streptomyces* species co-cultured with *Pseudonocardia* sp. PIP 161 (refer to 4.2.2). DKP used in this study were Cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro), which were supplied by Microbial Screening Technologies (MST) in freeze-dried powder form and cyclo (His-Pro) was purchased from Bachem (Bubendorf, Switzerland). The diketopiperazines from MST were then reconstituted into a final stock of 100 µg/ml which was filter sterilized. *Streptomyces* species inoculated (refer to 4.2.2) into 250 ml Ehrlenmeyer flasks containing 50 ml ISP-2 broth and consequently (or with a 2 day delay, with the flasks placed at 27°C with shaking at 150 rpm) DKPs added to a final concentration of 0.1, 0.3, 1 and 3 µg/ml. The flasks were incubated on a shaker at 150 rpm in 27°C for 7 days after which the broth and mycelial extracts were tested for antibiotic activity. A control to which only the highest amount of solvent used was also prepared. Cyclo (His-Pro) testing was conducted on *Streptomyces* sp. EUC 63 and *Streptomyces* sp. 36, with a co-culture control also prepared, and done in triplicate. Cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro) were only tested on *Streptomyces* sp. EUC 63, and done in duplicate, due to limitations of DKP supply.

6.2.8 Mycelial methanol extractions after DKP induction in liquid medium

Mycelial methanol extraction from liquid medium was similar to previous methods (refer to 4.2.3), with several alterations. The broth and mycelia were separated using centrifugation in a sterile 50 ml falcon tube at 3000 rpm for 5 minutes. The mycelium was extracted with 10 ml of methanol on a shaker at 150 rpm overnight. The methanol extracts were then centrifuged at 3000 rpm for 5 minutes, and 2 ml were transferred into an Eppendorf tube, and concentrated on a rotary evaporator for 4 hours. These were tested for antibiotic activity.

6.2.9 Spectrophotometry to determine colour change

The filtered broth of *Streptomyces* sp. SC 36 was tested for absorbance in 570 nm (to quantify purple colour) and 450 nm (to quantify orange-red colour) wavelength, following common procedures.

6.2.10 Statistical analysis

All statistical analysis was done using student t-test (one tailed, equal variance) in Excel, in which values were considered significant if p value is <0.05.

6.3 Results and Discussion

6.3.1 Methanolic fractions of *Pseudonocardia* sp. PIP 161 were able to induce sporulation and antibiotic production in *Streptomyces* sp.

Previous studies show that *Pseudonocardia* sp. PIP 161 broth was able to induce antibiotic production in liquid cultures of *Streptomyces* sp. EUC 63. A HPLC analysis of *Pseudonocardia* sp. PIP 161 observed a majority of the compounds produced in methanolic mycelial extract to be polar, as shown in figure 6.1.

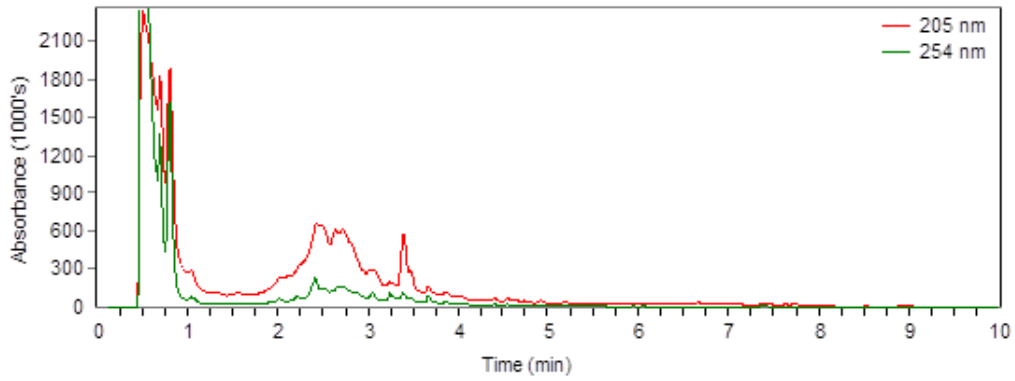


Figure 6.1 HPLC analysis of *Pseudonocardia* sp. PIP 161 methanolic mycelial extract.

Testing of the XAD-4 fractions with the disk diffusion assay, however, revealed very inconsistent results, as shown in figure 6.2. Positive results would only be confirmed if an increase of antibiotic activity was observed compared to the control on the same plate. As controls spots were placed at the same plate with a distance from the treatment spots, we suspect that some cross contamination occurred between the treatment and control, and therefore influencing the variability of the results obtained. Furthermore, as HPLC results show that multiple compounds were found in a single fraction, these compounds may have antagonistic or synergistic effects and therefore also influencing the results obtained. However, an increase in sporulation and a significant increase in antibiotic production were only observed when induced with fraction 4 and 5, which we repeated using a different method.

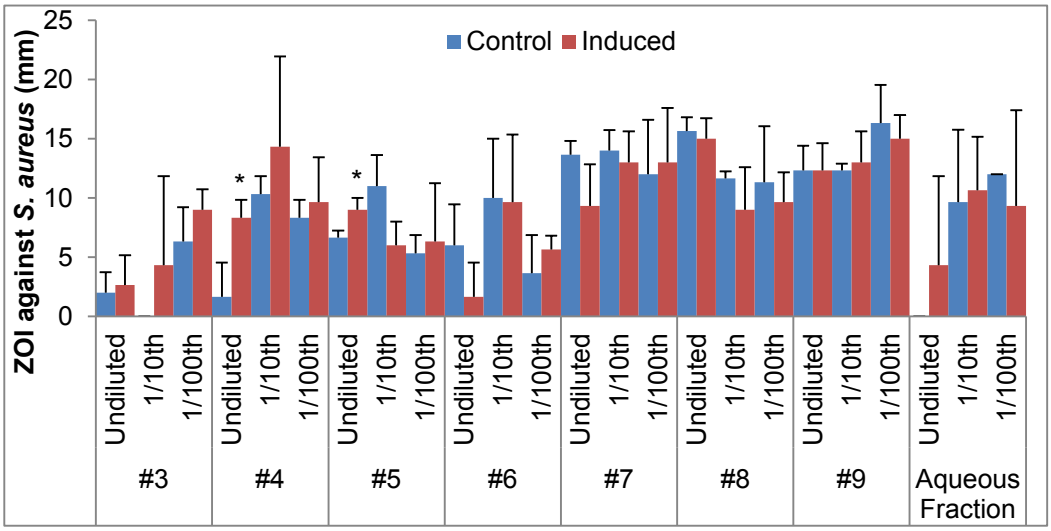


Figure 6.1 Antibiotic activity after disk diffusion assay of multiple methanolic (and aqueous) fractions from Pseudonocardia sp. PIP 161 broth on Streptomyces sp. EUC

63, showing mostly inconsistent and varied results, but with significant increase on fraction #4 and fraction #5. (*) denotes significant increases compared to control, which were found in undiluted fraction 4 and 5.

We then collected XAD-4 fractions and tested each fraction to induce sporulation and/or antibiotic production in *Streptomyces* sp. EUC 63 using a different assay. Early growth and development, as apparent from colony diameter and sporulation, of *Streptomyces* sp. EUM 356 and *Streptomyces* sp. EUC 63 was achieved after addition of fraction from XAD-4 to the monocultures, thus indicating active fractions, as shown in figure 6.3. Heavy sporulation was observed in *Streptomyces* sp. EUM 356 and *Streptomyces* sp. EUC 63 after induction with fractions 4 and 5, at 1/100th and/or 1/1000th dilutions after 6 days of incubation, with noticeable differences observed on the previous day.

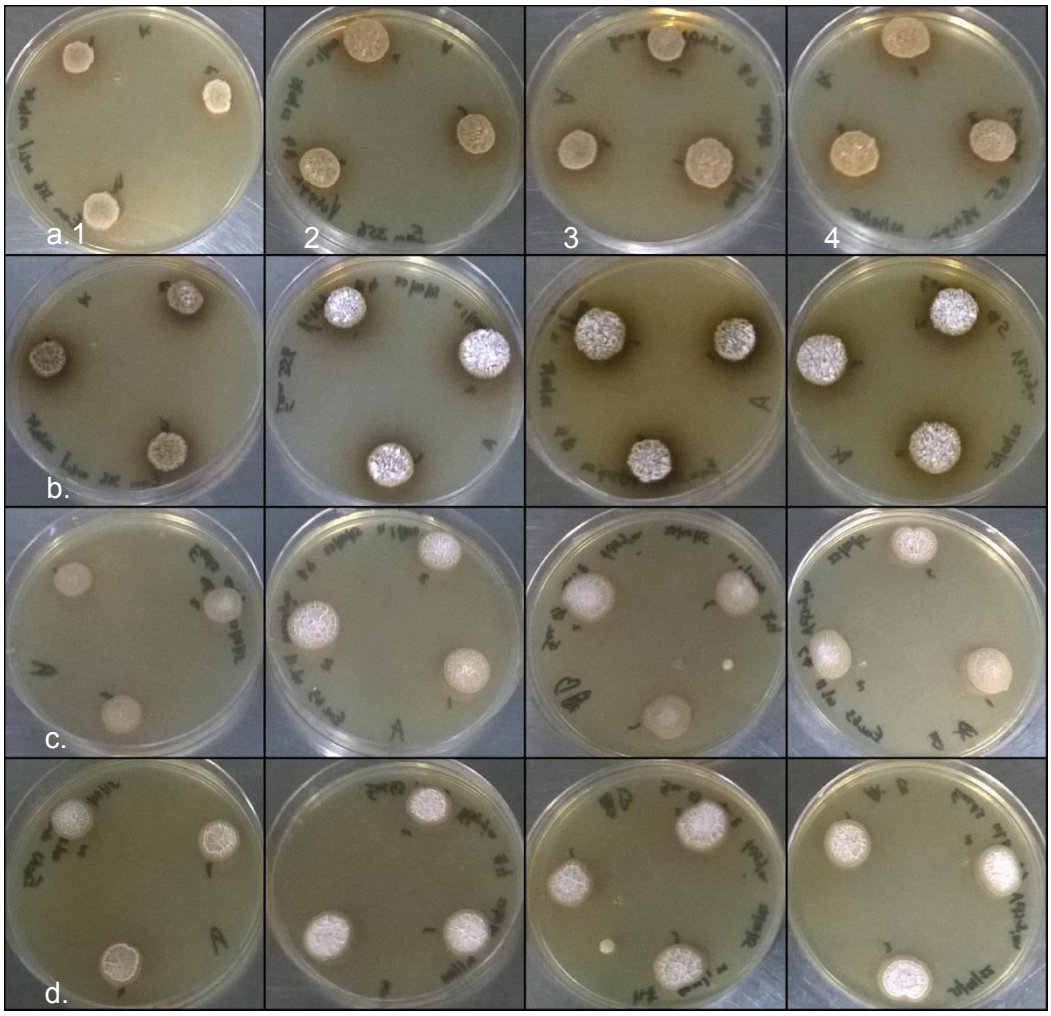


Figure 6.3 Induction of *Streptomyces* sp. EUM 356 at day 5 (row a) and day 6 (row b) and *Streptomyces* sp. EUC 63 at day 5 (row c) and day 6 (row d) with fraction 4 with 100 and 1000 time dilution (column 2 and 3, respectively), and fraction 5 with 100 time dilution (column 4), compared to their respective control (column 1).

Finally, antibiotic production was seen to significantly increase after induction with at least one of the fractions, suggesting the presence of the inducer compound, as shown in figure 6.4, in *Streptomyces* sp. EUC 63. As before, *Streptomyces* sp. EUM 356 showed no antibiotic production, with induction or otherwise. The results with this method were consistent and had relatively low variability.

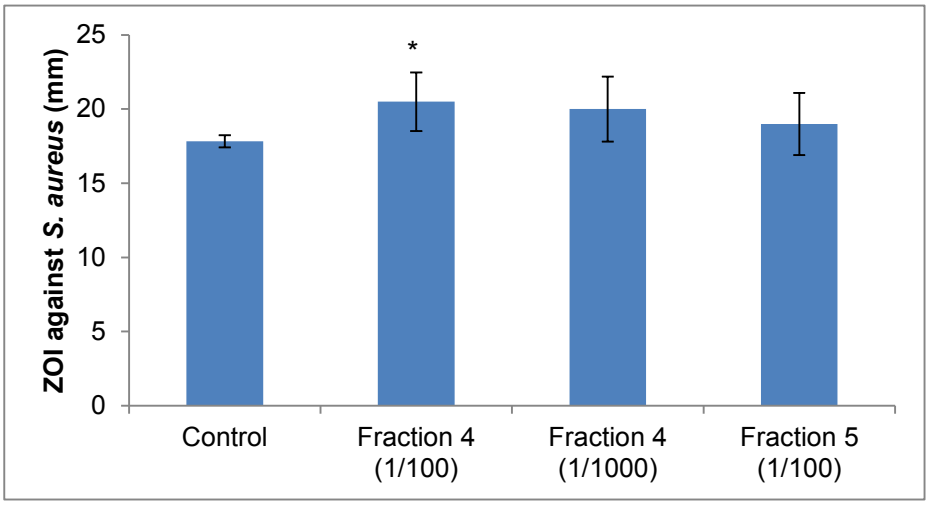


Figure 6.4 Increased antibiotic production from *Streptomyces* sp. EUC 63 induced with fractions from *Pseudonocardia* sp. PIP 161. A modest increase was seen with addition of Fraction 4 at 1/100 concentration, while other fractions did not show significant ($p<0.05$) results compared to un-induced control.

6.3.2 Tandem MS of *Pseudonocardia* sp. PIP 161 active fraction reveals DKP to be a major compound

The active fraction (fraction 4) was analysed using MS to reveal that a dominant compound having a mass-to-charge ratio (*m/z*) of 235.1, as shown in figure 6.7. Fraction 5 showed a different dominant compound of $[M+H^+]$ peak at *m/z* 255.1, with some compounds from the previous fraction also present, showing poor separation of these compounds using fractionation (figure 6.8). The reduced abundance of peak *m/z* 235.1 could explain the lower efficacy of the fraction 5 in inducing antibiotic production, as shown in previous tests.

We analysed the fragmentation pattern after tandem MS of peak *m/z* 235.1. The peaks that were observed were m/z 217.1, 207.1, 166.1, 162.1, 149.1, 138.1, 110.1, 82.1, and 70.1. Cyclo (His-Pro) MS/MS data retrieved by Huvenne *et al*. (2008) shows peaks with m/z of 69.7, 110.0, 137.7, 162.1, 165.9, 189.9, and 207.0, with a parent m/z of 234. Therefore, this suggests that the parent ion was a diketopiperazine, cyclo (His-Pro). This was further supported by studies on peptide fragmentation, in which immonium ions from peptide fragmentation of histidine (*m/z* 110) and proline (*m/z* 70)

matched the peaks observed in this sample (Wysocki *et al*. 2005). Based on this data and the total mass of the compound, we conclude that the compound was DKP Cyclo (His-Pro), which was supported by other studies (Furtado *et al*. 2007; Huvenne *et al*. 2008). Our proposed fragmentation of cyclo (His-Pro), based on the aforementioned literature, was shown in figure 6.9. The fragmentation of cyclo (His-Pro) was based on the fragmentation investigation of cyclo (L-Phe-L-Pro) by Furtado *et al*. (2007). Based on this investigation, a loss of CO from parent ion cyclo (His-Pro) formed a product ion at *m/z* 207.1. From this, the histidine immonium ion *m/z* 110.1 and product ion m/z 98.1 was produced by the breakage of a C-N bond, from which would result in a proline immonium ion m/z 70.1 after the loss of another CO. Alternatively, the formation of proline immonium ion m/z 70.1 may be formed by a loss of histidine CH_2NH from product ion m/z 207.1, followed by –CO. This would result in product ion *m/z* 138.1 and subsequent production of histidine immonium ion (as shown in figure 6.5).

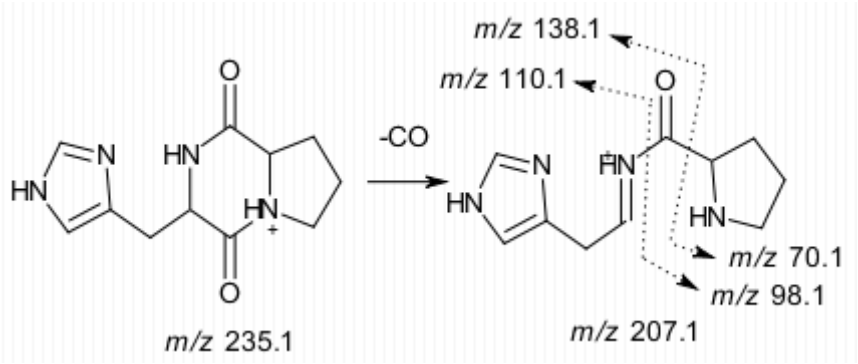


Figure 6.5 Proposed fragmentation of cyclo (His-Pro) based on investigation of cyclo (L-Phe-L-Pro) by Furtado *et al*. (2008).

At the time of writing, there were no reports of cyclo (His-Pro) isolated from actinobacteria. The earliest report of the discovery of cyclo (His-Pro) was endogenously from mammals in the late 1980s (Peterkofsky & Battaini 1980; Prasad, C. 1995). Cyclo (His-Pro) was discovered to be distributed and active in the central nervous systems, and acts as a neurostimulator through dopaminergic activity (Ikegami & Prasad 1990; Peterkofsky & Battaini 1980; Prasad, Chandan 1988), and suggested to be able mimic dopamine in many of its activities (Imamura & Prasad 2003). Other findings also show that the tertiary structure of cyclo (His-Pro) was required for binding to a liver receptor (Hirose, Ogawa & Mori 1985).

Another peak that was analysed using tandem MS was peak [$M+H^+$] *m/z* 254 in fraction 4 (figure 6.10). Based on the peak mass, immonium ions matching proline

(*m/z* 70 and 125) were observed. Comparison of the total mass of all proline based DKPs, show the possibility of it being cyclo (Arg-Pro). Recently, cyclo (Arg-Pro) was discovered to be transformed into a cyclo (Pro-pro) in marine sponges (Vergne *et al*. 2006). The fragmentation pattern of our compound showed peaks at *m/z* 195.1, 167.1, 125.1, 98.1 and 70.1, which was consistent with other cyclo (Pro-Pro) fragmentation discovered in other studies, showing *m/z* of 195.1, 98.1 and 70.1(Stark & Hofmann 2005). Based on Vergne *et al*. (2006), the fragmentation pattern of this compound was proposed to be first the loss of CN_3H_4, forming a cyclo (Pro-Pro) product ion at *m/z* 195.1. This was then proceeded with the loss of CO, resulting in a product ion at *m/z* 167.1, and the formation of proline immonium ions at *m/z* 70.1, as shown in figure 6.6 (Furtado *et al*. 2007). Thus, these studies support our conclusion that peak *m/z* 254.1 was cyclo (Arg-Pro).

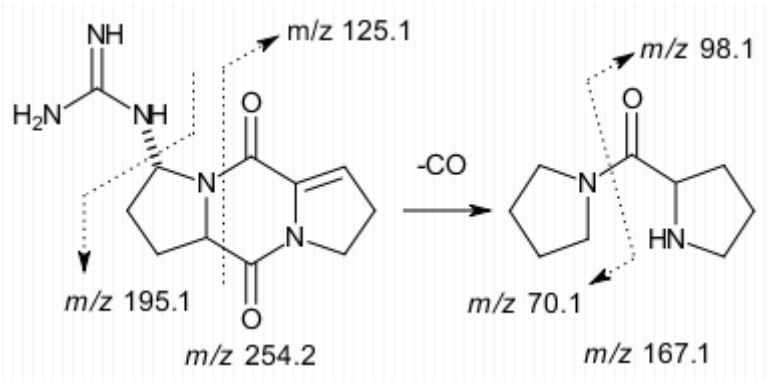


Figure 6.6 Proposed fragmentation of cyclo (Pro-Pro) based on investigation of by Furtado *et al*. (2008).

The number of compounds in the active fractions limits our observation to examine all compounds for more possible DKPs, or other compounds which may act as an inducer. A study discovered multiple DKPs from *Nocardiopsis* sp. RV163 monocultures (Dashti *et al*. 2014), thus indicating that several other compounds in the MS results may be other DKPs. At the time of publishing, no isolation of DKPs from *Pseudonocardia* sp. has been reported. Therefore, there may be a possibility that other DKPs are yet to be discovered at this point.

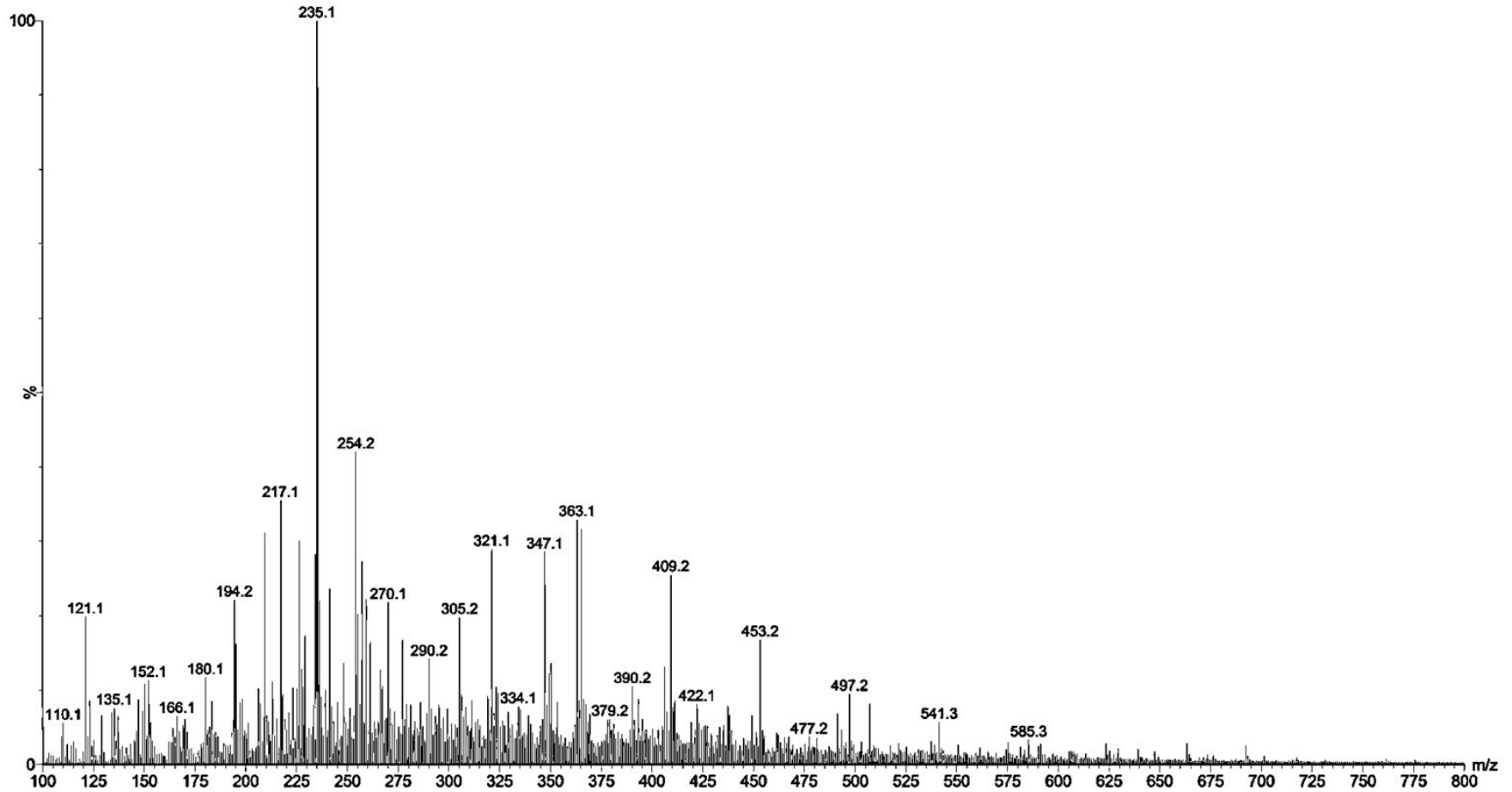


Figure 6.7 MS of XAD-4 methanolic fraction 4 of *Pseudonocardia* sp. PIP 161 broth medium, showing multiple compounds with a major peak at m/z 235.1, followed by 254.1. These two compounds were analysed with tandem MS.

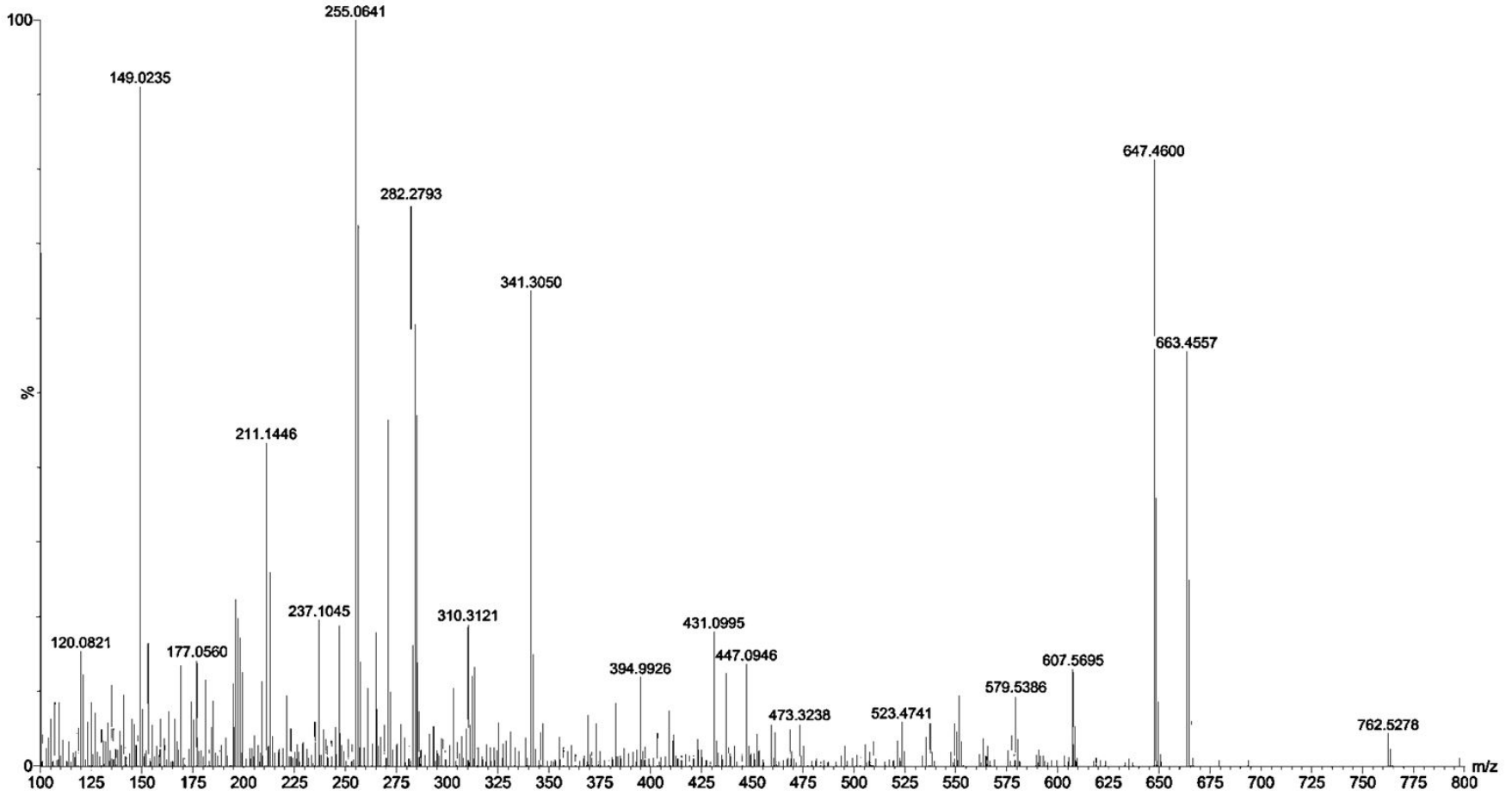


Figure 6.8 MS of XAD-4 methanolic fraction 5 of *Pseudonocardia* sp. PIP 161 broth medium, showing multiple compounds with a major peak m/z 255.1. Compounds from the previous fraction were also found in this fraction at a lower abundance.

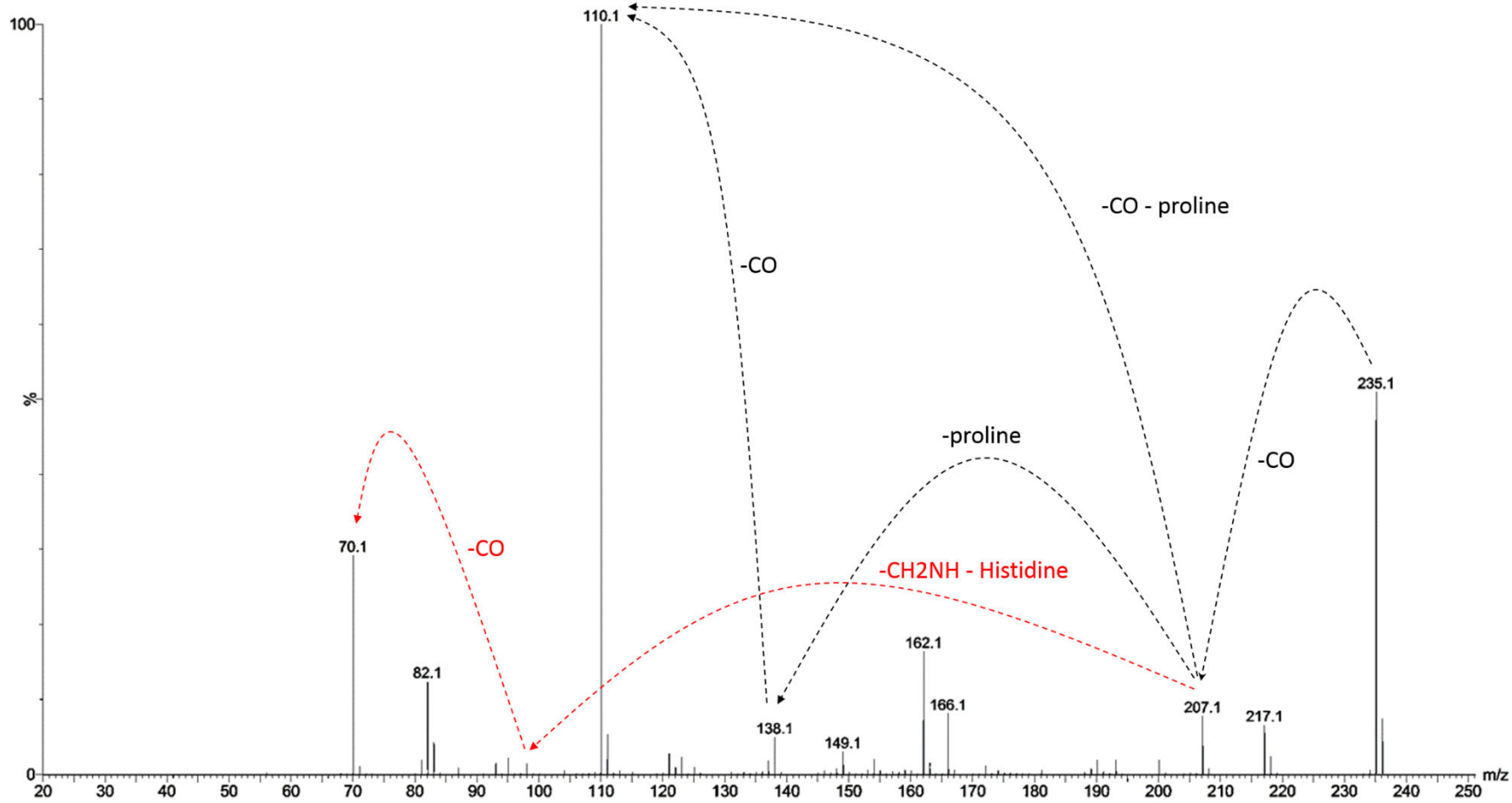


Figure 6.9 Hypothetical fragmentation of peak m/z 235.1 from active fraction 4, as supported by Furtado *et al*. (2007).

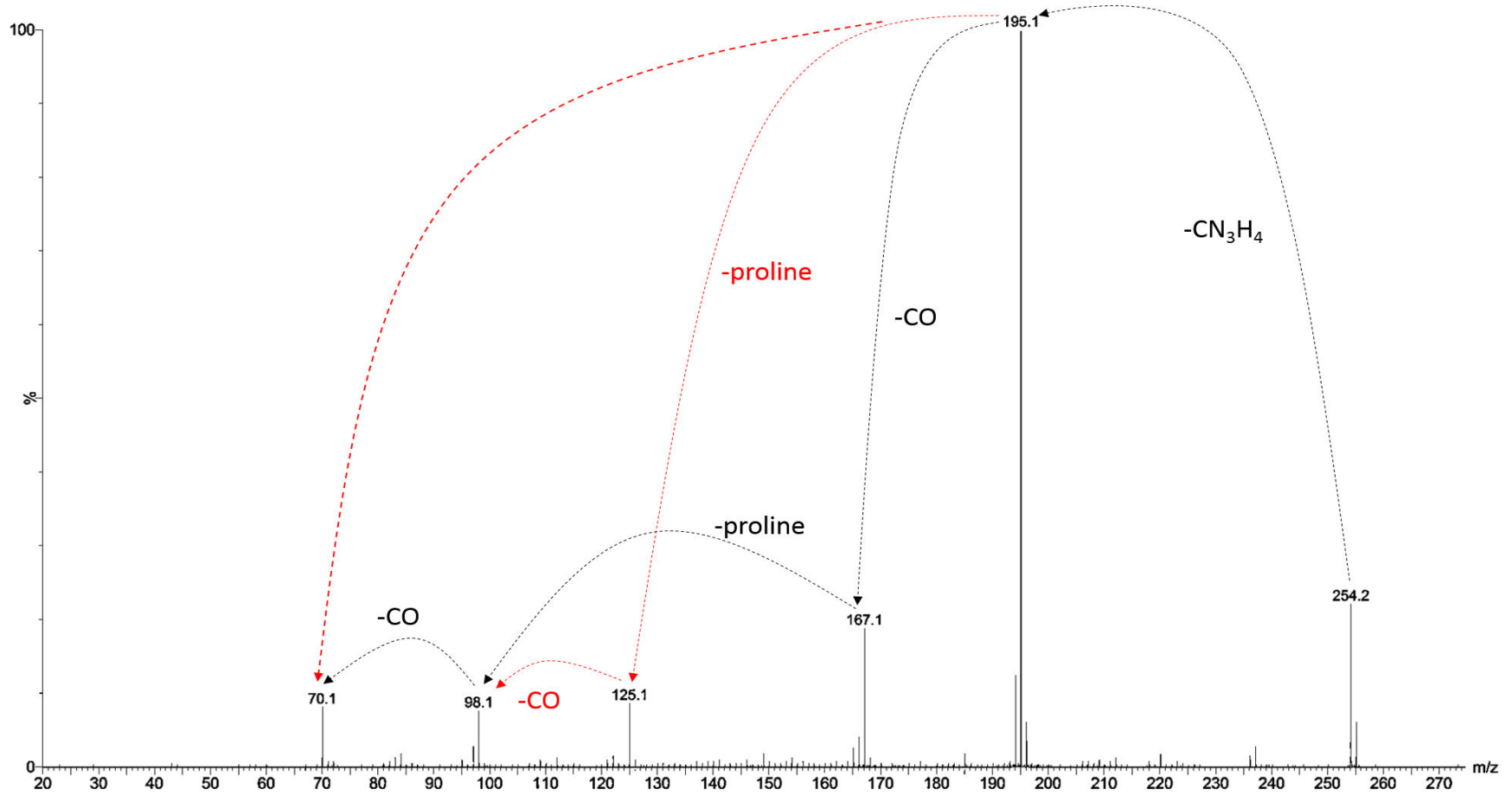


Figure 6.10 Hypothetical fragmentation of peak m/z 254.2 from active fraction 4, as supported by Vergne *et al*. (2006) and Stark and Hoffman (2005).

6.3.3 Cyclo (His-Pro) induced secondary metabolite changes in *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36 monocultures

In order to test the ability of cyclo (His-Pro) in inducing secondary metabolite changes in *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36, this compound was added to liquid medium and tested for secondary metabolite production. In order to compare the efficacy of cyclo (His-Pro), a comparison with monocultures of *Streptomyces* sp. EUC 63 and *Streptomyces* sp. SC 36 and co-cultured with *Pseudonocardia* sp. PIP 161 in the same batch was made. The result of the antibiotic activity of *Streptomyces* sp. EUC 63 after supplementation with cyclo (His-Pro) was shown in figure 6.11.

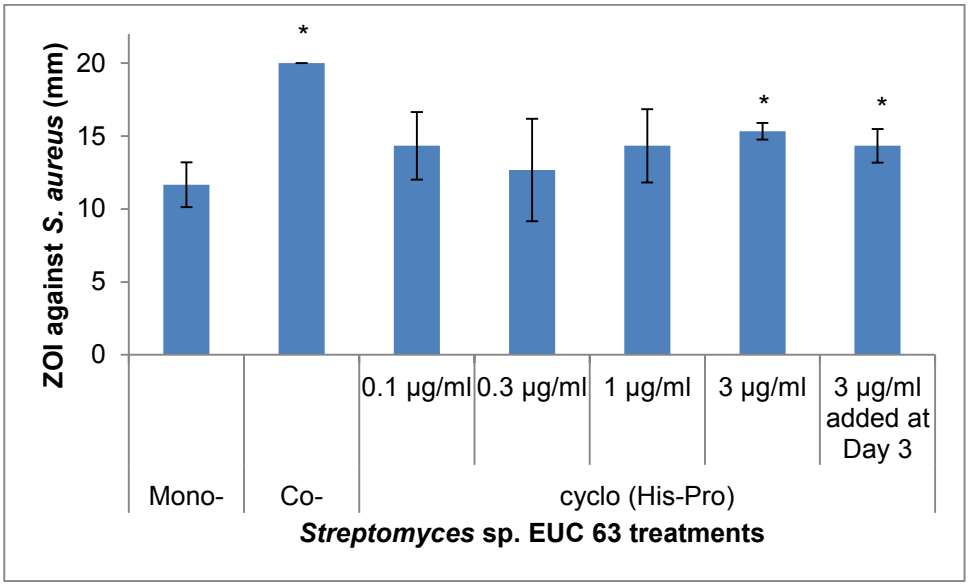
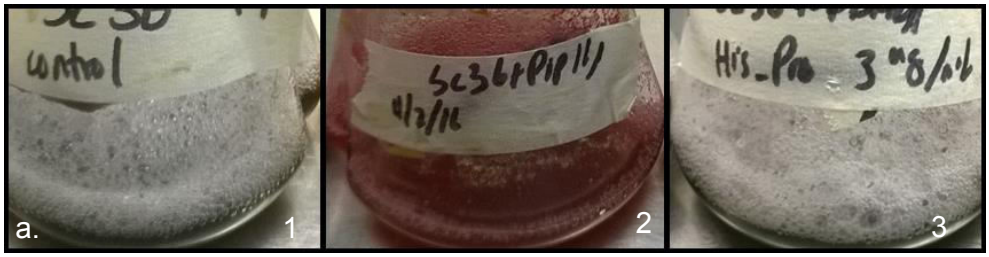


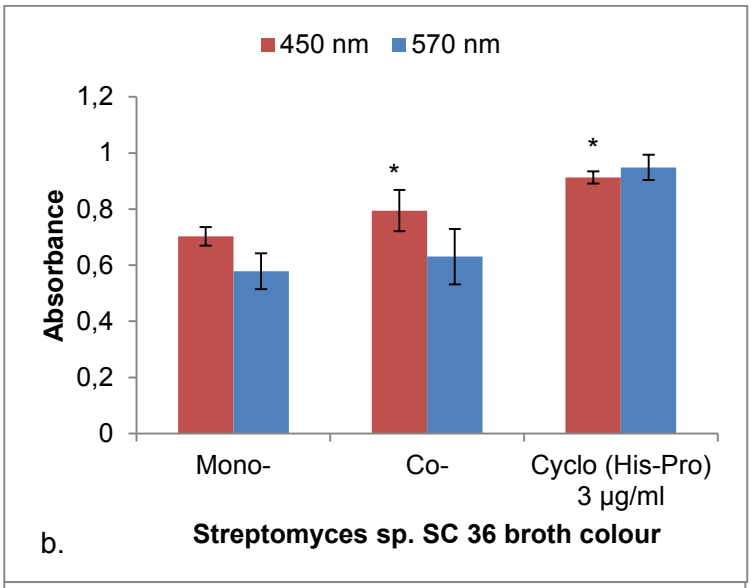
Figure 6.11 Comparison of antibiotic activity of methanol extracts of mycelium of *Streptomyces* sp. EUC 63 monocultured and co-cultured with *Pseudonocardia* sp. PIP 161 in ISP-2 medium and monocultures ISP-2 medium added at varying concentrations of cyclo (His-Pro). (*) denotes significant difference compared to the monoculture in ISP-2 medium.

A modest increase of antibiotic activity was observed when cyclo (His-Pro) was supplemented in *Streptomyces* sp. EUC 63 production medium when compared to monocultures. However, this level was significantly lower compared to *Streptomyces* sp. EUC 63 was co-cultured with *Pseudonocardia* sp. PIP 161. Unlike previous results where broth colour and mycelia colour changed when co-cultured with *Pseudonocardia* sp. PIP 161 (refer to chapter 4), no morphological changes were observed compared to the monocultures after supplementation with cyclo (His-Pro).

In *Streptomyces* sp. SC 36, however, a visible change of broth colour was observed after supplementation with cyclo (His-Pro), whereas the broth colour turned in to a

darker hue. However, co-culture with *Pseudonocardia* sp. PIP 161 resulted in a bright red colour change (as shown in figure 6.12a). This was then further quantified using a spectrophotometer (as shown in figure 6.12b). Finally, antibiotic activity of mycelial extracts of *Streptomyces* sp. SC 36 was also compared (as shown in figure 6.12c).





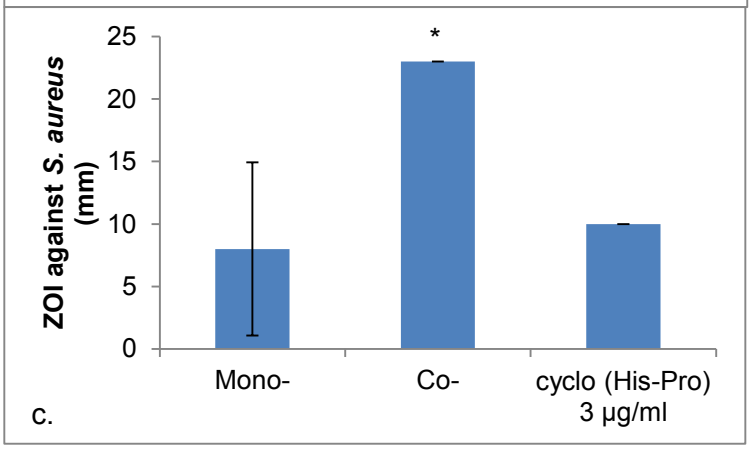


Figure 6.12 a. Broth colour of *Streptomyces* sp. SC 36 in (1) monoculture, (2) co-cultured with *Pseudonocardia* sp. PIP 161 and (3) with supplementation of cyclo (his-pro); b. quantification of colour change using spectrophotometer in 450 nm and 570 nm; c. antibiotic activity of mycelial extracts of *Streptomyces* sp. SC 36 against S. aureus. (*) denotes significant ($p<0.05$) difference compared to monocultures from the same wavelength measurement.

These results show that cyclo (His-Pro) was able to alter secondary metabolism in both *Streptomyces* species tested, with varying effects. While in *Streptomyces* sp. EUC 63 a slight increase in antibiotic activity was observed, no morphological changes or pigmentation was observed in the broth. In *Streptomyces* sp. SC 36, however, cyclo (His-Pro) supplementation had seemingly the opposite effect: an increased production of secreted pigments occurred, but no increase of antibiotic activity was observed. In both cases, supplementation of cyclo (His-Pro) alone failed to replicate the all changes that occurred when co-cultured with *Pseudonocardia* sp. PIP 161.

6.3.4 Low level of DKPs were able to increase antibiotic production in *Streptomyces* sp. EUC 63

Various DKPs available in our laboratory were then tested at different concentration on increase in antibiotic production in *Streptomyces* sp. EUC 63. Induction with two DKPs added separately, cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro), altered pigmentation, medium colour and extract colour in liquid cultivations of *Streptomyces* sp. EUC 63 compared to cultures without the addition of DKP. Induction with highest concentrations of cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro) increased red pigment formation in *Streptomyces* sp. EUC 63 to varying degrees, observed in liquid medium broth colour, indicating alteration in compounds produced during secondary metabolism, as shown in Figure 6.13.

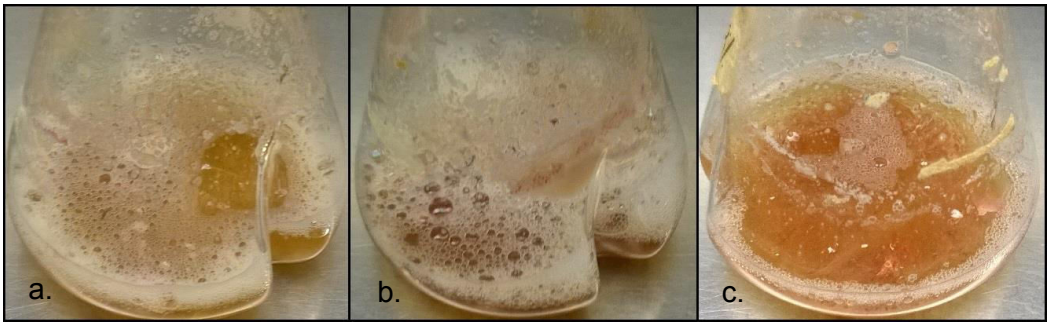


Figure 6.13 Observed colour changes in liquid broth of *Streptomyces* sp. EUC 63 in (a**)** un-induced, (b) 3 µg/ml cyclo (L-Tyr-L-Val) and (c) 3 µg/ml cyclo (L-Tyr-L-Pro) induction, whereas DKPs were added at day 0.

An increase in antibiotic production was observed in liquid cultures of *Streptomyces* sp. EUC 63 induced with the highest concentration of both cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro), as shown in figure 6.14. A higher induction was achieved when the inducer was added after 3 days of cultivation.

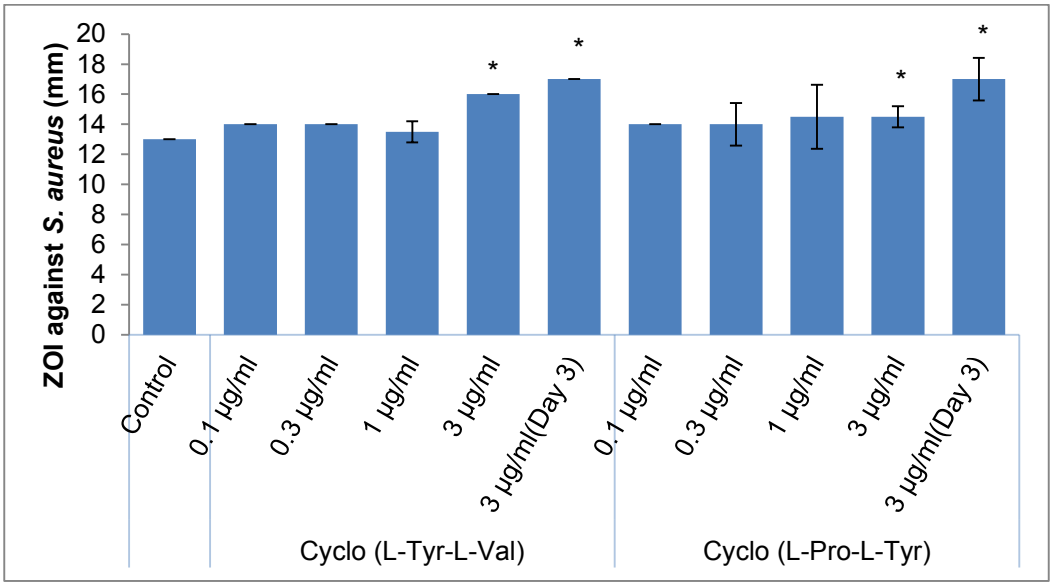


Figure 6.14 Increase of antibiotic production was observed when *Streptomyces* sp. EUC 63 was induced with different concentrations of cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro). The highest induction was achieved with the highest concentration of inducers added at day 3. (*) denotes significant ($p<0.05$) difference compared to control.

Based on these findings, it was concluded that DKPs were able to cause developmental changes and secondary metabolite production in *Streptomyces* sp. EUC 63 with different efficacies. As several DKPs were found in *Pseudonocardia* sp. PIP 161, it was likely that these DKPs were a mediator for the interaction between this species and *Streptomyces* sp. EUC 63, and can be used to induce secondary metabolite production from different *Streptomyces* species.

6.4 Conclusion

It was concluded that cyclo (His-Pro), which was secreted by *Pseudonocardia* sp. PIP 161, alters secondary metabolism in some *Streptomyces* species, and therefore is one of the factors that mediate the interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* species. However, cyclo (His-Pro) alone was not able to replicate the changes that were observed in co-culture with *Pseudonocardia* sp. PIP 161. At least one more DKP was discovered in a single fraction of *Pseudonocarida* sp. PIP

161 broth, and other known DKPs were also found to increase antibiotic production as well as pigment production in *Streptomyces* sp. EUC 63. These results indicate the importance of DKPs as inducers for secondary metabolism in *Streptomyces* species.

6.5 Acknowledgement

I acknowledge and thank Ern Lacey from Microbial Screening Technologies, Sydney, Australia, for his brilliant insight which led to the first examination of DKPs as inducers, as well as for supplying cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro) used in the bioassay. I also acknowledge Dr. Yadollah Bahrami for his help in conducting the mass spectrometry and chemical analysis.

CHAPTER 7. MAJOR FINDINGS AND FUTURE DIRECTIONS

7.1 Major findings

7.1.1 Interaction across genera in actinobacteria

A number of interactions between actinobacteria was observed in this study, with 45 positive and negative interactions discovered from 388 unique combinations of non-*Streptomyces* and *Streptomyces* strains. The interaction was in the form of changes in morphology, pigmentation, sporulation, and antibiotic production. The interaction that we focused on in this study was between *Streptomyces* spp., *Micromonospora* spp., and *Pseudonocardia* spp.

Streptomyces sp. EUM 76 was found to be induced by multiple actinobacteria from across genera (*Pseudonocardia*, *Nocardia*, and *Streptomyces*), characterising it as a 'receiver'-centric actinobacterium. *Pseudonocardia* spp. acted as an inducer of sporulation, pigment production and antibiotic production in actinobacteria from other genera. In particular, *Pseudonocardia* sp. PIP 161 was able to increase antibiotic production in 17 other *Streptomyces* spp, and therefore characterised as a 'sender'-centric actinobacterium.

7.1.2 *Pseudonocardia* sp. PIP 161 influences secondary metabolism in *Streptomyces* species

Pseudonocardia sp. PIP 161 was found to alter the metabolic profile of *Streptomyces* spp. by secreting chemical signalling compound(s). While these compounds increased the antibiotic production by the *Streptomyces* spp. tested, these compounds altered the metabolic profile differently. The effect of these chemical compounds on *Streptomyces* sp. PIP 146 only changed the production of several metabolites (increases on some and decreases on others), indicating a metabolic pathway shift. However, in *Streptomyces* sp. EUC 63, antibiotic activity increased and multiple antibiotic compounds were produced. Furthermore, almost all secondary metabolite production was increased, and a 7-fold increase in an enterobactin-like compound was observed.

In response to the finding that increased siderophore production was observed, a study as to how the iron levels influence secondary metabolite production was

conducted; in an attempt to understand the mechanism of the interaction. While iron was discovered to have an effect on the formation of aerial mycelium and other morphological characteristics, consistent with previous studies, pigment production and antibiotic production were found to be more influenced by co-culture with *Pseudonocardia* sp. PIP 161 compared to changes in iron levels. Furthermore, survivability of *Streptomyces* spp. was supported by co-culture in low iron levels, suggesting that the chemical compound from *Pseudonocardia* sp. PIP 161 either induced the production of siderophores or that siderophore-piracy between *Streptomyces* spp and *Pseudonocardia* sp. PIP 161 occurred.

7.1.3 Interaction between *Pseudonocardia* sp. PIP 161 and *Streptomyces* species is potentially mediated by diketopiperazines

Isolation of chemical signals from *Pseudonocardia* sp. PIP 161 indicate that the interaction with *Streptomyces* species, at least partially, was mediated by diketopiperazines, in particular, diketopiperazine cyclo (His-Pro). This is the first instance that cyclo (His-Pro) was isolated from actinobacteria, or more specifically, from *Pseudonocardia* sp. PIP 161. Furthermore, the results of exogenous cyclo (His-Pro) induction corroborated previous findings, in which different effects were observed in different *Streptomyces* species. For *Streptomyces* sp. EUC 63, an increase of antibiotic production was observed, while in *Streptomyces* sp. SC 36, an increase of blue pigment production was observed in the medium after induction with added cyclo (His-Pro).

While induction with cyclo (His-Pro) alone could not replicate all the changes observed during co-culture, other diketopiperazines tested were found to also induce secondary metabolism. Induction with different diketopiperazines (cyclo (L-Tyr-L-Val) and cyclo (L-Tyr-L-Pro)), at least with *Streptomyces* sp. EUC 63, also showed a modest increase in antibiotic production, and in some cases accompanied by changes in broth colour. Thus, the role of diketopiperazines as a mediator for interspecies cell-to-cell communication in actinobacteria was, therefore, established in this study.

7.2 Future directions

7.2.1 Further analysis of *Pseudonocardia* sp. PIP 161 compounds responsible for the interaction with other actinobacteria

The study of chemical signalling compounds secreted by *Pseudonocardia* sp. PIP 161 only explored the induction of the major compound in the active fraction isolated from *Pseudonocardia* sp. PIP 161 liquid medium. The same fraction showed at least one more known diketopiperazines, a cyclo (Arg-Pro), and several peaks within the size of known modified amino-acid diketopiperazines. As induction by cyclo (His-Pro) alone was not able to completely replicate the changes that occur in co-culture, it can be hypothesized that other diketopiperazines or a combination of diketopiperazines would cause the changes observed in co-culture to occur. Thus, a more detailed analysis and evaluation of the other fractions of compounds secreted by *Pseudonocardia* sp. PIP 161 could reveal other and possibly more potent inducers of secondary metabolism. Further studies would include the different diketopiperazine combinations and detecting the combinations which induces similar effects to *Pseudonocardia* sp. PIP 161 co-culture.

7.2.2 Understanding the mechanism of diketopiperazines in influencing secondary metabolism

Co-culture with *Pseudonocardia* sp. PIP 161 was observed to be able to increase secondary metabolite production in different *Streptomyces* spp. While antibiotic or pigment was seen to be also increased with the induction of exogenously added diketopiperazines, the molecular basis of the induction was un-explored in this study, due to a lack of time. Understanding whether a regulatory pathway which was influenced by diketopiperazine would further establish the position of diketopiperazines as quorum sensing molecules, and could provide an explanation of how *Pseudonocardia* sp. PIP 161 was able to communicate with various inter-genera (between actinobacteria) and even inter-kingdom (with fungal-ants and *Artemisia annua*, for instance) organisms. This can be achieved by using microarray techniques after induction of various diketopiperazines to measure gene expression in *Streptomyces* species, and therefore, uncovering genes that were upregulated (or downregulated) in the presence of different diketopiperazines.

7.2.3 Application of diketopiperazine induction in cryptic gene expression in *Streptomyces* spp. or other species

A major finding in this study was that diketopiperazines were able to induce antibiotic production in *Streptomyces* spp., signifying the potential for diketopiperazines to induce the expression of cryptic genes in *Streptomyces* spp. While evidence in other studies suggest that diketopiperazines acts on regulatory pathways in Gram negative bacteria, this has not been fully explored in the induction of novel antibiotic compounds from actinobacteria. Thus, findings from this study may suggest that the other diketopiperazines may be potent inducers for novel compound discovery from both known and unknown *Streptomyces* species.

CHAPTER 8. REFERENCES

Abdelmohsen, UR, Grkovic, T, Balasubramanian, S, Kamel, MS, Quinn, RJ & Hentschel, U 2015, 'Elicitation of secondary metabolism in actinomycetes', *Biotechnology Advances*, vol. 33, no. 6, Part 1, pp. 798-811.

Adegboye, MF & Babalola, OO 2012, 'Taxonomy and ecology of antibiotic producing actinomycetes', *African Journal of Agricultural Research*, vol. 7, no. 15, pp. 2255 - 61.

Adnani, N, Vazquez-Rivera, E, Adibhatla, SN, Ellis, GA, Braun, DR & Bugni, TS 2015, 'Investigation of Interspecies Interactions within Marine *Micromonospora*ceae Using an Improved Co-Culture Approach', *Marine Drugs*, vol. 13, no. 10, pp. 6082-98.

Ahmed, S, Craney, A, Pimentel-Elardo, SM & Nodwell, JR 2013, 'A Synthetic, Species-Specific Activator of Secondary Metabolism and Sporulation in *Streptomyces coelicolor*', *ChemBioChem*, vol. 14, no. 1, pp. 83-91.

Ahring, BK & Westermann, P 1987, 'Thermophilic Anaerobic Degradation of Butyrate by a Butyrate-Utilizing Bacterium in Coculture and Triculture with Methanogenic Bacteria', *Applied and Environmental Microbiology*, vol. 53, no. 2, pp. 429-33.

Alam, MT, Medema, MH, Takano, E & Breitling, R 2011, 'Comparative genome-scale metabolic modeling of actinomycetes: The topology of essential core metabolism', *FEBS Letters*, vol. 585, no. 14, pp. 2389-94.

Alanis, AJ 2005, 'Resistance to Antibiotics: Are We in the Post-Antibiotic Era?', *Archives of Medical Research*, vol. 36, no. 6, pp. 697-705.

Alexander, DB & Zuberer, DA 1991, 'Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria', *Biology and Fertility of Soils*, vol. 12, no. 1, pp. 39-45.

Alvan, G, Edlund, C & Heddini, A 2011, 'The global need for effective antibiotics—A summary of plenary presentations', *Drug Resistance Updates*, vol. 14, no. 2, pp. 70-6.

Andersen, SB, Hansen, LH, Sapountzis, P, Sørensen, SJ & Boomsma, JJ 2013, 'Specificity and stability of the *Acromyrmex–Pseudonocardia* symbiosis', *Molecular Ecology*, vol. 22, no. 16, pp. 4307-21.

Andersen, SB, Yek, SH, Nash, DR & Boomsma, JJ 2015, 'Interaction specificity between leaf-cutting ants and vertically transmitted *Pseudonocardia* bacteria', *BMC Evolutionary Biology*, vol. 15, p. 27.

Anderson, TB, Brian, P & Champness, WC 2001, 'Genetic and transcriptional analysis of absA, an antibiotic gene cluster-linked two-component system that regulates multiple antibiotics in *Streptomyces* coelicolor', *Mol Microbiol*, vol. 39.

Ando, N, Ueda, K & Horinouchi, S 1997, 'A *Streptomyces* griseus gene (sgaA) suppresses the growth disturbance caused by high osmolality and a high concentration of A-factor during early growth', *Microbiology*, vol. 143, no. 8, pp. 2715-23.

Arias, AA, Lambert, S, Martinet, L, Adam, D, Tenconi, E, Hayette, M-P, Ongena, M & Rigali, S 2015, 'Growth of desferrioxamine-deficient *Streptomyces* mutants through

xenosiderophore piracy of airborne fungal contaminations', *FEMS Microbiology Ecology*, vol. 91, no. 7.

Asamizu, S, Ozaki, T, Teramoto, K, Satoh, K & Onaka, H 2015, 'Killing of mycolic acid-containing bacteria aborted induction of antibiotic production by *Streptomyces* in combined-culture', *PLoS ONE*, vol. 10, no. 11, p. e0142372.

Atta, HM 2015, 'Biochemical studies on antibiotic production from *Streptomyces* sp.: Taxonomy, fermentation, isolation and biological properties', *Journal of Saudi Chemical Society*, vol. 19, no. 1, pp. 12-22.

Ayuso-Sacido, A & Genilloud, O 2005, 'New PCR primers for the screening of nrps and pks-i systems in actinomycetes: detection and distribution of these biosynthetic gene sequences in major taxonomic groups', *Microbial Ecology*, vol. 49, no. 1, pp. 10-24.

Azman, A-S, Othman, I, Velu, SS, Chan, K-G & Lee, L-H 2015, 'Mangrove rare actinobacteria: taxonomy, natural compound, and discovery of bioactivity', *Frontiers in Microbiology*, vol. 6, p. 856.

Bankar, SB & Singhal, RS 2010, 'Optimization of poly-ε-lysine production by *Streptomyces* noursei NRRL 5126', *Bioresource Technology*, vol. 101, no. 21, pp. 8370-5.

Barka, EA, Vatsa, P, Sanchez, L, Gaveau-Vaillant, N, Jacquard, C, Klenk, H-P, Clément, C, Ouhdouch, Y & van Wezel, GP 2016, 'Taxonomy, physiology, and natural products of actinobacteria', *Microbiology and Molecular Biology Reviews*, vol. 80, no. 1, pp. 1-43.

Barke, J, Seipke, R, Gruschow, S, Heavens, D, Drou, N, Bibb, M, Goss, R, Yu, D & Hutchings, M 2010, 'A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus'*, *BMC Biology*, vol. 8, no. 1, p. 109.

Barke, J, Seipke, RF, Grüschow, S, Heavens, D, Drou, N, Bibb, MJ, Goss, RJM, Yu, DW & Hutchings, MI 2010, 'A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus'*, *BMC Biology*, vol. 8, pp. 109-.

Barona-Gómez, F, Lautru, S, Francou, F-X, Leblond, P, Pernodet, J-L & Challis, GL 2006, 'Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambofaciens* ATCC 23877', *Microbiology*, vol. 152, no. 11, pp. 3355-66.

Baudoin, E, Benizri, E & Guckert, A 2003, 'Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere', *Soil Biology and Biochemistry*, vol. 35, no. 9, pp. 1183-92.

Béchet & Blondeau 1998, 'Iron deficiency-induced tetracycline production in submerged cultures by *Streptomyces aureofaciens*', *Journal of Applied Microbiology*, vol. 84, no. 5, pp. 889-94.

Bentley, SD, Chater, KF, Cerdeño-Tárraga, AM, Challis, GL, Thomson, NR, James, KD, Harris, DE, Quail, MA, Kieser, H & Harper, D 2002, 'Complete genome sequence of the model actinomycete *Streptomyces* coelicolor A3(2)', *Nature*, vol. 417.

Bentley, SD, Chater, KF, Cerdeno-Tarraga, AM, Challis, GL, Thomson, NR, James, KD, Harris, DE, Quail, MA, Kieser, H, Harper, D, Bateman, A, Brown, S, Chandra, G, Chen, CW, Collins, M, Cronin, A, Fraser, A, Goble, A, Hidalgo, J, Hornsby, T, Howarth, S, Huang, CH, Kieser, T, Larke, L, Murphy, L, Oliver, K, O'Neil, S, Rabbinowitsch, E, Rajandream, MA, Rutherford, K, Rutter, S, Seeger, K, Saunders, D, Sharp, S, Squares, R, Squares, S, Taylor, K, Warren, T, Wietzorrek, A, Woodward, J, Barrell, BG, Parkhill, J & Hopwood, DA 2002, 'Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2)', *Nature*, vol. 417, no. 6885, pp. 141-7.

Beppu, T 1992, 'Secondary metabolites as chemical signals for cellular differentiation', *Gene*, vol. 115, no. 1–2, pp. 159-65.

Bertrand, S, Bohni, N, Schnee, S, Schumpp, O, Gindro, K & Wolfender, J-L 2014, 'Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery', *Biotechnology Advances*, vol. 32, no. 6, pp. 1180-204.

Bibb, MJ 2005, 'Regulation of secondary metabolism in streptomycetes', *Curr Opin Microbiol*, vol. 8.

Bodenhausen, N, Horton, MW & Bergelson, J 2013, 'Bacterial Communities Associated with the Leaves and the Roots of *Arabidopsis thaliana*', *PLoS ONE*, vol. 8, no. 2, p. e56329.

Bogel, G, Schrempf, H & Ortiz de Orué Lucana, D 2008, 'The heme-binding protein HbpS regulates the activity of the *Streptomyces reticuli* iron-sensing histidine kinase SenS in a redox-dependent manner', *Amino Acids*, vol. 37, no. 4, pp. 681-91.

Borodina, I, Krabben, P & Nielsen, J 2005, 'Genome-scale analysis of *Streptomyces coelicolor* A3(2) metabolism', *Genome Research*, vol. 15, no. 6, pp. 820-9.

Bunet, R, Brock, A, Rexer, H-U & Takano, E 2006, 'Identification of genes involved in siderophore transport in *Streptomyces coelicolor* A3(2)', *FEMS Microbiology Letters*, vol. 262, no. 1, pp. 57-64.

Bush, K 2012, 'Improving known classes of antibiotics: an optimistic approach for the future', *Current Opinion in Pharmacology*, vol. 12, no. 5, pp. 527-34.

Cabaj, A & Kosakowska, A 2009, 'Iron-dependent growth of and siderophore production by two heterotrophic bacteria isolated from brackish water of the southern Baltic Sea', *Microbiological Research*, vol. 164, no. 5, pp. 570-7.

Cafaro, MJ, Poulsen, M, Little, AEF, Price, SL, Gerardo, NM, Wong, B, Stuart, AE, Larget, B, Abbot, P & Currie, CR 2011, 'Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria', *Proceedings of the Royal Society B: Biological Sciences*, vol. 278, no. 1713, pp. 1814-22.

Caffrey, P, Lynch, S, Flood, E, Finnan, S & Oliynyk, M 2001, 'Amphotericin biosynthesis in *Streptomyces nodosus*: deductions from analysis of polyketide synthase and late genes', *Chemistry & Biology*, vol. 8, no. 7, pp. 713-23.

Cano-Prieto, C, Losada, AA, Braña, AF, Méndez, C, Salas, JA & Olano, C 2015, 'Crosstalk of Nataxazole Pathway with Chorismate-Derived Ionophore Biosynthesis Pathways in *Streptomyces* sp. Tü 6176', *ChemBioChem*, vol. 16, no. 13, pp. 1925-32.

Carlsohn, MR, Groth, I, Spröer, C, Schütze, B, Saluz, H-P, Munder, T & Stackebrandt, E 2007, '*Kribbella aluminosa* sp. nov., isolated from a medieval alum slate mine', *International Journal of Systematic and Evolutionary Microbiology*, vol. 57, no. 9, pp. 1943-7.

Carr, G, Derbyshire, ER, Caldera, E, Currie, CR & Clardy, J 2012, 'Antibiotic and Antimalarial Quinones from Fungus-Growing Ant-Associated *Pseudonocardia* sp', *Journal of Natural Products*.

Challis, GL & Hopwood, DA 2003, 'Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species', *Proc Natl Acad Sci USA*, vol. 100, no. Suppl 2, pp. 14555-61.

Chan, Y-H, Fan, MM, Fok, C-M, Lok, ZL, Ni, M, Sin, C-F, Wong, K-K, Wong, S-M, Yeung, R, Yeung, TT, Chow, W-C, Lam, T-H & Schooling, CM 2012, 'Antibiotics nonadherence and knowledge in a community with the world's leading prevalence of antibiotics resistance: Implications for public health intervention', *American Journal of Infection Control*, vol. 40, no. 2, pp. 113-7.

Chankhamhaengdecha, S, Hongvijit, S, Srichaisupakit, A, Charnchai, P & Panbangred, W 2013, 'Endophytic Actinomycetes: A Novel Source of Potential Acyl Homoserine Lactone Degrading Enzymes', *BioMed Research International*, vol. 2013, p. 8.

Chen, H, Daletos, G, Abdel-Aziz, MS, Thomy, D, Dai, H, Brötz-Oesterhelt, H, Lin, W & Proksch, P 2015, 'Inducing secondary metabolite production by the soil-dwelling fungus Aspergillus terreus through bacterial co-culture', *Phytochemistry Letters*, vol. 12, no. 0, pp. 35-41.

Chen, S, Luo, J, Hu, M, Lai, K, Geng, P & Huang, H 2012, 'Enhancement of cypermethrin degradation by a coculture of Bacillus cereus ZH-3 and *Streptomyces aureus* HP-S-01', *Bioresource Technology*, vol. 110, pp. 97-104.

Choi, S-U, Lee, C-K, Hwang, Y-I, Kinosita, H & Nihira, T 2003, 'γ-Butyrolactone autoregulators and receptor proteins in non-*Streptomyces* actinomycetes producing commercially important secondary metabolites', *Archives of Microbiology*, vol. 180, no. 4, pp. 303-7.

Clardy, J, Fischbach, MA & Currie, CR 2009, 'The natural history of antibiotics', *Current Biology*, vol. 19, no. 11, pp. R437-R41.

Clarridge, JE 2004, 'Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases', *Clinical Microbiology Reviews*, vol. 17, no. 4, pp. 840-62.

Coisne, S, Béchet, M & Blondeau, R 1999, 'Actinorhodin production by *Streptomyces* coelicolor A3(2) in iron-restricted media', *Letters in Applied Microbiology*, vol. 28, no. 3, pp. 199-202.

Condon, BJ, Oide, S, Gibson, DM, Krasnoff, SB & Turgeon, BG 2014, 'Reductive Iron Assimilation and Intracellular Siderophores Assist Extracellular Siderophore-Driven Iron Homeostasis and Virulence', *Molecular Plant-Microbe Interactions*, vol. 27, no. 8, pp. 793-808.

Conn, VM & Franco, CMM 2004, 'Analysis of the Endophytic Actinobacterial Population in the Roots of Wheat (*Triticum aestivum* L.) by Terminal Restriction

Fragment Length Polymorphism and Sequencing of 16S rRNA Clones', *Applied and Environmental Microbiology*, vol. 70, no. 3, pp. 1787-94.

Cooley, M, Chhabra, SR & Williams, P 2008, 'N-Acylhomoserine Lactone-Mediated Quorum Sensing: A Twist in the Tail and a Blow for Host Immunity', *Chemistry & Biology*, vol. 15, no. 11, pp. 1141-7.

Cornelis, P, Wei, Q, Andrews, SC & Vinckx, T 2011, 'Iron homeostasis and management of oxidative stress response in bacteria', *Metallomics*, vol. 3, no. 6, pp. 540-9.

Cragg, GM & Newman, DJ 2013, 'Natural products: A continuing source of novel drug leads', *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1830, no. 6, pp. 3670-95.

Craig, M, Lambert, S, Jourdan, S, Tenconi, E, Colson, S, Maciejewska, M, Ongena, M, Martin, JF, van Wezel, G & Rigali, S 2012, 'Unsuspected control of siderophore production by N-acetylglucosamine in streptomycetes', *Environmental Microbiology Reports*, vol. 4, no. 5, pp. 512-21.

Crawford, DL, Lynch, JM, Whipps, JM & Ousley, MA 1993, 'Isolation and Characterization of Actinomycete Antagonists of a Fungal Root Pathogen', *Applied and Environmental Microbiology*, vol. 59, no. 11, pp. 3899-905.

Dashti, Y, Grkovic, T, Abdelmohsen, UR, Hentschel, U & Quinn, RJ 2014, 'Production of Induced Secondary Metabolites by a Co-Culture of Sponge-Associated *Actinomycetes*, *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163', *Marine Drugs*, vol. 12, no. 5, pp. 3046-59.

Davelos Baines, AL, Xiao, K & Kinkel, LL 2007, 'Lack of correspondence between genetic and phenotypic groups amongst soil-borne *streptomycetes'*, *FEMS Microbiology Ecology*, vol. 59, no. 3, pp. 564-75.

De Rosa, S, Mitova, M & Tommonaro, G 2003, 'Marine bacteria associated with sponge as source of cyclic peptides', *Biomolecular Engineering*, vol. 20, no. 4–6, pp. 311-6.

Degrassi, G, Aguilar, C, Bosco, M, Zahariev, S, Pongor, S & Venturi, V 2002, 'Plant growth-promoting Pseudomonas putida WCS358 produces and secretes four cyclic dipeptides: Cross-talk with quorum sensing bacterial sensors', *Current Microbiology*, vol. 45, no. 4, pp. 250-4.

Dietrich, LEP, Teal, TK, Price-Whelan, A & Newman, DK 2008, 'Redox-Active Antibiotics Control Gene Expression and Community Behavior in Divergent Bacteria', *Science (New York, N.Y.)*, vol. 321, no. 5893, pp. 1203-6.

Diggle, SP, Crusz, SA & Cámara, M 2007, 'Quorum sensing', *Current Biology*, vol. 17, no. 21, pp. R907-R10.

Du, Y-L, Shen, X-L, Yu, P, Bai, L-Q & Li, Y-Q 2011, 'Gamma-Butyrolactone Regulatory System of *Streptomyces chattanoogensis* Links Nutrient Utilization, Metabolism, and Development', *Applied and Environmental Microbiology*, vol. 77, no. 23, pp. 8415-26.

Duangmal, K, Thamchaipenet, A, Matsumoto, A & Takahashi, Y 2009, '*Pseudonocardia* acaciae sp. nov., isolated from roots of Acacia auriculiformis A.

Cunn. ex Benth', *International Journal of Systematic and Evolutionary Microbiology*, vol. 59, no. 6, pp. 1487-91.

Duraipandiyan, V, Sasi, AH, Islam, VIH, Valanarasu, M & Ignacimuthu, S 2010, 'Antimicrobial properties of actinomycetes from the soil of Himalaya', *Journal de Mycologie Médicale / Journal of Medical Mycology*, vol. 20, no. 1, pp. 15-20.

Dyson, P 2009, '*Streptomyces*', in S Editor-in-Chief: Moselio (ed.), *Encyclopedia of Microbiology (Third Edition)*, Academic Press, Oxford, pp. 318-32.

Escolar, L, Pérez-Martín, J & de Lorenzo, V 1999, 'Opening the Iron Box: Transcriptional Metalloregulation by the Fur Protein', *Journal of Bacteriology*, vol. 181, no. 20, pp. 6223-9.

Everest, GJ & Meyers, PR 2008, '*Kribbella hippodromi* sp. nov., isolated from soil from a racecourse in South Africa', *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 2, pp. 443-6.

Ezaki, M, Iwami, M, Yamashita, M, Komori, T, Umehara, K & Imanaka, H 1992, 'Biphenomycin A production by a mixed culture', *Applied and Environmental Microbiology*, vol. 58, no. 12, pp. 3879-82.

Fair, RJ & Tor, Y 2014, 'Antibiotics and Bacterial Resistance in the 21st Century', *Perspectives in Medicinal Chemistry*, vol. 6, pp. 25-64.

Fiedler, H-P, Krastel, P, Müller, J, Gebhardt, K & Zeeck, A 2001, 'Enterobactin: the characteristic catecholate siderophore of *Enterobacteriaceae* is produced by *Streptomyces* species', *FEMS Microbiology Letters*, vol. 196, no. 2, pp. 147-51.

Fischbach, MA & Walsh, CT 2006, 'Assembly-Line Enzymology for Polyketide and Nonribosomal Peptide Antibiotics: Logic, Machinery, and Mechanisms', *Chemical Reviews*, vol. 106, no. 8, pp. 3468-96.

—— 2009, 'Antibiotics for Emerging Pathogens', *Science*, vol. 325, no. 5944, pp. 1089-93.

Fischer, PM 2003, 'Diketopiperazines in peptide and combinatorial chemistry', *Journal of Peptide Science*, vol. 9, no. 1, pp. 9-35.

Flores, FJ & Martín, JF 2004, 'Iron-regulatory proteins DmdR1 and DmdR2 of *Streptomyces coelicolor* form two different DNA-protein complexes with iron boxes', *Biochemical Journal*, vol. 380, no. Pt 2, pp. 497-503.

Franco, C, Michelsen, P, Percy, N, Conn, V, Listiana, E, Moll, S, Loria, R & Coombs, J 2007, 'Actinobacterial endophytes for improved crop performance', *Australasian Plant Pathology*, vol. 36, no. 6, pp. 524-31.

Furtado, NAJC, Vessecchi, R, Tomaz, JC, Galembeck, SE, Bastos, JK, Lopes, NP & Crotti, AEM 2007, 'Fragmentation of diketopiperazines from *Aspergillus fumigatus* by electrospray ionization tandem mass spectrometry (ESI-MS/MS)', *Journal of Mass Spectrometry*, vol. 42, no. 10, pp. 1279-86.

Galet, J, Deveau, A, Hôtel, L, Frey-Klett, P, Leblond, P & Aigle, B 2015, 'Pseudomonas fluorescens Pirates both Ferrioxamine and Ferricoelichelin Siderophores from *Streptomyces ambofaciens*', *Applied and Environmental Microbiology*, vol. 81, no. 9, pp. 3132-41.

Gao, C, Hindra, Mulder, D, Yin, C & Elliot, MA 2012, 'Crp is a global regulator of antibiotic production in *Streptomyces*', *mBio*, vol. 3.

Gao, Y, Yu, L, Peng, C, Li, Z & Guo, Y 2010, 'Diketopiperazines from two strains of South China Sea sponge-associated microorganisms', *Biochemical Systematics and Ecology*, vol. 38, no. 5, pp. 931-4.

Geier, H, Mostowy, S, Cangelosi, GA, Behr, MA & Ford, TE 2008, 'Autoinducer-2 Triggers the Oxidative Stress Response in *Mycobacterium avium*, Leading to Biofilm Formation', *Applied and Environmental Microbiology*, vol. 74, no. 6, pp. 1798-804.

Gesheva, V, Ivanova, V & Gesheva, R 2005, 'Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus'*, *Microbiological Research*, vol. 160, no. 3, pp. 243-8.

Grayston, SJ, Vaughan, D & Jones, D 1997, 'Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability', *Applied Soil Ecology*, vol. 5, no. 1, pp. 29-56.

Gu, Q, Luo, H, Zheng, W, Liu, Z & Huang, Y 2006, '*Pseudonocardia oroxyli* sp. nov., a novel actinomycete isolated from surface-sterilized Oroxylum indicum root', *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 9, pp. 2193-7.

Guimarães, DO, Borges, WS, Vieira, NJ, de Oliveira, LF, da Silva, CHTP, Lopes, NP, Dias, LG, Durán-Patrón, R, Collado, IG & Pupo, MT 2010, 'Diketopiperazines produced by endophytic fungi found in association with two *Asteraceae* species', *Phytochemistry*, vol. 71, no. 11–12, pp. 1423-9.

Günter, K, Toupet, C & Schupp, T 1993, 'Characterization of an iron-regulated promoter involved in desferrioxamine B synthesis in *Streptomyces pilosus*: repressor-binding site and homology to the diphtheria toxin gene promoter', *Journal of Bacteriology*, vol. 175, no. 11, pp. 3295-302.

Guo, X, Liu, N, Li, X, Ding, Y, Shang, F, Gao, Y, Ruan, J & Huang, Y 2015, 'Red Soils Harbor Diverse Culturable Actinomycetes That are Promising Sources of Novel Secondary Metabolites', *Applied and Environmental Microbiology*, vol. 81, no. 9, pp. 3086-103.

Guyet, A, Benaroudj, N, Proux, C, Gominet, M, Coppée, J-Y & Mazodier, P 2014, 'Identified members of the *Streptomyces lividans* AdpA regulon involved in differentiation and secondary metabolism', *BMC Microbiology*, vol. 14, pp. 81-.

Haeder, S, Wirth, R, Herz, H & Spiteller, D 2009, 'Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 12, pp. 4742-6.

Hara, O & Beppu, T 1982, 'Mutants Blocked in Streptomycin Production in *Streptomyces griseus*-The Role of A-factor', *The Journal of Antibiotics*, vol. 35, no. 3, pp. 349-58.

Harvey, AL, Edrada-Ebel, R & Quinn, RJ 2015, 'The re-emergence of natural products for drug discovery in the genomics era', *Nat Rev Drug Discov*, vol. 14, no. 2, pp. 111-29.

Hasegawa, S, Meguro, A, Shimizu, M, Nishimura, T & Kunoh, H 2006, 'Endophytic Actinomycetes and Their Interactions with Host Plants', *Actinomycetologica*, vol. 20, no. 2, pp. 72-81.

Hibbing, ME, Fuqua, C, Parsek, MR & Peterson, SB 2010, 'Bacterial competition: surviving and thriving in the microbial jungle', *Nature reviews. Microbiology*, vol. 8, no. 1, pp. 15-25.

Higo, A, Hara, H, Horinouchi, S & Ohnishi, Y 2012, 'Genome-wide Distribution of AdpA, a Global Regulator for Secondary Metabolism and Morphological Differentiation in *Streptomyces*, Revealed the Extent and Complexity of the AdpA Regulatory Network', *DNA Research: An International Journal for Rapid Publication of Reports on Genes and Genomes*, vol. 19, no. 3, pp. 259-74.

Hirose, Y, Ogawa, N & Mori, A 1985, 'Properties of histidyl-proline diketopiperazine [cyclo(His-Pro)] binding sites in rat liver', *Regulatory Peptides*, vol. 12, no. 4, pp. 279-88.

Holden, MTG, Chhabra, SR, De Nys, R, Stead, P, Bainton, NJ, Hill, PJ, Manefield, M, Kumar, N, Labatte, M, England, D, Rice, S, Givskov, M, Salmond, GPC, Stewart, GSAB, Bycroft, BW, Kjelleberg, S & Williams, P 1999, 'Quorum-sensing cross talk: Isolation and chemical characterization of cyclic dipeptides from Pseudomonas aeruginosa and other Gram-negative bacteria', *Molecular microbiology*, vol. 33, no. 6, pp. 1254-66.

Hopwood, DA 2013, 'Imaging Mass Spectrometry Reveals Highly Specific Interactions between Actinomycetes To Activate Specialized Metabolic Gene Clusters', *mBio*, vol. 4, no. 5, pp. e00612-13.

Horinouchi, S 2002, 'A Microbial Hormone, A-Factor, as a Master Switch for Morphological Differentiation and Secondary Metabolism in *Streptomyces griseus*', *Frontiers in Bioscience*, vol. 7, pp. d2045-57.

Horinouchi, S & Beppu, T 1992a, 'Autoregulatory Factors and Communication in Actinomycetes', *Annual Review of Microbiology*, vol. 46, no. 1, pp. 377-92.

—— 1992b, 'Regulation of secondary metabolism and cell differentiation in *Streptomyces*: A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system', *Gene*, vol. 115, no. 1–2, pp. 167-72.

Horinouchi, S, Kito, M, Nishiyama, M, Furuya, K, Hong, S-K, Miyake, K & Beppu, T 1990, 'Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces* coelicolor A3(2)', *Gene*, vol. 95, no. 1, pp. 49-56.

Hoshino, S, Wakimoto, T, Onaka, H & Abe, I 2015, 'Chojalactones A–C, Cytotoxic Butanolides Isolated from *Streptomyces* sp. Cultivated with Mycolic Acid Containing Bacterium', *Organic Letters*, vol. 17, no. 6, pp. 1501-4.

Hoshino, S, Zhang, L, Awakawa, T, Wakimoto, T, Onaka, H & Abe, I 2015, 'Arcyriaflavin E, a new cytotoxic indolocarbazole alkaloid isolated by combined-culture of mycolic acid-containing bacteria and *Streptomyces* cinnamoneus NBRC 13823', *J Antibiot*, vol. 68, no. 5, pp. 342-4.

Huang, R-M, Yi, X-X, Zhou, Y, Su, X, Peng, Y & Gao, C-H 2014, 'An Update on 2,5-Diketopiperazines from Marine Organisms', *Marine Drugs*, vol. 12, no. 12, pp. 6213-35.

Huvenne, H, Goeminne, G, Maes, M & Messens, E 2008, 'Identification of quorum sensing signal molecules and oligolignols associated with watermark disease in willow (Salix sp.)', *Journal of Chromatography B*, vol. 872, no. 1–2, pp. 83-9.

Ibn Abubakar, BSU, Abdullah, N, Idris, A, Zakaria, MP & Shokur, MYA 2012, 'Optimisation of Pyrene Removal by a Mixed Culture in a Soil-Slurry Bioreactor using Response Surface Methodology (RSM)', *Procedia Engineering*, vol. 50, no. 0, pp. 786-99.

Ikegami, H & Prasad, C 1990, 'Neuropeptide-dopamine interactions. V. Cyclo(His-Pro) regulation of striatal dopamine transporter complex', *Peptides*, vol. 11, no. 1, pp. 145-8.

Imamura, M & Prasad, C 2003, 'Cyclo (His-Pro) potentiates GABA/ethanol-mediated chloride uptake by neurosynaptosomes', *Peptides*, vol. 24, no. 3, pp. 445-8.

Jafari, N, Behroozi, R, Farajzadeh, D, Farsi, M & Akbari-Noghabi, K 2014, 'Antibacterial Activity of *Pseudonocardia* sp. JB05, a Rare Salty Soil Actinomycete against Staphylococcus aureus', *BioMed Research International*, vol. 2014, p. 7.

Janda, JM & Abbott, SL 2007, '16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls', *Journal of Clinical Microbiology*, vol. 45, no. 9, pp. 2761-4.

Johnson, L 2008, 'Iron and siderophores in fungal–host interactions', *Mycological Research*, vol. 112, no. 2, pp. 170-83.

Jonsbu, E, McIntyre, M & Nielsen, J 2002, 'The influence of carbon sources and morphology on nystatin production by *Streptomyces* noursei', *Journal of Biotechnology*, vol. 95, no. 2, pp. 133-44.

Kaewkla, O- & Franco, CMM 2016, '*Kribbella* pittospori sp. nov., an endophytic actinobacterium isolated from the surface-sterilized stem of an Australian native apricot tree, Pittosporum angustifolium', *International Journal of Systematic and Evolutionary Microbiology*.

Kaewkla, O & Franco, CMM 2012, 'Rational Approaches to Improving the Isolation of Endophytic Actinobacteria from Australian Native Trees', *Microbial Ecology*, vol. 65, no. 2, pp. 384-93.

Kawai, K, Wang, G, Okamoto, S & Ochi, K 2007, 'The rare earth, scandium, causes antibiotic overproduction in *Streptomyces* spp', *FEMS Microbiology Letters*, vol. 274, no. 2, pp. 311-5.

Kitani, S, Hoshika, M & Nihira, T 2008, 'Disruption of sscR encoding a γ-butyrolactone autoregulator receptor in *Streptomyces* scabies NBRC 12914 affects production of secondary metabolites', *Folia Microbiologica*, vol. 53, no. 2, pp. 115-24.

Kobayashi, K, Fujikawa, M & Kozawa, T 2014, 'Oxidative stress sensing by the iron–sulfur cluster in the transcription factor, SoxR', *Journal of Inorganic Biochemistry*, vol. 133, pp. 87-91.

Kurosawa, H, Nomura, N & Tanaka, H 1989, 'Ethanol production from starch by a coimmobilized mixed culture system of Aspergillus awamori and Saccharomyces cerevisiae', *Biotechnology and Bioengineering*, vol. 33, no. 6, pp. 716-23.

Labeda, DP, Goodfellow, M, Brown, R, Ward, AC, Lanoot, B, Vanncanneyt, M, Swings, J, Kim, S-B, Liu, Z, Chun, J, Tamura, T, Oguchi, A, Kikuchi, T, Kikuchi, H, Nishii, T, Tsuji, K, Yamaguchi, Y, Tase, A, Takahashi, M, Sakane, T, Suzuki, KI & Hatano, K 2011, 'Phylogenetic study of the species within the family Streptomycetaceae', *Antonie van Leeuwenhoek*, vol. 101, no. 1, pp. 73-104.

Ladygina, N & Hedlund, K 2010, 'Plant species influence microbial diversity and carbon allocation in the rhizosphere', *Soil Biology and Biochemistry*, vol. 42, no. 2, pp. 162-8.

Lambert, S, Traxler, MF, Craig, M, Maciejewska, M, Ongena, M, van Wezel, GP, Kolter, R & Rigali, S 2014, 'Altered desferrioxamine-mediated iron utilization is a common trait of bald mutants of *Streptomyces coelicolor'*, *Metallomics*, vol. 6, no. 8, pp. 1390-9.

Laube, VM & Martin, SM 1981, 'Conversion of Cellulose to Methane and Carbon Dioxide by Triculture of *Acetivibrio cellulolyticus*, *Desulfovibrio* sp., and *Methanosarcina barkeri*', *Applied and Environmental Microbiology*, vol. 42, no. 3, pp. 413-20.

Lazzarini, A, Cavaletti, L, Toppo, G & Marinelli, F 2000, 'Rare genera of actinomycetes as potential producers of new antibiotics', *Antonie van Leeuwenhoek*, vol. 78, no. 3, pp. 399-405.

Le, HX 2015, 'Effects of endophytic actinobacteria on lucerne growth and the development of its N2-fixation symbiosis with rhizobia', *Unpublished PhD thesis. Flinders University.*

Lee, P-C, Umeyama, T & Horinouchi, S 2002, 'afsS is a target of AfsR, a transcriptional factor with ATPase activity that globally controls secondary metabolism in *Streptomyces coelicolor* A3(2)', *Molecular microbiology*, vol. 43, no. 6, pp. 1413-30.

Lee, SD, Kim, ES & Hah, YC 2000, 'Phylogenetic analysis of the genera *Pseudonocardia* and *Actinobispora* based on 16S ribosomal DNA sequences', *FEMS Microbiology Letters*, vol. 182, no. 1, pp. 125-9.

Li, J, Zhao, G-Z, Varma, A, Qin, S, Xiong, Z, Huang, H-Y, Zhu, W-Y, Zhao, L-X, Xu, L-H, Zhang, S & Li, W-J 2012, 'An Endophytic *Pseudonocardia* Species Induces the Production of Artemisinin in *Artemisia annua'*, *PLoS ONE*, vol. 7, no. 12, p. e51410.

Li, L, Qiao, B & Yuan, Y 2007, 'Nitrogen Sources Affect Streptolydigin Production and Related Secondary Metabolites Distribution of *Streptomyces lydicus* AS 4.2501', *Chinese Journal of Chemical Engineering*, vol. 15, no. 3, pp. 403-10.

Li, L, Zheng, H & Yuan, Y 2007, 'Effects of Propionate on Streptolydigin Production and Carbon Flux Distribution in *Streptomyces* lydicus AS 4.2501', *Chinese Journal of Chemical Engineering*, vol. 15, no. 2, pp. 143-9.

Li, W-J, Wang, D, Zhang, Y-Q, Schumann, P, Stackebrandt, E, Xu, L-H & Jiang, C-L 2004, '*Kribbella* antibiotica sp. nov., a Novel Nocardioform Actinomycete Strain Isolated from Soil in Yunnan, China', *Systematic and Applied Microbiology*, vol. 27, no. 2, pp. 160-5.

Li, W, Ying, X, Guo, Y, Yu, Z, Zhou, X, Deng, Z, Kieser, H, Chater, KF & Tao, M 2006, 'Identification of a Gene Negatively Affecting Antibiotic Production and Morphological Differentiation in *Streptomyces* coelicolor A3(2)', *Journal of Bacteriology*, vol. 188, no. 24, pp. 8368-75.

Liu, G, Chater, KF, Chandra, G, Niu, G & Tan, H 2013, 'Molecular Regulation of Antibiotic Biosynthesis in *Streptomyces*', *Microbiology and Molecular Biology Reviews*, vol. 77, no. 1, pp. 112-43.

Liu, N, Shang, F, Xi, L & Huang, Y 2013, 'Tetroazolemycins A and B, Two New Oxazole-Thiazole Siderophores from Deep-Sea *Streptomyces olivaceus* FXJ8.012', *Marine Drugs*, vol. 11, no. 5, pp. 1524-33.

Lucas, X, Senger, C, Erxleben, A, Grüning, BA, Döring, K, Mosch, J, Flemming, S & Günther, S 2013, 'StreptomeDB: a resource for natural compounds isolated from *Streptomyces* species', *Nucleic Acids Research*, vol. 41, no. Database issue, pp. D1130-D6.

Ludwig, W, Euzéby, J, Schumann, P, Busse, H-J, Trujillo, ME, Kämpfer, P & Whitman, WB 2012, 'Road map of the phylum Actinobacteria', in M Goodfellow, P Kämpfer, H-J Busse, ME Trujillo, K-i Suzuki, W Ludwig & WB Whitman (eds), *Bergey's Manual® of Systematic Bacteriology: Volume Five The Actinobacteria, Part A and B*, Springer New York, New York, NY, pp. 1-28.

Mao, X-M, Luo, S, Zhou, R-C, Wang, F, Yu, P, Sun, N, Chen, X-X, Tang, Y & Li, Y-Q 2015, 'Transcriptional Regulation of the Daptomycin Gene Cluster in *Streptomyces roseosporus* by an Autoregulator, AtrA', *Journal of Biological Chemistry*, vol. 290, no. 12, pp. 7992-8001.

March, JC & Bentley, WE 2004, 'Quorum sensing and bacterial cross-talk in biotechnology', *Current Opinion in Biotechnology*, vol. 15, no. 5, pp. 495-502.

Marschner, P, Yang, CH, Lieberei, R & Crowley, DE 2001, 'Soil and plant specific effects on bacterial community composition in the rhizosphere', *Soil Biology and Biochemistry*, vol. 33, no. 11, pp. 1437-45.

Martins, MB & Carvalho, I 2007, 'Diketopiperazines: biological activity and synthesis', *Tetrahedron*, vol. 63, no. 40, pp. 9923-32.

Matselyukh, B, Mohammadipanah, F, Laatsch, H, Rohr, J, Efremenkova, O & Khilya, V 2015, 'N-methylphenylalanyl-dehydrobutyrine diketopiperazine, an A-factor mimic that restores antibiotic biosynthesis and morphogenesis in *Streptomyces* globisporus 1912-B2 and *Streptomyces griseus* 1439', *J Antibiot*, vol. 68, no. 1, pp. 9-14.

McCleland, K, Milne, PJ, Lucieto, FR, Frost, C, Brauns, SC, Van De Venter, M, Du Plessis, J & Dyason, K 2004, 'An investigation into the biological activity of the selected histidine -containing diketopiperazines cyclo(His-Phe) and cyclo(His-Tyr)', *Journal of Pharmacy and Pharmacology*, vol. 56, no. 9, pp. 1143-53.

Meirelles, LA, Mendes, TD, Solomon, SE, Bueno, OC, Pagnocca, FC & Rodrigues, A 2014, 'Broad *Escovopsis*-inhibition activity of *Pseudonocardia* associated with *Trachymyrmex* ants', *Environmental Microbiology Reports*, vol. 6, no. 4, pp. 339-45.

Meneghini, R 1997, 'Iron Homeostasis, Oxidative Stress, and DNA Damage', *Free Radical Biology and Medicine*, vol. 23, no. 5, pp. 783-92.

Mingma, R, Pathom-aree, W, Trakulnaleamsai, S, Thamchaipenet, A & Duangmal, K 2013, 'Isolation of rhizospheric and roots endophytic actinomycetes from *Leguminosae* plant and their activities to inhibit soybean pathogen, *Xanthomonas* campestris pv. glycine', *World Journal of Microbiology and Biotechnology*, pp. 1-10.

Miyake, K, Kuzuyama, T, Horinouchi, S & Beppu, T 1990, 'The A-Factor-Binding Protein of *Streptomyces griseus* Negatively Controls Streptomycin Production and Sporulation', *Journal of Bacteriology*, vol. 172, no. 6, pp. 3003-8.

Modarresi, F, Azizi, O, Shakibaie, MR, Motamedifar, M, Mosadegh, E & Mansouri, S 2015, 'Iron limitation enhances acyl homoserine lactone (AHL) production and biofilm formation in clinical isolates of *Acinetobacter baumannii*', *Virulence*, vol. 6, no. 2, pp. 152-61.

Moelling, C, Oberschlacke, R, Ward, P, Karijolich, J, Borisova, K, Bjelos, N & Bergeron, L 2007, 'Metal-dependent repression of siderophore and biofilm formation in *Actinomyces naeslundii*', *FEMS Microbiology Letters*, vol. 275, no. 2, pp. 214-20.

Morel, CM & Mossialos, E 2010, 'Stoking the antibiotic pipeline', *BMJ*, vol. 340.

Mueller, U, Ishak, H, Lee, J, Sen, R & Gutell, R 2010, 'Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (*Pseudonocardia*ceae, Actinomycetales): a test of two symbiont-association models', *Antonie van Leeuwenhoek*, vol. 98, no. 2, pp. 195-212.

Mulé, MP, Giacalone, D, Lawlor, K, Golden, A, Cook, C, Lott, T, Aksten, E, O'Toole, GA & Bergeron, LJ 2015, 'Iron-dependent gene expression in *Actinomyces oris*', *Journal of Oral Microbiology*, vol. 7, p. 10.3402/jom.v7.29800.

Naeimpoor, F & Mavituna, F 2000, 'Metabolic Flux Analysis in *Streptomyces* coelicolor under Various Nutrient Limitations', *Metabolic Engineering*, vol. 2, no. 2, pp. 140-8.

Nakouti, I, Sihanonth, P & Hobbs, G 2012, 'A new approach to isolating siderophore-producing actinobacteria', *Letters in Applied Microbiology*, vol. 55, no. 1, pp. 68-72.

Nath, KR & Wagner, BJ 1973, 'Stimulation of Lactic Acid Bacteria by a *Micrococcus* Isolate: Evidence for Multiple Effects', *Applied Microbiology*, vol. 26, no. 1, pp. 49-55.

Neilands, JB 1995, 'Siderophores: Structure and Function of Microbial Iron Transport Compounds', *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 26723-6.

Nett, M, Ikeda, H & Moore, BS 2009, 'Genomic basis for natural product biosynthetic diversity in the actinomycetes', *Natural Product Reports*, vol. 26, no. 11, pp. 1362-84.

Netzker, T, Fischer, J, Weber, J, Mattern, DJ, König, CC, Valiante, V, Schroeckh, V & Brakhage, AA 2015, 'Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters', *Frontiers in Microbiology*, vol. 6, p. 299.

Neumann, T, Piepersberg, W & Distler, J 1996, 'Decision phase regulation of streptomycin production in *Streptomyces griseus*', *Microbiology*, vol. 142, no. 8, pp. 1953-63.

Newman, DJ & Cragg, GM 2016, 'Natural Products as Sources of New Drugs from 1981 to 2014', *Journal of Natural Products*.

Ng, TK, Ben-Bassat, A & Zeikus, JG 1981, 'Ethanol Production by Thermophilic Bacteria: Fermentation of Cellulosic Substrates by Cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*', *Applied and Environmental Microbiology*, vol. 41, no. 6, pp. 1337-43.

Niladevi, KN, Sukumaran, RK, Jacob, N, Anisha, GS & Prema, P 2009, 'Optimization of laccase production from a novel strain—*Streptomyces psammoticus* using response surface methodology', *Microbiological Research*, vol. 164, no. 1, pp. 105-13.

Ochi, K & Hosaka, T 2013, 'New strategies for drug discovery: activation of silent or weakly expressed microbial gene clusters', *Applied Microbiology and Biotechnology*, vol. 97, no. 1, pp. 87-98.

Ohnishi, Y, Kameyama, S, Onaka, H & Horinouchi, S 1999, 'The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus* : identification of a target gene of the A-factor receptor', *Molecular microbiology*, vol. 34, no. 1, pp. 102-11.

Ohnishi, Y, Seo, J-W & Horinouchi, S 2002, 'Deprogrammed sporulation in *Streptomyces*', *FEMS Microbiology Letters*, vol. 216, no. 1, pp. 1-7.

Ohnishi, Y, Yamazaki, H, Kato, J-y, Tomono, A & Horinouchi, S 2005, 'AdpA, a Central Transcriptional Regulator in the A-Factor Regulatory Cascade That Leads to Morphological Development and Secondary Metabolism in *Streptomyces griseus*', *Bioscience, Biotechnology, and Biochemistry*, vol. 69, no. 3, pp. 431-9.

Olano, C, Lombó, F, Méndez, C & Salas, JA 2008, 'Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering', *Metabolic Engineering*, vol. 10, no. 5, pp. 281-92.

Onaka, H, Mori, Y, Igarashi, Y & Furumai, T 2011, 'Mycolic Acid-Containing Bacteria Induce Natural-Product Biosynthesis in *Streptomyces* Species', *Applied and Environmental Microbiology*, vol. 77, no. 2, pp. 400-6.

Park, S-Y, Kang, H-O, Jang, H-S, Lee, J-K, Koo, B-T & Yum, D-Y 2005, 'Identification of Extracellular N-Acylhomoserine Lactone Acylase from a *Streptomyces* sp. and Its Application to Quorum Quenching', *Applied and Environmental Microbiology*, vol. 71, no. 5, pp. 2632-41.

Park, SW, Park, ST, Lee, JE & Kim, YM 2008, '*Pseudonocardia* carboxydivorans sp. nov., a carbon monoxide-oxidizing actinomycete, and an emended description of the genus *Pseudonocardia*', *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 11, pp. 2475-8.

Peláez, F 2006, 'The historical delivery of antibiotics from microbial natural products—Can history repeat?', *Biochemical Pharmacology*, vol. 71, no. 7, pp. 981-90.

Pelchovich, G, Omer-Bendori, S & Gophna, U 2013, 'Menaquinone and Iron Are Essential for Complex Colony Development in Bacillus subtilis', *PLoS ONE*, vol. 8, no. 11, p. e79488.

Pereira, CS, Thompson, JA & Xavier, KB 2013, 'AI-2-mediated signalling in bacteria', *FEMS Microbiology Reviews*, vol. 37, no. 2, pp. 156-81.

Peterkofsky, A & Battaini, F 1980, 'The biological activities of the neuropeptide histidyl-proline diketopiperazine', *Neuropeptides*, vol. 1, no. 2, pp. 105-18.

Polkade, AV, Mantri, SS, Patwekar, UJ & Jangid, K 2016, 'Quorum Sensing: An Under-Explored Phenomenon in the Phylum Actinobacteria', *Frontiers in Microbiology*, vol. 7, p. 131.

Poulsen, M, Cafaro, M, Boomsma, JJ & Currie, CR 2005, 'Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants', *Molecular Ecology*, vol. 14, no. 11, pp. 3597-604.

Poulsen, M, Cafaro, MJ, Erhardt, DP, Little, AEF, Gerardo, NM, Tebbets, B, Klein, BS & Currie, CR 2010, 'Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants', *Environmental Microbiology Reports*, vol. 2, no. 4, pp. 534-40.

Poulsen, M, Erhardt, DP, Molinaro, DJ, Lin, T-L & Currie, CR 2007, 'Antagonistic Bacterial Interactions Help Shape Host-Symbiont Dynamics within the Fungus-Growing Ant-Microbe Mutualism', *PLoS ONE*, vol. 2, no. 9, p. e960.

Poulsen, M, Maynard, J, Roland, DL & Currie, CR 2011, 'The Role of Symbiont Genetic Distance and Potential Adaptability in Host Preference Towards *Pseudonocardia* Symbionts in Acromyrmex Leaf-Cutting Ants', *Journal of Insect Science*, vol. 11, p. 120.

Prabahar, V, Dube, S, Reddy, GSN & Shivaji, S 2004, '*Pseudonocardia antarctica* sp. nov. an Actinomycetes from McMurdo Dry Valleys, Antarctica', *Systematic and Applied Microbiology*, vol. 27, no. 1, pp. 66-71.

Prasad, C 1988, 'Neuropeptide-dopamine interactions. II. Cyclo (His-Pro) augmentation of amphetamine- but not apomorphine-induced stereotypic behavior', *Behavioural Brain Research*, vol. 27, no. 3, pp. 241-5.

Prasad, C 1995, 'Bioactive cyclic dipeptides', *Peptides*, vol. 16, no. 1, pp. 151-64.

Purohit, M, Sassi-Gaha, S & Rest, RF 2010, 'Rapid sporulation of Bacillus anthracis in a high iron, glucose-free medium', *Journal of Microbiological Methods*, vol. 82, no. 3, pp. 282-7.

Qin, S, Su, Y-Y, Zhang, Y-Q, Wang, H-B, Jiang, C-L, Xu, L-H & Li, W-J 2008, '*Pseudonocardia* ailaonensis sp. nov., isolated from soil in China', *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 9, pp. 2086-9.

Quinn, RJ, Carroll, AR, Pham, NB, Baron, P, Palframan, ME, Suraweera, L, Pierens, GK & Muresan, S 2008, 'Developing a Drug-like Natural Product Library', *Journal of Natural Products*, vol. 71, no. 3, pp. 464-8.

Raina, S, De Vizio, D, Palonen, EK, Odell, M, Brandt, AM, Soini, JT & Keshavarz, T 2012, 'Is quorum sensing involved in lovastatin production in the filamentous fungus *Aspergillus terreus*?', *Process Biochemistry*, vol. 47, no. 5, pp. 843-52.

Raina, S, Odell, M & Keshavarz, T 2010, 'Quorum sensing as a method for improving sclerotiorin production in *Penicillium sclerotiorum'*, *Journal of Biotechnology*, vol. 148, no. 2–3, pp. 91-8.

Raymond, KN, Dertz, EA & Kim, SS 2003, 'Enterobactin: An archetype for microbial iron transport', *Proceedings of the National Academy of Sciences*, vol. 100, no. 7, pp. 3584-8.

Recio, E, Colinas, Á, Rumbero, Á, Aparicio, JF & Martín, JF 2004, 'PI Factor, a Novel Type Quorum-sensing Inducer Elicits Pimaricin Production in *Streptomyces natalensis*', *Journal of Biological Chemistry*, vol. 279, no. 40, pp. 41586-93.

Retzlaff, L & Distler, J 1995, 'The regulator of streptomycin gene expression, StrR, of *Streptomyces griseus* is a DNA binding activator protein with multiple recognition sites', *Molecular microbiology*, vol. 18, no. 1, pp. 151-62.

Ridley, CP & Khosla, C 2009, 'Polyketides', in S Editor-in-Chief: Moselio (ed.), *Encyclopedia of Microbiology (Third Edition)*, Academic Press, Oxford, pp. 472-81.

Rigali, S, Titgemeyer, F, Barends, S, Mulder, S, Thomae, AW, Hopwood, DA & van Wezel, GP 2008, 'Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by *Streptomyces*', *EMBO reports*, vol. 9, no. 7, pp. 670-5.

Rodrigues, A, Bacci Jr, M, Mueller, UG, Ortiz, A & Pagnocca, FC 2008, 'Microfungal "Weeds" in the Leafcutter Ant Symbiosis', *Microbial Ecology*, vol. 56, no. 4, pp. 604-14.

Saez, JM, Benimeli, CS & Amoroso, MJ 2012, 'Lindane removal by pure and mixed cultures of immobilized actinobacteria', *Chemosphere*, vol. 89, no. 8, pp. 982-7.

Saha, M, Sarkar, S, Sarkar, B, Sharma, BK, Bhattacharjee, S & Tribedi, P 2015, 'Microbial siderophores and their potential applications: a review', *Environmental Science and Pollution Research*, pp. 1-16.

Sakaguchi, G & Tohyama, Y 1955, 'Studies On The Toxin Production Of Clostridium Botulinum Type E Ii. The Mode Of Action Of The Contaminant Organisms To Promote Toxin Production Of Type E Organisms', *Japanese Journal of Medical Science and Biology*, vol. 8, no. 3, pp. 255-62.

Sakiyama, Y, Thao, NKN, Vinh, HV, Giang, NM, Miyadoh, S, Hop, DV & Ando, K 2010, '*Pseudonocardia* babensis sp. nov., isolated from plant litter', *International Journal of Systematic and Evolutionary Microbiology*, vol. 60, no. 10, pp. 2336-40.

Santos-Beneit, F, Rodríguez-García, A & Martín, JF 2012, 'Identification of different promoters in the absA1–absA2 two-component system, a negative regulator of antibiotic production in *Streptomyces coelicolor*', *Molecular Genetics and Genomics*, vol. 288, no. 1, pp. 39-48.

Schalk, IJ, Mislin, GLA & Brillet, K 2012, 'Chapter Two - Structure, Function and Binding Selectivity and Stereoselectivity of Siderophore–Iron Outer Membrane Transporters', in MA José & L Svetlana (eds), *Current Topics in Membranes*, Academic Press, vol. Volume 69, pp. 37-66.

Scherlach, K & Hertweck, C 2009, 'Triggering cryptic natural product biosynthesis in microorganisms', *Organic & Biomolecular Chemistry*, vol. 7, no. 9, pp. 1753-60.

Schirmer, A, Gadkari, R, Reeves, CD, Ibrahim, F, DeLong, EF & Hutchinson, CR 2005, 'Metagenomic Analysis Reveals Diverse Polyketide Synthase Gene Clusters in Microorganisms Associated with the Marine Sponge Discodermia dissoluta', *Applied and Environmental Microbiology*, vol. 71, no. 8, pp. 4840-9.

Schoenian, I, Spiteller, M, Ghaste, M, Wirth, R, Herz, H & Spiteller, D 2011, 'Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 5, pp. 1955-60.

Schroeckh, V, Scherlach, K, Nützmann, H-W, Shelest, E, Schmidt-Heck, W, Schuemann, J, Martin, K, Hertweck, C & Brakhage, AA 2009, 'Intimate bacterial–fungal interaction triggers biosynthesis of archetypal polyketides in Aspergillus

nidulans', *Proceedings of the National Academy of Sciences*, vol. 106, no. 34, pp. 14558-63.

Seipke, RF, Barke, J, Brearley, C, Hill, L, Yu, DW, Goss, RJM & Hutchings, MI 2011, 'A Single *Streptomyces* Symbiont Makes Multiple Antifungals to Support the Fungus Farming Ant Acromyrmex octospinosus', *PLoS ONE*, vol. 6, no. 8, p. e22028.

Sen, R, Ishak, HD, Estrada, D, Dowd, SE, Hong, E & Mueller, UG 2009, 'Generalized antifungal activity and 454-screening of *Pseudonocardia* and Amycolatopsis bacteria in nests of fungus-growing ants', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 42, pp. 17805-10.

Seyedsayamdost, MR, Traxler, MF, Clardy, J & Kolter, R 2012, 'Chapter Five - Old Meets New: Using Interspecies Interactions to Detect Secondary Metabolite Production in Actinomycetes', in AH David (ed.), *Methods in Enzymology*, Academic Press, vol. Volume 517, pp. 89-109.

Shen, B 2003, 'Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms', *Current Opinion in Chemical Biology*, vol. 7, no. 2, pp. 285-95.

Shin, SH, Lim, Y, Lee, SE, Yang, NW & Rhee, JH 2001, 'CAS agar diffusion assay for the measurement of siderophores in biological fluids', *Journal of Microbiological Methods*, vol. 44, no. 1, pp. 89-95.

Shirling, EB & Gottlieb, D 1966, 'Methods for characterization of *Streptomyces* species', *International Journal of Systematic Bacteriology*, vol. 16, no. 3, pp. 313-40.

Shu, C-H, Tsai, C-C, Chen, K-Y, Liao, W-H & Huang, H-C 2013, 'Enhancing high quality oil accumulation and carbon dioxide fixation by a mixed culture of Chlorella sp. and Saccharomyces cerevisiae', *Journal of the Taiwan Institute of Chemical Engineers*, vol. 44, no. 6, pp. 936-42.

Song, MK, Hwang, IK, Rosenthal, MJ, Harris, DM, Yamaguchi, DT, Yip, I & Go, VLW 2003, 'Anti-Hyperglycemic Activity of Zinc Plus Cyclo (His-Pro) in Genetically Diabetic Goto-Kakizaki and Aged Rats', *Experimental Biology and Medicine*, vol. 228, no. 11, pp. 1338-45.

Spellberg, B, Powers, JH, Brass, EP, Miller, LG & Edwards, JE 2004, 'Trends in Antimicrobial Drug Development: Implications for the Future', *Clinical Infectious Diseases*, vol. 38, no. 9, pp. 1279-86.

Stackebrandt, E, Rainey, Fa & Ward-Rainey, NI 1997, 'Proposal for a New Hierarchic Classification System, Actinobacteria classis nov', *International Journal of Systematic and Evolutionary Microbiology*, vol. 47, no. 2, pp. 479-91.

Stark, T & Hofmann, T 2005, 'Structures, Sensory Activity, and Dose/Response Functions of 2,5-Diketopiperazines in Roasted Cocoa Nibs (Theobroma cacao)', *Journal of Agricultural and Food Chemistry*, vol. 53, no. 18, pp. 7222-31.

Sterling, T & Irwin, JJ 2015, 'ZINC 15 – Ligand Discovery for Everyone', *Journal of Chemical Information and Modeling*, vol. 55, no. 11, pp. 2324-37.

Sun, J, Daniel, R, Wagner-Döbler, I & Zeng, A-P 2004, 'Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways', *BMC Evolutionary Biology*, vol. 4, pp. 36-.

Sun, Z, He, X, Brancaccio, VF, Yuan, J & Riedel, CU 2014, 'Bifidobacteria Exhibit LuxS-Dependent Autoinducer 2 Activity and Biofilm Formation', *PLoS ONE*, vol. 9, no. 2, p. e88260.

Takano, E 2006, 'γ-Butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation', *Current Opinion in Microbiology*, vol. 9, no. 3, pp. 287-94.

Tanaka, A, Takano, Y, Ohnishi, Y & Horinouchi, S 2007, 'AfsR Recruits RNA Polymerase to the afsS Promoter: A Model for Transcriptional Activation by SARPs', *Journal of Molecular Biology*, vol. 369, no. 2, pp. 322-33.

Tanaka, H, Kurosawa, H & Murakami, H 1986, 'Ethanol production from starch by a coimmobilized mixed culture system of Aspergillus awamori and Zymomonas mobilis', *Biotechnology and Bioengineering*, vol. 28, no. 12, pp. 1761-8.

Tanaka, Y, Hosaka, T & Ochi, K 2010, 'Rare earth elements activate the secondary metabolite-biosynthetic gene clusters in *Streptomyces* coelicolor A3(2)', *J Antibiot*, vol. 63, no. 8, pp. 477-81.

Tanaka, Y, Kasahara, K, Hirose, Y, Murakami, K, Kugimiya, R & Ochi, K 2013, 'Activation and Products of the Cryptic Secondary Metabolite Biosynthetic Gene Clusters by Rifampin Resistance (rpoB) Mutations in Actinomycetes', *Journal of Bacteriology*, vol. 195, no. 13, pp. 2959-70.

Taniguchi, M, Nakazawa, H, Takeda, O, Kaneko, T, Hoshino, K & Tanaka, T 1998, 'Production of a Mixture of Antimicrobial Organic Acids from Lactose by Co-Culture of Bifidobacterium longum and Propionibacterium freudenreichii', *Bioscience, Biotechnology, and Biochemistry*, vol. 62, no. 8, pp. 1522-7.

Tanvir, R, Sajid, I, Hasnain, S, Kulik, A & Grond, S 2016, 'Rare actinomycetes Nocardia caishijiensis and *Pseudonocardia* carboxydivorans as endophytes, their bioactivity and metabolites evaluation', *Microbiological Research*, vol. 185, pp. 22-35.

Tian, X-P, Long, L-J, Li, S-M, Zhang, J, Xu, Y, He, J, Li, J, Wang, F-Z, Li, W-J, Zhang, C-S & Zhang, S 2013, '*Pseudonocardia* antitumoralis sp. nov., a deoxynyboquinone-producing actinomycete isolated from a deep-sea sediment', *International Journal of Systematic and Evolutionary Microbiology*, vol. 63, no. 3, pp. 893-9.

Tierrafría, VH, Ramos-Aboites, HE, Gosset, G & Barona-Gómez, F 2011, 'Disruption of the siderophore-binding desE receptor gene in *Streptomyces* coelicolor A3(2) results in impaired growth in spite of multiple iron–siderophore transport systems', *Microbial Biotechnology*, vol. 4, no. 2, pp. 275-85.

Touati, D 2000, 'Iron and Oxidative Stress in Bacteria', *Archives of Biochemistry and Biophysics*, vol. 373, no. 1, pp. 1-6.

Traxler, MF, Seyedsayamdost, MR, Clardy, J & Kolter, R 2012, 'Interspecies modulation of bacterial development through iron competition and siderophore piracy', *Molecular microbiology*, vol. 86, no. 3, pp. 628-44.

Traxler, MF, Watrous, JD, Alexandrov, T, Dorrestein, PC & Kolter, R 2013a, 'Interspecies Interactions Stimulate Diversification of the *Streptomyces* coelicolor Secreted Metabolome', *mBio*, vol. 4, no. 4.

—— 2013b, 'Interspecies Interactions Stimulate Diversification of the *Streptomyces* coelicolor Secreted Metabolome', *mBio*, vol. 4, no. 4, pp. e00459-13.

Trujillo, ME, Kroppenstedt, RM, Schumann, P & Martínez-Molina, E 2006, '*Kribbella* lupini sp. nov., isolated from the roots of Lupinus angustifolius', *International Journal of Systematic and Evolutionary Microbiology*, vol. 56, no. 2, pp. 407-11.

Ueda, K, Kawai, S, Ogawa, H-o, Kiyama, A, Kubota, T, Kawanobe, H & Beppu, T 2000, 'Wide distribution of interspecific stimulatory events on antibiotic production and sporulation among *Streptomyces* species', *The Journal of Antibiotics*, vol. 53, no. 9, pp. 979-82.

van Wezel, GP & McDowall, KJ 2011, 'The regulation of the secondary metabolism of *Streptomyces*: new links and experimental advances', *Natural Product Reports*, vol. 28, no. 7, pp. 1311-33.

Vergne, C, Boury-Esnault, N, Perez, T, Martin, M-T, Adeline, M-T, Tran Huu Dau, E & Al-Mourabit, A 2006, 'Verpacamides A−D, a Sequence of C11N5 Diketopiperazines Relating Cyclo(Pro-Pro) to Cyclo(Pro-Arg), from the Marine Sponge Axinella vaceleti: Possible Biogenetic Precursors of Pyrrole-2-aminoimidazole Alkaloids', *Organic Letters*, vol. 8, no. 11, pp. 2421-4.

Vetsigian, K, Jajoo, R & Kishony, R 2011, 'Structure and Evolution of <italic>*Streptomyces*</italic> Interaction Networks in Soil and In Silico', *PLoS Biol*, vol. 9, no. 10, p. e1001184.

Wang, X, Tabudravu, J, Jaspars, M & Deng, H 2013, 'Tianchimycins A–B, 16-membered macrolides from the rare actinomycete Saccharothrix xinjiangensis', *Tetrahedron*, vol. 69, no. 30, pp. 6060-4.

Wang, Y-H, Yang, B, Ren, J, Dong, M-L, Liang, D & Xu, A-L 2005, 'Optimization of medium composition for the production of clavulanic acid by *Streptomyces* clavuligerus', *Process Biochemistry*, vol. 40, no. 3–4, pp. 1161-6.

Wang, Y-X, Zhang, Y-Q, Xu, L-H & Li, W-J 2008, 'Actinopolymorpha rutila sp. nov., isolated from a forest soil', *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 10, pp. 2443-6.

Wang, Y, Wang, L, Zhuang, Y, Kong, F, Zhang, C & Zhu, W 2014, 'Phenolic Polyketides from the Co-Cultivation of Marine-Derived Penicillium sp. WC-29-5 and *Streptomyces* fradiae 007', *Marine Drugs*, vol. 12, no. 4, pp. 2079-88.

Wang, YM, Zhang, ZS, Xu, XL, Ruan, JS & Wang, Y 2001, 'Actinopolymorpha singaporensis gen. nov., sp. nov., a novel actinomycete from the tropical rainforest of Singapore', *International Journal of Systematic and Evolutionary Microbiology*, vol. 51, no. 2, pp. 467-73.

Wang, Z, Yan, M, Chen, X, Li, D, Qin, L, Li, Z, Yao, J & Liang, X 2013, 'Mixed culture of Saccharomyces cerevisiae and Acetobacter pasteurianus for acetic acid production', *Biochemical Engineering Journal*, vol. 79, no. 0, pp. 41-5.

Watrous, JD, Phelan, VV, Hsu, C-C, Moree, WJ, Duggan, BM, Alexandrov, T & Dorrestein, PC 2013, 'Microbial metabolic exchange in 3D', *The ISME Journal*, vol. 7, no. 4, pp. 770-80.

Watve, MG, Tickoo, R, Jog, MM & Bhole, BD 2001, 'How many antibiotics are produced by the genus *Streptomyces*?', *Archives of Microbiology*, vol. 176, no. 5, pp. 386-90.

Wedderhoff, I, Kursula, I, Groves, MR & Ortiz de Orué Lucana, D 2013, 'Iron Binding at Specific Sites within the Octameric HbpS Protects Streptomycetes from Iron-Mediated Oxidative Stress', *PLoS ONE*, vol. 8, no. 8, p. e71579.

Wenzel, RP 2004, 'The Antibiotic Pipeline — Challenges, Costs, and Values', *New England Journal of Medicine*, vol. 351, no. 6, pp. 523-6.

WHO 2008, *The Global Burden of Disease: 2004 Update*, World Health Organization, Geneva.

Williams, P 2007, 'Quorum sensing, communication and cross-kingdom signalling in the bacterial world', *Microbiology*, vol. 153, no. 12, pp. 3923-38.

Woo, PCY, Lau, SKP, Teng, JLL, Tse, H & Yuen, KY 2008, 'Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories', *Clinical Microbiology and Infection*, vol. 14, no. 10, pp. 908-34.

Wu, W-M, Jain, MK & Zeikus, JG 1994, 'Anaerobic Degradation of Normal- and Branched-Chain Fatty Acids with Four or More Carbons to Methane by a Syntrophic Methanogenic Triculture', *Applied and Environmental Microbiology*, vol. 60, no. 7, pp. 2220-6.

Wysocki, VH, Resing, KA, Zhang, Q & Cheng, G 2005, 'Mass spectrometry of peptides and proteins', *Methods*, vol. 35, no. 3, pp. 211-22.

Yagüe, P, Rodríguez-García, A, López-García, MT, Rioseras, B, Martín, JF, Sánchez, J & Manteca, A 2014, 'Transcriptomic Analysis of Liquid Non-Sporulating *Streptomyces* coelicolor Cultures Demonstrates the Existence of a Complex Differentiation Comparable to That Occurring in Solid Sporulating Cultures', *PLoS ONE*, vol. 9, no. 1, p. e86296.

Yamanaka, K, Oikawa, H, Ogawa, H-o, Hosono, K, Shinmachi, F, Takano, H, Sakuda, S, Beppu, T & Ueda, K 2005, 'Desferrioxamine E produced by *Streptomyces* griseus stimulates growth and development of *Streptomyces* tanashiensis', *Microbiology*, vol. 151, no. 9, pp. 2899-905.

Yang, Y, Harris, DP, Luo, F, Wu, L, Parsons, AB, Palumbo, AV & Zhou, J 2008, 'Characterization of the Shewanella oneidensis Fur gene: roles in iron and acid tolerance response', *BMC Genomics*, vol. 9, no. Suppl 1, pp. S11-S.

Yoon, V & Nodwell, JR 2013, 'Activating secondary metabolism with stress and chemicals', *Journal of Industrial Microbiology & Biotechnology*, vol. 41, no. 2, pp. 415-24.

Zarins-Tutt, JS, Barberi, TT, Gao, H, Mearns-Spragg, A, Zhang, L, Newman, DJ & Goss, RJM 2016, 'Prospecting for new bacterial metabolites: a glossary of approaches for inducing, activating and upregulating the biosynthesis of bacterial cryptic or silent natural products', *Natural Product Reports*, vol. 33, no. 1, pp. 54-72.

Zhang, D-F, Jiang, Z, Li, L, Liu, B-B, Zhang, X-M, Tian, X-P, Zhang, S & Li, W-J 2014, '*Pseudonocardia* sediminis sp. nov., isolated from marine sediment', *International Journal of Systematic and Evolutionary Microbiology*, vol. 64, no. 3, pp. 745-50.

Zhang, F, Ding, J, Zhang, Y, Chen, M, Ding, Z-W, van Loosdrecht, MCM & Zeng, RJ 2013, 'Fatty acids production from hydrogen and carbon dioxide by mixed culture in the membrane biofilm reactor', *Water Research*, vol. 47, no. 16, pp. 6122-9.

Zhao, G-Z, Li, J, Qin, Y-L, Miao, C-P, Wei, D-Q, Zhang, S, Xu, L-H & Li, W-J 2012, '*Pseudonocardia* antimicrobica sp. nov., a novel endophytic actinomycete associated with Artemisia annua L. (sweet wormwood)', *J Antibiot*, vol. 65, no. 9, pp. 469-72.

Zuck, KM, Shipley, S & Newman, DJ 2011, 'Induced Production of N-Formyl Alkaloids from Aspergillus fumigatus by Co-culture with *Streptomyces* peucetius', *Journal of Natural Products*, vol. 74, no. 7, pp. 1653-7.

APPENDICES: MEDIA LIST

The media used are described as follows, with RO water added to make 1liter unless indicated otherwise. Adjustment of pH was done before agar was added. The media described was sterilized by autoclave at 121°C for 35 minutes holding time. When required, unless indicated otherwise, trace elements were added from stocks before autoclave.

ISP-2:		
Glucose	4	g/l
Yeast extract	4	g/l
Malt extract	10	g/l
Agar	15	g/l
pH	7.2 ± 0.2	

ISP-3:		
Oatmeal	20	g/l
Trace elements (see below)	1	ml/l
Agar	15	g/l
pH	7.2 ± 0.2	

ISP-5 (Glycerol-asparagine agar):		
L-asparagine	1	g/l
Glycerol	10	g/l
K2HPO4	1	g/l
Trace elements (see below)	1	ml/l
pH	7.2 ± 0.2	
Agar	15	g/l

IM-22		
Glucose anhydrous	15	g/l
Soya meal	15	g/l
Corn Steep Liquor	5	g/l
NaCl	5	g/l
$CaCO_3$	2	g/l
pH	7.2 ± 0.2	

Half Strength potato dextrose agar (HPDA):		
Potato dextrose agar	9.75	g/l
Agar	7.5	g/l
Mannito-Soya Agar (MSA):		
Mannitol	10	g/l
Soya flour	10	g/l
Agar	15	g/l
pH	7.2 ± 0.2	
Tryptone soy agar (DIFCO)		
Tryptone soy medium (DIFCO)	30	g/l
Agar	15	g/l
Antibiotic medium no 1 (OXOID)		
Antibiotic medium no 1	27	g/l
Trace elements		
$FeSO_4.7H_2O$	0.1	g/l
$MnCl_2.4H_2O$	0.1	g/l
$ZnSO_4.7H_2O$	0.1	g/l
RO water	100	ml