UNIVERSITY OF CALIFORNIA, SAN DIEGO

Ecology and Evolution of Hybrid Isoprenoid Secondary Metabolite Production in a *Streptomyces* Lineage

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

Kelley Ann Gallagher

Committee in Charge:

Paul Jensen, Chair
Susan Golden
Bradley Moore
Brian Palenik
Gregory Rouse
Gregory Wanger

2015

The Dissertation of Kelley Ann Gallagher is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2015

DEDICATION

For my parents

EPIGRAPH

Leave the door open for the unknown, the door into the dark.
That's where the most important things come from,
where you yourself came from, and where you will go.

Rebecca Solnit, A Field Guide to Getting Lost

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

8-amino-flaviolin	8-amino-2,5,7-trihydroxynapthalene-1,4-dione
ACE	abundance coverage estimator
atpD	gene encoding ATP synthase, F1 complex, β subunit
BLAST	basic local alignment search tool
bp	base pairs
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethyl sulfoxide
E°'	formal reduction potential
eDNA	environmental DNA
fur	furaquinocin biosynthesis pathway
gyrB	gene encoding DNA gyrase, subunit B
HGT	horizontal gene transfer
HI	hybrid isoprenoid
HIGC	hybrid isoprenoid gene cluster
HMBC	heteronuclear multiple-bond correlation
hmgr	gene encoding 3-hydroxy-3-methyl-glutaryl-CoA reductase
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence spectroscopy
IPP	isopentenyl diphosphate
LC-MS	liquid chromatography-mass spectrometry
mev	mevalonate pathway
ML	maximum likelihood
MP	maximum parsimony
NADH	nicotinamide adenine dinucleotide
nap	napyradiomycin biosynthesis pathway
NHE	normal hydrogen electrode
NMR	nuclear magnetic resonance spectroscopy
OMZ	oxygen minimum zone
Orf2 PTases	sub-group within the ABBA preyltransferases
OTU	operational taxonomic unit
PCR	polymerase chain reaction
phz	phenazine biosynthesis pathway
PTase	prenyltransferase
PTC	prenyltransferase clade
recA	gene encoding DNA-dependent ATPase
rpoB	gene encoding RNA polymerase, beta subunit
rRNA	ribosomal RNA
TFA	trifluoroacetic acid
trpB	gene encoding tryptophan synthase, β subunit

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ACKNOWLEDGEMENTS

I'd like to thank my adviser, Paul Jensen, for his unending support, advice and guidance. He supported me not only in the research aspects of my dissertation, but also in my goals for my future career. I would also like to thank past and present Jensen lab members for all of their valuable input on my research and presentations, and for creating such a productive and friendly lab atmosphere. In particular, thanks to Nadine Ziemert for her advice, inspiration, and encouragement through the entire course of my graduate studies. I would also like to acknowledge Chambers Hughes and Greg Wanger for their role as collaborators on my dissertation work, and for teaching me techniques that I would not otherwise have had the opportunity to learn. Lastly, I'd like to acknowledge my dissertation committee for all of the advice they have given me throughout my graduate studies.

The Center for Marine Biotechnology and Biomedicine and the Scripps Graduate Department have provided me with many opportunities that have been instrumental in my development as a researcher. I would like to specifically thank Beth Masek for her help in countless situations. I am very grateful for departmental financial support that allowed me to attend several conferences as well as a molecular evolution workshop in the Czech Republic. I would also like to thank everyone in the Graduate Department for their continuous personal and professional assistance, especially for their support in

initiating the SIO Student Symposium. Thanks also to the UC ShipFunds Program, which provided me with the opportunity to participate in the student-led San Diego Coastal Expedition. Finally, I would like to acknowledge the financial support of the Claude Zobell Fellowship in Marine Microbiology and the National Institutes of Health Marine Biotechnology Training Program.

Chapter 2, in full, is a reprint of the material as it appears in Current Opinion in Biotechnology, 2010. Kelley Gallagher, William Fenical, and Paul Jensen. The dissertation author was the primary author of this review paper.

Chapter 3, in full, is a reprint of the material as it appears in Applied and Environmental Microbiology, 2013. Kelley Gallagher, Kristin Rauscher, Laura Pavan Ioca, and Paul Jensen. The dissertation author was the primary investigator and author of this paper.

Chapter 4 is currently being formulated into a manuscript that will be submitted for publication in 2015. Kelley Gallagher and Paul Jensen. The dissertation author was the primary investigator on these studies.

Chapter 5 is currently being formulated into a manuscript that will be submitted for publication in 2015. Kelley Gallagher, Greg Wanger, Chambers Hughes, Jane Henderson, Mark Llorente, and Paul Jensen. The dissertation author was the primary investigator on these studies.

VITA

EDUCATION

2009	B.S., with Honors Major – Biological Sciences Minors – Chemistry, French Language University Pittsburgh
2015	Ph.D. in Marine Biology Scripps Institution of Oceanography University of California, San Diego

RESEARCH EXPERIENCE

Ph.D. Student at University of California, San Diego 2009-2015
Scripps Institution of Oceanography, La Jolla, CA, USA
Supervisor: Dr. Paul Jensen
Thesis: Ecology and evolution of hybrid isoprenoid secondary metabolites in marine streptomycete bacteria

Undergraduate Researcher at University of Pittsburgh 2005-2009
Department of Biological Sciences, Pittsburgh, PA, USA
Supervisor: Prof. Brian Traw
Undergraduate Honors thesis: Role of glucosinolates in *Arabidopsis thaliana* defense against bacterial pathogens

Volunteer Research Assistant at University of Maryland School of Medicine 2007
Department of Physiology, Baltimore, MD, USA
Supervisor: Prof. Stuart Martin
Research project: Effects of two PI3 kinase inhibitors on apoptosis of breast cancer cells.

Field Research Assistant at University of Pittsburgh 2006
Pymatuning Laboratory of Ecology, Pymatuning, PA, USA
Supervisor: Prof. Tia-Lynn Ashman
Duties: Experimental set up and data collection for field studies on evolution of *Fragaria virginiana*.

PUBLICATIONS

Gallagher KA, Rauscher K, Ioca L, Jensen PR: Phylogenetic and Chemical Diversity of a Hybrid Isoprenoid-producing Streptomycete Lineage. *Applied and Environmental Microbiology* 2013, 22:6894-6902.

Gallagher KA, Fenical W, Jensen PR. Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes. *Current Opinion in Biotechnology* 2010, 21:794-800.

SCHOLARSHIPS/AWARDS

2013 Claude E. Zobell Fellowship for Marine Microbiology

2012-2014 NIH Marine Biotechnology Predoctoral Trainee

2012 UC ShipFunds Program - Funding for two 11-day student-led cruises aboard the *R/V Melville*

Summer 2008 HHMI Undergraduate Research Fellow

2006 Wilma Binder Zeder Memorial Scholarship

2006 Order of the Eastern Star, Inc. Educational Scholarship

2005-2009 University Honors College Academic Scholarship

2005-2009 Alumni Association Legacy Scholar

2005 Ralph B. Canterbury Scholar

ABSTRACT OF THE DISSERTATION

Ecology and Evolution of Hybrid Isoprenoid
Secondary Metabolite Production in a *Streptomyces* Lineage

by

Kelley Ann Gallagher

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2015

Paul R. Jensen, Chair

The production of secondary metabolites is a fundamental way that bacteria interact with their environments, yet little is known about the fitness benefits conferred by specific molecules or the dynamics influencing the distribution of biosynthetic gene clusters among bacterial lineages. This

dissertation consists of six chapters that address these topics in an unusual class of secondary metabolites called hybrid isoprenoids (HIs). The first chapter introduces microbial chemical ecology and secondary metabolism, followed by four research-oriented chapters and a summary chapter.

Chapter 2 presents a literature review of the distribution of HI production within the actinobacterial genus *Streptomyces*. Reports of HI production were phylogenetically very scattered, with the exception of a putative marine *Streptomyces* lineage called the 'MAR4' clade. Following from this, Chapter 3 presents an in-depth study of the phylogenetic diversity of the MAR4 clade. Sequence data from a variety of sources led to a considerable expansion of the MAR4 clade, and representative strains were shown to produce 1-4 classes of HI molecules, an ability that is unmatched elsewhere in the streptomycetes.

Chapter 4 presents an analysis of 120 *Streptomyces* genomes, including 12 MAR4 strains, which provides strong support for the hypothesis that this clade is enriched in gene clusters for HI production. Phylogenetic analysis of a HI biosynthesis gene suggest that vertical inheritance, horizontal gene transfer, duplication followed by functional divergence, and gene rearrangements have all contributed to the striking number of HI gene clusters found in MAR4 genomes.

The final research chapter addresses the fact that none of these molecules have an experimentally characterized natural function, which is a

major obstacle to understanding the unusual distribution of HI gene clusters. Because many HIs resemble characterized electron shuttles, the hypothesis that they function in redox cycling was explored. A redox-active pigment that was upregulated in low-oxygen conditions was isolated from a MAR4 strain. This compound was identified as an intermediate in a HI gene cluster, which largely goes to completion in aerobic culture. This provides an unusual example of how a single biosynthetic cluster can be used to generate products with distinct properties depending on culture conditions.

Chapter 1: Introduction

1.1 Microbial chemical ecology

Microbes use small molecules to sense and respond to their environment and mediate interactions among themselves and with other members of the community. The earliest studies showing that microbes produce molecules that may have effects on other members of their communities occurred during the foundational years of microbiology (Traxler and Kolter, 2012). Following from the seminal discovery of penicillin from a fungal isolate, many compounds with antimicrobial properties were isolated from bacteria and fungi (Lewis, 2012). Since these early discoveries, many of the bioactive compounds isolated from bacterial cultures have been hypothesized to function as chemical warfare agents in nature, helping producing organisms to defeat competitors and avoid grazers (Wietz et al., 2013). As the field of microbial ecology developed, however, it has become increasingly clear that bacterially-produced small molecules have functions beyond allelopathy. Small molecules are now known to mediate a wide range of bacterial behaviors including extracellular communication, control of gene expression, and nutrient acquisition. Microbes live in complex assemblages that occur in dynamic environments, and small molecules are one way in which they coordinate behavior and adapt to variable conditions (Price-Whelan et al., 2006). Some of the best-studied functions for bacterially produced compounds, which will be summarized here, include iron scavenging, quorum sensing, intracellular signaling, redox cycling, and antagonism.

1.1.1 Iron scavenging

Owing to its aqueous insolubility, ferric iron is frequently a limiting nutrient controlling the growth of bacteria in the environment (Saha et al., 2013). In response to iron limitation, many bacteria excrete diffusible ferric iron-scavenging molecules called siderophores. These molecules are structurally diverse, but typically contain catecholate or hydroxamate moieties that form coordination complexes with ferric iron (Saha et al., 2013). Iron-siderophore complexes are selectively imported into the cell via ATP-dependent transporters (Schneider and Hantke, 1993), where iron is released and can be used as a cofactor in various cellular functions. The transcription of siderophore biosynthesis genes is under the control of iron-dependent repressors, which ensure that these molecules are only produced in iron-limited conditions. In high GC-content Gram-positive bacteria, homologs of the diphtheria toxin repressor protein DtxR have been shown to carry out this iron-dependent regulatory role (Schmitt et al., 1995), while in Gram-negative bacteria and low-GC content Gram-positive bacteria the global repressor Fur controls siderophore production (Saha et al., 2013).

Because siderophores are secreted, they can also mediate inter-species interactions. For example, some organisms that do not make siderophores nevertheless possess transporters to import iron-siderophore complexes, and can use iron chelators produced by other organisms (Lesuisse et al., 2001). Interestingly, members of the genus *Streptomyces* have been observed to produce multiple distinct siderophores. The coproduced molecules are thought to

function contingently, where there are multiple independent routes for iron uptake that offset the utilization of siderophores by other community members (Challis and Hopwood, 2003).

1.1.2 Quorum sensing

One important function for bacterially produced small molecules is cell-to-cell communication. Bacteria secrete small hormone-like molecules called autoinducers to coordinate population-wide behavior in a process called quorum sensing (Waters and Bassler, 2005). The amount of autoinducer molecules in an environment will reflect population density, which is detected by individual bacterial cells. After a certain threshold concentration of autoinducer is detected, bacteria alter gene expression, thus synchronizing cell density-dependent behaviors such as morphological development or toxin production (Waters and Bassler, 2005).

A canonical example of quorum sensing is the regulation of light production by *Vibrio fischeri* living symbiotically with the bobtail squid *Euprymna scolopes*. *V. fischeri* cells colonize the light organ of the squid where upon reaching high cell density, as sensed by concentrations of acyl-homoserine lactone (AHL) autoinducers, they bioluminesce and provide counter-illumination for the squid host. AHL molecules have since been found to function as autoinducers in many other Gram-negative bacteria. Variations in the side chain of the molecule impart specificity to systems used by different species (Fuqua and Greenberg, 2002). Gram-positive bacterial species have been shown to use

autoinducers that are oligopeptides or acylated lactones (Dunny and Leonard, 1997). Bacteria are frequently found to possess multiple quorum sensing systems that can operate in parallel, in series, or antagonistically. Signals have also been found that facilitate inter-species or even inter-domain communication (Waters and Bassler, 2005). Chemical communication is clearly a very important process shaping microbial communities, and elucidating complex quorum sensing networks remains a major goal in microbial chemical ecology.

1.1.3 Intracellular signaling

In all bacterial cells, small nucleotide-based molecules function as second messengers that mediate cellular responses to changing conditions. In general, extracellular conditions sensed by membrane-bound receptor proteins trigger the breakdown or synthesis of second messengers. These intracellular signals allosterically regulate diverse effector proteins that produce global responses including differentiation, antibiotic production, virulence, motility, use of alternative carbon sources, biofilm formation, and cell cycle progression (Camilli and Bassler, 2006; Hengge, 2009; Pesavento and Hengge, 2009).

The best-studied second messengers in bacteria are cyclic adenosine monophosphate (cAMP), which is synthesized in response to carbon limitation, guanosine tetraphosphate (ppGpp), which is synthesized in response to nutrient limitation and triggers the stringent response, and cyclic di-guanosine monophosphate (c-di-GMP), a ubiquitous second messenger that regulates various complex processes including developmental and lifestyle transitions

(Pesavento and Hengge, 2009). Understanding the interactions between second messenger cascades as well as the functional and structural diversity of second messengers in divergent bacteria are important objectives related to these fundamental intracellular signals.

1.1.4 Redox cycling

Many bacteria are able to reduce substrates outside of the cell in a process called extracellular electron transfer. This occurs when terminal electron acceptors cannot diffuse into the cell owing to their size (e.g. humic substances) or solubility (e.g. oxidized minerals such as Mn(IV) or Fe(III)) (Gralnick and Newman, 2007). The transfer of electrons to these substrates is thought to occur either through direct contact between cellular structures and electron acceptors or through the use of redox-active small molecule mediators called electron shuttles. In the latter case, electron shuttles can be present in the environment (exogenous) or produced by bacteria (endogenous) (Hernandez and Newman, 2001). Electron shuttling has been shown to dissipate excess reducing equivalents in low-oxygen conditions, thus supporting ATP generation by substrate-level phosphorylation (Brutinel and Gralnick, 2012; Glasser et al., 2014). This process could also theoretically connect quinone pools from the electron transfer chain to extracellular electron acceptors, though this has yet to be proven experimentally (Brutinel and Gralnick, 2012).

Though not as well studied, the use of electron shuttles by bacteria is, in many ways, analogous to the use of secreted siderophores for iron acquisition. In

both cases, molecules are energetically expensive to produce and secrete, but can be recycled by members of the population. In addition, similar to siderophore utilization by non-producers, bacteria that do not endogenously produce shuttles have been shown to use shuttles produced by other species (Pham et al., 2008). Redox cycling by small molecules will be discussed in more detail in Chapter 5.

1.1.5 Antagonism

Knowledge of chemically mediated antagonistic interactions between bacteria has developed considerably since the early discoveries of antibiotic-producing bacteria. Studies of bacterial-bacterial interactions are typically performed in culture-based assays by challenging an antibiotic-producing isolate with a strain thought to be a competitor. Inhibitory interactions have been reported among a variety of microbes including marine invertebrate-associated communities (Strahl et al., 2002; Mangano et al., 2009; Rypien et al., 2010) and those inhabiting terrestrial soils (Vetsigian et al., 2011; Vaz Jauri and Kinkel, 2014) and marine snow particles (Long and Azam, 2001). Interestingly, several of these studies independently report that bacteria are more likely to inhibit strains isolated from the same sampling site compared with strains from distinct locations (Long and Azam, 2001; Vetsigian et al., 2011; Vaz Jauri and Kinkel, 2014), which supports the idea that chemical antagonism mediates niche-specific competition among bacteria. Molecules produced by bacteria have also been reported to disrupt vital population-level behaviors, including the inhibition of

biofilm formation (Nithya et al., 2011; Cude et al., 2012) and quorum sensing ('quorum quenching') (Waters and Bassler, 2005).

Inhibitory compounds are likely to play a large role in structuring microbial communities, however there is much that remains to be learned about the role of specific molecules *in situ*. Antibiotic production can be induced by the presence of competitors (Trischman et al.; Slattery et al., 2001; Angell et al., 2006) and compounds are frequently inhibitory only to particular groups of bacteria (Yoshikawa et al., 1997; Xiao et al., 2011), which implies that compound production can target specific community members. In addition, a lack of knowledge of the environmental concentrations of antibiotics makes it difficult to properly assess their impact. Finally, the effect of inhibitory molecules on community structure certainly extends beyond competition among bacteria, as chemical antagonism is known to play a role in interactions between bacteria and eukaryotes and in the protection of invertebrate host organisms by bacterial symbionts (Wietz et al., 2013).

1.1.6 Functions of bacterial 'secondary' metabolites

Small molecules synthesized by microbes at late stages of growth are frequently referred to as 'secondary' metabolites, a term that is meant to convey that these compounds are not required for growth and survival of the producing organisms. Knowledge of the functional diversity of small molecules has increased considerably in recent years, yet the vast majority of molecules that are isolated from bacteria do not have a known function. Because many

molecules are isolated in the context of drug discovery and possess antibiotic or cytotoxic properties, they are often thought to function in antagonism. One of the major problems with this assumption stems from the fact that some bioactive molecules have variable effects on bacteria depending upon dose (Davies et al., 2006). While there is compelling evidence that some secondary metabolites function in antagonism, as previously discussed, it is important to consider that bacteria may use small molecules to interact with each other and their environments in myriad ways.

It is worth noting that if many of the molecules described in the previous sections had been randomly isolated from bacteria culture broths, they likely would have been classified as 'secondary' metabolites. It is only because they were discovered in context that they are more accurately classified as, for example, autoinducers or second messengers. The limitations of the term secondary metabolism have been the subject of much recent discussion (Price-Whelan et al., 2006; Davies, 2013), and one of the alternative names that is currently gaining momentum in the literature is 'specialized' metabolism. In keeping with convention, I will be referring to small molecules produced by bacteria as secondary metabolites, though it will be used as a pragmatic (as opposed to theoretical) term.

1.2 The genus *Streptomyces*

The Gram-positive actinobacterial genus *Streptomyces* is one of the most well studied bacterial genera in the context of secondary metabolism.

Streptomycetes are filamentous and grow as branching hyphae that form a vegetative mycelium and disperse via spores (Flardh and Buttner, 2009). They are found ubiquitously in terrestrial soils, where they produce the volatile terpenoid molecule geosmin (Gerber and Lechevalier, 1965) which imparts the typical earthy scent of soils. The considerable contribution of *Streptomyces*-derived molecules to the pharmaceutical industry attests to their uniqueness in nature as prolific secondary metabolite producers (Watve et al., 2001; Berdy, 2005; Pelaez, 2006). Despite the plethora of molecules isolated from *Streptomyces* cultures, the vast majority do not have an experimentally characterized function, making this genus an important system for studies of microbial chemical ecology.

Secondary metabolite discovery from *Streptomyces* was pioneered by Selman Waksman, who in 1952 was awarded the Nobel Prize in Medicine for his discovery of the antibiotic streptomycin from a *Streptomyces griseus* isolate (Schatz et al., 1944). Waksman was a soil microbiologist, and thus much of the foundational work on the genus focused on terrestrial environments. More recently, it has become apparent that *Streptomyces* occupy varied ecological niches, including both free-living and symbiotic lifestyles (Seipke, Kaltenpoth, et al., 2012). In many cases, the production of secondary metabolites is hypothesized to be a major factor contributing to successful niche occupation. *Streptomyces* spp. have been observed in mutualisms with invertebrate and plant species (Seipke, Kaltenpoth, et al., 2012), where they are thought to chemically

defend their hosts through the production of antibacterial or antifungal secondary metabolites. In addition to protective mutualisms, *Streptomyces* spp. have also been isolated from the guts of various arthropod species where they may be responsible for degradation of polymeric carbohydrates (Pasti and Belli, 1985; Pasti et al., 1990; Gebhardt et al., 2002; Watanabe et al., 2003; Jayasinghe and Parkinson, 2009).

Protective mutualisms between *Streptomyces* spp. and invertebrate hosts have been proposed for marine sponges (Taylor et al., 2007), cone snails (Peraud et al., 2009), solitary digger wasps (Nechitaylo et al., 2014), and several groups of fungus-growing insects including attine ants (Currie et al., 1999), southern pine beetles (Scott et al., 2008), and *Allomerus* ants (Seipke, Barke, et al., 2012). In many of these cases, symbiotic *Streptomyces* spp. are closely related to free-living isolates, suggesting that mutualists are recruited from the environment (Barke et al., 2010). One fascinating exception to this is found in the symbionts of the solitary digger wasps of the genera *Philanthus*, *Philanthinus* and *Trachypus*. 'Ca. *Streptomyces philanthi*' living in specialized antennal glands of these insects produce multiple antibiotics that protect larvae from pathogenic fungi. In contrast to other symbiotic streptomycetes, '*S. philanthi*' forms a monophyletic clade and shows strong evidence for co-evolution with the host genera (Kaltenpoth et al., 2012; Nechitaylo et al., 2014).

Streptomycetes have been identified as important components of agricultural soil communities that suppress soil-borne plant pathogens (Weller et

al., 2002; Bakker et al., 2010). This phenomenon is hypothesized to be a result of antimicrobial production, though no specific molecules have yet been implicated in pathogen suppression. Soil-dwelling streptomycetes have also been shown to stimulate the growth of ectomycorrhizal fungi (Maier et al., 2004; Schrey et al., 2005), which promotes nutrient acquisition in plants. Endophytic streptomycetes occur in a wide variety of plants (Taechowisan et al.; Seipke, Kaltenpoth, et al., 2012), and have been linked with pathogen suppression (Taechowisan et al.; Coombs et al., 2004; Conn et al., 2008). They may also promote plant growth through the production of the plant hormone auxin (Igarashi et al., 2002; Seipke, Kaltenpoth, et al., 2012) and by stimulating *Rhizobium* spp. root nodule formation (Tokala et al., 2002).

Pathogenic lifestyles are generally rare among streptomycetes, but several species are known to infect humans and plants. *S. sudanensis* and *S. somaliensis* are the causative agents of actinomycetoma, an infection of skin, subcutaneous tissue, and bone. Actinomyceteoma is little-studied but nevertheless debilitating and sometimes fatal disease that largely affects regions within Sudan (Quintana et al., 2008; Kirby et al., 2012). Streptomycete plant pathogens cause a disease called common scab, which mainly affects root and tuber crops such as potato (Lerat et al., 2009). Species causing the disease are polyphyletic but share a pathogenicity island that is transferred horizontally among streptomycetes and confers virulence. The primary virulence factor found on the pathogenicity island is a gene cluster for the biosynthesis of the phytotoxin

thaxtomin, which inhibits the synthesis of cellulose thereby compromising cell wall integrity (Kers et al., 2005).

The genus *Streptomyces* is highly adaptable, and the number of known environments occupied by this genus will undoubtedly continue to increase in the future. Though secondary metabolites are predicted to drive much of the niche adaptation described above (Demain and Fang, 2000), in many cases the specific molecules involved have not been identified. Still, the incredible diversity of compounds that have been structurally characterized from *Streptomyces* spp. makes the genus an ideal system to examine the roles of secondary metabolites in nature. Notably, most of the predicted functions for streptomycete metabolites in the interactions described above are as antagonists. While inhibition undoubtedly is an important component of *Streptomyces* chemical ecology, it remains important to consider additional ecological functions if we are to truly understand their importance in streptomycete biology (Davies et al., 2006; Martínez, 2008).

1.3 Marine *Streptomyces*

The existence of marine actinobacteria was initially not widely accepted among microbiologists. One of the reasons for this is that many terrestrial actinomycetes form resistant spores that can be transported into the ocean, and therefore it was thought that actinobacterial isolates from the ocean may be simply the recovery of washed-in dormant forms (Bull et al., 2000). Some of the best evidence for active marine actinobacteria has come from the discovery of

marine-specific actinobacterial lineages. Marine-specific actinobacteria have now been described within the genera *Dietzia*, *Rhodococcus*, *Marinophilus*, *Solwaraspora*, *Salinibacterium*, *Aeromicrobium marinum*, *Williamsia maris* and *Verrucosispora* (Lam, 2006). Additionally, all members of the actinobacterial genus *Salinispora* are obligate marine, and to date have not been observed outside of the marine environment (Jensen and Mafnas, 2006). Interestingly, some marine sediment-derived actinobacterial isolates require seawater supplemented to growth media, which suggest that they are marine-adapted (Prieto-Davo et al., 2008).

In addition to the above genera, there is compelling evidence that suggests the existence of marine-specific *Streptomyces* lineages. Some of the first evidence for indigenous marine *Streptomyces* came from a 1995 study by Moran and colleagues who found that rRNA from vegetatively growing *Streptomyces* accounted for 2-5% of the total community ribosomal RNA in marine sediment samples (Moran et al., 1995). Additionally, the amount of *Streptomyces* rRNA varied in response to sediment incubation with different substrates, providing further support that these bacteria are active in sediment communities (Moran et al., 1995). Subsequent culturing of marine streptomycetes by other groups has resulted in the isolation of many novel secondary metabolites. In a period from 2003-2005 alone, twelve novel compounds were structurally characterized from marine-derived *Streptomyces* spp. (Lam, 2006), including the antimicrobial ester bonactin from a sediment-

derived strain (Schumacher et al., 2003), the antibiotic frigocyclinone from a *S. griseus* strain derived from Terra Nova Bay in Antarctica (Bruntner et al., 2005), the neuritogentic molecule komodoquinone A from a sediment-derived strain (Itoh et al., 2003), and the highly unusual prenylated pyrrole glaciapyrrole from an Alaskan marine sediment-drive strain (Macherla et al., 2005). Novel compounds have also been isolated from marine invertebrate-derived *Streptomyces* isolates (Han et al.; Lee et al., 1998; Lin et al., 2010; Pimentel-Elardo et al., 2010; Schneemann et al., 2010).

So far, very little is known about specific adaptations of *Streptomyces* to the marine environment. Abiotic conditions in marine sediments are highly heterogeneous, and estimates of bacterial abundance in these environments are as high as 10^9 cells per cm^3 (Torsvik et al., 1996). The roles of secondary metabolites in marine sediment community structure, function, and interactions have yet to be studied in detail, but could be important factors contributing to *Streptomyces* adaptation to this complex and variable environment.

1.4 Secondary metabolite biosynthesis and gene cluster evolution

Details of the molecular genetics of secondary metabolite biosynthesis have been the subject of much study in recent years (Fischbach and Walsh, 2006). In combination with the increased availability of genome sequence data, a wide range of novel techniques for studies of secondary metabolite diversity and evolution have been developed. Enzymes for the biosynthesis of secondary metabolites are often modular and operate in an assembly line-like manner,

where monomer units are iteratively incorporated onto the growing molecule through a series of condensation steps (Fischbach and Walsh, 2006; Hertweck, 2009). Individual protein domains of the assembly line determine what monomer units are incorporated and what tailoring steps are used to synthesize the secondary metabolite. Two of the best-studied classes of secondary metabolites that are generally synthesized in this manner are polyketides, which are usually built from carboxylic acid monomers, and nonribosomal peptides, which are usually built from amino acid monomers (Fischbach and Walsh, 2006; Hertweck, 2009). This modular nature means that simple mutations within the assembly line or changes to the set of tailoring enzymes present in the cluster create novel structural diversity that could result in improved or altered fitness of the producer (Fischbach et al., 2008).

Genes for the biosynthesis of secondary metabolites are typically clustered in microbial genomes along with specific regulatory, transport, and resistance genes (Fischbach et al., 2008). According to the Selfish Operon Model, the clustering of genes that encode a selectable phenotype allows for horizontal gene transfer of the collective to new genomes because the survival of the cluster is independent of the fate of the host bacterium (Lawrence, 1999). Consistent with this, closely related secondary metabolite clusters have been observed in divergent bacteria and these clusters are frequently associated with mobile elements and genomic islands (Fischbach et al., 2008; Ziemert et al., 2014). The clustering of secondary metabolite biosynthesis genes also facilitates

their identification in microbial genomes. In 2002, the first streptomycete genome, *S. coelicolor* A3(2), was reported and found to encode more biosynthetic pathways than were expressed under normal culture conditions (Bentley et al., 2002). Subsequent genome sequencing of *Streptomyces* isolates revealed that these organisms consistently dedicate a relatively large fraction of their coding capacity (5-10%) to secondary metabolite biosynthetic gene clusters, the majority of which are cryptic with no known product (Baltz, 2008).

Studying the relationships among secondary metabolite genes can provide insight into the evolutionary mechanisms that lead to structural diversity, including horizontal gene transfer, recombination, and vertical inheritance. For example, a recent study of over 10,000 biosynthetic gene clusters found that events such as insertions, deletions, and duplications occur more frequently within these clusters when compared with genes for primary metabolism, supporting the idea that these elements evolve relatively quickly (Medema et al., 2014). The same study also reported that concerted evolution can play a role in gene cluster evolution, where recombination of repetitive elements within a single cluster results in homogenization of similar protein domains (Medema et al., 2014).

Ideally, it would be possible to overlay the functions of secondary metabolites onto the phylogenies of the genes responsible for their production in order to test hypotheses about how new functional diversity arises. For example, SapB is a surfactant peptide that allows streptomycetes to overcome surface

tension at the air-water interface and produce aerial mycelia during the process of sporulation (Kodani et al., 2004). Its structure falls within a major group of ribosomally-synthesized peptides known as type III lantibiotics, which often have strong antimicrobial activity and are predicted to function in antagonism (Völler et al., 2012). Given the functional role of this peptide in development, it might be expected that lantibiotic biosynthesic gene clusters evolved from the SapB gene cluster (Kodani et al., 2004), yet the lanthipeptide synthetase responsible for SapB production clades within a larger group of synthetases responsible for the production of type III lantibiotics including avermipeptin, catenulipeptin, and griseopeptin (Zhang et al., 2012). This suggests that the genes that produce the developmental SapB protein could have evolved from a cluster for antimicrobial production. As more is learned about the *in situ* roles of secondary metabolites, it will be interesting to examine if evolutionarily related gene clusters produce molecules with distinct functions. Relating the evolutionary history of biosynthetic gene clusters with the chemical structures they create and the fitness benefit conferred on the producer remains an important goal in microbial chemical ecology.

1.5 Overview of the dissertation

This dissertation examines the distributions and ecology of an unusual class of secondary metabolites called hybrid isoprenoids (HIs) in the genus *Streptomyces*. Chapter 2 presents a literature review synthesizing reports of hybrid isoprenoid production by actinobacteria. This summary revealed that the

genus *Streptomyces* is responsible for the majority of known actinobacterial HIs, and that within the genus a single lineage consistently produced diverse molecules within this class. The clade had previously been identified as a putative marine *Streptomyces* lineage assigned the designation 'MAR4' (Fenical and Jensen, 2006). This chapter was published in its entirety as a review paper in Current Opinion in Biotechnology in 2010 (Gallagher et al., 2010).

Chapter 3 presents an in-depth study of MAR4 phylogenetic diversity and HI production. This study was published in its entirety in Applied and Environmental Microbiology in 2013 (Gallagher et al., 2013). The goals of this chapter were to establish a phylogeny of the MAR4 clade, to use culture-dependent and culture-independent methods to assess MAR4 environmental diversity, and to test the hypothesis that MAR4 strains consistently produce HI molecules. By incorporating sequence data from diverse sources including marine sediment samples collected off the coast of Santa Cruz Island, California, 57 cultured isolates and 180 clone sequences that belong to the MAR4 clade were identified, most of which are marine-derived. Consistent with the literature-based observations in Chapter 2, the culture extracts from representative MAR4 strains were found to contain at least one HI molecule, and some strains even produced up to four distinct types of HI molecules, an ability that has not been reported elsewhere in the streptomycetes. There was also some evidence for lineage-specific production of HI molecules. Overall, Chapter 3 significantly increases knowledge of the diversity and distributions of the MAR4 clade and

provides further support for the hypothesis that this group is enriched in HI production.

Chapter 4 presents a genomic analysis of the potential for HI production in 120 *Streptomyces* genomes, including 12 MAR4 strains. This study in its entirety is currently being prepared for publication. Full genome sequences provided the opportunity to rigorously test the hypothesis that members of the MAR4 clade will possess more HI gene clusters than other *Streptomyces* strains. A key group of enzymes in the biosynthesis of HIs called ABBA prenyltransferases were consistently found in higher abundance in MAR4 genomes compared with non-MAR4 streptomycetes. Thirteen gene clusters for HI production were identified in MAR4 genomes, the majority of which have not been linked to a secondary metabolic product. This implies that MAR4 strains have the genetic capacity to produce even more HIs than have been isolated to date. A phylogenetic analysis of ABBA prenyltransferases was used to assess the evolutionary history of HI gene clusters in MAR4, which provides insights into the processes that have led to the accumulation of HI gene clusters in this clade.

So far, no streptomycete-produced HI has an experimentally confirmed natural function. To address this, Chapter 5 presents the results of a study examining a functional hypothesis for these molecules. The study in its entirety is currently being prepared for publication. Because many HIs resemble the electron shuttles employed in primary and secondary metabolism, the hypothesis that they function in redox cycling in low-oxygen environments was examined.

Secondary metabolite production by a MAR4 strain was tracked in high- and low-oxygen conditions. A highly pigmented redox-active molecule significantly up-regulated in low-oxygen conditions was isolated and identified as 8-amino-flaviolin. Interestingly, the molecule was known to be a precursor product of the *nap* gene cluster, the end product of which is the HI molecule napyradiomycin, which was significantly down-regulated in low-oxygen culture. This provides a unique example of how a single biosynthetic cluster may produce products with distinct properties depending on culture conditions.

Chapter 6 is a concluding chapter providing a synthesis and discussion of the results of the dissertation as a whole. It also looks to future directions for the projects initiated by this dissertation and the field of microbial chemical ecology.

1.6 References

Angell, S., Bench, B.J., Williams, H., and Watanabe, C.M.H. (2006) Pyocyanin isolated from a marine microbial population: synergistic production between two distinct bacterial species and mode of action. *Chem. Biol.* **13**: 1349–59.

Bakker, M.G., Glover, J.D., Mai, J.G., and Kinkel, L.L. (2010) Plant community effects on the diversity and pathogen suppressive activity of soil streptomycetes. *Appl. Soil Ecol.* **46**: 35–42.

Baltz, R.H. (2008) Renaissance in antibacterial discovery from actinomycetes. *Curr. Opin. Pharmacol.* **8**: 557–63.

Barke, J., Seipke, R.F., Grueschow, S., Heavens, D., Drou, N., Bibb, M.J., Goss, R.J.M., Yu, D.W., and Hutchings, M.I. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *Bmc Biol.* **8**: 109.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., and Hopwood, D.A. (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**: 141-147.

Berdy, J. (2005) Bioactive microbial metabolites - A personal view. *J. Antibiot. (Tokyo).* **58**: 1–26.

Bruntner, C., Binder, T., Pathom-aree, W., Goodfellow, M., Bull, A.T., Potterat, O., Puder, C., Horer, S., Schmid, A., Bolek, W., Wagner, K., Mihm, G., and Fiedler, H.-P. (2005) Frigocyclinone, a novel angucyclinone antibiotic produced by a *Streptomyces griseus* strain from Antarctica. *J. Antibiot. (Tokyo).* **58**: 346–9.

Brutinel, E.D. and Gralnick, J.A. (2012) Shuttling happens: soluble flavin mediators of extracellular electron transfer in *Shewanella*. *Appl. Microbiol. Biotechnol.* **93**: 41–48.

Bull, A.T., Ward, A.C., and Goodfellow, M. (2000) Search and discovery strategies for biotechnology: the paradigm shift. *Microbiol. Mol. Biol. Rev.* **64**: 573–606.

Camilli, A. and Bassler, B.L. (2006) Bacterial small-molecule signaling pathways. *Science* **311**: 1113–6.

Challis, G.L. and Hopwood, D.A. (2003) Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci. U. S. A.* **100**: 14555–14561.

Conn, V.M., Walker, A.R., and Franco, C.M.M. (2008) Endophytic Actinobacteria Induce Defense Pathways in *Arabidopsis thaliana*. **2**: 208-218.

Coombs, J.T., Michelsen, P.P., and Franco, C.M.M. (2004) Evaluation of endophytic actinobacteria as antagonists of *Gaeumannomyces graminis* var. *tritici* in wheat. *Biol. Control* **29**: 359–366.

Cude, W.N., Mooney, J., Tavanaei, A.A., Hadden, M.K., Frank, A.M., Gulvik, C.A., May, A.L., and Buchan, A. (2012) Production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization by the marine roseobacter *Phaeobacter* sp. strain Y4I. *Appl. Environ. Microbiol.* **78**: 4771–80.

Currie, C.R., Scott, J.A., Summerbell, R.C., and Malloch, D. (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**: 701-704.

Davies, J. (2013) Specialized microbial metabolites: functions and origins. *J. Antibiot. (Tokyo).* **66**: 361–4.

Davies, J., Spiegelman, G.B., and Yim, G. (2006) The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.* **9**:445-453.

Demain, A.L. and Fang, A. (2000) The natural functions of secondary metabolites. *Adv. Biochem. Eng. Biotechnol.* **69**: 1–39.

Dunny, G.M. and Leonard, B.A. (1997) Cell-cell communication in gram-positive bacteria. *Annu. Rev. Microbiol.* **51**: 527–64.

Fenical, W. and Jensen, P.R. (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat. Chem. Biol.* **2**: 666–673.

Fischbach, M.A. and Walsh, C.T. (2006) Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chem. Rev.* **106**: 3468–96.

Fischbach, M.A., Walsh, C.T., and Clardy, J. (2008) The evolution of gene collectives: How natural selection drives chemical innovation. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 4601–4608.

Flardh, K. and Buttner, M.J. (2009) *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. *Nat. Rev. Microbiol.* **7**: 36–49.

Fuqua, C. and Greenberg, E.P. (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nat. Rev. Mol. Cell Biol.* **3**: 685–95.

Gallagher, K.A., Fenical, W., and Jensen, P.R. (2010) Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes. *Curr. Opin. Biotechnol.* **21**: 794–800.

Gallagher, K.A., Rauscher, K., Pavan Ioca, L., and Jensen, P.R. (2013) Phylogenetic and Chemical Diversity of a Hybrid Isoprenoid-producing Streptomycete Lineage. *Appl. Environ. Microbiol.* **79**: 6894–902.

Gebhardt, K., Schimana, J., Krastel, P., Dettner, K., Rheinheimer, J., Zeeck, A., and Fiedler, H.-P.P. (2002) Endophenazines A similar to D, new phenazine antibiotics from the arthropod associated endosymbiont *Streptomyces anulatus* I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot. (Tokyo).* **55**: 794–800.

Gerber, N.N. and Lechevalier, H.A. (1965) Geosmin, an earthly-smelling substance isolated from actinomycetes. *Appl. Microbiol.* **13**: 935–8.

Glasser, N.R., Kern, S.E., and Newman, D.K. (2014) Phenazine redox cycling enhances anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a proton-motive force. *Mol. Microbiol.* **92**: 399–412.

Gralnick, J.A. and Newman, D.K. (2007) Extracellular respiration. *Mol. Microbiol.* **65**: 1-11.

Han, Y., Yang, B., Zhang, F., Miao, X., and Li, Z. Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with South China Sea sponge *Craniella australiensis. Mar. Biotechnol. (NY).* **11**: 132–40.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* **7**: 263–73.

Hernandez, M.E. and Newman, D.K. (2001) Extracellular electron transfer. *Cell. Mol. Life Sci.* **58**: 1562–1571.

Hertweck, C. (2009) The biosynthetic logic of polyketide diversity. *Angew. Chem. Int. Ed. Engl.* **48**: 4688–716.

Igarashi, Y., Iida, T., Yoshida, R., and Furumai, T. (2002) Pteridic Acids A and B, Novel Plant Growth Promoters with Auxin-like Activity from Streptomyces hygroscopicus TP-A0451. *J. Antibiot. (Tokyo).* **55**: 764–767.

Itoh, T., Kinoshita, M., Aoki, S., and Kobayashi, M. (2003) Komodoquinone A, a novel neuritogenic anthracycline, from marine *Streptomyces* sp. KS3. *J. Nat. Prod.* **66**: 1373–7.

Jayasinghe, B.A.T.D. and Parkinson, D. (2009) Earthworms as the vectors of actinomycetes antagonistic to litter decomposer fungi. *Appl. Soil Ecol.* **43**: 1–10.

Jensen, P.R. and Mafnas, C. (2006) Biogeography of the marine actinomycete *Salinispora*. *Environ. Microbiol.* **8**: 1881–1888.

Kaltenpoth, M., Yildirim, E., Guerbuez, M.F., Herzner, G., and Strohm, E. (2012) Refining the Roots of the Beewolf-*Streptomyces* Symbiosis: Antennal Symbionts in the Rare Genus *Philanthinus* (Hymenoptera, Crabronidae). *Appl. Environ. Microbiol.* **78**: 822–827.

Kers, J.A., Cameron, K.D., Joshi, M. V, Bukhalid, R.A., Morello, J.E., Wach, M.J., Gibson, D.M., and Loria, R. (2005) A large, mobile pathogenicity island confers plant pathogenicity on *Streptomyces* species. *Mol. Microbiol.* **55**: 1025–33.

Kirby, R., Sangal, V., Tucker, N.P., Zakrzewska-Czerwinska, J., Wierzbicka, K., Herron, P.R., Chu, C.-J., Chandra, G., Fahal, A.H., Goodfellow, M., Hoskisson, P.A. (2012) Draft genome sequence of the human pathogen *Streptomyces somaliensis*, a significant cause of actinomycetoma. *J. Bacteriol.* **194**: 3544–3545.

Kodani, S., Hudson, M.E., Durrant, M.C., Buttner, M.J., Nodwell, J.R., and Willey, J.M. (2004) The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene ramS in *Streptomyces coelicolor*. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 11448–11453.

Lam, K.S. (2006) Discovery of novel metabolites from marine actinomycetes. *Curr. Opin. Microbiol.* **9**: 245–251.

Lawrence, J. (1999) Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. *Curr. Opin. Genet. Dev.* **9**: 642–648.

Lee, H.K., Lee, D.-S., Lim, J., Kim, J.S., Im, K.S., and Jung, J.H. (1998) Topoisomerase i inhibitors from the *Streptomyces* sp. strain KM86-9B isolated from a marine sponge. *Arch. Pharm. Res.* **21**: 729–733.

Lerat, S., Simao-Beaunoir, A.-M., and Beaulieu, C. (2009) Genetic and physiological determinants of *Streptomyces scabies* pathogenicity. *Mol. Plant Pathol.* **10**: 579–585.

Lesuisse, E., Blaiseau, P.L., Dancis, A., and Camadro, J.M. (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. *Microbiology* **147**: 289–98.

Lewis, K. (2012) Antibiotics: Recover the lost art of drug discovery. *Nature* **485**: 439–40.

Lin, Z., Antemano, R.R., Hughen, R.W., Tianero, M.D.B., Peraud, O., Haygood, M.G., Concepcion, G.P., Olivera, B.M., Light, A., and Schmidt, E.W. (2010) Pulicatins A-E, neuroactive thiazoline metabolites from cone snail-associated bacteria. *J. Nat. Prod.* **73**: 1922–6.

Long, R.A. and Azam, F. (2001) Antagonistic interactions among marine pelagic bacteria. *Appl. Environ. Microbiol.* **67**: 4975–83.

Macherla, V.R., Liu, J.N., Bellows, C., Teisan, S., Nicholson, B., Lam, K.S., and Potts, B.C.M. (2005) Glaciapyrroles A, B, and C, pyrrolosesquiterpenes from a *Streptomyces* sp. isolated from an Alaskan marine sediment. *J. Nat. Prod.* **68**: 780–783.

Maier, A., Riedlinger, J., Fiedler, H.-P., and Hampp, R. (2004) Actinomycetales bacteria from a spruce stand: Characterization and effects on growth of root symbiotic and plant parasitic soil fungi in dual culture. *Mycol. Prog.* **3**: 129–136.

Mangano, S., Michaud, L., Caruso, C., Brilli, M., Bruni, V., Fani, R., and Lo Giudice, A. (2009) Antagonistic interactions between psychrotrophic cultivable bacteria isolated from Antarctic sponges: a preliminary analysis. *Res. Microbiol.* **160**: 27–37.

Martínez, J.L. (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**: 365–367.

Medema, M.H., Cimermancic, P., Sali, A., Takano, E., and Fischbach, M.A. (2014) A Systematic Computational Analysis of Biosynthetic Gene Cluster Evolution: Lessons for Engineering Biosynthesis. *PLoS Comput. Biol.* **10**: e1004016.

Moran, M.A., Rutherford, L.T., and Hodson, R.E. (1995) Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. *Appl. Environ. Microbiol.* **61**: 3695–3700.

Nechitaylo, T.Y., Westermann, M., and Kaltenpoth, M. (2014) Cultivation reveals physiological diversity among defensive "*Streptomyces philanthi*" symbionts of beewolf digger wasps (Hymenoptera, Crabronidae). *BMC Microbiol.* **14**: 202.

Nithya, C., Devi, M.G., and Karutha Pandian, S. (2011) A novel compound from the marine bacterium *Bacillus pumilus* S6-15 inhibits biofilm formation in gram-positive and gram-negative species. *Biofouling* **27**: 519–28.

Pasti, M.B. and Belli, M.L. (1985) Cellulolytic activity of Actinomycetes isolated from termites (Termitidae) gut. *FEMS Microbiol. Lett.* **26**: 107–112.

Pasti, M.B., Pometto, A.L., Nuti, M.P., and Crawford, D.L. (1990) Lignin-solubilizing ability of actinomycetes isolated from termite (Termitidae) gut. *Appl. Environ. Microbiol.* **56**: 2213–8.

Pelaez, F. (2006) The historical delivery of antibiotics from microbial natural products - Can history repeat? *Biochem. Pharmacol.* **71**: 981–990.

Peraud, O., Biggs, J.S., Hughen, R.W., Light, A.R., Concepcion, G.P., Olivera, B.M., and Schmidt, E.W. (2009) Microhabitats within venomous cone snails contain diverse actinobacteria. *Appl. Environ. Microbiol.* **75**: 6820–6826.

Pesavento, C. and Hengge, R. (2009) Bacterial nucleotide-based second messengers. *Curr. Opin. Microbiol.* **12**: 170–176.

Pham, T.H., Boon, N., Aelterman, P., Clauwaert, P., De Schamphelaire, L., Vanhaecke, L., De Maeyer, K., Hofte, M., Verstraete, W., and Rabaey, K. (2008) Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer. *Appl. Microbiol. Biotechnol.* **77**: 1119–1129.

Pimentel-Elardo, S.M., Kozytska, S., Bugni, T.S., Ireland, C.M., Moll, H., and Hentschel, U. (2010) Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. *Mar. Drugs* **8**: 373–80.

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2006) Rethinking "secondary" metabolism: physiological roles for phenazine antibiotics (vol 2, pg 71, 2006). *Nat. Chem. Biol.* **2**: 221.

Prieto-Davo, A., Fenical, W., and Jensen, P.R. (2008) Comparative actinomycete diversity in marine sediments. *Aquat. Microb. Ecol.* **52**: 1–11.

Quintana, E.T., Wierzbicka, K., Mackiewicz, P., Osman, A., Fahal, A.H., Hamid, M.E., Zakrzewska-Czerwinska, J., Maldonado, L.A., and Goodfellow, M. (2008) *Streptomyces sudanensis* sp. nov., a new pathogen isolated from patients with actinomycetoma. *Antonie Van Leeuwenhoek* **93**: 305–13.

Rypien, K.L., Ward, J.R., and Azam, F. (2010) Antagonistic interactions among coral-associated bacteria. *Environ. Microbiol.* **12**: 28–39.

Saha, R., Saha, N., Donofrio, R.S., and Bestervelt, L.L. (2013) Microbial siderophores: a mini review. *J. Basic Microbiol.* **53**: 303–17.

Schatz, A., Bugle, E., and Waksman, S.A. (1944) Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria. *Exp. Biol. Med.* **55**: 66–69.

Schmitt, M., Predich, M., Doukhan, L., Smith, I., and Holmes, R. (1995) Characterization of an iron-dependent regulatory protein (IdeR) of *Mycobacterium tuberculosis* as a functional homolog of the diphtheria toxin repressor (DtxR) from *Corynebacterium diphtheriae. Infect. Immun.* **63**: 4284–4289.

Schneemann, I., Kajahn, I., Ohlendorf, B., Zinecker, H., Erhard, A., Nagel, K., Wiese, J., and Imhoff, J.F. (2010) Mayamycin, a cytotoxic polyketide from a *Streptomyces* strain isolated from the marine sponge Halichondria panicea. *J. Nat. Prod.* **73**: 1309–12.

Schneider, R. and Hantke, K. (1993) Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as part of a binding protein-dependent transport system. *Mol. Microbiol.* **8**: 111–121.

Schrey, S.D., Schellhammer, M., Ecke, M., Hampp, R., and Tarkka, M.T. (2005) Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. *New Phytol.* **168**: 205–16.

Schumacher, R.W., Talmage, S.C., Miller, S.A., Sarris, K.E., Davidson, B.S., and Goldberg, A. (2003) Isolation and structure determination of an antimicrobial ester from a marine sediment-derived bacterium. *J. Nat. Prod.* **66**: 1291–3.

Scott, J.J., Oh, D.-C., Yuceer, M.C., Klepzig, K.D., Clardy, J., and Currie, C.R. (2008) Bacterial protection of beetle-fungus mutualism. *Science* **322**: 63.

Seipke, R.F., Barke, J., Ruiz-Gonzalez, M.X., Orivel, J., Yu, D.W., and Hutchings, M.I. (2012) Fungus-growing *Allomerus* ants are associated with antibiotic-producing actinobacteria. *Antonie Van Leeuwenhoek* **101**: 443–7.

Seipke, R.F., Kaltenpoth, M., and Hutchings, M.I. (2012) *Streptomyces* as symbionts: an emerging and widespread theme? *Fems Microbiol. Rev.* **36**: 862–876.

Slattery, M., Rajbhandari, I., and Wesson, K. (2001) Competition-Mediated Antibiotic Induction in the Marine Bacterium *Streptomyces tenjimariensis*. *Microb. Ecol.* **41**: 90–96.

Strahl, E.D., Dobson, W.E., and Lundie, L.L. (2002) Isolation and screening of brittlestar-associated bacteria for antibacterial activity. *Curr. Microbiol.* **44**: 450–459.

Taechowisan, T., Peberdy, J.F., and Lumyong, S. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.* **19**: 381–385.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. *Microbiol. Mol. Biol. Rev.* **71**: 295-347.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H., Deobald, L.A., Bailey, J.F., and Morra, M.J. (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). *Appl. Environ. Microbiol.* **68**: 2161–71.

Torsvik, V., Sørheim, R., and Goksøyr, J. (1996) Total bacterial diversity in soil and sediment communities—A review. *J. Ind. Microbiol. Biotechnol.* **17**: 170–178.

Traxler, M.F. and Kolter, R. (2012) A massively spectacular view of the chemical lives of microbes. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 10128–9.

Trischman, J.A., Oeffner, R.E., de Luna, M.G., and Kazaoka, M. Competitive induction and enhancement of indole and a diketopiperazine in marine bacteria. *Mar. Biotechnol. (NY).* **6**: 215–20.

Vaz Jauri, P. and Kinkel, L.L. (2014) Nutrient overlap, genetic relatedness and spatial origin influence interaction-mediated shifts in inhibitory phenotype among *Streptomyces* spp. *FEMS Microbiol. Ecol.* **90**: 264–75.

Vetsigian, K., Jajoo, R., and Kishony, R. (2011) Structure and evolution of *Streptomyces* interaction networks in soil and in silico. *PLoS Biol.* **9**: e1001184.

Völler, G.H., Krawczyk, J.M., Pesic, A., Krawczyk, B., Nachtigall, J., and Süssmuth, R.D. (2012) Characterization of new class III lantibiotics--erythreapeptin, avermipeptin and griseopeptin from *Saccharopolyspora erythraea*, *Streptomyces avermitilis* and *Streptomyces griseus* demonstrates stepwise N-terminal leader processing. *Chembiochem* **13**: 1174–83.

Watanabe, Y., Shinzato, N., and Fukatsu, T. (2003) Isolation of actinomycetes from termites' guts. *Biosci. Biotechnol. Biochem.* **67**: 1797–801.

Waters, C.M. and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.* **21**: 319–46.

Watve, M.G., Tickoo, R., Jog, M.M., and Bhole, B.D. (2001) How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* **176**: 386–390.

Weller, D.M., Raaijmakers, J.M., Gardener, B.B.M., and Thomashow, L.S. (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* **40**: 309–48.

Wietz, M., Duncan, K., Patin, N. V, and Jensen, P.R. (2013) Antagonistic interactions mediated by marine bacteria: the role of small molecules. *J. Chem. Ecol.* **39**: 879–91.

Xiao, Y., Wei, X., Ebright, R., and Wall, D. (2011) Antibiotic production by myxobacteria plays a role in predation. *J. Bacteriol.* **193**: 4626–33.

Yoshikawa, K., Takadera, T., Adachi, K., Nishijima, M., and Sano, H. (1997) Korormicin, a novel antibiotic specifically active against marine gram-negative bacteria, produced by a marine bacterium. *J. Antibiot. (Tokyo).* **50**: 949–53.

Zhang, Q., Yu, Y., Vélasquez, J.E., and van der Donk, W.A. (2012) Evolution of lanthipeptide synthetases. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 18361–6.

Ziemert, N., Lechner, A., Wietz, M., Millán-Aguiñaga, N., Chavarria, K.L., and Jensen, P.R. (2014) Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proc. Natl. Acad. Sci. U. S. A.* **111**: E1130–9.

Chapter 2: Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes

2.1 Abstract

Terpenoids are among the most ubiquitous and diverse secondary metabolites observed in nature. Although actinomycete bacteria are one of the primary sources of microbially derived secondary metabolites, they rarely produce compounds in this biosynthetic class. The terpenoid secondary metabolites that have been discovered from actinomycetes are often in the form of biosynthetic hybrids called hybrid isoprenoids (HIs). HIs include significant structural diversity and biological activity and thus are important targets for natural product discovery. Recent screening of marine actinomycetes has led to the discovery of a new lineage that is enriched in the production of biologically active HI secondary metabolites. These strains represent a promising resource for natural product discovery and provide unique opportunities to study the evolutionary history and ecological functions of an unusual group of secondary metabolites.

2.2 Introduction

Terpenoids are a large and ubiquitous class of metabolites that provide diverse functions in both primary and secondary metabolism (Gershenzon and Dudareva, 2007). Terpenoids are assembled from five-carbon isoprene subunits into a wide assortment of structures that generally range from monoterpenes (two isoprene units) to triterpenes (six isoprene units) but in certain cases can also be larger. In addition, terpenoid moieties can be appended to molecules produced

via non-terpenoid biosynthetic routes forming products known as hybrid isoprenoids (HIs). As secondary metabolites, terpenoids are common in plants, insects, fungi, and some marine invertebrates such as soft corals (Gershenzon and Dudareva, 2007; Paul and Ritson-Williams, 2008). They are known to play diverse ecological roles in signaling and chemical defense and are responsible for many of the common odors and flavors associated with plants and fruits (Langenheim, 1994; Gershenzon and Dudareva, 2007; Bohlmann and Keeling, 2008). For reasons that are not understood, neither terpenoid nor HI secondary metabolites are commonly observed among prokaryotes (Kuzuyama and Seto, 2003). In cases where bacterial HI secondary metabolites have been reported, the most common sources are actinomycetes, and the products include a number of structurally diverse and biomedically important compounds making them rational targets for drug discovery. This review explores the phylogenetic diversity and environmental origins of actinomycetes that produce HI secondary metabolites with a particular focus on the recent observation that specific groups of marine-derived strains are enriched in the production of this class of compounds. It is our hope that a synthesis of this information may provide new insight into strategies for the discovery of bacterial HI secondary metabolites and the evolutionary processes driving HI biosynthesis.

2.3 Actinomycete terpenoids

The actinomycetes comprise a group of Gram-positive bacteria within the Order Actinomycetales. They generally have a complex life cycle, filamentous

morphology, and are renowned for the production of structurally diverse and biologically active small organic molecules commonly referred to as secondary metabolites. In some cases, actinomycetes produce primary metabolites of terpenoid origin. Well known among these are the hopanoids, which are thought to function in bacterial membranes in a manner analogous to that of cholesterol in eukaryotes (Ourisson et al., 1987). Actinomycetes are also known to produce terpenoid secondary metabolites, although this is uncommon relative to compounds of other biosynthetic origins such as polyketides and non-ribosomal peptides. Examples of terpenoid secondary metabolites include carotenoid pigments (Krugel et al., 1999) and the volatile compounds geosmin and 2-methylisoborneol, which are responsible for the distinct earthy odors often associated with soil actinomycetes (Nett et al., 2009). Actinomycete terpenoid secondary metabolites also include HIs, which are the focus of this review.

2.4 Hybrid isoprenoids

Hybrid isoprenoids are metabolites in which terpenoid moieties of varying levels of complexity are attached to such diverse molecules as polyketides, ribosomal and non-ribosomal peptides, phenazines and pyrroles. The attachment of an isoprenoid to another molecule is referred to as ‘prenylation’ and can increase biological activity, perhaps due to the increased affinity of the isoprene unit for biological membranes (Botta et al., 2005). Isoprenoid units are attached to non-terpenoid small molecules via prenyltransferase enzymes (PTases). The point of attachment can be a carbon, oxygen, or nitrogen atom or another

isoprene unit, in which case head to tail assembly is the norm but reverse prenylation is also observed (Heide, 2009). Collectively, these processes result in the generation of extraordinary chemical diversity. Although HIs are generally thought of in the context of secondary metabolism, not all fall into this category. Among the best-known actinomycete HI primary metabolites are the respiratory menaquinones. These compounds function in the electron transport chain and have been used as a chemotaxonomic trait to distinguish actinomycetes from other groups of bacteria (Collins et al., 1977).

Although reports of HI secondary metabolites from actinomycetes are relatively rare, the compounds that have been discovered are significant in terms of both structural novelty and biological activity. Tables 2.1 and 2.2 provide a list of many of the important HI secondary metabolites that have been isolated from actinomycetes. The first compound discovered was novobiocin (**1**) in 1955 from *Streptomyces spheriodes* (Harris et al., 1955; Leopold et al., 1957). Novobiocin is a potent inhibitor of DNA gyrase and continues to be the subject of active research related to its antibiotic (Walsh et al., 1993; Palaniappan and Holley, 2010) and anti-cancer (Burlison et al., 2008; Donnelly et al., 2008) activities. Subsequently, at least 36 additional HI secondary metabolites have been reported from actinomycetes (Tables 2.1 and 2.2). These compounds encompass considerable structural diversity (Figure 2.1), which is often driven by the architecture of the terpenoid substituents as observed in napthterpin (**2**), a napyradiomycin analog (**3**), and neomarinone (**4**), all of which share similar

polyketide scaffolds. To date, HI secondary metabolites have been reported from at least five actinomycete genera. However, of the 36 compounds listed in Tables 2.1 and 2.2, 32 are from the genus *Streptomyces* making this taxon the single most important source of actinomycete-derived HI secondary metabolites. The biosynthetic origin of the terpenoid portion of actinomycete HIs has been an active area of research and was most recently reviewed by Kuzuyama and Seto in 2003 (Kuzuyama and Seto, 2003). Ongoing research into the PTase enzymes responsible for generating HIs has shed new insight into substrate specificity and created interest in the rational engineering of these catalysts in an effort to generate new structural diversity (Tello et al., 2008; Takahashi et al., 2010).

Table 2.1 Select hybrid isoprenoids of actinomycete origin. Strains in bold are marine-derived.

Compound	Original producing strain	Year	Non-terpene scaffold	Reference
(**1**) novobiocin	*S. sphereoides* (=*S. niveus*)	1955	Aminocoumarin	(Harris et al., 1955; Leopold et al., 1957)
(**2**) naphterpin	*Streptomyces* sp. CL190	1990	Naphthoquinone	(Shinya et al., 1990)
(**3**) napyradiomycin	*Chania rubra* MG802-AF1	1986	Naphthoquinone	(Shiomi et al., 1986)
(**4**) neomarinonone	***Streptomyces* sp. CNH-099**	2000	Naphthoquinone	(Hardt et al., 2000)
(**5**) cyclomarin	***Streptomyces* sp. CNB-982**	1999	Indole/peptide	(Renner et al., 1999)
(**6**) monoterpene phenazine ether	***Streptomyces sp*. CNQ-509**	2010	Phenazine	Unpublished
(**7**) nitropyrrolin	***Streptomyces* sp. CNQ-509**	2010	Pyrrole	Submitted
(**8**) lavanducyanin	*Streptomyces* sp. CL190	1989	Phenazine	(Imai et al., 1989)
(**9**) marinone	***Streptomyces* sp. CNB-632**	1992	Naphthoquinone	(Pathirana et al., 1992)
(**10**) azamerone	***Streptomyces* sp. CNQ-766**	2006	Pyridazine	(Cho et al., 2006)

Table 2.2 Chronological reporting of actinomycete-derived hybrid isoprenoids including additional reports of strains producing each compound and references if available. Strains in bold are marine-derived.

Compound	Original producing strain	Year	Non-terpene scaffold	Additional producing strains
novobiocin (originally cathomycin/ streptonivicin) (Harris et al., 1955; Leopold et al., 1957)	*S. sphereoides* sp. (= *S. niveus*)	1955	aminocoumarin	*S. niveus* sp. (Smith et al., 1956), *S. spheroides* NCIMB 11891 (Steffensky et al., 2000), *S. niveus* ATCC 19793 (Hoggarth et al., 1994), *S. niveus* BC-345 (Kominek, 1972), *S. griseoflavus* sp., *S. griseus* sp. (Orihara et al., 1998)
teleocidin (Takashima and Sakai, 1960)	*Streptomyces* 2A 1563	1960	indole/peptide	*S. blastmyceticum* NA34-17 (Irie et al., 1998)
moenomycin (Wallhausser et al., 1966) [10]	*S. bambergiensis*	1966	phospho-glycerate	*S. ghanaensis* NRRL B-12104 (Zazopoulos et al., 2003), *S. bambergiensis* S712 (Zotchev et al., 1995)
chlorobiocin (Mancy et al., 1974)	*S. roseo-chromogenes* var. oscitans NRRL 3504	1974	aminocoumarin	*S. hygroscopucus* NRRL 3418, *S. albocinerescens* NRRL 3419 (Mancy et al., 1974)
napyradiomycin (Shiomi et al., 1986)	*Chania rubra* MG802-AF1	1986	naphthoquinone	***Streptomyces* sp. CNQ-525** (Soria-Mercado et al., 2005), *S. aculeolatus* NRRL 18422 (Winter et al., 2007), *S. antimycoticus* NT17 (Motohashi, Sue, et al., 2008), ***Streptomyces* sp. CNQ-260, CNS-335, CNQ-907, CNQ-766, CNQ-381, CNT-371, CNQ-904, CNQ-865** (unpubl)
SF2415 (Shomura et al., 1987)	*S. aculeolatus* SF2415	1987	naphthoquinone	
lavanducyanin (Imai et al., 1989)	*Streptomyces* sp. CL190	1989	phenazine	***Streptomyces* sp. CNH-099** (unpubl)
furaquinocin (Komiyama et al., 1990)	*Streptomyces* sp. KO-3988	1990	naphthoquinone	
A80915 (Fukuda et al., 1990)	*S. aculeolatus* A80915	1990	naphthoquinone	***Streptomyces* sp. MS239** (Motohashi, et al., 2008)

Table 2.2 Chronological reporting of actinomycete-derived hybrid isoprenoids, Continued.

Compound	Original producing strain	Year	Non-terpene scaffold	Additional producing strains
naphthomevalin (Henkel and Zeeck, 1991)	*Streptomyces* sp. Gö 28	1990	naphthoquinone	
naphterpin (Shinya et al., 1990)	*Streptomyces* sp. CL190	1990	naphthoquinone	***Streptomyces* sp. CNQ-509** (unpubl)
benthocyanin (Shinya et al., 1991)	*S. prunicolor*	1991	phenazine	
furanonaph-thoquinone (Sedmera et al., 1991)	*S. cinna-monensis* DSM 1042	1991	naphthoquinone	
longestin (KS-505a) (Nakanishi et al., 1992)	*S. argenteolus* A-2	1992	glucuronic acid, succinyl benzoate	
carquinostatin (Shinya et al., 1993)	*S. exfoliatus* 2419-SVT2	1993	carbazole	
pyrrolostatin (Kato et al., 1993)	*S. chresto-myceticus*	1993	pyrrole	
aestivophoenin (Shinya et al., 1995)	*S. purpeofuscus* 2887-SVS2	1995	phenazine	***Streptomyces* sp. HB122** (Schneemann et al., 2010)
lavanduquinocin (Shinya et al., 1995)	*S. viridо-chromogenes* 2942-SVS3	1995	carbazole	
BE-40644 (Torigoe et al., 1996)	*Actinoplanes* sp. A40644	1995	benzoquinone	
naphthablin (Umezawa et al., 1995)	*Streptomyces* sp. MK15-42F22	1995	naphthoquinone	
benzastatin (Kim et al., 1996)	*S. nitrosporeus* 30643	1996	amino-benzamide or tetrahydro-quinoline	

Table 2.2 Chronological reporting of actinomycete-derived hybrid isoprenoids, Continued.

Compound	Original producing strain	Year	Non-terpene scaffold	Additional producing strains
BU-4664L (diazepinomicin) (Ohkuma and Kobaru, 1996)	*Micromonospora* sp. M990-6	1996	dibenzo-diazepine	*Micromonospora* sp. TP-A0860 (Igarashi et al., 2005), *Micromonospora* sp. 046-ECO11 (Bachmann et al., 2003), ***Micromonospora*** **DPJ12** (Charan et al., 2004), ***Micromonospora*** **sp.** (Fiedler et al., 2005)
CJ-13, 136 (Dekker et al., 1998)	*Pseudonocardia* sp. CL38489	1998	quinolone	
brasilicardin (Komaki et al., 1999)	*Nocardia brasiliensis* (= *N. terpenica*) IFM 0406	1999	sugar, amino acid, hydroxy-benzoate	
cyclomarin (Renner et al., 1999)	***Streptomyces*** **sp. CNB-982**	1999	indole/peptide	***Salinispora arenicola*** **CNS-205** (Schultz et al., 2010)
neomarinonone (Hardt et al., 2000)	***Streptomyces*** **sp. CNH-099**	2000	naphthoquinone	***Streptomyces*** **sp. CNB-632, CNR-927** (unpubl)
endophenazine (Gebhardt et al., 2002)	*S. anulatus* 9663	2002	phenazine	*S. anulatus* 9843, 9958, 10099 (Gebhardt et al., 2002) *S. cinnamonensis* DSM 1042 (Bringmann et al., 2007)
6-dimethyl-allylindole (Sasaki et al., 2002)	*Streptomyces* sp. TP-A0595	2002	indole	***Streptomyces*** **sp. BL-49-58-005** (Sanchez Lopez et al., 2003) *Streptomyces* sp. SN-593 (Takahashi et al., 2010)
phenalinolactone (Durr et al., 2006)	*Streptomyces* sp. Tü6071	2003	sugar, pyrrole, γ-butyrolactone	
fumaquinone (Charan et al., 2005)	*S. fumanus* LL-F42248	2005	naphthoquinone	***Streptomyces*** **sp. Sp080513GE-23** (Khan et al., 2010)
glaciapyrroles (Macherla et al., 2005)	***Streptomyces*** **sp. NPS008187**	2005	pyrrole	
azamerone (Cho et al., 2006)	***Streptomyces*** **sp. CNQ-766**	2006	pyridazine	

Table 2.2 Chronological reporting of actinomycete-derived hybrid isoprenoids, Continued.

Compound	Original producing strain	Year	Non-terpene scaffold	Additional producing strains
oxaloterpin (Motohashi et al., 2007)	*Streptomyces* sp. KO-3988	2007	carbamate or oxalic acid derivatives	
5-dimethyl-allylindole-3-carboxylic acid (Motohashi, Irie, et al., 2008)	***Streptomyces* sp. MS239**	2008	indole	
cyclomarazine (Schultz et al., 2008)	***Salinispora arenicola* CNS-205**	2008	peptide	
JBIR-46 (Izumikawa et al., 2010)	***Streptomyces* SpC080624SC-11**	2010	phenazine	***Streptomyces* sp. SpA080624GE-02** (Khan et al., 2010)
marinoterpin (unpubl.)	***Streptomyces* sp. CNQ-253**	2010	quinolone	
monoterpene phenazine ether (unpubl.)	***Streptomyces sp.* CNQ509**	2010	phenazine	***Streptomyces* sp. CNQ-384** (unpubl)
nitropyrrolin (submitted)	***Streptomyces* sp. CNQ-509**	2010	pyrrole	

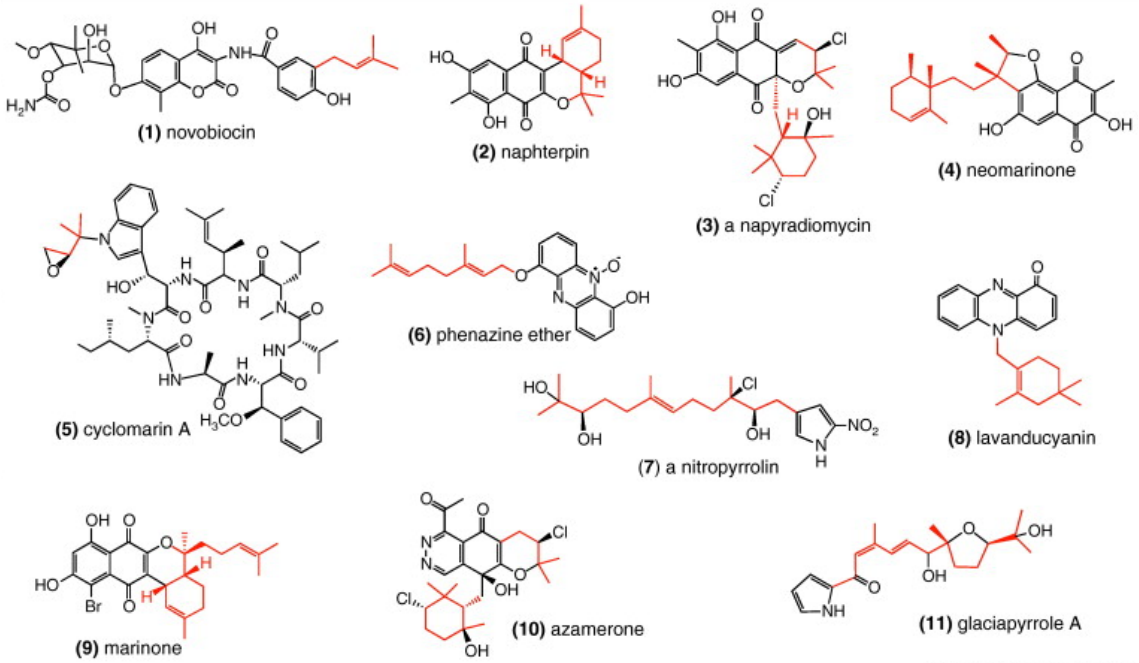


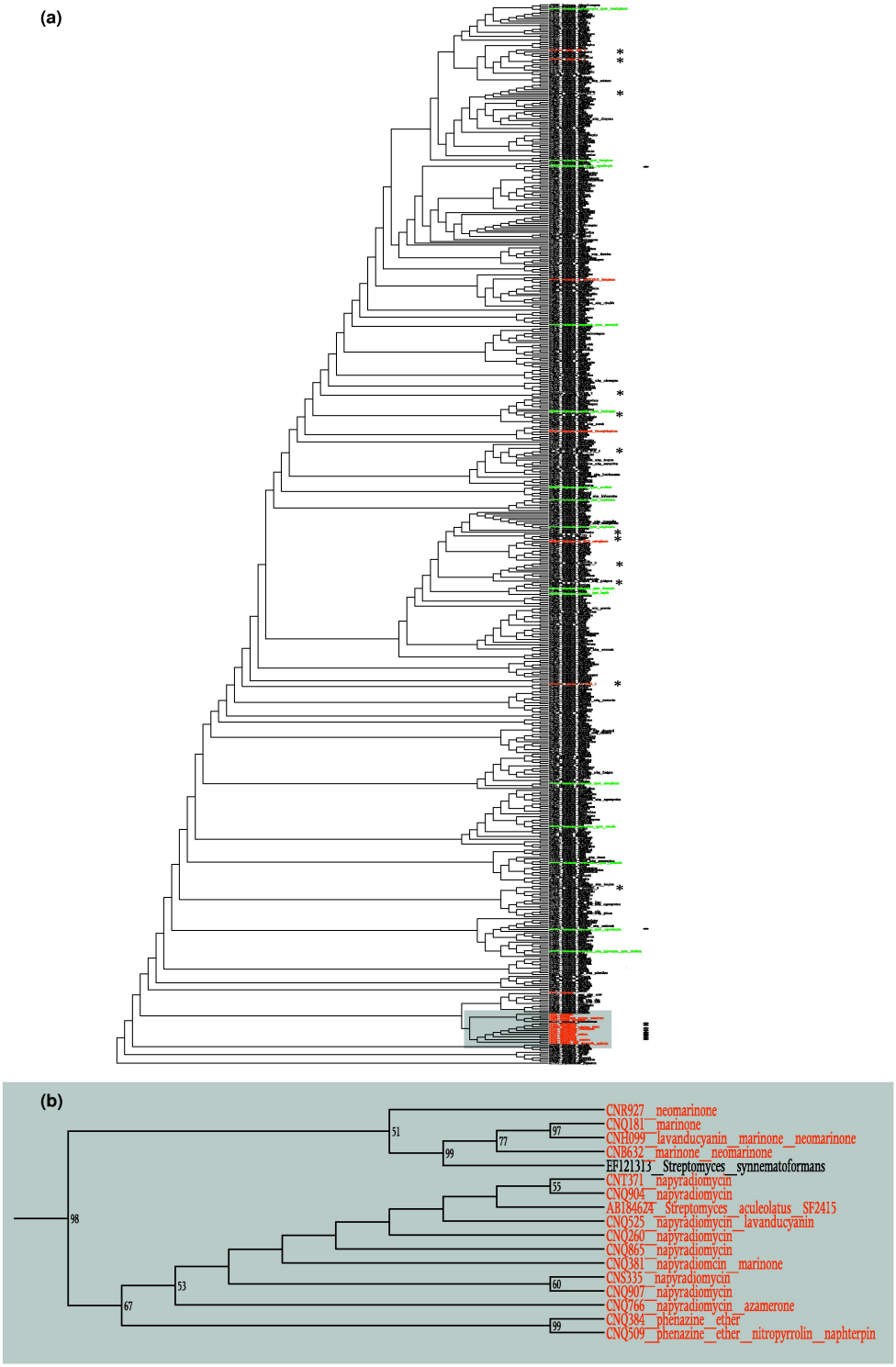
Figure 2.1 Structures of HIs produced by actinomycetes. Isoprenoid portions of compounds are indicated in red.

2.5 Phylogenetic distribution of HI producers

A phylogenetic analysis of HI producing *Streptomyces* species reveals them to be sporadically distributed throughout the genus Figure 2.2. This observation supports previous statements that HI production is rare among actinomycetes (Kawasaki et al., 2003) and is not what would be expected if the associated biosynthetic pathways had been inherited vertically from a common ancestor. The suggestion that HI biosynthetic pathways are exchanged by horizontal gene transfer (HGT) is further supported by the production of compounds in the napyradiomycin class by distantly related streptomycetes (Figure 2.2). Possibly the clearest example of HGT comes from the prenylated depsipeptide cyclomarin A (**5**), which was originally reported from a marine-derived *Streptomyces* sp. (Renner et al., 1999) and was subsequently isolated

from the marine actinomycete *Salinispora arenicola* (Schultz et al., 2008), which resides in a different family

Figure 2.2 16S rRNA gene phylogeny of HI producing streptomycetes. **(A)** Neighbor-joining cladogram constructed in SeaView (Gouy et al., 2010) including all HI producing streptomycetes listed in Tables 2.1 and 2.2 for which sequence data were available (red) or the type strain for the producing species (green). The cladogram also includes all type strains for the genus *Streptomyces* as identified using the List of Prokaryotic names with Standing in Nomenclature (LPSN) database (Euzeby, 1997), and 12 *Streptomyces* full or partial genome sequenced strains for which 16S rRNA sequence data were available (*). All sequence data were aligned using CLUSTALW (Thompson et al., 1994) to the actinomycete 16S sequence alignment available from the Ribosomal Database Project (Cole et al., 2009). Strains that produce napyradiomycins are indicated with a dash (–). **(B)** Detail of the MAR4 clade. Bootstrap values (in percent) calculated from 1000 resamplings are shown at the respective nodes for values >50%.


(a)
(b)
CNR927__neomarinone
CNQ181__marinone
CNH099__lavanducyanin__marinone__neomarinone
CNB632__marinone__neomarinone
EF121313__Streptomyces__synnematoformans
CNT371__napyradiomycin
CNQ904__napyradiomycin
AB184624__Streptomyces__aculeolatus__SF2415
CNQ525__napyradiomycin__lavanducyanin
CNQ260__napyradiomycin
CNQ865__napyradiomycin
CNQ381__napyradiomcin__marinone
CNS335__napyradiomycin
CNQ907__napyradiomycin
CNQ766__napyradiomycin__azamerone
CNQ384__phenazine__ether
CNQ509__phenazine__ether__nitropyrrolin__naphterpin
51
97
77
99
55
98
53
60
67
99

In an interesting example of convergent evolution, actinomycetes use two distinct pathways for the production of isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the five-carbon precursors of the isoprene incorporated into terpenoids (Chappell, 1995; Fischbach et al., 2008). Of these, the non-mevalonate or MEP pathway is commonly observed in bacteria and thought to be the primary source of the isoprene incorporated into primary metabolites (Kuzuyama and Seto, 2003). By contrast, the mevalonate (MVA) pathway is widely distributed in archaea and eukaryotes but is only sporadically found in bacteria. In actinomycetes, the presence of the MVA pathway in addition to the MEP pathway has been linked to the production of HI secondary metabolites (Kuzuyama and Seto, 2003). The genes associated with the MVA pathway often flank those responsible for HI production (Dairi, 2005), and feeding experiments have shown it to be the major source of the IPP incorporated into the streptomycete-derived HIs naphterpin (**2**), napyradiomycin A, BE-40644, furanoquinocin, and terpentecin (Kuzuyama and Seto, 2003). These observations led to the hypothesis that the presence of the MVA pathway is a predictor of HI production in actinomycetes (Dairi, 2005). A screening technique targeting type I HMG CoA reductase (*hmgr*), a key enzyme in the MVA pathway (Kuzuyama and Seto, 2003), was used successfully to capitalize on this concept and led to the isolation of the novel prenylated compounds JBIR-46 (Izumikawa et al., 2010) and 5-dimethylallylindole-3-carboxylic acid (Motohashi, et al., 2008). In another example, the discovery of a second *hmgr* containing gene cluster in the

furaquinocin-producer *Streptomyces* sp. KO-3988 led to the isolation of the novel HI oxaloterpin (Motohashi et al., 2007). However, the isoprene incorporated into actinomycete HI secondary metabolites can also be derived from the MEP pathway (e.g. (Kalaitzis et al., 2003)) and thus it is not clear that screening for *hmgr* is in itself sufficient to identify all strains with the potential to produce HI secondary metabolites. Incongruent phylogenies generated from 16S rRNA and *hmgr* gene sequences provide additional evidence that HGT has played a major role in the evolution of HI biosynthesis (Khan et al., 2010).

To better assess the distribution of the MVA pathway among *Streptomyces* species, we analyzed 25 partial or complete genome sequences. Blast searches targeting *hmgr* yielded only one match in *S. griseoflavus* Tu4000; however terpenoid secondary metabolites have not been reported from this strain. In addition, Blast searches querying nine experimentally characterized PTases against this same set of 25 *Streptomyces* genome sequences yielded only six matches with *e*-values $< 1e^{-10}$ (Table 2.3). All of these matches were annotated as 'hypothetical' or 'conserved hypothetical' proteins. In the case of one of the query sequences (gene SC07190 from the *S. coelicolor* A3 genome), *in vitro* PTase activity has been demonstrated; however the prenylated substrate has not been identified (Kuzuyama et al., 2005). Even with the inclusion in the 16S rRNA tree of the three PTase positive strains for which 16S data are available (Figure 2.2A), the results support the conclusion that the biosynthetic

machinery associated with HI secondary metabolite production is uncommon among *Streptomyces* spp.

Table 2.3 BLAST matches (e-values <1e^{-10}) to actinomycete prenyltransferases found in 25 *Streptomyces* genome sequences (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Prenyltrasferase queries: NapT8 (ABS50489), NapT9 (ABS50490), CloQ (AF329398), NovQ (AAF67510), NphB (BAE00106), Fnq26 (CAL34104), Fur7 (BAF02319), SCO7190 (from *S. coelicolor* A3(2)), and CymD (Sare_4565). *16S sequence data not available.

Gene (accession number)	Genome strain	Highest matching characterized PTase	% identity
EFL38182	*S. griseoflavus* Tu4000*	NapT8	46%
EFD65019	*S. lividans* TK24	SCO7190	99%
SCO7467	*S. coelicolor* A3(2)	IptA	58%
EDY48936	*S. clavuligerus* ATCC27064	CymD	42%
EFL04354	*Streptomyces* sp. AA4*	IptA	36%
EFD64739	*S. lividans* TK24	IptA	58%

Although HI producers are distributed throughout the *Streptomyces* phylogenetic tree, a robust clade enriched in strains that produce this class of compounds is observed (Figure 2.2B). Previous phylogenetic studies identified this clade as a marine lineage within the genus *Streptomyces*, and it was given the designation MAR4 (Fenical and Jensen, 2006). The MAR4 clade currently includes 17 strains, 15 of which were isolated from marine sediments. The only non-marine MAR4 strains reported to date are the type strains of *S. aculeolatus* (Shomura et al., 1987) and *S. synnematoformans* (Hozzein and Goodfellow, 2007), which were isolated from a soil sample and a sand dune, respectively. The 17 members of the MAR4 clade share as little as 96.2% 16S rRNA gene

sequence identity and thus encompass as much phylogenetic diversity as many bacterial genera. The HI secondary metabolites discovered from these strains (Table 2.2) include napyradiomycins (**3**) (Winter et al., 2007), azamerone (**10**) (Cho et al., 2006), an unpublished monoterpene phenazine ether (**6**), nitropyrrolin (submitted) (**7**), naphterpin (**2**), marinone (**9**) (Pathirana et al., 1992), neomarinone (**4**) (Hardt et al., 2000), and lavanducyanin (**8**). In addition, the terrestrially derived *S. aculeolatus* strain produces SF2415 (Shomura et al., 1987), which is structurally similar to napyradiomycin. These compounds are not only structurally diverse but also display antitumor, antibiotic, antioxidant, and other biological activities supporting their value as a resource for pharmaceutical screening.

Interestingly, the compounds produced by the MAR4 strains show some evidence of phylogenetic clustering, with all of the napyradiomycin producers falling into the clade represented by *S. aculeolatus* (Figure 2.2B, eighth strain from the top). Most notable among these strains is CNQ-509, which is able to produce HI compounds incorporating phenazine and pyrrole scaffolds in addition to compounds in the napyradiomycin class. The production of such diverse HIs by one strain is remarkable considering the relative rarity of terpenoid chemistry in actinomycete secondary metabolism. Six additional MAR4 strains produce HIs that incorporate at least two different scaffolds (unpublished data, Table 2.2) revealing that the production of diverse HIs is a common feature of this group. Additional phylogenetic clustering is observed in the *S. synnematoformans*

lineage of the MAR4 clade (upper clade Figure 2.2B, *S. synnematoformans* in black) where all of the marinone producers reside with the exception of strain CNQ-381. These correlations between 16S phylotype and secondary metabolite production provide evidence of vertical inheritance within more recently evolved lineages. The observation that all MAR4 strains, with the exception of *S. synnematoformans*, have demonstrable capacities to produce HI secondary metabolites indicates that the selective cultivation of new MAR4 diversity represents a logical and potentially productive strategy for the isolation of new HI secondary metabolites. No other similarly related cluster of strains within the 16S tree shows a comparable prolificacy for HI production. Because little information is available about the ecological roles of actinomycete HI secondary metabolites, their sporadic occurrence remains a mystery.

2.6 HI producing strains from the marine environment

There has been recent interest in marine actinomycetes as a source of novel natural products (Fiedler et al., 2005; Bull and Stach, 2007; Prieto-Davo et al., 2008). This interest coincides with an increase in the discovery of new HIs as well as the observation that strains from both marine and non-marine sources can produce the same HI secondary metabolites (Tables 2.1 and 2.2). The first HI discovered from a marine-derived actinomycete was marinone (**9**) in 1992 (Pathirana et al., 1992). Subsequently, nine additional HIs have been reported from marine-derived strains (Tables 2.1 and 2.2) including two that were isolated from more than one strain. Likewise, there are 23 HIs that have only been

reported from terrestrial-derived strains and an additional eight that have been reported from both environments. Of the 11 new HIs isolated from marine-derived actinomycetes, six were isolated from MAR4 strains (**3**, **4**, **6**, **7**, **9**, **10**). In addition, recent examples of HI production by marine actinomycetes outside of the MAR4 clade include cyclomarin A (**5**) (Renner et al., 1999), cyclomarazine (Schultz et al., 2008), 5-dimethylallylindole-3-carboxylic acid (Motohashi et al., 2008), glaciapyrroles (**11**) (Macherla et al., 2005), marinoterpin (unpublished), and JBIR-46 (Izumikawa et al., 2010). Actinomycetes isolated from marine invertebrates have also been shown to produce previously reported HIs (Sanchez Lopez et al., 2003; Charan et al., 2004; Schneemann et al., 2010), however the phylogenetic relationships of these strains are unknown because of limited 16S sequence availability. A 2003 study by Sigmund *et al*. (Sigmund et al., 2003) provided the first evidence that marine strains may be enriched in HI secondary metabolite production with the observation that all four *hmgr* positive strains, out of 385 screened, were from marine sources. Similarly, a 2010 study by Khan *et al.* (Khan et al., 2010) identified six actinomycete strains containing the *hmgr* gene out of 523 marine-derived strains screened. The structural novelty of many of the compounds originating from marine strains, coupled with the observation that the MAR4 clade is largely marine-derived, raises the intriguing possibility that the production of these molecules has adaptive significance for actinomycetes in the marine environment. In the case of phenazine-containing HIs, these functions may include acting as electron acceptors during respiration in low oxygen

conditions or reducing iron oxides to improve iron availability (Price-Whelan et al., 2006).

2.7 Conclusions

All available evidence suggest that the biosynthesis of HIs is not a common feature of actinomycete secondary metabolism, with producing strains being largely restricted to but scattered throughout the genus *Streptomyces*. The sporadic distribution of these compounds, coupled with evidence that the associated biosynthetic genes are subject to HGT, suggests that complex processes and undefined selective pressures are driving pathway evolution. One particularly interesting and recent observation about HI secondary metabolites is their apparent enrichment among marine inhabiting strains. The MAR4 group of marine actinomycetes provides a striking example of this phenomenon, and it is intriguing to speculate that HI secondary metabolite production represents an adaptation to life in the marine environment. While experimental work is needed to support such hypotheses, increasing access to genome sequence data will expand our understanding of the taxonomic and environmental distributions of the genes associated with the biosynthesis of this broad class of compounds. Given our present state of knowledge, it is clear that the marine environment represents an excellent source of HI producing actinomycetes and that additional studies of these strains will probably yield new structures that can be screened for useful pharmaceutical properties.

2.8 Acknowledgements

The authors acknowledge Bradley S. Moore for helpful comments on the manuscript. This work was supported by the NOAA California Sea Grant College Program Project R/NMP-100 (Grant NA100AR4170060 to P.R.J.) through NOAA'S National Sea Grant College Program, U.S. Dept. of Commerce. The statements, findings, conclusions and recommendations are those of the author(s) and do not necessarily reflect the views of California Sea Grant or the U.S. Dept. of Commerce.

Chapter 2, in full, is a reprint of the material as it appears in Current Opinion in Biotechnology, 2010. Kelley Gallagher, William Fenical, and Paul Jensen. The dissertation author was the primary author of this review paper.

2.9 References

Bachmann, B.O., McAlpine, J.B., Zazopoulos, E., Farnet, C.M., and Piraee, M. (2003) Farnesyl dibenzodiazepinones, processes for their production and their use as pharmaceuticals. US Patent.

Bohlmann, J. and Keeling, C.I. (2008) Terpenoid biomaterials. *Plant J.* **54**: 656–669.

Botta, B., Vitali, A., Menendez, P., Misiti, D., and Delle Monache, G. (2005) Prenylated flavonoids: Pharmacology and biotechnology. *Curr. Med. Chem.* **12**: 713–739.

Bringmann, G., Haagen, Y., Gulder, T.A.M., Gulder, T., and Heide, L. (2007) Biosynthesis of the isoprenoid moieties of furanonaphthoquinone I and endophenazine A in *Streptomyces cinnamonensis* DSM 1042. *J. Org. Chem.* **72**: 4198–4204.

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

Burlison, J.A., Avila, C., Vielhauer, G., Lubbers, D.J., Holzbeierlein, J., and Blagg, B.S.J. (2008) Development of novobiocin analogues that manifest anti-proliferative activity against several cancer cell lines. *J. Org. Chem.* **73**: 2130–2137.

Chappell, J. (1995) The biochemistry and molecular biology of isoprenoid metabolism. *Plant Physiol.* **107**: 1.

Charan, R.D., Schlingmann, G., Bernan, V.S., Feng, X.D., and Carter, G.T. (2005) Fumaquinone, a new prenylated naphthoquinone from *Streptomyces fumanus*. *J. Antibiot. (Tokyo).* **58**: 271–274.

Charan, R.D., Schlingmann, G., Janso, J., Bernan, V., Feng, X.D., and Carter, G.T. (2004) Diazepinomicin, a new antimicrobial alkaloid from a marine *Micromonospora* sp. *J. Nat. Prod.* **67**: 1431–1433.

Cho, J.Y., Kwon, H.C., Williams, P.G., Jensen, P.R., and Fenical, W. (2006) Azamerone, a terpenoid phthalazinone from a marine-derived bacterium related to the genus *Streptomyces* (actinomycetales). *Org. Lett.* **8**: 2471–2474.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* **37**: D141–D145.

Collins, M.D., Pirouz, T., Goodfellow, M., and Minnikin, D.E. (1977) Distribution of menaquinones in actinomycetes and corynebacteria. *J. Gen. Microbiol.* **100**: 221–230.

Dairi, T. (2005) Studies on biosynthetic genes and enzymes of isoprenoids produced by actinomycetes. *J. Antibiot. (Tokyo).* **58**: 227–243.

Dekker, K.A., Inagaki, T., Gootz, T.D., Huang, L.H., Kojima, Y., Kohlbrenner, W.E., Matsunaga, Y., McGuirk, P.R., Nomura, E., Sakakibara, T., Sakemi, S., Suzuki, Y., Yamauchi, Y., and Kojima, N. (1998) New quinolone compounds from *Pseudonocardia* sp. with selective and potent anti-*Helicobacter pylori* activity: Taxonomy of producing strain, fermentation, isolation, structural elucidation and biological activities. *J. Antibiot. (Tokyo).* **51**: 145–152.

Donnelly, A.C., Mays, J.R., Burlison, J.A., Nelson, J.T., Vielhauer, G., Holzbeierlein, J., and Blagg, B.S.J. (2008) The Design, Synthesis, and Evaluation of Coumarin Ring Derivatives of the Novobiocin Scaffold that Exhibit Antiproliferative Activity. *J. Org. Chem.* **73**: 8901–8920.

Durr, C., Schnell, H.J., Luzhetskyy, A., Murillo, R., Weber, M., Welzel, K., Vente, A., and Bechthold, A. (2006) Biosynthesis of the terpene phenalinolactone in *Streptomyces* sp. Tu6071: Analysis of the gene cluster and generation of derivatives. *Chem. Biol.* **13**: 365–377.

Euzeby, J.P. (1997) List of bacterial names with standing in nomenclature: A folder available on the Internet. *Int. J. Syst. Bacteriol.* **47**: 590–592.

Fenical, W. and Jensen, P.R. (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat. Chem. Biol.* **2**: 666–673.

Fiedler, H.-P., Bruntner, C., Bull, A.T., Ward, A.C., Goodfellow, M., Potterat, O., Puder, C., and Mihm, G. (2005) Marine actinomycetes as a source of novel secondary metabolites. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **87**: 37–42.

Fischbach, M.A., Walsh, C.T., and Clardy, J. (2008) The evolution of gene collectives: How natural selection drives chemical innovation. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 4601–8.

Fukuda, D.S., Mynderse, J.S., Baker, P.J., Berry, D.M., Boeck, L.D., Yao, R.C., Mertz, F.P., Nakatsukasa, W.M., Mabe, J., Ott, J., Counter, F.T., Ensminger, P.W., Allen, N.E., Alborn, W.E., and Hobbs, J.N. (1990) A80915, A new antibiotic complex produced by Streptomyces aculeolatus - Discovery, taxonomy, fermentation, isolation, characterization, and antibacterial evaluation. *J. Antibiot. (Tokyo).* **43**: 623–633.

Gebhardt, K., Schimana, J., Krastel, P., Dettner, K., Rheinheimer, J., Zeeck, A., and Fiedler, H.-P.P. (2002) Endophenazines A similar to D, new phenazine antibiotics from the arthropod associated endosymbiont *Streptomyces anulatus* I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot. (Tokyo).* **55**: 794–800.

Gershenzon, J. and Dudareva, N. (2007) The function of terpene natural products in the natural world. *Nat. Chem. Biol.* **3**: 408–414.

Gouy, M., Guindon, S., and Gascuel, O. (2010) SeaView Version 4: A Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree Building. *Mol. Biol. Evol.* **27**: 221–224.

Hardt, I.H., Jensen, P.R., and Fenical, W. (2000) Neomarinone, and new cytotoxic marinone derivatives, produced by a marine filamentous bacterium (actinomycetales). *Tetrahedron Lett.* **41**: 2073–2076.

Harris, D.A., Reagan, M.A., Ruger, M., Wallick, H., and Woodruff, H.B. (1955) Discovery and antimicrobial properties of cathomycin, a new antibiotic produced by *Streptomyces spheroides* n. sp. *Antibiot. Annu.* **3**: 909–917.

Heide, L. (2009) Prenyl transfer to aromatic substrates: genetics and enzymology. *Curr. Opin. Chem. Biol.* **13**: 171–179.

Henkel, T. and Zeeck, A. (1991) Secondary metabolites by chemical screening 15. Structure and absolute configuration of naphthomevalin, a new dihydro-naphthoquinone antibiotic from *Streptomyces* sp. *J. Antibiot. (Tokyo).* **44**: 665–669.

Hoggarth, J.H., Cushing, K.E., Mitchell, J.I., and Ritchie, D.A. (1994) Induction of resistance to novobiocin in the novobiocin-producing organism *Streptomyces niveus*. *FEMS Microbiol. Lett.* **116**: 131–136.

Hozzein, W.N. and Goodfellow, M. (2007) *Streptomyces synnematoformans* sp nov., a novel actinomycete isolated from a sand dune soil in Egypt. *Int. J. Syst. Evol. Microbiol.* **57**: 2009–2013.

Igarashi, Y., Miyanaga, S., Onaka, H., Takeshita, M., and Furumai, T. (2005) Revision of the structure assigned to the antibiotic BU-4664L from *Micromonopora*. *J. Antibiot. (Tokyo).* **58**: 350–352.

Imai, S., Furihata, K., Hayakawa, Y., Noguchi, T., and Seto, H. (1989) Lavanducyanin, a new antitumor substance produced by *Streptomyces* sp. *J. Antibiot. (Tokyo).* **42**: 1196–1198.

Irie, K., Nakagawa, Y., Tomimatsu, S., and Ohigashi, H. (1998) Biosynthesis of the monoterpenoid moiety of teleocidins via the non-mevalonate pathway in *Streptomyces*. *Tetrahedron Lett.* **39**: 7929–7930.

Izumikawa, M., Khan, S.T., Takagi, M., and Shin-Ya, K. (2010) Sponge-Derived *Streptomyces* Producing Isoprenoids via the Mevalonate Pathway. *J. Nat. Prod.* **73**: 208–212.

Kalaitzis, J.A., Hamano, Y., Nilsen, G., and Moore, B.S. (2003) Biosynthesis and structural revision of neomarinone. *Org. Lett.* **5**: 4449–4452.

Kato, S., Shindo, K., Kawai, H., Odagawa, A., Matsuoka, M., and Mochizuki, J. (1993) Pyrrolostatin, a novel lipid peroxidation inhibitor from *Streptomyces chrestomyceticus* - Taxonomy, fermentation, isolation, structure elucidation and biological properties. *J. Antibiot. (Tokyo).* **46**: 892–899.

Kawasaki, T., Kuzuyama, T., Furihata, K., Itoh, N., Seto, H., and Dairi, T. (2003) A relationship between the mevalonate pathway and isoprenoid production in actinomycetes. *J. Antibiot. (Tokyo).* **56**: 957–966.

Khan, S.T., Izumikawa, M., Motohashi, K., Mukai, A., Takagi, M., and Shin-Ya, K. (2010) Distribution of the 3-hydroxyl-3-methylglutaryl coenzyme A reductase gene and isoprenoid production in marine-derived Actinobacteria. *FEMS Microbiol. Lett.* **304**: 89–96.

Kim, W.G., Kim, J.P., Kim, C.J., Lee, K.H., and Yoo, I.D. (1996) Benzastatins A, B, C, and D: New free radical scavengers from *Streptomyces nitrosporeus* 30643 .1. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *J. Antibiot. (Tokyo).* **49**: 20–25.

Komaki, H., Nemoto, A., Tanaka, Y., Takagi, H., Yazawa, K., Mikami, Y., Shigemori, H., Ando, A., and Nagata, Y. (1999) Brasilicardin A, a new terpenoid antibiotic from pathogenic Nocardia brasiliensis: Fermentation, isolation and biological activity. *J. Antibiot. (Tokyo).* **52**: 13–19.

Kominek, L.A. (1972) Biosynthesis of novobiocin by *Streptomyces niveus*. *Antimicrob. Agents Chemother.* **1**: 123–134.

Komiyama, K., Funayama, S., Anraku, Y., Ishibashi, M., Takahashi, Y., and Omura, S. (1990) Novel antibiotics, furaquinocin A and furaquinocin B - Taxonomy, fermentation, isolation, and physicochemical and biological characteristics. *J. Antibiot. (Tokyo).* **43**: 247–252.

Krugel, H., Krubasik, P., Weber, K., Saluz, H.P., and Sandmann, G. (1999) Functional analysis of genes from Streptomyces griseus involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. *Biochim. Biophys. Acta-Molecular Cell Biol. Lipids* **1439**: 57–64.

Kuzuyama, T., Noel, J.P., and Richard, S.B. (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* **435**: 983–987.

Kuzuyama, T. and Seto, H. (2003) Diversity of the biosynthesis of the isoprene units. *Nat. Prod. Rep.* **20**: 171–183.

Langenheim, J.H. (1994) Higher plant terpenoids - a phytocentric overview of their ecological roles. *J. Chem. Ecol.* **20**: 1223–1280.

Leopold, I.H., Paul, S.D., and Sery, T.W. (1957) Novobiocin, a new antibiotic; ocular penetration and tolerance. *AMA Arch. Ophthalmol.* **57**: 100–109.

Macherla, V.R., Liu, J.N., Bellows, C., Teisan, S., Nicholson, B., Lam, K.S., and Potts, B.C.M. (2005) Glaciapyrroles A, B, and C, pyrrolosesquiterpenes from a *Streptomyces* sp. isolated from an Alaskan marine sediment. *J. Nat. Prod.* **68**: 780–783.

Mancy D, Ninet, L., and Preud Homme, J.: Antibiotic 18. 631 RP. US Patent 1974.

Motohashi, K., Irie, K., Toda, T., Matsuo, Y., Kasai, H., Sue, M., Furihata, K., and Seto, H. (2008) Studies on terpenoids produced by actinomycetes - 5-dimethylallylindole-3-carboxylic acid and A80915G-8 -acid produced by marine-derived *Streptomyces* sp. MS239. *J. Antibiot. (Tokyo).* **61**: 75–80.

Motohashi, K., Sue, M., Furihata, K., Ito, S., and Seto, H. (2008) Terpenoids produced by actinomycetes: Napyradiomycins from *Streptomyces antimycoticus* NT17. *J. Nat. Prod.* **71**: 595–601.

Motohashi, K., Ueno, R., Sue, M., Furihata, K., Matsumoto, T., Dairi, T., Omura, S., and Seto, H. (2007) Studies on terpenoids produced by actinomycetes: Oxaloterpins A, B, C, D, and E, Diterpenes from *Streptomyces* sp. KO-3988. *J. Nat. Prod.* **70**: 1712–1717.

Nakanishi, S., Osawa, K., Saito, Y., Kawamoto, I., Kuroda, K., and Kase, H. (1992) KS-505A, a novel inhibitor of bovine brain Ca2+ and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Streptomyces argenteolus*. *J. Antibiot. (Tokyo).* **45**: 341–347.

Nett, M., Ikeda, H., and Moore, B.S. (2009) Genomic basis for natural product biosynthetic diversity in the actinomycetes. *Nat. Prod. Rep.* **26**: 1362–1384.

Ohkuma, H. and Kobaru, S. (1996) Compound produced by a strain of *Micromonospora*. US Patent.

Orihara, N., Kuzuyama, T., Takahashi, S., Furihata, K., and Seto, H. (1998) Studies on the biosynthesis of terpenoid compounds produced by actinomycetes - 3. Biosynthesis of the isoprenoid side chain of novobiocin via the non-mevalonate pathway in *Streptomyces niveus*. *J. Antibiot. (Tokyo).* **51**: 676–678.

Ourisson, G., Rohmer, M., and Poralla, K. (1987) Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annu. Rev. Microbiol.* **41**: 301–333.

Palaniappan, K. and Holley, R.A. (2010) Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. *Int. J. Food Microbiol.* **140**: 164–168.

Pathirana, C., Jensen, P.R., and Fenical, W. (1992) Marinone and debromomarinone - antibiotic sesquiterpenoid naphthoquinones of a new structure class from a marine bacterium. *Tetrahedron Lett.* **33**: 7663–7666.

Paul, V.J. and Ritson-Williams, R. (2008) Marine chemical ecology. *Nat. Prod. Rep.* **25**: 662–695.

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2006) Rethinking “secondary” metabolism: physiological roles for phenazine antibiotics (vol 2, pg 71, 2006). *Nat. Chem. Biol.* **2**: 221.

Prieto-Davo, A., Fenical, W., and Jensen, P.R. (2008) Comparative actinomycete diversity in marine sediments. *Aquat. Microb. Ecol.* **52**: 1–11.

Renner, M.K., Shen, Y.C., Cheng, X.C., Jensen, P.R., Frankmoelle, W., Kauffman, C.A., Fenical, W., Lobkovsky, E., and Clardy, J. (1999) Cyclomarins A-C, new antiinflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.). *J. Am. Chem. Soc.* **121**: 11273–11276.

Sanchez Lopez, J.M., Martinez Insua, M., Perez Baz, J., Fernandez Puentes, J.L., and Canedo Hernandez, L.M. (2003) New cytotoxic indolic metabolites from a marine *Streptomyces*. *J. Nat. Prod.* **66**: 863–864.

Sasaki, T., Igarashi, Y., Ogawa, M., and Furumai, T. (2002) Identification of 6-prenylindole as an antifungal metabolite of *Streptomyces* sp. TP-A0595 and synthesis and bioactivity of 6-substituted indoles. *J. Antibiot. (Tokyo).* **55**: 1009–1012.

Schneemann, I., Nagel, K., Kajahn, I., Labes, A., Wiese, J., and Imhoff, J.F. (2010) Comprehensive investigation of marine Actinobacteria associated with the sponge *Halichondria panicea*. *Appl. Environ. Microbiol.* **76**: 3702–3714.

Schultz, A.W., Lewis, C.A., Luzung, M.R., Baran, P.S., and Moore, B.S. (2010) Functional Characterization of the Cyclomarin/Cyclomarazine Prenyltransferase CymD Directs the Biosynthesis of Unnatural Cyclic Peptides. *J. Nat. Prod.* **73**: 373–377.

Schultz, A.W., Oh, D.C., Carney, J.R., Williamson, R.T., Udwary, D.W., Jensen, P.R., Gould, S.J., Fenical, W., and Moore, B.S. (2008) Biosynthesis and structures of cyclomarins and cyclomarazines, prenylated cyclic peptides of marine actinobacterial origin. *J. Am. Chem. Soc.* **130**: 4507–4516.

Sedmera, P., Pospisil, S., and Novak, J. (1991) New furanonaphthoquinone from *Streptomyces cinnamonensis*. *J. Nat. Prod.* **54**: 870–872.

Shin-Ya, K., Shimizu, S., Kunigami, T., Furihata, K., and Seto, H. (1995) A new neuronal cell protecting substance, lavanduquinocin, produced by *Streptomyces viridochromogenes*. *J. Antibiot. (Tokyo).* **48**: 574–8.

Shinya, K., Furihata, K., Hayakawa, Y., Seto, H., Kato, Y., and Clardy, J. (1991) The structure of benthocyanin A - A new free radical scavenger of microbial origin. *Tetrahedron Lett.* **32**: 943–946.

Shinya, K., Imai, S., Furihata, K., Hayakawa, Y., Kato, Y., Vanduyne, G.D., Clardy, J., and Seto, H. (1990) Isolation and structural elucidation of an antioxidative agent, naphterpin. *J. Antibiot. (Tokyo).* **43**: 444–447.

Shinya, K., Shimizu, S., Kunigami, T., Furihata, K., Hayakawa, Y., and Seto, H. (1995) Novel neuronal cell protecting substances, aestivophoenin A and aestivophoenin B, produced by *Streptomyces purpeofuscus*. *J. Antibiot. (Tokyo).* **48**: 1378–1381.

Shinya, K., Tanaka, M., Furihata, K., Hayakawa, Y., and Seto, H. (1993) Structure of carquinostatin A, a new neuronal cell protecting substance produced by *Streptomyces exfoliatus*. *Tetrahedron Lett.* **34**: 4943–4944.

Shiomi, K., Iinuma, H., Hamada, M., Naganawa, H., Manabe, M., Matsuki, C., Takeuchi, T., Umezawa, H. (1986) Novel antibiotics napyradiomycins - Production, isolation, physicochemical properties and biological activity. *J. Antibiot. (Tokyo).* **39**: 487–493.

Shomura, T., Gomi, S., Ito, M., Yoshida, J., Tanaka, E., Amano, S., Hiro-Omi, W., Ohuchi, S., Itoh, J., Sezaki, M., Takebe, H., and Uotani, K. (1987) Studies on new antibiotics SF2415 1. Taxonomy, fermentation, isolation, physicochemical properties and biological activities. *J. Antibiot. (Tokyo).* **40**: 732–739.

Sigmund, J.M., Clark, D.C., Rainey, F.A., and Anderson, A.S. (2003) Detection of eubacterial 3-hydroxy-3-methylglutaryl coenzyme a reductases from natural populations of actinomycetes. *Microb. Ecol.* **46**: 106–112.

Smith, C.G., Dietz, A., Sokolski, W.T., and Savage, G.M. (1956) Streptonivicin, a new antibiotic. I. Discovery and biologic studies. *Antibiot and Chemother* **6**: 135–141.

Soria-Mercado, I.E., Prieto-Davo, A., Jensen, P.R., and Fenical, W. (2005) Antibiotic terpenoid chloro-dihydroquinones from a new marine actinomycete. *J. Nat. Prod.* **68**: 904–910.

Steffensky, M., Muhlenweg, A., Wang, Z.X., Li, S.M., and Heide, L. (2000) Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. *Antimicrob. Agents Chemother.* **44**: 1214–1222.

Takahashi, S., Takagi, H., Toyoda, A., Uramoto, M., Nogawa, T., Ueki, M., Sakaki, Y., and Osada, H. (2010) Biochemical Characterization of a Novel Indole Prenyltransferase from *Streptomyces* sp. SN-593. *J. Bacteriol.* **192**:2839-2851.

Takashima, M. and Sakai, H. (1960) A new toxic substance, teleocidin, produced by *Streptomyces*. 1. Production, isolation, and chemical studies. *Bull. Agric. Chem. Soc. Japan* **24**: 647–651.

Tello, M., Kuzuyama, T., Heide, L., Noel, J.P., and Richard, S.B. (2008) The ABBA family of aromatic prenyltransferases: broadening natural product diversity. *Cell. Mol. Life Sci.* **65**: 1459–1463.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL-W - Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.

Torigoe, K., Wakasugi, N., Sakaizumi, N., Ikejima, T., Suzuki, H., Kojiri, K., and Suda, H. (1996) BE-40644, a new human thioredoxin system inhibitor isolated from *Actinoplanes* sp. A40644. *J. Antibiot. (Tokyo).* **49**: 314–317.

Umezawa, K., Masuoka, S., Ohse, T., Naganawa, H., Kondo, S., Ikeda, Y., Kinoshita, N., Hamada, M., Sawa, T., and Takeuchi, T. (1995) Isolation from *Streptomyces* of a novel naphthoquinone compound, naphthablin, that inhibits ABL oncogene functions. *J. Antibiot. (Tokyo).* **48**: 604–607.

Wallhausser, K.H., Nesemann, G., Prave, P., and Steigler, A. (1966) Moenomycin, a new antibiotic. I. Fermentation and isolation. *Antimicrob Agents Chemother.* **1965**: 734–736.

Walsh, T.J., Standiford, H.C., Reboli, A.C., John, J.F., Mulligan, M.E., Ribner, B.S., Montgomerie, J.Z., Goetz, M.B., Mayhall, C.G., and Rimland, D. (1993) Randomized double-blinded trial of rifampin with either novobiocin or trimethoprim-sulfamethoxazole against methicillin-resistant *Staphylococcus aureus* colonization - Prevention of antimicrobial resistance and effect of host factors on outcome. *Antimicrob. Agents Chemother.* **37**: 1334–1342.

Winter, J.M., Moffitt, M.C., Zazopoulos, E., McAlpine, J.B., Dorrestein, P.C., and Moore, B.S. (2007) Molecular basis for chloronium-mediated meroterpene cyclization - Cloning, sequencing, and heterologous expression of the napyradiomycin biosynthetic gene cluster. *J. Biol. Chem.* **282**: 16362–16368.

Zazopoulos, E., Huang, K.X., Staffa, A., Liu, W., Bachmann, B.O., Nonaka, K., Ahlert, J., Thorson, J.S., Shen, B., and Farnet, C.M. (2003) A genomics-guided approach for discovering and expressing cryptic metabolic pathways. *Nat. Biotechnol.* **21**: 187–190.

Zotchev, S.B., Schrempf, H., and Hutchinson, C.R. (1995) Identification of a methyl-specific restriction system mediated by a conjugative element from *Streptomyces bambergiensis*. *J. Bacteriol.* **177**: 4809–4812.

Chapter 3: Phylogenetic and chemical diversity of a hybrid isoprenoid-producing streptomycete lineage

3.1 Abstract

Streptomyces species dedicate a large portion of their genomes to secondary metabolite biosynthesis. A diverse and largely marine-derived lineage within this genus has been designated as 'MAR4' and identified as a prolific source of hybrid isoprenoid (HI) secondary metabolites. These terpenoid-containing compounds are common in nature but rarely observed as bacterial secondary metabolites. To assess the phylogenetic diversity of the MAR4 lineage, complementary culture-based and culture-independent techniques were applied to marine sediment samples collected off the Channel Islands, California. The results, including an analysis of publically available sequence data and strains isolated as part of prior studies, placed 40 new strains in the MAR4 clade, of which 32 originated from marine sources. When combined with sequences cloned from environmental DNA, 28 MAR4 operational taxonomic units (0.01% genetic distance) were identified. Of these, 82% consisted exclusively of either cloned sequences or cultured strains, supporting the complementarity of these two approaches. Chemical analyses of diverse MAR4 strains revealed the production of five different HI structure classes. All 21 MAR4 strains tested produced at least one HI class with most strains producing from two to four classes. The two major clades within the MAR4 lineage displayed distinct patterns in the structural classes and the number and amount of HIs produced suggesting a relationship between taxonomy and secondary metabolite production. The production of HI secondary metabolites appears to be a

phenotypic trait of the MAR4 lineage, which represents an emerging model with which to study the ecology and evolution of HI biosynthesis.

3.2 Introduction

The genus *Streptomyces* is extraordinarily diverse, with nearly 600 formally described species (http://www.ezbiocloud.net). These filamentous, Gram-positive bacteria have been heavily exploited for their ability to produce structurally diverse secondary metabolites, many of which have become useful pharmaceutical agents (Watve et al., 2001; Berdy, 2005). *Streptomyces* spp. are generally saprophytic and are known soil inhabitants, where they play important ecological roles in the breakdown of recalcitrant organic materials (Zimmermann, 1990) and the suppression of plant pathogens (Weller et al., 2002). They are also widely distributed in marine sediments (Moran et al., 1995; Maldonado et al., 2005), form symbiotic relationships with plants (Strobel and Daisy, 2003) and insects (Seipke et al., 2012), and are observed in association with marine sponges (Seipke et al., 2012). Some streptomycete lineages have been observed broadly both in soils and in marine sediments (Prieto-Davo et al., 2008), suggesting a high degree of environmental flexibility.

The ecological functions of most streptomycete secondary metabolites have not been defined, and thus, potential links between secondary metabolite production and environmental adaptation remain largely unknown. One example where such links have been reported is the production of thaxtomins by the phytopathogen *Streptomyces scabies*. These phytotoxins are the causal agent of

potato scab disease, and the transfer of the associated biosynthetic genes among related streptomycetes allows strains to emerge as new pathogens (Lerat et al., 2009). Another example is the production of the antifungal agent candicidin by *Streptomyces* spp. involved in the tripartite mutualism with attine ants and their fungal gardens (Barke et al., 2010). Although streptomycetes have been observed broadly in ocean sediments, we are aware of no evidence linking the production of a specific class of secondary metabolites to the survival of *Streptomyces* spp. in the marine environment.

The MAR4 streptomycete lineage was first recognized as part of a survey of cultured marine actinobacteria (Jensen et al., 2005). Prior to the present study, the *S. aculeolatus* and *S. synnematoformans* type strains were the only nonmarine strains recognized within this clade (Gallagher et al., 2010). These strains were derived from a terrestrial soil sample and a sand dune sample, respectively, and are the only formally described species within the clade.

The MAR4 lineage has been linked to the production of hybrid isoprenoid (HI) secondary metabolites (Gallagher et al., 2010), which are of mixed biosynthetic origin and include an isoprene-derived moiety. The attachment of isoprene, the building block of terpenes, to a secondary metabolite can occur on a variety of biosynthetically distinct chemical scaffolds, including phenazines, polyketides, peptides, aminocoumarins, phenylpropanoids, and alkaloids (Tello et al., 2008). While terpenoids are relatively common plant secondary metabolites (Gershenzon and Dudareva, 2007), they are considered a rare part of

actinomycete secondary metabolism (Izumikawa et al., 2010). Nonetheless, actinomycete-derived HIs, such as the commercially important aminocoumarin antibiotics (Heide, 2009), can display potent biological activities and thus are relevant targets for drug discovery efforts.

The major goals of this study are to expand our understanding of the diversity and distributions of actinomycetes in the MAR4 lineage and to test for correlations between MAR4 phylogeny and HI production. To assess the diversity within this group, complementary culture-based and culture-independent methods were applied to marine sediment samples collected off the Channel Islands, CA. In addition, strains previously identified as MAR4 candidates on the basis of prior research or derived from publically available sequence data were also analyzed.

3.3 Materials and methods

3.3.1 Sample collection

Two hundred thirty-two near-shore sediment samples were collected from five sites (34°02.67′N, 119°32.67′W; 33°59.65′N, 119°33.39′W; 34°03.08′N, 119°34.51′W; 34°01.99′N, 119°42.14′W; 34°03.29′N, 119°49.16′W) surrounding Santa Cruz Island, CA, from 20 to 22 September 2010. Surface sediment samples from water depths of 8 to 12 m were collected in sterile 4-oz Whirl-Pak bags (Nasco, Modesto, CA) by divers. Two cores were collected at one site (34°01.99′N, 119°42.14′W) using a handheld coring device. These were cut into 1- to 3-cm sections immediately after collection and stored frozen. A subset of

the samples from each site was processed for actinomycete cultivation immediately after collection. All remaining samples were stored at –20°C before further processing.

Sixty-eight additional surface sediment samples were collected from 53 sites along the coast between Ventura and Long Beach, CA, and surrounding Santa Cruz Island. These samples were collected at depths from 5 to 833 m using a modified surface-deployed sampler (model 214WA110; Kahlsico, El Cajon, CA) on 4 to 5 December 2010. Samples were stored on ice for at most 3 days before processing and storage at –20°C.

3.3.2 Strain isolation

All samples were processed using previously described methods, including treatments that select for spore-forming actinomycetes (Mincer et al., 2005; Gontang et al., 2007; Freel et al., 2012). The 232 samples collected in September 2010 were processed using up to four methods. These included serial dilution, heat shock, drying, and enrichment culturing (Mincer et al., 2005), followed by plating on agar media. The 68 samples collected in December 2010 were processed by serial dilution and plating. All samples were inoculated onto the following media prepared with 18 g agar: A1 (10 g soluble starch, 4 g yeast extract, 2 g peptone, 1 liter seawater), 50% A1, 20% A1, B1 (2.5 g peptone, 1.5 g yeast extract, 1.5 ml glycerol, 1 liter seawater), Difco marine broth 2215 (Becton, Dickinson and Company, Sparks, MD), and SWA (1 liter seawater, 1 ml trace

mineral solution (Lee et al., 2000)). All plates contained 100 µg/ml cycloheximide to reduce fungal growth.

Serial dilutions (1:50, 1:1,000, and 1:10,000) were made in sterile seawater. Heat shock was performed on samples diluted 1:4 in sterile seawater, followed by heating at 55°C in a water bath for 6 to 9 min. Twenty-five microliters of each dilution was inoculated onto agar medium and spread with a sterile glass rod. For the drying treatment, sediment samples were air dried in a laminar flow hood for at least 24 h and stamped onto agar medium using a sterile foam plug. The enrichment cultures consisted of 1.5 ml of sediment in a 15-ml test tube containing 10 ml of either sterile seawater and 1% chitin or 20% A1 supplemented with either polymyxin B sulfate (5 µg/ml), novobiocin (25 µg/ml), kanamycin (20 µg/ml), or no antibiotic. The tubes were incubated without shaking at room temperature for 100 days. The supernatant was then serially diluted as described above, and 25 µl was plated prior to treatment and following heat shock treatment. The underlying sediment was dried and stamped as described above.

All plates were incubated for up to 90 days at room temperature following inoculation. Actinomycete colonies were identified by colony morphology, texture, and spore formation and transferred with sterile toothpicks onto medium A1. This process was repeated until pure cultures were obtained, as evidenced by uniform morphology. Pure strains were cultured in liquid A1 and stored at –80°C with 10% glycerol added as a cryoprotectant.

3.3.3 DNA extraction, PCR amplification, and sequencing

Genomic DNA was isolated using the DNeasy protocol (Qiagen Inc., Valencia, CA) with previously described modifications (Gontang et al., 2007) and stored at –20°C. The 16S rRNA gene was PCR amplified using primers FC27 and RC1492 (Table 3.1). Each 20-µl PCR mixture contained 20 to 50 ng genomic DNA, 200 µM (each) the forward and reverse primers, 2.5 mM $MgCl_2$, PCR buffer (Applied Biosystems), 2 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 400 µM deoxynucleoside triphosphate mixture, and 7% dimethyl sulfoxide. The PCR protocol consisted of 12.5 min at 94°C and 35 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C, followed by 7 min at 72°C. The PCR products were purified using a Zymo Clean and Concentrator kit (Zymo Research) according to the manufacturer's protocol and sequenced by Sanger sequencing (SeqXcel, Inc., San Diego, CA).

3.3.4 eDNA extraction and PCR

Environmental DNA (eDNA) was extracted and purified from frozen sediment samples according to the PowerSoil DNA isolation protocol (MoBio, Carlsbad, CA), with the bead-beating step extended to 1 h. Purified DNA was stored at –20°C. Two seminested PCR protocols were used to specifically amplify MAR4 16S rRNA genes from eDNA (Tables 3.1, 3.2). The primer sets were designed on the basis of a 16S rRNA alignment of previously identified MAR4 strains and several non-MAR4 *Streptomyces* spp. Primer specificity was confirmed using the Probe Match program (Cole et al., 2005)

(http://rdp.cme.msu.edu/probematch/search.jsp). PCRs were carried out as described above with various annealing temperatures (Table 3.2). Due to the low yields of eDNA, only 5 to 10 ng was added to each 20-µl PCR mixture during the first round of amplification. PCR products from the first round were purified with the Zymo Clean and Concentrator kit (Zymo Research) and immediately used in the second round of amplification with an annealing temperature of 60°C.

Table 3.1 Primers used in this study

Primer name	Forward or Reverse	Specificity	Sequence 5'- 3'
FC27	F	General bacteria	AGAGTTTGATCCTGGCTGGCTCAG
RC1492	R	General bacteria	TACGGCTACCTTGTTACGACTT
F7	F	Actinobacteria	TGCACTCTGGGACAAGCCCT
MAR4_R3	R	MAR4	AGTCTCCCGTGAGTCCCCAC
MAR4_F5	F	MAR4	ACYWYYGGGCGCATGYCTG
MAR4_F6	F	MAR4	WYYGGGCGCATGYCTGDKR

Table 3.2 Primers used for nested PCR amplification of MAR4 16S rRNA gene sequences from environmental samples. + Indicates primers sets that yielded PCR products. In some cases, two separate nested PCR reactions were performed using different second round primers.

Sample no.	1st round FC27/RC1492	1st round F7/MAR4_R3	2nd round MAR4_F5/ MAR4_R3	2nd round MAR4_F6/ MAR4_R3
26	+	-	-	+
153	-	+	-	+
280	-	+	+	+
407	+	-	+	+

3.3.5 Clone libraries

Purified PCR products derived from eDNA were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol with the following modifications. The 6-µl cloning reaction mixtures contained 4 µl PCR product, 1 µl salt solution, and 1 µl TOPO vector. The entire volume was used in the transformation of competent TOP10 cells. Plasmids were purified and sequenced by Sanger sequencing using primer M13 (SeqXcel, San Diego, CA, and Beckman Coulter Genomics, Danvers, MA). Forward and reverse reads were obtained for each clone, yielding approximately 950 bp per clone. Chimeric clones were identified using the Bellerophon program (Huber et al., 2004) and removed from the analysis.

3.3.6 Identification of MAR4 sequences

Phylogenetic analyses included previously identified MAR4 16S rRNA gene sequences (Gallagher et al., 2010), as well as strains cultured as part of the present study and strains cultured as part of prior studies (Jensen et al., 2005; Gontang et al., 2007; Prieto-Davo et al., 2008) that were subsequently considered MAR4 candidates on the basis of sequence data or the production of HI secondary metabolites. Diverse MAR4 sequence representatives (accession numbers KC261629, KC261602, EF121313, KC261627, KC261626, X87316, NR041166, EF538742, KC261604, JF346436, EF581384, KC261611, and KC261615) were additionally used as BLASTn queries of the NCBI database. Redundancies were removed from the top 100 hits, and the remaining

sequences were subjected to phylogenetic analysis along with the cloned sequences generated as part of the present study. 16S rRNA gene sequences were aligned using the MUSCLE program (Edgar, 2004). Maximum likelihood phylogenies were built using raxmlGUI (Silvestro and Michalak, 2011) and the GTR+G substitution model with 100 thorough bootstraps. Maximum parsimony trees were created using PAUP* (Swofford 2003). The tree topology and position of the previously identified MAR4 strains within the least inclusive node were used to identify the MAR4 lineage. The strains within this lineage were clustered into operational taxonomic units (OTUs) on the basis of a 16S rRNA sequence distance of 0.01 using the mothur program (Schloss et al., 2009; Schloss and Westcott, 2011) with no terminal gap penalty and an average neighbor algorithm with precision set at 100.

3.3.7 Secondary metabolite analysis

Twenty-one MAR4 strains, up to five strains from each available OTU, were chosen for secondary metabolite analysis. Nine strains were cultured in duplicate to assess the reproducibility of the analyses. Each strain was cultured in 25 ml medium A1 at 27°C with shaking at 230 rpm for 4 to 7 days, after which 10 ml was inoculated into 1 liter of medium A1 in a 2.8-liter Fernbach flask. Following 3, 5, and 7 days of growth, 45 ml was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was separated and dried under vacuum. On day 9, the remainder of the culture was extracted and dried.

The extracts were analyzed by liquid chromatography (LC)-mass spectrometry (MS) (Hewlett-Packard series 1100) using UV detection, a reversed-phase C_{18} column (4.6 by 100 mm; pore size, 5 μm; Phenomenex Luna), and a solvent gradient from 10% to 100% CH_3CN in water. Low-resolution mass spectra were obtained in the positive mode (electrospray ionization [ESI] voltage, 6.0 kV; capillary temperature, 200°C; auxiliary and sheath gas pressure, 5 units and 70 lb/in^2, respectively). LC traces were generated at 210 and 254 nm, and the UV absorbance spectra associated with each peak were evaluated by comparison to an in-house spectral library (Figure 3.1). Peaks were assigned to a particular compound class if they had a UV matching score of 900 or greater, as reported using the Agilent Technologies (Santa Clara, CA) ChemStation software, and a mass within the range previously reported for compounds in that class. Positive scores ranged from 903 to 999 (mean, 969.2; median, 973.5). Some peaks assigned to the napyradiomycin and marinone classes eluted with 100% CH_3CN and were assigned solely on the basis of highly diagnostic UV spectra. Peaks with absorption spectra characteristic for phenazines but with masses that could not be assigned to compounds in the lavanducyanin class were analyzed by high-resolution LC-tandem MS (MS/MS; 6530 Accurate Mass quadrupole time-of-flight [Q-TOF] system; Agilent Technologies) using the column and conditions described above. High-resolution MS/MS data were obtained in the positive mode (ESI voltage, 4.5 kV; ion source and drying gas temperatures, 350°C; drying gas pressure, 11.0 liters/min; collision energy, 20

V). The total number of peaks associated with each compound class was recorded for each strain. The area under each peak was measured at 254 nm for the napyradiomycin and phenazine classes and 210 nm for the marinone, lavanducyanin, and nitropyrrolin classes. In cases where multiple peaks were detected within one compound class, the area under the peaks (mass absorbance units multiplied by time) was summed and divided by the total number of peaks, resulting in an average area per peak for each compound class.

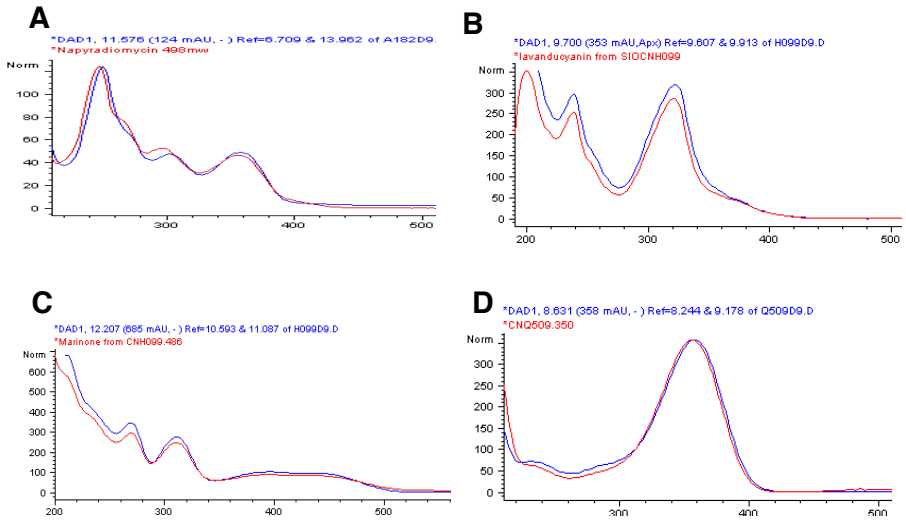


Figure 3.1 UV spectra for compounds in culture extracts (blue) in comparison with known HIs (red). (A) napyradiomycin A2, match score = 983; (B) lavanducyanin, match score = 989; (C) marinone, match score = 996; and (D) nitropyrrolin A, match score = 983. Match scores >900 were considered positive identification for that compound class. Match scores ranged from 903-999 and averaged 969.

3.3.8 Nucleotide sequence accession numbers

The GenBank accession numbers generated as part of this study are KC261602 to KC261629 (cultured strains) and KC477442 to KC477620 (cloned sequences).

3.4 Results

3.4.1 MAR4 diversity

The 16S rRNA gene sequence diversity of the MAR4 clade was evaluated using cultured strains and cloned sequences generated from marine sediment samples collected off the Channel Islands, CA. In addition, strains cultured as part of prior studies and sequence data from public databases were also evaluated. Cultivation efforts applied to 300 sediment samples yielded 155 strains with morphological features typical of the genus *Streptomyces*, including the production of aerial hyphae, tough, leathery colonies, and spores. NCBI BLAST analyses of 16S rRNA gene sequences indicated that 125 of these strains were most closely related to *Streptomyces* spp. A maximum likelihood phylogeny placed five of these strains (CNY-714, CNY-715, CNY-716, CNY-717, and CNY-718) within a consistent and well-supported node that contained the 17 previously identified MAR4 strains (Gallagher et al., 2010) (Figure 3.2). These strains were obtained using a variety of media and processing methods (Table 3.3). Phylogenetic placement within this node was used to assign these and other strains and cloned sequences to the MAR4 lineage.

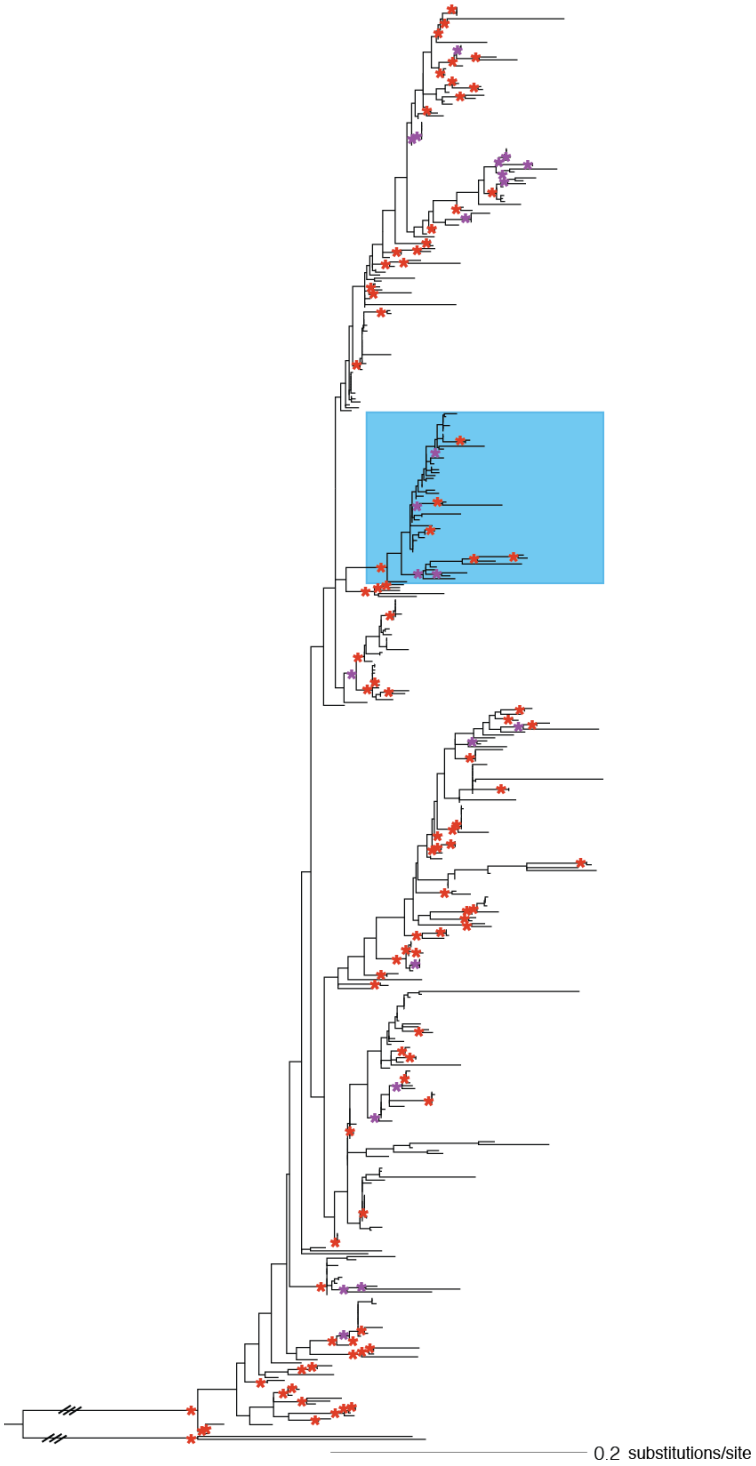


Figure 3.2 16S rRNA gene maximum likelihood phylogeny of MAR4 strains and BLAST matches. The MAR4 clade is highlighted in blue. No conflicting nodes were detected using parsimony methods. Red asterisks indicate nodes supported by ≥50% using both maximum likelihood (bootstrap) and parsimony (jackknife) methods. A purple asterisk indicates nodes supported by only one of these methods.

Table 3.3 MAR4 isolates from Channel Islands sediments.

Strain number	Location	Processing method	Isolation media	NCBI accession number
CNY-714	34° 02.67' N, 119° 32.67' W	dry, stamp	trace mineral + cycloheximide	KC261606
CNY-715	33° 58.39' N, 118° 57.22' W	no pretreatment	sea water agar + cycloheximide	KC261604
CNY-716	34° 02.67' N, 119° 32.67' W	enrichment culture	1/5 A1 + cycloheximide	KC261615
CNY-717	34° 01.99' N, 119° 42.14' W	dry, stamp	trace mineral + cycloheximide	KC261622
CNY-718	33° 58.39' N, 118° 57.22' W	no pretreatment	sea water agar + cycloheximide	KC261611

Culture-independent analyses performed on four of the Channel Island sediment samples using MAR4-specific 16S rRNA primers yielded 196 nonchimeric sequences, of which 179 fell within the MAR4 clade (GenBank accession numbers KC477442 to KC477620). An additional 15 strains, isolated as part of prior studies (Jensen et al., 2005; Gontang et al., 2007; Prieto-Davo et al., 2008) and considered candidates for the MAR4 clade on the basis of preliminary sequence data or secondary metabolite analysis, were also confirmed to be members of this lineage. To further expand the search, 13 diverse MAR4 sequences were used as queries of the NCBI database. The top 100 BLAST matches yielded 465 nonredundant sequences, of which 21 fell within the MAR4 lineage. These consisted of 20 cultured strains and one cloned sequence (Table 3.4). Slightly more that 50% of these were of marine origin.

Table 3.4 Sources of MAR4 strains and sequences identified in this study
[a] This proportion considers only the 38 strains for which an environmental origin was available.
[b] From Jensen et al., 2005; Gontang et al., 2007; and Prieto-Davo et al., 2008

Source	No. of cultured isolates (% marine)	No. of cloned sequences (% marine)
Channel Islands	5 (100)	179 (100)
Prior studies[b]	15 (100)	0
NCBI	20 (55)	1 (0)
Total	40 (79[a])	180 (99)

In total, 801 candidate MAR4 sequences were analyzed as part of this study. Phylogenetic analyses placed 220 of these sequences in the MAR4 clade (Table 3.4). The inclusion of the 17 previously identified MAR4 strains (Gallagher et al., 2010) with the 40 strains identified here raises the total number of cultured strains that can be placed in this lineage to 57. A comprehensive phylogeny of the 57 cultured strains and representative cloned sequences defines the extant diversity of the MAR4 lineage (Figure 3.3), which encompasses 4.1% 16S rRNA sequence divergence within the genus *Streptomyces*. The MAR4 lineage includes the *S. synnematoformans* and *S. aculeolatus* type strains, which are the only named species within the clade. It consistently bifurcates into two distinct yet poorly supported subclades, each of which contains one of the type strains. Only one strain within the MAR4 node (the strain with accession number X87316) does not fall within these two subclades.

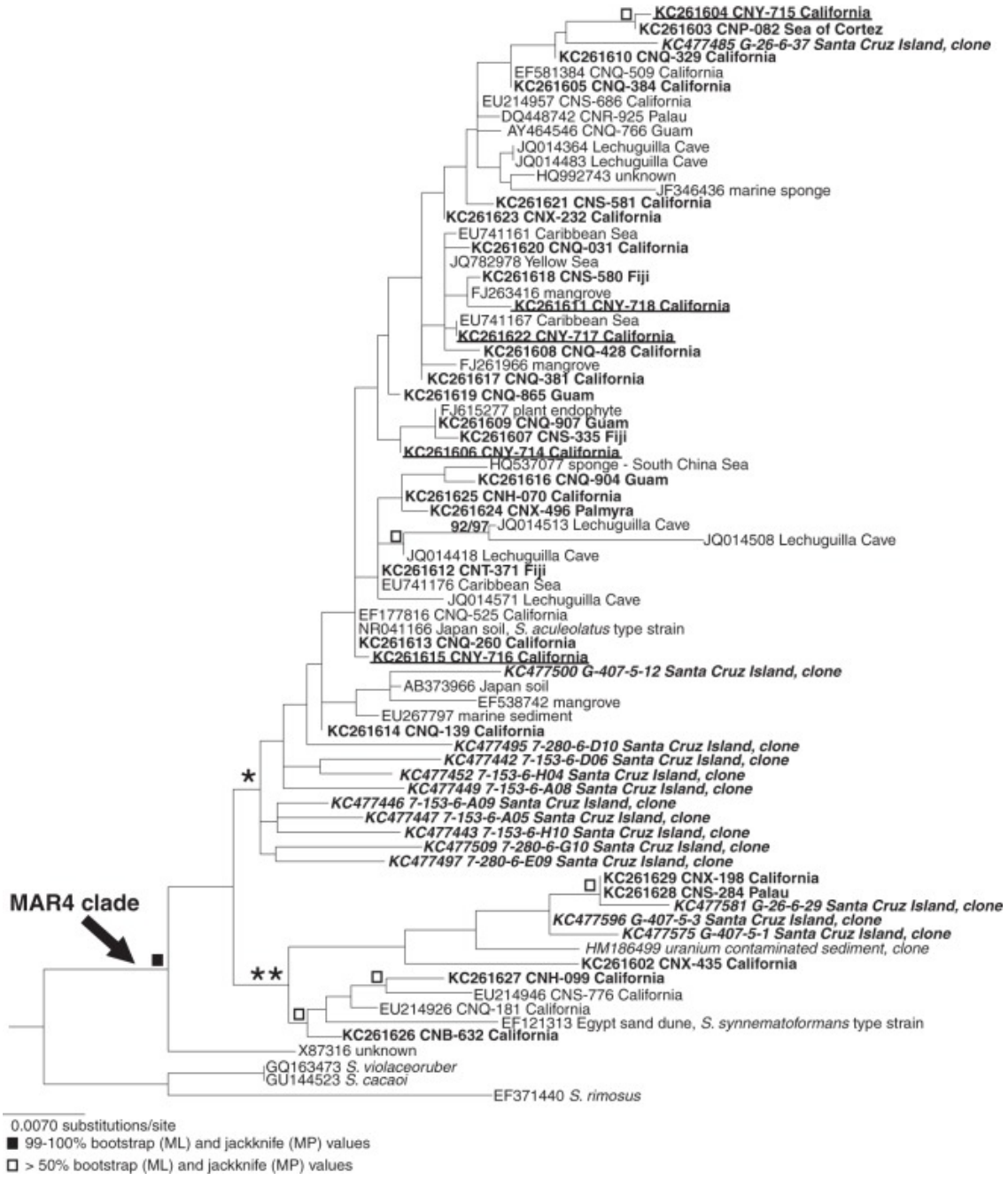


Figure 3.3 16S rRNA gene phylogeny of the MAR4 clade. The maximum likelihood (ML) phylogeny includes all cultured MAR4 strains and a representative from each OTU (Figure 3.4) that contains only cloned sequences. Sequences are identified by NCBI accession number followed by the strain number and source, when available. Sequences added to the NCBI database as a result of this study are indicated in bold. * and ** indicate that the clades represented by the *S. aculeolatus* and *S. synnematoformans* type strains, respectively. Maximum likelihood and maximum parsimony (MP) trees had no conflicting nodes. Bootstrap (maximum likelihood) and jackknife (maximum parsimony) values higher than 50% are indicated.

The MAR4 lineage as currently described is largely of marine origin, with 46 of 57 cultured strains and 179 of 180 cloned sequences originating from the marine environment (Table 3.5). The 12 sequences that are not linked to the marine environment include 1 sequence cloned from a uranium-contaminated subsurface sediment (GenBank accession number HM186499), three strains isolated from terrestrial sources (accession numbers NR041166, EF121313, and AB373966), six strains isolated from Lechuguilla Cave, NM (accession numbers JQ014364, JQ014418, JQ014483, JQ014508, JQ014513, and JQ014571), and two sequence deposits that include no environmental source information (accession numbers X87316 and HQ992743).

Table 3.5 Cultured and cloned MAR4 sequences. Bold locations indicate that strains are from marine samples. Strains numbers beginning with "CN" are maintained in the SIO culture collection. *All cloned sequences are from Santa Cruz Island, except where indicated. ** Strains included in secondary metabolite analysis.

OTU no.	No. clones*	Cultured strains	Source of cultured strains	Reference
1	0	CNX-435 (KC261602)**	**California**	
2	2	0		
3	0	CNS-776 (EU214946)**	**California**	(Prieto-Davo et al., 2008)
4	0	CNY-715 (KC261604)**	**California**	
		CNP-082 (KC261603)**	**California**	
5	HM186499 (Washington)	0		(Lin et al., 2012)
6	1	0		
7	3	CNB-632 (KC261626)**	**California**	
8	0	(EF121313)	Egypt sand dune	(Hozzein et al. 2007)
9	2	CNH-099 (KC261627)**	**California**	
		CNQ-181 (EU214926)**	**California**	(Prieto-Davo et al., 2008)

Table 3.5 Cultured and cloned MAR4 sequences, Continued.

OTU no.	No. clones*	Cultured strains	Source of cultured strains	Reference
10	7	0		
11	1	0		
12	8	(JQ014483)	Lechuguilla Cave	
		(JQ014364)	Lechuguilla Cave	
		CNQ-329	**California**	
		(KC261610)		
		CNS-581	**California**	
		(KC261621)**		
		CNQ-509	**California**	(Prieto-Davo et al., 2008)
		(EF581384)**		
		CNS-686	**California**	(Prieto-Davo et al., 2008)
		(EU214957)**		
		CNQ-384	**California**	
		(KC261605)		
		CNR-925	**Palau**	(Gontang et al., 2007)
		(DQ448742)		
		CNQ-766	**Guam**	(Jensen and Fenical, 2005)
		(AY464546)**		
13	105	(NR_041166)	Japan soil	(Labeda 2012)
		CNQ-260	**California**	
		(KC261613)		
		CNQ-525	**California**	(Soria-Mercado et al., 2005)
		(EF177816)**		
		CNY-716	**California**	
		(KC261615)**		
		CNQ-139	**California**	
		(KC261614)**		
14	0	(JQ014513)	Lechuguilla Cave	
		(JQ014571)	Lechuguilla Cave	
		CNH-070	**California**	
		(KC261625)		
		CNX-496	**Palmyra**	
		(KC261624)		
		(EU741176)	**Caribbean beach sand**	(Solano et al., 2009)
		(JQ014418)	Lechuguilla Cave	
		CNT-371	**Fiji**	
		(KC261612)		
		CNQ-904	**Guam**	
		(KC261616)		
		(HQ537077)	**sponge, South China Sea**	(Wang et al., 2011)
		(HQ992743)	unknown	
		(JQ782978)	**Yellow Sea**	
		(JF346436)	**marine sponge**	
		CNY-717	**California**	
		(KC261622)**		
		CNQ-031	**California**	
		(KC261620)		

Table 3.5 Cultured and cloned MAR4 sequences, Continued.

OTU no.	No. clones*	Cultured strains	Source of cultured strains	Reference
14, Cont.		(EU741167)	**Caribbean beach sand**	(Solano et al., 2009)
		(EU741161)	**Caribbean beach sand**	(Solano et al., 2009)
		(FJ263416)	**mangrove**	
		CNS-580 (KC261618)	**Fiji**	
		CNY-718 (KC261611)**	**California**	
		CNQ-428 (KC261608)	**California**	
		(FJ261966)	**mangrove**	
		CNQ-865 (KC261619)**	**Guam**	
		CNX-232 (KC261623)	**California**	
		CNQ-381 (KC261617)	**California**	
		(FJ615277)	plant endophyte	(Qin et al., 2009)
		CNQ-907 (KC261609)	**Guam**	
		CNS-335 (KC261607)**	**Fiji**	
		CNY-714 (KC261606)**	**California**	
15	0	(EF538742)	**mangrove**	
		(EU267797)	**Xiaoping Islands**	(Zhao et al., 2009)
		(AB373966)	Japan soil	
16	1	0		
17	37	CNX-198 (KC261629)**	**California**	
		CNS-284 (KC261628)**	**Palau**	
18	3	0		
19	0	(X87316)	unknown	
20	0	(JQ014508)	Lechuguilla Cave	
21	1	0		
22	1	0		
23	1	0		
24	1	0		
25	1	0		
26	1	0		
27	1	0		
28	2	0		

3.4.2 Cultured and culture-independent MAR4 diversity

The 16S rRNA sequences within the MAR4 clade were clustered at a genetic distance of 0.01 (Hughes et al., 2002), a value commonly used to delineate actinomycete taxonomic units (Stach and Maldonado, 2003; Prieto-Davó et al., 2013). This resulted in 28 OTUs (Figure 3.4), of which 14 were comprised entirely of cloned sequences, 8 were comprised entirely of cultured sequences, and 6 were comprised of both cultured and cloned sequences. Thus, most OTUs (82%) were comprised entirely of either cultured strains or cloned sequences, with little overlap between the two. OTUs 13 and 17 contained 105 and 37 clones, respectively, which accounted for 71% of the cloned sequences. Each of these OTUs also contained 3 to 5 cultured strains. Approximately one-half of the cultured strains fell within OTU 14, which did not include any cloned sequences. Overall, 88% of the MAR4 strains and cloned sequences were assigned to 3 of the 28 OTUs. The five MAR4 strains cultured as part of this study all fell within OTUs containing previously isolated strains, while the 179 cloned sequences fell within 20 OTUs, of which 14 were composed entirely of Channel Islands clone sequences.

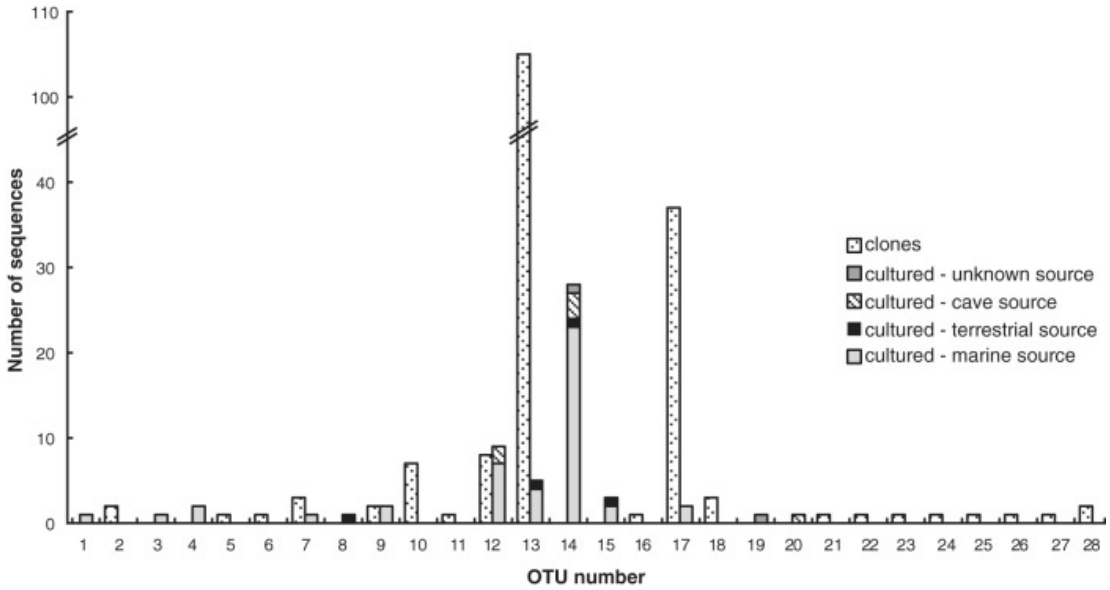


Figure 3.4 Distribution of MAR4 sequences among OTUs with an average neighbor clustering distance of 0.01.

A rarefaction curve generated from the combined MAR4 OTU data reveals a steep slope, indicating that considerable diversity has yet to be sampled (Figure 3.5) (Hughes et al., 2002). This result is supported by the Chao1 and abundance coverage estimator (ACE) richness estimators, which predict that continued sequencing will yield from 58 to 86 OTUs. Individual rarefaction curves of cultured strains and cloned sequences similarly revealed that more sampling will yield additional MAR4 diversity.

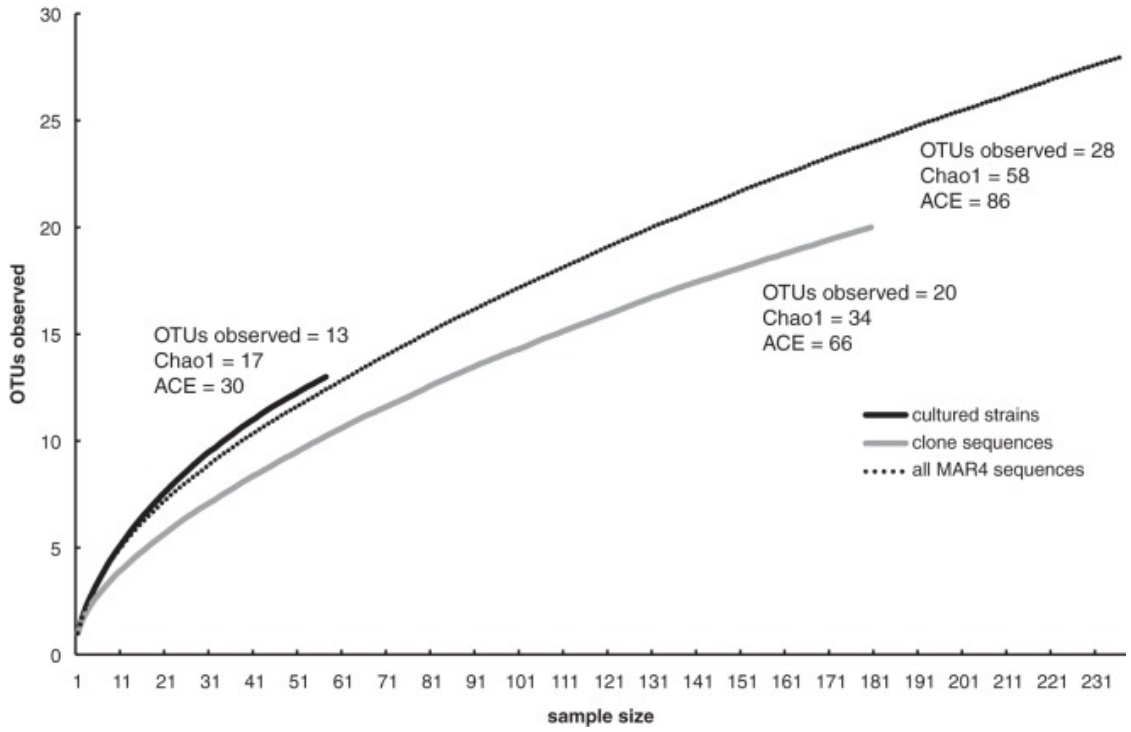


Figure 3.5 Rarefaction curve for MAR4 cultured, cloned, and combined sequences. Chao1 and ACE richness estimates are indicated.

3.4.3 HI production by MAR4 strains

A phenotypic trait previously associated with the MAR4 clade is the production of HI secondary metabolites (Gallagher et al., 2010). To further explore the relationships between HI production and MAR4 phylogeny, we analyzed the culture extracts of 21 diverse MAR4 strains over a 9-day cultivation period. These strains belonged to 9 OTUs that were broadly distributed throughout the MAR4 clade and included representatives from both the *S. synnematoformans* and *S. aculeolatus* clades (Table 3.5). Based on UV absorbance and mass spectral data, we observed the production of compounds in four previously reported HI classes. These classes were the napyradiomycins (Shiomi et al., 1986), the marinones (Pathirana et al., 1992; Hardt et al., 2000),

the lavanducyanins (Imai et al., 1989), and the nitropyrrolins (Kwon et al., 2010) (Figure 3.6). In addition, a compound with a UV spectrum that was diagnostic for phenazines but that did not match any of these four classes was also detected. Further investigation of this compound by high-resolution MS/MS revealed a parent ion, $[M + H]^+$, with an exact mass of 377.2236 and a fragmentation pattern consistent with phenazine-1-carboxylic acid (Watson et al., 1988; Moree et al., 2012) with methyl and monoterpene substituents (Figure 3.7). These results provide preliminary evidence that the unknown compound is a prenylated phenazine, which we have tentatively assigned to a fifth HI class while further structural characterization is ongoing.

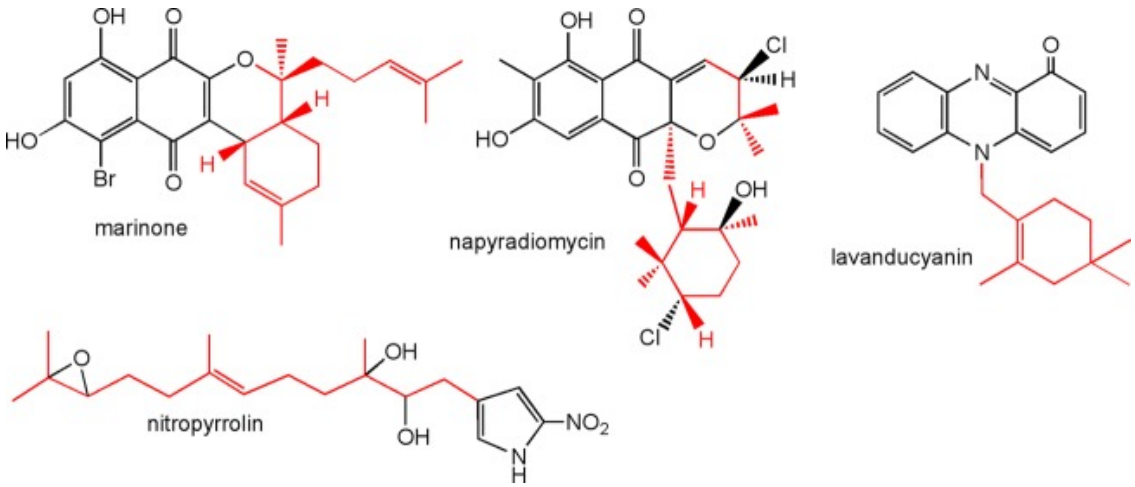


Figure 3.6 Previously reported hybrid isoprenoid structure classes detected from MAR4 strains. The terpene-derived portion of each molecule is highlighted in red.

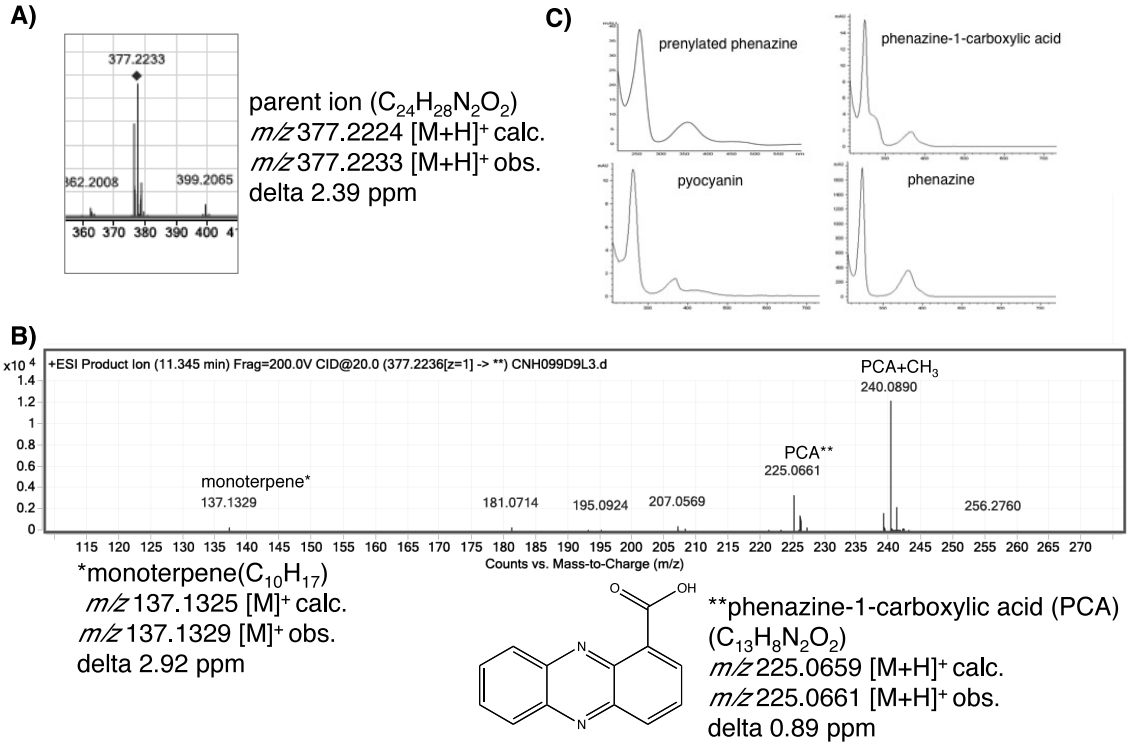


Figure 3.7 Identification of prenylated phenazine. (A) Parent ion. (B) MS2 fragmentation of parent ion. Fragments coresponding to a monoterpene*, phenazine-1-carboxylic acid (PCA**), and PCA with a methyl substituent are observed. (C) UV spectra for the prenylated phenazine observed in this study and related compounds (Moree et al., 2012).

All 21 of the MAR4 strains investigated produced at least one HI and, in most cases, produced more than one (Figure 3.8). In many cases, multiple derivatives within the same structural class were observed from a single strain. The *S. aculeolatus* and *S. synnematoformans* lineages showed differences in the numbers and types of HI secondary metabolites produced, with the former including the only four strains observed to produce nitropyrrolins. In addition, the majority of the strains tested in the *S. aculeolatus* clade (10 of 14) produced napyradiomycins, whereas only 1 strain in the *S. synnematoformans* clade produced a napyradiomycin. Eight of the *S. aculeolatus* strains also produced

more than one napyradiomycin derivative; in comparison, the *S. synnematoformans* strain produced only one. The opposite pattern was observed with the marinones, with all of the *S. synnematoformans* strains producing multiple derivatives but the *S. aculeolatus* strains producing only one. Interestingly, no strain produced multiple compounds in both the napyradiomycin and marinone classes.

In cases where strains produced multiple derivatives within a compound class, they generally produced more of each derivative than strains that produced only a single compound. This is best observed with napyradiomycin production, in which case the *S. aculeolatus* clade produced more peaks per strain (Figure 3.8) and the average area under each peak was 25.3 times larger than that in the *S. synnematoformans* clade (Table 3.6). Similarly, the average area under each marinone peak in the *S. synnematoformans* clade, within which all strains produced multiple derivatives, was 4.3 times larger than that in the *S. aculeolatus* clade, where only one derivative was produced. The relative amounts and number of derivatives of the lavanducyanins and the uncharacterized prenylated phenazine produced by both clades were similar; however, both compound classes were observed more consistently from members of the *S. synnematoformans* clade. No major differences were observed between duplicate extracts except for strain CNQ-181, where a lavanducyanin peak was observed in only one of the replicates (data not shown).

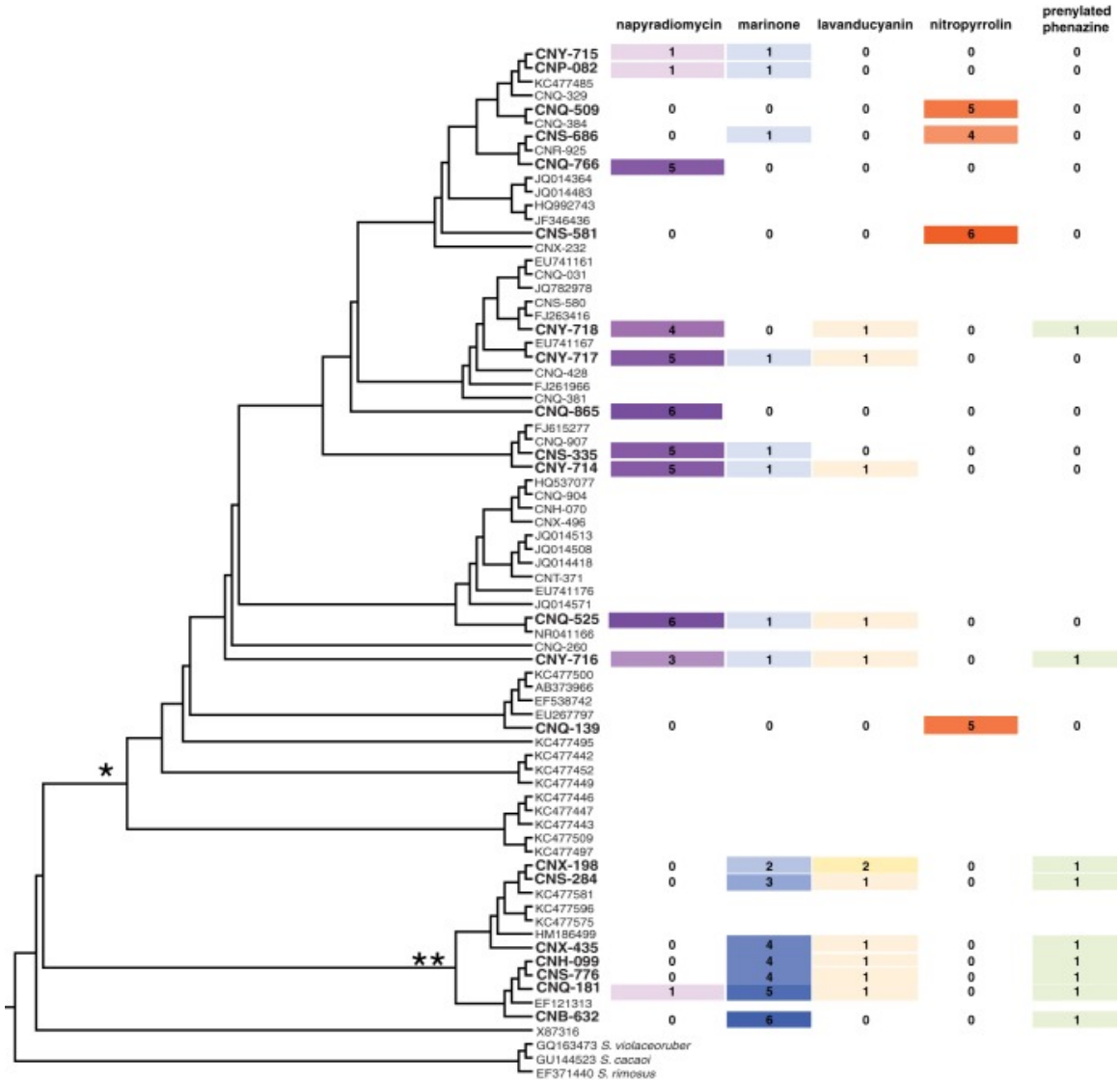


Figure 3.8 Lineage-specific HI production by MAR4 strains. The 16S rRNA gene phylogeny shown in Figure 3.3 is displayed as a cladogram. Strains analyzed for HI production are in bold. For each strain analyzed, the number of compounds detected within each class is indicated by number and color intensity according to the number detected. * and ** indicate the clades represented by the *S. aculeolatus* and *S. synnematoformans* type strains, respectively.

Table 3.6 Relative compound production for members of the *S. aculeolatus* and *S. synnematoformans* clades. Values represent the average area under the peaks associated with each compound class ± standard error of the mean. ND, not detected.

Compound	Avg area under the peak (no. of peaks) for:		Ratio
	***S. aculeolatus* clade**	***S. synnematoformans* clade**	
Napyradiomycin	1,451 ± 312 (41)	57 (1)	25.6:1
Marinone	380 ± 234 (8)	1,638 ± 470 (28)	1:4.3
Lavanducyanin	2,216 ± 1,120 (5)	1,347 ± 474 (7)	1.6:1
Nitropyrrolin	447 ± 102 (20)	ND	
Prenylated phenazine	661 ± 364 (2)	878 ± 364 (7)	1:1.3

3.5 Discussion

The MAR4 lineage of streptomycetes has been reported to be largely of marine origin and recognized as a source of the rare HI class of bacterial secondary metabolites (Fenical and Jensen, 2006; Gallagher et al., 2010). The aim of the present study was to further explore the diversity and distribution of this lineage through the application of culture-dependent and culture-independent methods and through the mining of publically available sequence data. Secondary aims were to characterize the numbers, types, and relative amounts of HI secondary metabolites produced by these bacteria and to search for correlations between MAR4 phylogeny and HI production.

The MAR4 lineage is defined on the basis of a well-supported node observed in the 16S rRNA gene phylogeny (Figure 3.3). The present study expands the number of cultured strains within this lineage from 17 to 57. Seventy-nine percent of these strains, including strains reported from eight independent research groups and strains from a range of sample types (Table 3.5), originated from marine sources, suggesting that the lineage may

preferentially occur in marine habitats. At the level of resolution obtained in this study, there is no evidence for specific marine or terrestrial lineages within the MAR4 clade, suggesting that strains are broadly distributed across these environments. Interestingly, the six strains isolated from Lechuguilla Cave, NM, do not cluster together, suggesting that the cave system has been colonized multiple times. However, a more highly resolved phylogeny generated using less conserved loci will be needed before conclusions can be drawn about intraclade relationships and biogeographic patterns within the MAR4 lineage.

Of the 125 *Streptomyces* spp. cultured as part of this study, only 5 fell within the MAR4 clade, suggesting that these bacteria are rare members of the streptomycete community. The 20 additional MAR4 strains identified on the basis of NCBI sequence deposits further support the rarity of this group, given the nearly 30,000 NCBI sequence deposits labeled "*Streptomyces* 16S." While it remains possible that the selective cultivation methods typically employed for *Streptomyces* spp. are not particularly effective for this group, only one MAR4 clone was detected among the 589 environmental *Streptomyces* 16S sequence deposits in the NCBI database. Despite this apparent rarity, the results of a culture-independent analysis performed on a subset of the Channel Island sediment samples revealed that considerable MAR4 OTU diversity has yet to be obtained in culture. Surprisingly, 82% of the OTUs were comprised entirely of either cloned or cultured sequences. These included several OTUs from the Channel Island sites with which both techniques were performed yet only

cultured OTUs were observed. Similar observations have been made for soil samples (Shade et al., 2012) and could reflect the presence of readily cultured members of the rare biosphere that remain undetected in clone libraries due to low abundance. These observations support the application of both culture and culture-independent methods in the assessment of MAR4 diversity.

The results presented here further establish the production of HI secondary metabolites as a phenotypic trait of the MAR4 lineage, with all 21 of the strains tested producing HI secondary metabolites. These compounds could be delineated into five structural classes, including a prenylated phenazine that could not be assigned to a known chemical class. Surprisingly, many strains produced multiple HI classes, suggesting that they possess more than one pathway associated with HI biosynthesis.

Despite the lack of statistical support for the intraclade relationships in the MAR4 phylogeny, distinct patterns were observed in the production of HIs by the two clades defined by the *S. aculeolatus* and the *S. synnematoformans* type strains. These patterns suggest that an investment in the production of either the napyradiomycin or marinone class appears to be a phenotypic trait that differentiates the two clades. The relationship between napyradiomycin production and the *S. aculeolatus* clade is further supported by an independent study of the MAR4 strains CNH-070 and CNQ-329, which yielded six novel and three known napyradiomycin derivatives (Cheng et al., 2013). In addition, the *S. synnematoformans* clade preferentially produces the prenylated phenazine, while

the only strains observed to produce the nitropyrrolin class were in the *S. aculeolatus* clade. Prenylated pyrroles are rare in nature; to the best of our knowledge, the only other streptomycete-produced compounds of this type are the glaciapyrroles isolated from a marine sediment-derived *Streptomyces* sp. (Macherla et al., 2005) and a pyrrolostatin produced by a Brazilian soil streptomycete (Kato et al., 1993). Future comparative genomic studies will provide better resolution of the lineage specificity of the biosynthetic pathways associated with these compound classes as well as the full HI biosynthetic potential of MAR4 strains.

The enrichment of HI secondary metabolites in the MAR4 lineage provides evidence that positive selection has favored their production. While the natural functions of MAR4 HIs have yet to be defined, it will be interesting to evaluate nonmarine MAR4 strains to determine if this enrichment represents a marine adaptation. Ultimately, identifying the ecological functions of HI secondary metabolites and how they may enhance survival in specific habitats remain important goals for future studies of MAR4 bacteria.

3.6 Acknowledgements

Chambers Hughes and William Fenical are gratefully acknowledged for guidance on analytical chemistry. This work was supported by NOAA California Sea Grant College Program project R/NMP-100 (grant NA100AR4170060 to Paul Jensen) through NOAA's National Sea Grant College Program, U.S. Department of Commerce. Paul Jensen acknowledges NIH for support to acquire high-

resolution LC-MS instrumentation under grant S10 OD010640-01. Kelley Gallagher acknowledges support from an NIH Training Grant in Marine Biotechnology (GM067550).

Chapter 3, in full, is a reprint of the material as it appears in Applied and Environmental Microbiology, 2013. Kelley Gallagher, Kristin Rauscher, Laura Pavan Ioca, and Paul Jensen. The dissertation author was the primary investigator and author of this paper.

3.7 References

Barke, J., Seipke, R.F., Grueschow, S., Heavens, D., Drou, N., Bibb, M.J., Goss, R.J.M., Yu, D.W., and Hutchings, M.I. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus. Bmc Biol.* **8**: 109.

Berdy, J. (2005) Bioactive microbial metabolites - A personal view. *J. Antibiot. (Tokyo).* **58**: 1–26.

Cheng, Y.-B., Jensen, P.R., and Fenical, W. (2013) Cytotoxic and Antimicrobial Napyradiomycins from Two Marine-Derived, MAR4 *Streptomyces* Strains. *European J. Org. Chem.* **2013**: 3751-3757.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., and Tiedje, J.M. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* **33**: D294-D296.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Fenical, W. and Jensen, P.R. (2006) Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat. Chem. Biol.* **2**: 666–673.

Freel, K.C., Edlund, A., and Jensen, P.R. (2012) Microdiversity and evidence for high dispersal rates in the marine actinomycete "*Salinispora pacifica*." *Environ. Microbiol.* **14**: 480-493.

Gallagher, K.A., Fenical, W., and Jensen, P.R. (2010) Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes. *Curr. Opin. Biotechnol.* **21**: 794–800.

Gershenzon, J. and Dudareva, N. (2007) The function of terpene natural products in the natural world. *Nat. Chem. Biol.* **3**: 408–414.

Gontang, E.A., Fenical, W., and Jensen, P.R. (2007) Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. *Appl. Environ. Microbiol.* **73**: 3272–3282.

Hardt, I.H., Jensen, P.R., and Fenical, W. (2000) Neomarinone, and new cytotoxic marinone derivatives, produced by a marine filamentous bacterium (actinomycetales). *Tetrahedron Lett.* **41**: 2073–2076.

Heide, L. (2009) Genetic engineering of antibiotic biosynthesis for the generation of new aminocoumarins. *Biotechnol. Adv.* **27**: 1006–14.

Hozzein, W.N. and Goodfellow, M. (2007) *Streptomyces synnematoformans* sp nov., a novel actinomycete isolated from a sand dune soil in Egypt. *Int. J. Syst. Evol. Microbiol.* **57**: 2009–2013.

Huber, T., Faulkner, G., and Hugenholtz, P. (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.

Hughes, J.B., Hellmann, J.J., Ricketts, T.H., and Bohannan, B.J.M. (2002) Counting the uncountable: Statistical approaches to estimating microbial diversity (vol 67, pg 4399, 2001). *Appl. Environ. Microbiol.* **68**: 448.

Imai, S., Furihata, K., Hayakawa, Y., Noguchi, T., and Seto, H. (1989) Lavanducyanin, a new antitumor substance produced by *Streptomyces* sp. *J. Antibiot. (Tokyo).* **42**: 1196–1198.

Izumikawa, M., Khan, S.T., Takagi, M., and Shin-Ya, K. (2010) Sponge-Derived Streptomyces Producing Isoprenoids via the Mevalonate Pathway. *J. Nat. Prod.* **73**: 208–212.

Jensen, P.R. and Fenical, W. (2005) New natural-product diversity from marine actinomycetes. *Nat. Prod. Drug Discov. Ther. Med.* 315–328.

Jensen, P.R., Gontang, E., Mafnas, C., Mincer, T.J., and Fenical, W. (2005) Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. *Environ. Microbiol.* **7**: 1039–48.

Kato, S., Shindo, K., Kawai, H., Odagawa, A., Matsuoka, M., and Mochizuki, J. (1993) Pyrrolostatin, a novel lipid peroxidation inhibitor from *Streptomyces chrestomyceticus* - Taxonomy, fermentation, isolation, structure elucidation and biological properties. *J. Antibiot. (Tokyo).* **46**: 892–899.

Kwon, H.C., Espindola, A.P.D.M., Park, J.-S., Prieto-Davo, A., Rose, M., Jensen, P.R., and Fenical, W. (2010) Nitropyrrolins A-E, Cytotoxic Farnesyl-alpha-nitropyrroles from a Marine-Derived Bacterium within the Actinomycete Family Streptomycetaceae. *J. Nat. Prod.* **73**: 2047–2052.

Labeda, D.P., Goodfellow, M., Brown, R., Ward, A.C., 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, K.I., and Hatano, K. (2012) Phylogenetic study of the species within the family Streptomycetaceae. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **101**: 73–104.

Lee, S.D., Kang, S.O., and Hah, Y.C. (2000) *Catellatospora koreensis* sp nov., a novel actinomycete isolated from a gold-mine cave. *Int. J. Syst. Evol. Microbiol.* **50**: 1103-1111.

Lerat, S., Simao-Beaunoir, A.-M., and Beaulieu, C. (2009) Genetic and physiological determinants of *Streptomyces scabies* pathogenicity. *Mol. Plant Pathol.* **10**: 579–585.

Lin, X., Kennedy, D., Fredrickson, J., Bjornstad, B., and Konopka, A. (2012) Vertical stratification of subsurface microbial community composition across geological formations at the Hanford Site. *Environ. Microbiol.* **14**: 414–425.

Macherla, V.R., Liu, J.N., Bellows, C., Teisan, S., Nicholson, B., Lam, K.S., and Potts, B.C.M. (2005) Glaciapyrroles A, B, and C, pyrrolosesquiterpenes from a *Streptomyces* sp. isolated from an Alaskan marine sediment. *J. Nat. Prod.* **68**: 780–783.

Maldonado, L.A., Stach, J.E.M., Pathom-aree, W., Ward, A.C., Bull, A.T., and Goodfellow, M. (2005) Diversity of cultivable actinobacteria in geographically widespread marine sediments. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **87**: 11–18.

Mincer, T.J., Fenical, W., and Jensen, P.R. (2005) Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora*. *Appl. Environ. Microbiol.* **71**:7019-7028.

Moran, M.A., Rutherford, L.T., and Hodson, R.E. (1995) Evidence for indigenous *Streptomyces* populations in a marine environment determined with a 16S rRNA probe. *Appl. Environ. Microbiol.* **61**: 3695–3700.

Moree, W.J., Phelan, V. V, Wu, C.-H., Bandeira, N., Cornett, D.S., Duggan, B.M., and Dorrestein, P.C. (2012) Interkingdom metabolic transformations captured by microbial imaging mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 13811–13816.

Pathirana, C., Jensen, P.R., and Fenical, W. (1992) Marinone and debromomarinone - antibiotic sesquiterpenoid naphthoquinones of a new structure class from a marine bacterium. *Tetrahedron Lett.* **33**: 7663–7666.

Prieto-Davo, A., Fenical, W., and Jensen, P.R. (2008) Comparative actinomycete diversity in marine sediments. *Aquat. Microb. Ecol.* **52**: 1–11.

Prieto-Davó, A., Villarreal-Gómez, L.J., Forschner-Dancause, S., Bull, A.T., Stach, J.E.M., Smith, D.C., Rowley, D.C., and Jensen, P.R. (2013) Targeted search for actinomycetes from nearshore and deep-sea marine sediments. *FEMS Microbiol. Ecol.* **84**: 510–8.

Qin, S., Li, J., Chen, H.-H., Zhao, G.-Z., Zhu, W.-Y., Jiang, C.-L., Xu, L.-H., and Li, W.-Jun. (2009) Isolation, Diversity, and Antimicrobial Activity of Rare Actinobacteria from Medicinal Plants of Tropical Rain Forests in Xishuangbanna, China. *Appl. Environ. Microbiol.* **75**: 6176–6186.

Schloss, P.D. and Westcott, S.L. (2011) Assessing and Improving Methods Used in Operational Taxonomic Unit-Based Approaches for 16S rRNA Gene Sequence Analysis. *Appl. Environ. Microbiol.* **77**: 3219–3226.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., and Weber, C.F. (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl. Environ. Microbiol.* **75**: 7537–7541.

Seipke, R.F., Kaltenpoth, M., and Hutchings, M.I. (2012) *Streptomyces* as symbionts: an emerging and widespread theme? *Fems Microbiol. Rev.* **36**: 862–876.

Shade, A., Hogan, C.S., Klimowicz, A.K., Linske, M., McManus, P.S., and Handelsman, J. (2012) Culturing captures members of the soil rare biosphere. *Environ. Microbiol.* **14**: 2247-2252.

Shiomi, K., Iinuma, H., Hamada, M., Naganawa, H., Manabe, M., Matsuki, C., Takeuchi, T., and Umezawa, H. (1986) Novel antibiotics napyradiomycins - Production, isolation, physicochemical properties and biological activity. *J. Antibiot. (Tokyo).* **39**: 487–493.

Silvestro, D. and Michalak, I. (2011) raxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* **12**: 335–337.

Solano, G., Rojas-Jimenez, K., Jaspars, M., and Tamayo-Castillo, G. (2009) Study of the diversity of culturable actinomycetes in the North Pacific and Caribbean coasts of Costa Rica. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **96**: 71–78.

Soria-Mercado, I.E., Prieto-Davo, A., Jensen, P.R., and Fenical, W. (2005) Antibiotic terpenoid chloro-dihydroquinones from a new marine actinomycete. *J. Nat. Prod.* **68**: 904–910.

Stach, J. and Maldonado, L. (2003) Statistical approaches for estimating actinobacterial diversity in marine sediments. *Appl. Environ. Microbiol.* **69**: 6189-6200.

Strobel, G. and Daisy, B. (2003) Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* **67**: 491–502.

Swofford, D.L. PAUP*: phylogenetic analysis using parsimony, version 4.0b10. 2003.

Tello, M., Kuzuyama, T., Heide, L., Noel, J.P., and Richard, S.B. (2008) The ABBA family of aromatic prenyltransferases: broadening natural product diversity. *Cell. Mol. Life Sci.* **65**: 1459–1463.

Wang, H., Liu, N., Xi, L., Rong, X., Ruan, J., and Huang, Y. (2011) Genetic Screening Strategy for Rapid Access to Polyether Ionophore Producers and Products in Actinomycetes. *Appl. Environ. Microbiol.* **77**: 3433–3442.

Watson, D., Taylor, G.W., Wilson, R., Cole, P.J., and Rowe, C. (1988) Thermospray mass-spectrometric analysis of phenazines. *Biomed. Environ. Mass Spectrom.* **17**: 251–255.

Watve, M.G., Tickoo, R., Jog, M.M., and Bhole, B.D. (2001) How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* **176**: 386–390.

Weller, D.M., Raaijmakers, J.M., Gardener, B.B.M., and Thomashow, L.S. (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* **40**: 309–48.

Zhao, X.-Q., Jiao, W.-C., Jiang, B., Yuan, W.-J., Yang, T.-H., and Hao, S. (2009) Screening and identification of actinobacteria from marine sediments: Investigation of potential producers for antimicrobial agents and type I polyketides. *World J. Microbiol. Biotechnol.* **25**: 859–866.

Zimmermann, W. (1990) Degradation of lignin by bacteria. *J. Biotechnol.* **13**: 119–130.

Chapter 4: Diversity and distribution of hybrid isoprenoid biosynthetic gene clusters in the MAR4 clade of streptomycetes

4.1 Abstract

Here we examine the distribution of gene clusters predicted to encode the biosynthesis of hybrid isoprenoids (HIs) in the actinobacterial genus *Streptomyces*. This unusual class of structurally diverse bacterial secondary metabolites is characterized by a mixed biosynthetic origin that includes the incorporation of a terpene moiety. A largely marine-derived lineage within the genus *Streptomyces* called the 'MAR4' clade has previously been observed to produce diverse molecules within this structural class. Using a set of 120 *Streptomyces* genomes, including twelve MAR4 strains, we searched for HI biosynthetic gene clusters using ABBA prenyltransferases (PTases) as queries. These enzymes are responsible for a key step in HI biosynthesis. We found ABBA PTase homologs in 21% of the non-MAR4 *Streptomyces* genomes yet all of the MAR4 genomes contained these enzymes. In addition, MAR4 genomes averaged more PTases per genome than other streptomycetes. Phylogenetic analyses suggest that MAR4 PTase diversity has arisen by a combination of horizontal gene transfer and gene duplication. Identification of putative HI gene clusters in MAR4 strains reveals many clusters with unknown products, indicating there are as-yet unidentified HIs produced by these strains. Strikingly, some orthologous PTases were found in different gene clusters in closely related MAR4 strains, suggesting they represent highly mobile genetic elements. These rearrangements could reflect a mechanism by which HI structural diversity is

created. Finally, we examine the distribution of the mevalonate pathway in *Streptomyces*, which has previously been hypothesized to provide isoprene for secondary metabolism. We confirm that, when present, this pathway is always associated with HI or terpenoid biosynthetic enzymes, however it is not a consistent marker by which to predict the presence of HI gene clusters.

4.2 Introduction

Microbes produce an incredible diversity of small molecules as a part of secondary metabolism. In bacteria, the enzymes responsible for the biosynthesis of these complex structures are typically clustered on the chromosome. Biosynthetic gene clusters for small molecule production evolve rapidly relative to other genetic elements (Fischbach et al., 2008; Medema et al., 2014), which probably has contributed to the chemical diversity of their products. Many of these small molecules are thought to have diverse functions in allelopathy, signaling, or iron scavenging, but it is rare for a secondary metabolite to be experimentally connected to its natural function. Because of this, it remains unclear *why* such a diversity of small molecules has evolved. Recently, however, the availability of large numbers of genome sequences has allowed researchers to begin to identify the evolutionary mechanisms governing *how* structural novelty is created in secondary metabolism (Fischbach et al., 2008; Medema et al., 2014; Ziemert et al., 2014).

Members of the Gram-positive actinobacterial genus *Streptomyces* produce numerous and diverse molecules as a part of secondary metabolism

(Watve et al., 2001; Berdy, 2005). With nearly 600 named species, the genus is phylogenetically diverse and taxonomically complex. Streptomycetes are typically saprophytic and are found in terrestrial soils and marine sediments, and as plant endophytes, invertebrate mutualists, and human or plant pathogens (Jensen and Fenical, 2005; Seipke et al., 2012). Recently, a largely marine-derived lineage within the streptomycetes has been described, called the 'MAR4' clade. The lineage includes 57 cultured isolates and 180 clone sequences that encompass 4.1% 16S rRNA divergence. Members of the group consistently form two sub-clades represented by the type strains *S. aculeolatus* and *S. synnematoformans* (Gallagher et al., 2013). These are the only named species within the clade.

The MAR4 clade has previously been linked to the production of compounds broadly classified hybrid isoprenoids (HIs). These compounds are biosynthetic hybrids that derive part of their structures from five-carbon isoprene units (Gallagher et al., 2010). The addition of isoprene ("prenylation") can occur on a variety of chemical scaffolds, thus creating considerable diversity within this class. HIs frequently possess biological activity and thus their discovery is of interest to the pharmaceutical industry. Based on literature reports, the production of HIs appears to be scattered throughout the streptomycete phylogeny and is a relatively rare part of secondary metabolism (Gallagher et al., 2010). In contrast, some members of the MAR4 clade have been observed to produce up to three distinct HI classes and all MAR4 strains tested to date produce at least one HI, an ability that has not been reported elsewhere in the

genus (Gallagher et al., 2013). So far, MAR4 strains have been reported to produce the prenylated napthoquionone molecules napyradiomycin (Winter et al., 2007), marinone, debromomarinone, and neomarinone (Pathirana et al., 1992; Hardt et al., 2000), the prenylated pthalazinone azamerone (Cho et al., 2006), the prenylated phenazine molecules lavanducyanin (Kondratyuk et al., 2012) and marinophenazine (Zeyhle et al., 2014), and the highly unusual prenylated pyrrole nitropyrrolin (Kwon et al., 2010).

Prenyltransferases (PTases) are a key group of enzymes in the biosynthesis of His. They are responsible for the attachment of isoprene to a variety of acceptor molecules. PTases are widespread in the biosynthesis of both primary and secondary metabolites including, for example, membrane sterols, carotenoids, and lipoquinones (Heide, 2009). One sub-group of PTases that is specific to secondary metabolism are the ABBA PTases, named in reference to the ααββ structural repeats that form a large β-barrel fold comprising the active center of the enzyme. ABBA PTases attach isoprenoid molecules to aromatic substrates, and to date all characterized ABBA PTases are involved in the biosynthesis of HI secondary metabolites (Bonitz et al., 2011).

The ABBA PTases can be further divided into two sub-groups, both of which are found in fungi and bacteria. The first group is called the indole ABBA PTases, which are responsible for the prenylation of indole substrates. One bacterial example is the enzyme CymD, which is involved in the biosynthesis of the cyclic peptide cyclomarin (Schultz et al., 2010). The second group, termed

'Orf2' PTases, attach isoprene to a variety of aromatic substrates, including phenazines, napthoquinones, and aminocoumarins. This group is named for the first characterized member, which is involved in the biosynthesis of naphterpin (this enzyme was later renamed 'NphB') (Kuzuyama et al., 2005). Although the indole and Orf2 PTases bear little sequence homology, they are thought to have arisen from a common ancestor (Bonitz et al., 2011).

The isoprene units that are precursors in terpenoid production are generated in bacteria by either the mevalonate or non-mevalonate pathways. Generally, *Streptomyces* use the non-mevalonate pathway for isoprenoid biosynthesis. Where the mevalonate (*mev*) pathway has been found, however, it is always flanking a HI pathway (Kawasaki et al., 2003). Feeding experiments have confirmed that the *mev* pathway can provide some or all of the isoprene for the production of these molecules (Kuzuyama and Seto, 2003; Bringmann et al., 2007; Winter et al., 2009; Izumikawa et al., 2010). These observations have led to the hypothesis that the *mev* pathway is a marker for HI production (Kawasaki et al., 2003). The availability of genome sequences from a large number of *Streptomyces* strains now provides the opportunity to test this long-standing hypothesis.

To date, evidence for the accumulation of HI pathways in MAR4 strains is based on literature reports and the analysis of MAR4 culture extracts. Here we use full genome sequences to more rigorously test the hypothesis that MAR4 strains are enriched in HI pathways relative to other streptomycetes. Using ABBA

PTases as a marker for HI production, we show that MAR4 strains possess a higher biosynthetic potential for HI production than do other streptomycetes, and that the *mev* pathway is generally not associated with HI gene clusters. We also found that most MAR4 PTases are very closely related, and that they have undergone both horizontal gene transfer and gene duplication events. We observed evidence that orthologous PTases are subject to rearrangement and can occur in different biosynthetic pathways in closely related strains. The evolutionary history of HI pathways in MAR4 streptomycetes provides insight into how chemical diversity is generated in microbial secondary metabolism.

4.3 Materials and methods

4.3.1 Genome sequencing

Eleven MAR4 strains were cultured in 200 mL of A1 medium (10 g soluble starch, 4 g yeast extract, 2 g peptone, 750 mL seawater, 250 mL deionized water) as previously described (Ziemert et al., 2014). DNA was extracted according to the Joint Genome Institute (JGI) standard protocol (http://my.jgi.doe.gov/general/protocols.html). Genome sequencing, annotation, and assembly were carried out as previously described by the Joint Genome Institute (Ziemert et al., 2014). An additional MAR4 genome (CNQ-509) was provided by Prof. Lutz Heide (University of Tuebingen, Germany) and will be made public as part of an upcoming publication (Leipoldt et al.).

4.3.2 Identification of ABBA prenyltransferase homologs

All biochemically characterized ABBA PTases of bacterial origin (Bonitz et al., 2011) were used as query sequences in a BLASTp search of the MAR4 genomes and an additional 108 *Streptomyces* genomes that were publicly available as of March 2014 (Table 4.1) using the BLAST interface at IMG/ER (https://img.jgi.doe.gov/er/), with an e-value cutoff of 1e-5. The MAR4 strain CNQ-509, which was not available through JGI, was individually searched with the same query set using BLAST+ (Camacho et al., 2009). Multiple sequence alignments of all BLAST hits to characterized indole and Orf2 PTases were constructed using MUSCLE (Edgar, 2004). For each alignment, a maximum likelihood phylogeny was constructed using raxmlGUI (Silvestro and Michalak, 2011) using the GTR+G model. For the Orf2 PTase phylogeny, two outgroups were included from the indole PTase family. Likewise, for the indole family, two Orf2 PTases were used as outgroups. A BLAST hit was confirmed to be an ABBA PTase if it formed a clade with all previously characterized PTases and if it did so without an excessively long branch length.

Table 4.1 List of accession numbers for the genome sequences investigated in this study. The twelve MAR4 strains are indicated at the beginning of the list.
*Genome provided by Prof. Lutz Heide at the University of Tuebingen, Germany.
**Genome no longer available from JGI

Genome	IMG taxon ID	NCBI taxon ID
Streptomyces sp. CNB-632 (MAR4)	2558860984	1408314
Streptomyces sp. CNH-099 (MAR4)	2515154205	1137269
Streptomyces sp. CNP-082 (MAR4)	2526164782	1137270
Streptomyces sp. CNQ-525 (MAR4)	2561511112	418855
Streptomyces sp. CNQ-329 (MAR4)	2528311045	1298879
Streptomyces sp. CNQ-509 (MAR4)*	N/A	N/A
Streptomyces sp. CNQ-766 (MAR4)	2517572165	1169157
Streptomyces sp. CNQ-865 (MAR4)	2524023247	1288081
Streptomyces sp. CNS-335 (MAR4)	2517572166	1169160
Streptomyces sp. CNX-435 (MAR4)	2558860985	1408316
Streptomyces sp. CNY-243 (MAR4)	2518285564	1169161
Streptomyces sp. CNT-371 (MAR4)	2515154206	1136433
Streptomyces acidiscabies 84-104	2547132143	1116232
Streptomyces afghaniensis 772	2545824777	1283301
Streptomyces albulus CCRC 11814	2545824724	1316445
Streptomyces albus J1074	2541047081	457425
Streptomyces aurantiacus JA 4570	2545824776	1286094
Streptomyces auratus AGR0001	2531839540	1160718
Streptomyces avermitilis MA-4680	637000304	227882
Streptomyces bingchenggensis BCW-1	646862346	749414
Streptomyces bottropensis ATCC 25435	2517572239	1054862
Streptomyces canus 299MFChir4.1	2521172643	1172183
Streptomyces cattleya ATCC 35852	2504756050	29303
Streptomyces chartreusis NRRL 12338	2547132122	1079986
Streptomyces chartreusis NRRL 3882	2547132121	1079985
Streptomyces clavuligerus ATCC 27064	651324105	443255
Streptomyces coelicoflavus ZG0656	2534682028	1120227
Streptomyces coelicolor A3(2)	637000305	100226
Streptomyces collinus Tu 365	2554235367	1214242
Streptomyces davawensis JCM 4913	2561511188	1214101
Streptomyces flavidovirens DSM 40150	2522572192	1123319
Streptomyces fulvissimus DSM 40593	2554235391	1303692
Streptomyces gancidicus BKS 13-15	2537561976	1284664
Streptomyces ghanaensis ATCC 14672	645058824	566461

Table 4.1 List of accession numbers for genome sequences, Continued.

Genome	IMG taxon ID	NCBI taxon ID
Streptomyces globisporus C-1027	2548877066	1172567
Streptomyces griseoaurantiacus M045	651324106	996637
Streptomyces griseoflavus Tu4000	645058728	467200
Streptomyces griseus griseus NBRC 13350	641522653	455632
Streptomyces hygroscopicus ATCC 53653	645058857	457427
Streptomyces hygroscopicus jinggangensis 5008	2561511169	1133850
Streptomyces hygroscopicus jinggangensis TL01	2561511180	1203460
Streptomyces ipomoeae 91-03	2537561795	698759
Streptomyces lividans TK24	645058856	457428
Streptomyces lysosuperificus ATCC 31396	2547132120	1079984
Streptomyces mobaraensis NBRC 13819	2537561865	1223523
Streptomyces pristinaespiralis ATCC 25486	648861016	457429
Streptomyces purpureus ATCC 21405	2516493006	1054860
Streptomyces rimosus rimosus ATCC 10970	2541047971	1265868
Streptomyces roseosporus NRRL 11379	645058827	457430
Streptomyces roseosporus NRRL 15998	645058822	457431
Streptomyces scabiei 87.22	646564576	680198
Streptomyces scabrisporus DSM 41855	2515154197	1123320
Streptomyces somaliensis DSM 40738	2548876818	1134445
Streptomyces sp. SirexAA-E	2523533511	862751
Streptomyces sp. 142MFCol3.1	2524614552	1172179
Streptomyces sp. 303MFCol5.2	2521172626	1172181
Streptomyces sp. 351MFTsu5.1	2521172628	1172180
Streptomyces sp. AA0539	2551306164	1210045
Streptomyces sp. AA1529	2551306127	1203257
Streptomyces sp. XyelbKG-1 1	647000328	649189
Streptomyces sp. Amel2xB2	2524614578	1305829
Streptomyces sp. Amel2xC10	2524614730	1305826
Streptomyces sp. C	645058853	253839
Streptomyces sp. CNB-091	2518285535	1169156
Streptomyces sp. CNH-189	2515154150	1136432
Streptomyces sp. CNH-287	2524023245	1288082
Streptomyces sp. CNR-698	2516143117	1206101
Streptomyces sp. CNS-606	2526164783	1305837
Streptomyces sp. CNS-615	2518285536	1169158
Streptomyces sp. CNS-654	2561511106	1931
Streptomyces sp. CNT-302	2517572190	1169155
Streptomyces sp. CNT-318	2524614559	1288079

Table 4.1 List of accession numbers for genome sequences, Continued.

Genome	IMG taxon ID	NCBI taxon ID
Streptomyces sp. CNT-360	2524614560	1288080
Streptomyces sp. CNT-372	2517572167	1169154
Streptomyces sp. CNY-228	2517572184	1169159
Streptomyces sp. DpondAA-B6	2524614581	682311
Streptomyces sp. DvalAA-21	2524614543	1305824
Streptomyces sp. e14	647533235	645465
Streptomyces sp. FXJ7.023	2554235020	579932
Streptomyces sp. HGB0020	2541046998	1078086
Streptomyces sp. HPH0547	2541047017	1203592
Streptomyces sp. HrubLS-53	2504756065	60912
Streptomyces sp. KhCrAH-337	2524614544	1305839
Streptomyces sp. KhCrAH-40	2524614547	1305838
Streptomyces sp. KhCrAH-43	2524614576	1305827
Streptomyces sp. LaPpAH-201	2526164526	1305823
Streptomyces sp. LCC**	N/A	N/A
Streptomyces sp. Mg1	642791623	465541
Streptomyces sp. PAMC26508	2561511190	1265601
Streptomyces sp. PgraA7	2524614673	1157641
Streptomyces sp. PP-C42	2547132312	986330
Streptomyces sp. PsTaAH-130	2524614577	1305828
Streptomyces sp. PsTaAH-137	2524614580	1305830
Streptomyces sp. S4	2547132081	889487
Streptomyces sp. SA3_actG	649990018	683219
Streptomyces sp. ScaeMP-e122	2524614542	1305825
Streptomyces sp. SPB74	647533234	465543
Streptomyces sp. SPB78	645951849	591157
Streptomyces sp. SS	2551306143	260742
Streptomyces sp. TAA-040	2524614564	1288083
Streptomyces sp. TAA-204	2524614565	1289387
Streptomyces sp. TAA-486	2524614850	1298880
Streptomyces sp. TOR3209	2547132111	1073567
Streptomyces sp. Tu6071	651285011	355249
Streptomyces sp. W007	2514752031	1055352
Streptomyces sp. WMMB 322	2522125135	1286821
Streptomyces sp. WMMB 714	2522125136	1286822
Streptomyces sulphureus DSM 40104	2518645610	1123321
Streptomyces sviceus ATCC 29083	648861017	463191
Streptomyces tsukubaensis NRRL 18488	2529292926	1114943

Table 4.1 List of accession numbers for genome sequences, Continued.

Genome	IMG taxon ID	NCBI taxon ID
Streptomyces turgidiscabies Car8	2541046981	698760
Streptomyces venezuelae ATCC 10712	2524023215	953739
Streptomyces violaceusniger SPC6	2554235005	1306406
Streptomyces violaceusniger Tu 4113	648276750	653045
Streptomyces viridochromogenes DSM 40736	645058855	591159
Streptomyces viridochromogenes Tue57	2531839509	1160705
Streptomyces viridosporus T7A, ATCC 39115	2518285526	665577
Streptomyces vitaminophilus DSM 41686	2515154142	1123322
Streptomyces xinghaiensis S187, NRRL B24674	2548876513	1038929
Streptomyces zinciresistens K42	2531839181	700597

4.3.3 *Streptomyces* phylogeny

Five housekeeping genes (*recA, atpD, rpoB, gyrB*, and *trpB*) that have previously been used in streptomycete multi-locus sequence typing (Doroghazi and Buckley, 2010) were identified in the set of 120 *Streptomyces* genomes using a BLAST search for each gene. In order to confirm the identity of these housekeeping genes, the amino acid sequences for each gene set were aligned using MUSCLE, and individual maximum likelihood phylogenies for each of the housekeeping genes built using raxmlGUI (Silvestro and Michalak, 2011). Housekeeping genes from two *Pseudonocardia* strains were included as outgroups (*P. dioxanivorans* CB1190 and *P. asaccharolytica* DSM44247). BLAST hits were confirmed to represent housekeeping genes if they claded monophyletically.

Of the five housekeeping genes examined, only *rpoB* and *atpD* were present in single copy in all of the 120 genomes. These two housekeeping genes were thus selected to build a *Streptomyces* phylogeny. Multiple sequence

alignments of these two genes were trimmed by hand so that all sequences were of the same length and concatenated. raxmlGUI was used to build a maximum likelihood phylogeny of the resulting multiple sequence alignment. LG+I+G was selected as the best-fit model for the data set based on a ProtTest (Darriba et al., 2011) analysis of the concatenated alignment.

4.3.4 Orf2 prenyltransferase phylogeny

An amino acid phylogeny of the Orf2 ABBA prenyltransferases was constructed using all of the homologs identified in genome sequences as well a set of characterized Orf2 prenyltransferases (Bonitz et al., 2011). The sequences were aligned using MUSCLE (Edgar, 2004). A maximum likelihood phylogeny was built using raxmlGUI (Silvestro and Michalak, 2011) as describe above. Based on a ProtTest (Darriba et al., 2011) analysis, LG was chosen as the best-fit model for the data set. A preliminary tree using two indole prenyltransferases as outgroups (SCO7190, Accession number WP_011031680; CymD, Accession number SARE_4565) was built to confirm that all of the Orf2 prenyltransferase sequences were monophyletic. To improve the alignment quality, the outgroups were removed, the sequences re-aligned, and a final tree built using identical methods with midpoint rooting.

4.3.5 HI gene cluster identification

Gene clusters associated with all MAR4 PTases were analyzed using a combination of BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and IMG/ER, (https://img.jgi.doe.gov/cgi-bin/er/main.cgi). Clusters were defined by shared

content and gene annotation. Gene clusters in different strains were compared using MultiGeneBlast (Medema et al., 2013) supplied with a database of the 12 MAR4 genome strains. Gene clusters were considered to be the same if they shared >85% gene content and had a MultiGeneBlast cumulative BLAST bit score >80% of the query sequences' score to itself. Cumulative BLAST bit scores dropped precipitously in strains that did not contain the cluster. Uncharacterized pathways were assigned a unique identifier that we termed a "Hybrid Isoprenoid biosynthetic Gene Cluster" number (HIGC 1-11). The previously characterized napyradiomycin (*nap*) pathway was assembled with the aid of the published sequence (Winter et al., 2007).

4.3.6 MAR4 strain phylogeny

The five housekeeping genes that were used in a prior *Streptomyces* phylogeny (Doroghazi and Buckley, 2010) were all present in single copy in the 12 MAR4 genomes and used to build a more robust MAR4 phylogeny. Nucleotide-based multiple sequence alignments for each of these five genes, as well as the 16S rRNA gene, were individually built using MUSCLE (Edgar, 2004), and trimmed by hand so that all sequences were of the same length. The six alignments were then concatenated and a maximum likelihood phylogeny was built using raxmlGUI (Silvestro and Michalak, 2011) with 100 bootstrap replicates using the GTR+G substitution model. A maximum parsimony tree was built using PAUP* (Swofford) with 100 bootstrap replicates. The two strains that were found to clade most closely with the MAR4 clade in the *Streptomyces* phylogeny (*S.*

vitaminophilus DSM 41686 and *S. bottropensis* ATCC 25435) were included in the analysis as outgroups. Acquisition points of HIGCs within the MAR4 lineage were predicted using the trace character history function in Mesquite (Maddison and Maddison, 2011) as previously described (Ziemert et al., 2014). Likelihood scores >50% were used to predict the points of HIGC acquisition in the MAR4 phylogeny.

4.3.7 Mevalonate pathway identification

To identify the mevalonate (*mev*) pathway, the 3-hydroxy-3-methyl-glutaryl-CoA reductase gene from *S. aculeolatus* NRRL 18422 (ABS50444) was used as a query in a BLASTp search of the *Streptomyces* genomes. This gene has previously been used as a marker for the *mev* pathway (Izumikawa et al., 2010; Khan et al., 2010). Once located, the neighboring genes were manually examined to confirm the presence of the remaining five genes of the pathway (3-hydroxy-3-methyl-glutaryl-CoA synthase, isopentenyl pyrophosphate isomerase, phosphomevalonate kinase, mevalonate decarboxylase, and mevalonate kinase). It was also confirmed that these six genes were syntenic.

4.4 Results

4.4.1 Identifying ABBA prenyltransferases in 120 *Streptomyces* genomes

One hundred and twenty publicly available *Streptomyces* genomes, including twelve MAR4 genomes (Table 4.1), were examined for the presence of HI biosynthetic pathways using ABBA PTases as a diagnostic marker for the ability to produce HIs. Of the 120 genomes searched, 29.2% (35 strains)

contained at least one ABBA PTase, including all of the 12 MAR4 strains. Of the non-MAR4 strains, one strain contained three ABBA PTases, five strains contained two ABBA PTases, and the remainder (17 strains) contained only one of these enzymes. The indole and Orf2 classes of ABBA prenyltransferases were found in similar numbers (12 Orf2, 16 indole) in non-MAR4 genomes. In contrast, the 12 MAR4 genomes contained exclusively Orf2 prenyltransferases (3-8 per genome) with the exception of a single indole prenyltransferase in strain CNQ-509 (Figure 4.1).

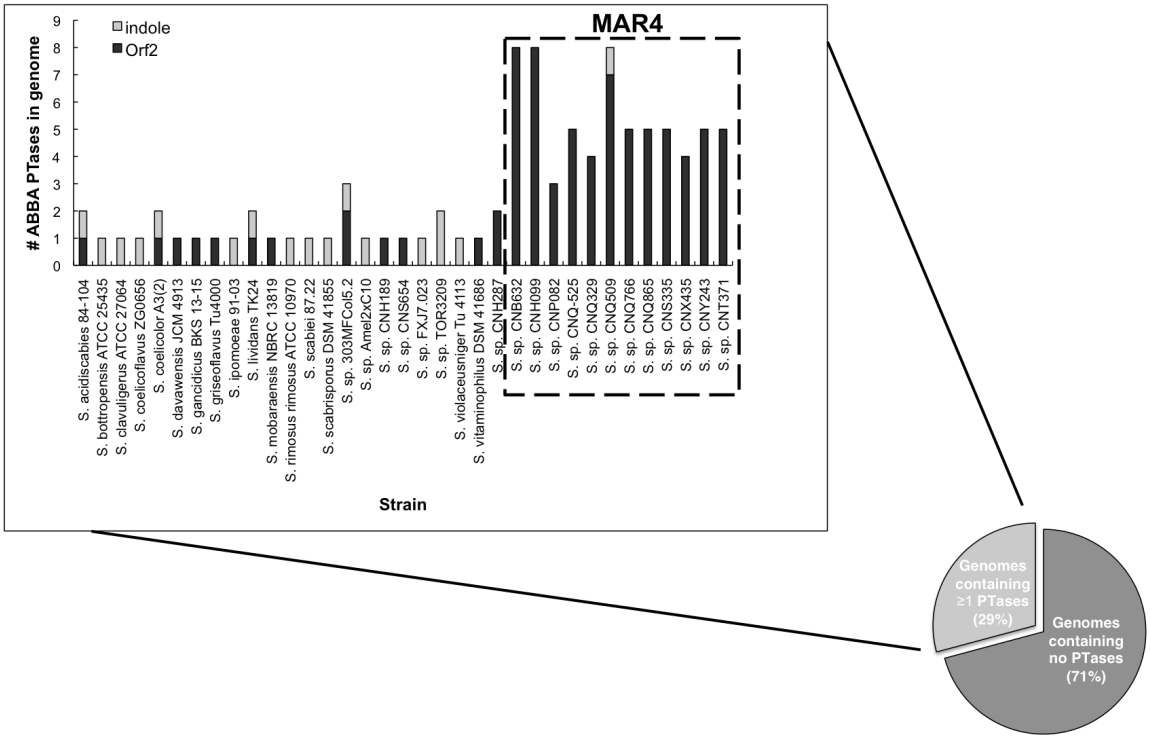


Figure 4.1 Homologs of indole and Orf2 ABBA prenyltransferases found in 120 *Streptomyces* genomes. The pie chart illustrates the percentage of genomes found to contain at least one ABBA PTase homolog. The majority of genomes (85) contained no prenyltransferases. For the 35 *Streptomyces* genomes containing at least one prenyltransferase, the number of total ABBA prenyltransferases is indicated. The MAR4 clade is also indicated.

4.4.2 Distribution of PTases in *Streptomyces* spp.

The distribution of PTases in the genus *Streptomyces* was examined for the 120 genome strains using a phylogeny generated from two housekeeping genes. By mapping the occurrence of these genes onto the tree, it became clear that PTases are sparsely distributed throughout the genus with the exception of the MAR4 clade and a distantly related clade that includes *S. coelicolor* A3(2). These two clades are composed entirely of PTase-containing strains (Figure 4.2). Members of the *S. coelicolor* clade contain on average one ABBA PTase per genome, while members of the MAR4 clade contain an average of five ABBA PTases per genome. Interestingly, the closest relatives of the MAR4 clade do not consistently contain ABBA PTases.

Figure 4.2 Maximum likelihood phylogeny of all genomes examined in this study based on AtpD and RpoB amino acid sequences. Nodes supported by bootstrap values greater than 50% are indicated (based on 100 replicates). Colors of taxa names indicate the number of PTases found in each genome. The MAR4 and *S. coelicolor* clades are indicated.


Streptomyces sp. TOR3209
Streptomyces sp. FXJ7.023
Streptomyces coelicoflavus ZG0656
Streptomyces lividans TK24
Streptomyces coelicolor A3(2)
Streptomyces griseoflavus Tu4000
Streptomyces gancidicus BKS 13-15
Streptomyces sp. CNH-189
S. coelicolor clade
Streptomyces ghanaensis ATCC 14672
Streptomyces viridosporus T7A
Streptomyces sp. e14
Streptomyces hygroscopicus jinggangensis TL01
Streptomyces hygroscopicus jinggangensis 5008
Streptomyces sp. LCC
Streptomyces sp. PsTaAH-130
Streptomyces collinus Tu 365
Streptomyces afghaniensis 772
Streptomyces chartreusis NRRL 12338
Streptomyces viridochromogenes DSM 40736
Streptomyces sp. HGB0020
Streptomyces ipomoeae 91-03
Streptomyces davawensis JCM 4913
Streptomyces chartreusis NRRL 3882
Streptomyces viridochromogenes Tue57
Streptomyces sp. PsTaAH-137
Streptomyces sp. 303MFCol5.2
Streptomyces griseoaurantiacus M045
Streptomyces acidiscabies 84-104
Streptomyces bottropensis ATCC 25435
Streptomyces scabiei 87.22
Streptomyces sp. AmelDxC10
Streptomyces zinciresistens K42
Streptomyces sp. 351MFTsu5.1
Streptomyces sviceus ATCC 29083
Streptomyces canus 299MFChir4.1
Streptomyces sp. 142MFCol3.1
Streptomyces avermitilis MA-4680
Streptomyces turgidiscabies Car8
Streptomyces aurantiacus JA 4570
Streptomyces sp. SPB78
Streptomyces sp. SA3_actG
Streptomyces sp. Tu6071
Streptomyces sp. HrubLS-53
Streptomyces sp. SPB74
Streptomyces albus J1074
Streptomyces sp. LaPpAH-201
Streptomyces sp. CNY-228
Streptomyces sp. PP-C42
Streptomyces sp. S4
Streptomyces sp. W007
Streptomyces sp. CNB-091
Streptomyces globisporus C-1207
Streptomyces griseus griseus NBRC 13350
Streptomyces sp. XyelDKG-1 1
Streptomyces sp. PgraA7
Streptomyces sp. CNS-654
Streptomyces roseosporus NRRL 11379
Streptomyces roseosporus NRRL 15998
Streptomyces fulvissimus DSM 40593
Streptomyces sp. ScaeMP-e122
Streptomyces sp. DvalAA-21
Streptomyces sp. DpondAA-B6
Streptomyces sp. SirexAA-E
Streptomyces flavidovirens DSM 40150
Streptomyces sp. PAMC26508
Streptomyces sp. KhCrAH-43
Streptomyces sp. KhCrAH-40
Streptomyces sp. KhCrAH-337
Streptomyces pristinaespiralis ATCC 25486
Streptomyces lysosuperificus ATCC 31396
Streptomyces sp. Mg1
Streptomyces sp. C
Streptomyces purpureus KA281
Streptomyces sp. CNT-372
Streptomyces violaceusniger SPC6
Streptomyces somaliensis DSM 40738
Streptomyces sp. SS
Streptomyces venezuelae ATCC 10712
Streptomyces sp. CNT-302
Streptomyces sp. CNR-698
Streptomyces sp. CNS-615
Streptomyces clavuligerus ATCC 27064
Streptomyces tsukubaensis NRRL 18488
Streptomyces mobaraensis NBRC 13819
Streptomyces sp. WMMB 322
Streptomyces sp. TAA-204
Streptomyces sp. TAA-486
Streptomyces sp. WMMB 714
Streptomyces sp. AmelDxB2
Streptomyces sulphureus DSM 40104
Streptomyces sp. CNT-318
Streptomyces sp. AA1529
Streptomyces sp. CNT-360
Streptomyces sp. HPH0547
Streptomyces sp. CNH-287
Streptomyces sp. CNS-606
Streptomyces sp. TAA-040
Streptomyces sp. AA0539
Streptomyces xinghaiensis S187
Streptomyces sp. CNQ-865
Streptomyces sp. CNY-243
Streptomyces sp. CNT-571
Streptomyces sp. CNQ-766
Streptomyces sp. CNS-355
Streptomyces sp. CNP-082
Streptomyces sp. CNQ-329
Streptomyces sp. CNQ-525
Streptomyces sp. CNQ-509
Streptomyces sp. CNX-435
Streptomyces sp. CNB-632
Streptomyces sp. CNH-099
MAR4 clade
Streptomyces bingchenggensis BCW-1
Streptomyces violaceusniger Tu 4113
Streptomyces hygroscopicus ATCC 53653
Streptomyces auratus AGR0001
Streptomyces rimosus rimosus ATCC 10970
Streptomyces albulus CCRC 11814
Streptomyces cattleya ATCC 35852
Streptomyces vitaminophilus DSM 41686
Streptomyces scabrisporus DSM 41855
Pseudonocardia dioxanivorans CB1190
Pseudonocardia asaccharolytica DSM 44247
0.4 substitutions per site
0 1 2 3 4 5 6 7 8
No. PTases found in genome

4.4.3 Orf2 PTase phylogeny

Because all but one of the MAR4 ABBA PTases fall into the Orf2 class, an Orf2 phylogeny was constructed to assess the evolutionary history of these sequences (Figure 4.3). This phylogeny included all of the identified Orf2 homologs, as well as all previously characterized Orf2 PTases (Bonitz et al., 2011). Two of the experimentally characterized MAR4 prenyltransferases (ABS50462 and ABS50461) are from the type strain *S. aculeolatus* NRRL 18422. Both of these are from the *nap* pathway responsible for napyradiomycin biosynthesis (Winter et al., 2007). A genome sequence is not available for this MAR4 strain so the total number of PTases remains unknown.

The initial bifurcation in the Orf2 phylogeny reveals two clades, the lower and smaller of which clade contains the previously characterized CloQ and NovQ PTases, which are responsible for the prenylation of the aminocoumarin molecules chlorobiocin and novobiocin, respectively (Saleh et al., 2009).The upper and larger clade consists of characterized prenyltransferases known to prenylate napthoquinone (e.g. NphB, Fur7, and Fnq26 (Bonitz et al., 2011)) and phenazine (EpzP and PpzP (Bonitz et al., 2011)) scaffolds. All of the PTases found in the MAR4 genomes fell within this large clade. The MAR4 PTases could be further delineated into thirteen highly supported (100% bootstrap) sub-clades that represent groups of orthologous PTases. These sub-clades were each assigned "PrenylTransferase Clade" (PTC) numbers (Figure 4.3). Only PTC5 contains a single prenyltransferase, found in strain CNQ-509.

The Orf2 phylogeny indicates that horizontal gene transfer (HGT) and gene duplication have both played a role in PTase evolution. The presence of closely related Orf2 PTases in distantly related strains suggests that these genes have been subject to HGT within the streptomycetes. For example, a PTase from *Streptomyces* sp. CNH-189, which is found in the *S. coelicolor* clade on the genus phylogeny, clades most closely with PTC7 and PTC8, both of which are found in MAR4 strains (Figure 4.3). In contrast, the presence of closely related PTases found in the same strains indicates that they are the result of gene duplication. For example, the phylogenies within the sister clades PTC7 and PTC8 are congruent (Figure 4.3), suggesting that they are the result of gene duplication followed by vertical inheritance.

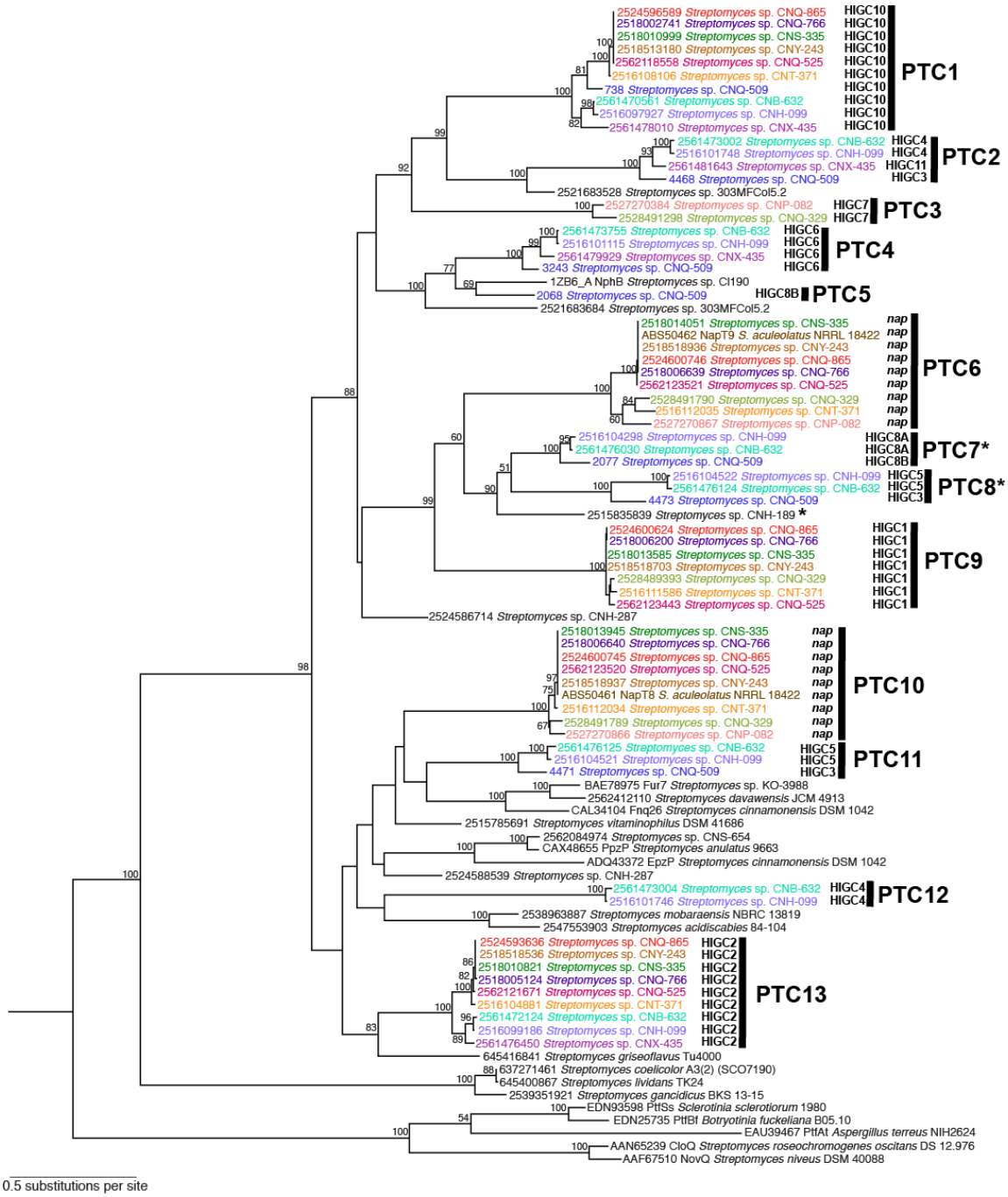


Figure 4.3 Maximum likelihood phylogeny of Orf2 PTases with mid-point rooting. The phylogeny contains all of the Orf2 ABBA PTases identified in *Streptomyces* genomes as well as all experimentally characterized Orf2 PTases. Homologs found in non-MAR4 genomes are in black, while those from MAR4 strains are in color-coded based on strain. MAR4 PTases have been delineated into 13 prenyltransferase clades (PTCs). Each MAR4 PTase is also assigned a hybrid isoprenoid gene cluster (HIGC) number, which defines the gene cluster in which it was observed. Nodes supported by bootstrap values greater than 50% are indicated (based on 100 replicates). Asterisks highlight the examples of horizontal gene transfer and gene duplication discussed in text.

To compare each PTC phylogeny with that of the strains in which the sequences were observed, five housekeeping genes (three genes in addition to those used to build the *Streptomyces* phylogeny) were used to construct a robust phylogeny of the genome-sequenced MAR4 strains (Figure 4.4). A likelihood analysis was used to predict the acquisition points for each PTC. Based on this analysis, no PTCs are predicted to have been present in the MAR4 ancestor. Five PTCs are found in seven or more MAR4 strains (PTCs 1,6,9,10,13). All other PTCs (2-5, 7-8, and 11-12) are present in four or fewer MAR4 strains. In all cases, the phylogeny of each individual PTC is largely congruent with that of the MAR4 strain phylogeny (Figures 4.3, 4.4).

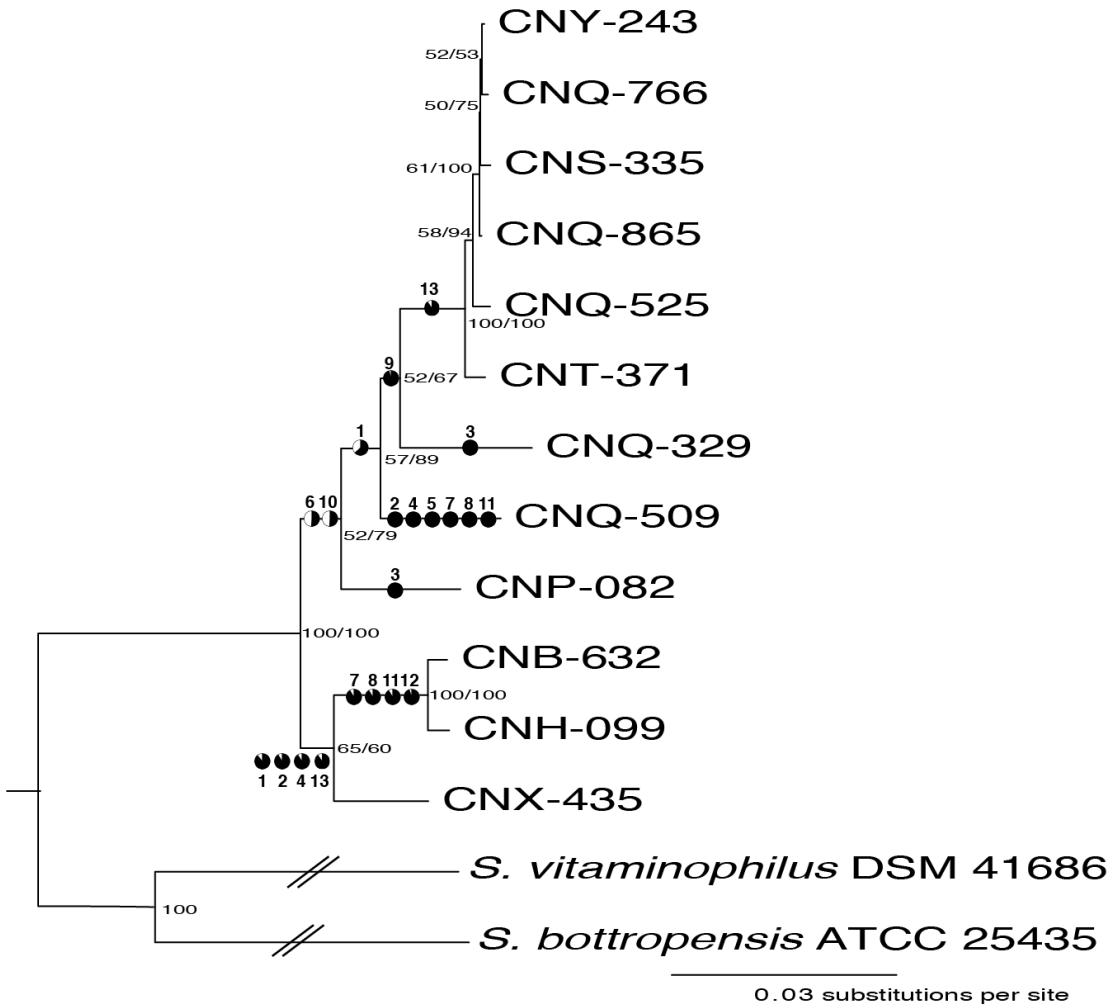


Figure 4.4 Phylogeny of MAR4 genome-sequenced strains. Tree was built from a multiple sequence alignment containing concatenated DNA sequences of five single-copy housekeeping genes (*atpD*, *rpoB*, *trpB*, *recA*, and *gyrB*) found in all MAR4 strains for which genome sequences were available. Maximum likelihood (ML) and maximum parsimony (MP) trees showed the same topology. Bootstrap values are indicated at each node (MP/ML) based on 100 replicates. Circles indicate predicted acquisition points for each PTC based on a likelihood analysis, with the proportional likelihood that the PTC was present at the predicted nodes indicated by the % fill of the circle.

4.4.4 HI gene clusters in MAR4

Analyses of the gene neighborhoods surrounding each of the PTases identified in the 12 MAR4 strains for which genome sequences were available predict the presence of 13 distinct gene clusters. Of these, 12 have not been characterized and each was assigned a HIGC (Hybrid Isoprenoid Gene Cluster)

number (Table 4.2) based on shared gene content and annotation. The remaining cluster was identified as *nap* (Winter et al., 2007) based on alignment to the previously defined sequence. The *nap* pathway was observed in 8 of the 12 MAR4 strains, although in each case the cluster was split onto several contigs.

Four of the HIGCs contain multiple PTases (Figure 4.3, Table 4.2). In each of these cases, the PTCs found in the same cluster are distant from each other on the Orf2 PTase phylogeny (i.e. they are never sister). While all of the clusters contain genes that are frequently associated with secondary metabolism (Figure 4.5, Table 4.2), generally it is very difficult to predict the scaffold that will be prenylated based on HIGC gene content. Two of the pathways, HIGC8A and 8B, do not meet the criteria to be assigned to the same pathway, but were given the same number owing to their similarity. Specifically, all of the genes found in HIGC8A are also present in HIGC8B, but this second gene cluster contains additional genes including a second PTase and a type III polyketide synthase. The products of two of the HIGCs could be predicted based on gene annotation and distribution of HIGCs in MAR4 strains previously observed to produce specific HI compounds (Gallagher et al., 2013). HIGC8B is predicted to be responsible for production of the known MAR4 HI compound marinone, while HIGC10 is predicted to be responsible for production of the known compound lavanducyanin (Table 4.2).

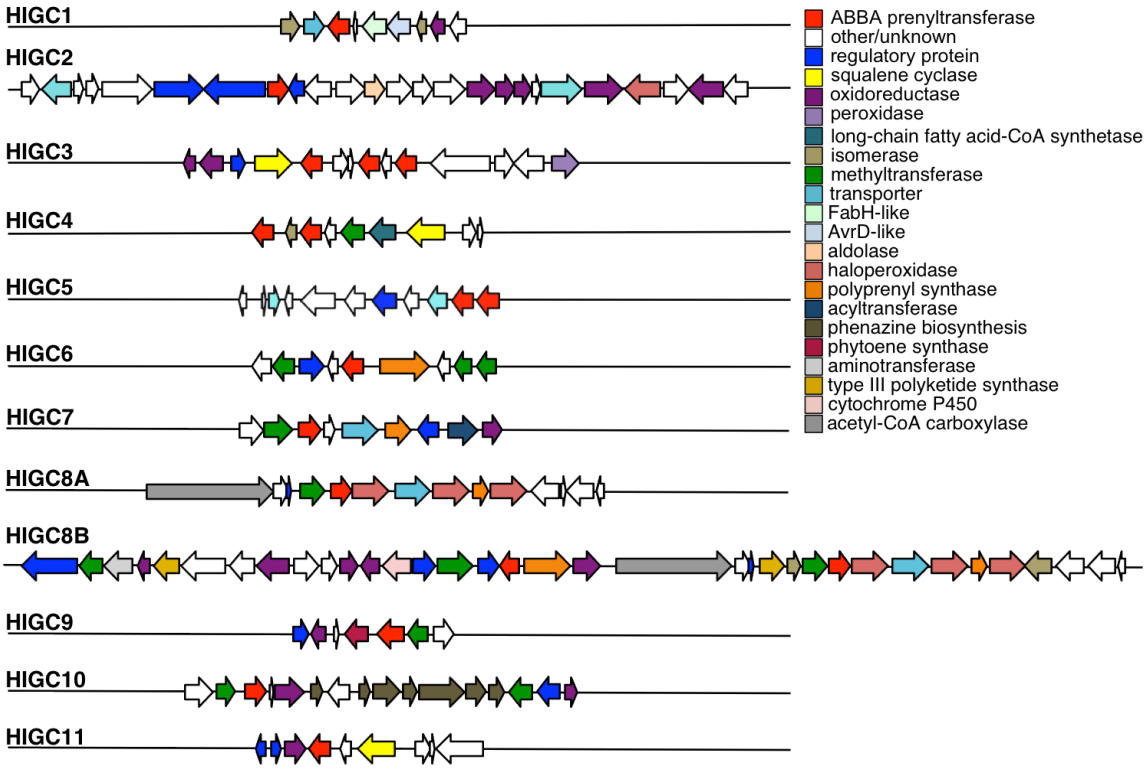


Figure 4.5 Putative HI gene clusters (HIGCs) identified in MAR4 strains. Putative gene functions (predicted by pfam annotations) are indicated by color.

Table 4.2 Summary of putative HI gene clusters (HIGCs) identified in MAR4 strains. For each HIGC, the PTC(s) present in the cluster are identified. A brief description of key genes relevant to secondary metabolism is provided for each HIGC. All clusters contained additional predicted genes frequently associated with secondary metabolism (i.e. reductase, methyltransferase, monooxygenase, cytochrome P450, isomerase, etc.). Where possible, the predicted HI product of the gene cluster is indicated.

Gene cluster	PTC(s) present	Description of gene content	Predicted product
nap	PTC10, PTC6	Characterized gene cluster; produces napyradiomycin, azamerone, and 8-amino-flaviolin	Unknown
HIGC1	PTC9	FabH-like protein, AvrD-like protein	Unknown
HIGC2	PTC13	haloperoxidase	Unknown
HIGC3	PTC2, PTC8, PTC11	squalene-hopene cyclase	Unknown
HIGC4	PTC2, PTC12	squalene-hopene cyclase	Unknown
HIGC5	PTC8, PTC11	Found on very short contig, additional genes in this cluster may exist	Unknown
HIGC6	PTC4	isopentenyl pyrophosphate synthase	Unknown
HIGC7	PTC3	polyprenyl synthase	Unknown
HIGC8A	PTC7	three haloperoxidases; found on very short contig, additional genes in this cluster may exist	Unknown
HIGC8B	PTC7, PTC5	Two type III polyketide synthases; portion of this HIGC is identical to HIGC8A	marinone
HIGC9	N/A	indole PTase, phytoene synthase	Unknown
HIGC10	PTC1	full suite of phenazine biosynthesis genes (*phzABCDEFG*)	lavanducyanin
HIGC11	PTC2	squalene-hopene cyclase	Unknown

4.4.5 Evidence for PTase rearrangement within the MAR4 clade

In most cases, all of the PTases that fall within a single PTC occur in the same HIGC (Figure 4.3). There are, however, three notable exceptions (PTCs 2, 8, and 11), where orthologous PTases are found in different gene clusters. Most notably, the four sequences that clade together in PTC2 are found in three different HIGCs (Figure 4.6). In strain CNQ-509, the PTC2 sequence is found in an HIGC that contains two additional PTCs (11 and 8), while in CNB-632 and

CNH-099 the orthologous PTC2 enzymes are found in the same cluster as a PTC12 ortholog. In CNX-435, PTC2 is the only PTase observed. The presence of the same PTase different gene clusters suggests they are involved in the production of distinct HIs.

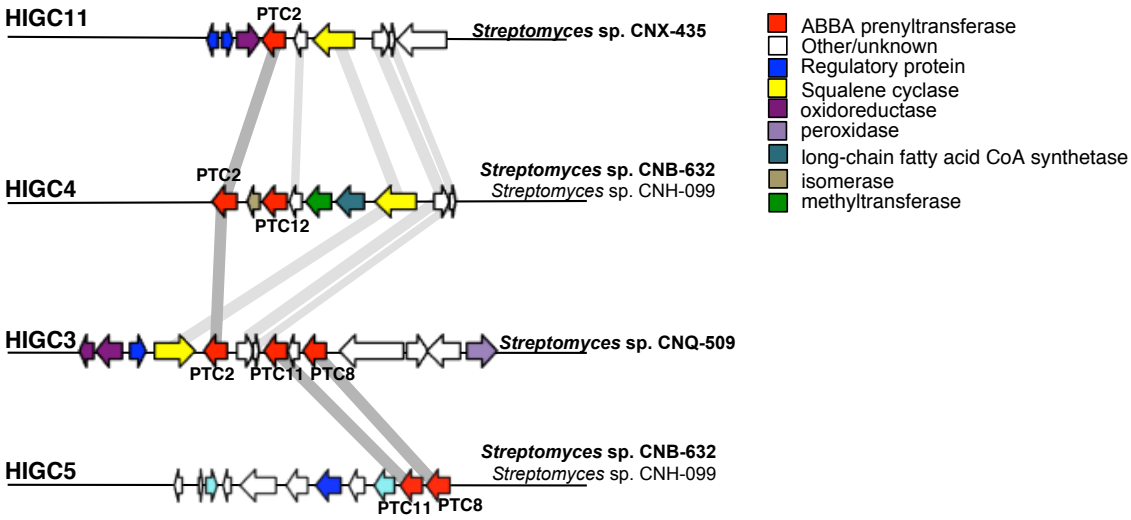


Figure 4.6 PTase rearrangement within MAR4 strains. Four HIGCs are illustrated with putative gene functions (predicted by pfam annotations) indicated by color. Homologs are indicated by gray bars. ABBA PTases are shown in red, and the PTC associated with each PTase indicated. The strain(s) containing each HIGC are labeled.

4.4.6 Mevalonate pathway distribution

The 120 *Streptomyces* genomes were examined for the presence of the six genes that compose the *mev* pathway to determine if it is frequently associated with HI gene clusters. Thirteen genomes were found to contain the complete *mev* pathway (Table 4.3), which was never found more than once in a single genome. Of these thirteen strains, eleven also contained at least one ABBA PTase. In all of these cases the *mev* pathway was found in close proximity to a PTase. In MAR4 strains, the *mev* pathway was often found on a very short contig, and it was only present in strains containing the *nap* gene cluster. Two

marine-derived strains, *Streptomyces* sp. TAA-040 and *Streptomyces* sp. CNT-372, contain the mevalonate pathway, but no ABBA PTase. In these strains the mevalonate pathway was found in close proximity to genes that may be involved in terpene biosynthesis (i.e. terpene synthase, non-ABBA PTases). Thus, it appears that when present, the *mev* pathway is generally associated with genes for secondary metabolism.

Table 4.3 List of strains containing a complete set of mevalonate pathway genes, the location of the pathway in each genome, and the distance from the pathway to the nearest ABBA PTase.

Strain	Location of mevalonate pathway (JGI gene IDs)	Proximity to ABBA PTases
Streptomyces davawensis JCM 4913	2562412117-2562412112	1303 bp (1 ORF)
Streptomyces griseoflavus Tu4000	645416842-645416847	69 bp (0 ORFs)
Streptomyces sp. 303MFCol5.2	2521683690-2521683695	7,931 bp (5 ORFs)
Streptomyces sp. CNH-189	2515835853-2515835858	18,320 bp (13 ORFs)
Streptomyces sp. CNQ-865 (MAR4)	2524600726-2524600731	16,532 (13 ORFs)
Streptomyces sp. CNP-082 (MAR4)	2527270597-2527270602	Unknown - short contig
Streptomyces sp. CNQ-766 (MAR4)	2518006507-2518006512	Unknown - short contig
Streptomyces sp. CNS-335 (MAR4)	2518013953-2518013958	Unknown - short contig
Streptomyces sp. CNT-371 (MAR4)	2516111891-2516111896	Unknown - short contig
Streptomyces sp. CNQ-525 (MAR4)	2562123501-2562123506	16,498 bp (13 ORFs)
Streptomyces sp. CNY-243 (MAR4)	2518518916-2518518922	Unknown - short contig
Streptomyces sp. TAA-040	2524957321-2524957326	N/A
Streptomyces sp. CNT-372	2518017234-2518017240	N/A

4.5 Discussion

Remarkable progress has been made in recent years using microbial genome sequences to define the mechanisms that govern the evolution of secondary metabolite biosynthetic pathways (Doroghazi et al., 2014; Medema et al., 2014; Ziemert et al., 2014). For the most part, these studies have focused on major classes of the best-characterized biosynthetic gene clusters, such as those responsible for the biosynthesis of non-ribosomal peptides and polyketides. The distribution and evolution of HI pathways, in contrast, has not previously been

examined, and PTases are not currently used as markers in tools aimed at locating secondary metabolite gene clusters, such as NaPDos (Ziemert et al., 2012) and antiSMASH (Blin et al., 2013). Additionally, few previous studies have focused on the genomes of closely related *Streptomyces* strains, which provides opportunities to address enzyme and pathway evolution.

By using a large dataset of streptomycete genomes, we were able to test the hypotheses that the ability to produce HIs is relatively rare in streptomycetes and that MAR4 streptomycetes are enriched in genes encoding HI production. The analysis does not include the unrelated PTase CnqPT1, which is membrane-bound and responsible for the O-prenylation of phenazine in the biosynthesis of marinophenazine (Zeyhle et al., 2014). To the best of our knowledge, CnqPT1 is the only non-ABBA PTase known to be involved in the production of HI secondary metabolites.

Analysis of genome sequences revealed that a minority of streptomycetes (29%) contain ABBA PTases and that MAR4 strains possess considerably more than other *Streptomyces* strains (Figure 4.1). This observation suggests that MAR4 strains produce more HIs than other streptomycetes. Interestingly, all but one of the MAR4 PTases falls within the Orf2 class, perhaps because the products of gene clusters containing Orf2 PTases provide a selective advantage specific to the environmental niche occupied by MAR4 strains. A phylogeny of the 120 *Streptomyces* strains that were a part of this study revealed that the distribution of PTase sequences is relatively scattered throughout the genus, with

the exceptions of the MAR4 clade and the clade of eight strains that includes the model organism *S. coelicolor* A3(2) (Figure 4.2). It would be interesting to determine if HI production is serving a similar function for members of these two clades. The largely scattered distribution of ABBA PTases in the genus phylogeny supports the hypothesis that these genes are not a conserved feature of the genus, and suggests that their presence in individual genomes is the result of horizontal gene transfer (HGT). Importantly, the closest relatives of the MAR4 clade do not consistently contain ABBA PTases. This suggests the relatively large number of these genes in MAR4 strains is the result of HGT at some point during the evolutionary history of the lineage.

A PTase phylogeny was generated to assess the evolutionary history of these genes in relation to the strains in which they reside. Although the Orf2 and indole ABBA PTases are thought to share a common ancestry, they do not share enough sequence homology to construct a reliable phylogeny of the two classes together. A phylogeny of the Orf2 PTases alone (Figure 4.3) reveals that there has been extensive HGT of PTases within the *Streptomyces* genus as a whole, as the overall PTase phylogeny bears little congruence to the *Streptomyces* genus phylogeny (Figure 4.2). Additionally, none of the MAR4 PTCs clade with PTases found in closely related *Streptomyces* strains, which provides further support for the hypothesis that HGT led to PTase acquisition by the MAR4 clade. For example, the Orf2 phylogeny suggests that HGT has occurred between

MAR4 strains and a strain (*S*. sp. CNH-189) in the distant *S. coelicolor* clade (Figure 4.3).

None of the PTCs contain more than one sequence from a single MAR4 strain (Figure 4.3) eliminating the possibility that PTase diversity arose from relatively recent duplication events. Two PTases are present only in strain CNQ-509 (PTC5 in the Orf2 phylogeny and the single indole prenyltransferase), which is likely the result of recent acquisition events. A likelihood analysis predicts that none of the PTCs were present in the MAR4 ancestor (Figure 4.4). An alternative hypothesis to this is that PTases were present in the MAR4 ancestor and subsequently lost in certain lineages. Regardless of when the PTCs were acquired, the fact that individual PTC phylogenies are largely congruent with the MAR4 phylogeny indicates that once acquired, these enzymes were subsequently inherited vertically. The maintenance of certain PTases in most MAR4 strains suggests that the functions of the HIs they produce are important for these organisms.

Gene duplication also appears to have played an important role in the evolutionary history of the MAR4 PTases. The clearest case of this is the sister clades PTC7 and PTC8. The phylogenies of the PTases found in these two clades are congruent, indicating that these have arisen as a result of duplication prior to MAR4 diversification (Figure 4.3). Interestingly, members of these two paralogous clades are found in different HIGCs. This suggests that they have

moved into different HIGCs subsequent to the duplication event and are now involved in the prenylation of different HI molecules.

Surprisingly, we found that some orthologous PTases occur in different HIGCs, which implies they are involved in the biosynthesis of distinct molecules (Figure 4.6). Thus, PTase phylogeny is not a good predictor of HIGC content and therefore secondary metabolite production. ABBA PTases frequently accept a range of substrates *in vitro* (Bonitz et al., 2011), which could mean they are easily incorporated into new biosynthetic gene clusters where they are responsible for the prenylation of a different scaffold. PTase rearrangement in the genomes of such closely related strains could be a mechanism by which novelty in HI secondary metabolites has arisen.

Lavanducyanin, nitropyrrolin, and marinone are HIs produced by MAR4 strains that have not been connected with a gene cluster. All of the experimentally characterized PTases that are related to MAR4 PTases prenylate phenazines and napthoquinones (Bonitz et al., 2011). Only two of the identified HIGCs contain the biosynthetic machinery required to build these types of molecules (Table 4.2), HIGC10 and HIGC8B. HIGC10 contains all of the genes required to build phenazines (*phzA-G*), but surprisingly the PTase in this cluster (PTC1) does not clade with the characterized sequences EpzP and PpzP, which are known to prenylate phenazine scaffolds (Bonitz et al., 2011). HIGC8B contains a type III polyketide synthase, suggesting that this cluster may be responsible for production of the napthoquinone molecule marinone, which is

known to be produced by strain CNQ-509 (Gallagher et al., 2013). Careful mutagenesis experiments will be required to confirm this hypothesis. Interestingly, the strains containing HIGC8A are also known to produce marinones (Gallagher et al., 2013). Because this pathway is found on a very short contig (Table 4.2), further sequencing efforts could reveal biosynthetic machinery similar to that found in HIGC8B.

With the exceptions of *phz* and type III polyketide-containing pathways, it is difficult to predict the types of molecules produced by HIGCs. There are previous examples of PTases that are not clustered with the genes required to produce the remainder of the HI scaffold (Haagen et al., 2006; Zeyhle et al., 2014), which could also be the case for some of the identified HIGCs. One interesting pattern revealed by this analysis is that when multiple PTases are found in the same HIGC, as is the case for HIGCs 3, 4, 5, and 8B, they are distantly related to each other on the PTase phylogeny (Figure 4.3). There are no examples of duplicated PTases in the same HIGC, suggesting that that clusters containing multiple PTases acquired each separately through genetic re-arrangement or HGT.

Here we also report the distribution of the *mev* pathway in the set of 120 *Streptomyces* genomes (Table 4.3). Only thirteen strains contained the *mev* pathway, including 11 of the 35 strains that contain an ABBA PTase. Seven MAR4 strains are predicted to contain the *mev* pathway. In many of these strains the genes of the *mev* pathway were found on very short contigs, making it

impossible to determine if the mevalonate pathway is flanking a HI gene cluster. However, the same seven MAR4 strains also contain the *nap* pathway, which is known to use the mevalonate pathway to supply isoprene (Winter et al., 2009). It is therefore likely that in these strains the mevalonate pathway is associated with the *nap* gene cluster, though confirmation of this would require further sequencing efforts. One additional MAR4 strain, CNQ-329, contains the *nap* gene cluster as well as five out of the six genes of the mevalonate pathway (data not shown). For the purposes of this study, this strain did not meet the criteria required to confirm a match to the mevalonate pathway, but further sequencing efforts would likely show that this strain does in fact contain the entire pathway associated with the *nap* cluster. In the two strains that contain the *mev* pathway but no ABBA PTase, the proximity of the *mev* pathway to genes associated with production of terpenoids suggests that these strains could be producing a secondary metabolite using isoprene derived from the mevalonate pathway, or that an HI is being produced by an as-yet uncharacterized type of PTase. In all other cases, the mevalonate pathway was found in very close proximity to an ABBA PTase (Table 4.3). These observations lend support to the hypothesis that when present in streptomycete genomes, the mevalonate pathway provides isoprene for secondary metabolism. The infrequency of the pathway in these genomes, including a majority (68%) of the ABBA PTase-containing genomes, indicates that this pathway is not an ideal marker for HI production.

Overall, the results of this study support the hypothesis that MAR4 strains contain more HIs than other streptomycetes. We also show that gene duplication, HGT, and gene rearrangement are likely involved in the evolution of distinct HIGCs and, by extension, the generation of HI chemical novelty. Secondary metabolites have previously been linked to functional adaptation in actinobacteria (Penn et al., 2009), therefore the accumulation of HIs in this clade could be related to colonization of a particular environmental niche. The goal of understanding why such a diversity of HI pathways have evolved and been maintained in MAR4 strains will require the challenging task of linking these molecules to ecological roles in the environment.

4.6 Acknowledgements

Thanks to Lutz Heide for providing the genome sequence of strain CNQ-509, and to the Joint Genome Institute for providing sequencing, assembly, and annotation of eleven genomes. I also thank Nadine Ziemert and Greg Rouse for helpful discussions about the data. Anindita Sarkar, Natalie Millán-Aguiñaga, Krystle Chavarria, Kevin Penn, Kelle Freel, Nadine Ziemert, and Eun Ju Choi assisted with DNA extractions.

Chapter 4 is currently being formulated into a manuscript that will be submitted for publication in 2015. Kelley Gallagher and Paul Jensen. The dissertation author was the primary investigator on these studies.

4.7 References

Berdy, J. (2005) Bioactive microbial metabolites - A personal view. *J. Antibiot. (Tokyo).* **58**: 1–26.

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013) antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Res.* **41**: W204–W212.

Bonitz, T., Alva, V., Saleh, O., Lupas, A.N., and Heide, L. (2011) Evolutionary relationships of microbial aromatic prenyltransferases. *PLoS One* **6**: e27336.

Bringmann, G., Haagen, Y., Gulder, T.A.M., Gulder, T., and Heide, L. (2007) Biosynthesis of the isoprenoid moieties of furanonaphthoquinone I and endophenazine A in *Streptomyces cinnamonensis* DSM 1042. *J. Org. Chem.* **72**: 4198–4204.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009) BLAST+: architecture and applications. *BMC Bioinformatics* **10**: 421.

Cho, J.Y., Kwon, H.C., Williams, P.G., Jensen, P.R., and Fenical, W. (2006) Azamerone, a terpenoid phthalazinone from a marine-derived bacterium related to the genus *Streptomyces* (actinomycetales). *Org. Lett.* **8**: 2471–2474.

Darriba, D., Taboada, G.L., Doallo, R., and Posada, D. (2011) ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* **27**: 1164–5.

Doroghazi, J.R., Albright, J.C., Goering, A.W., Ju, K.-S., Haines, R.R., Tchalukov, K.A., Labeda, D.P., Kelleher, N.L., and Metcalf, W.W. (2014) A roadmap for natural product discovery based on large-scale genomics and metabolomics. *Nat. Chem. Biol.* **10**: 963–8.

Doroghazi, J.R. and Buckley, D.H. (2010) Widespread homologous recombination within and between *Streptomyces* species. *ISME J.* **4**: 1136–1143.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Fischbach, M.A., Walsh, C.T., and Clardy, J. (2008) The evolution of gene collectives: How natural selection drives chemical innovation. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 4601–4608.

Gallagher, K.A., Fenical, W., and Jensen, P.R. (2010) Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes. *Curr. Opin. Biotechnol.* **21**: 794–800.

Gallagher, K.A., Rauscher, K., Pavan Ioca, L., and Jensen, P.R. (2013) Phylogenetic and Chemical Diversity of a Hybrid Isoprenoid-producing Streptomycete Lineage. *Appl. Environ. Microbiol.* **79**: 6894–902.

Haagen, Y., Glueck, K., Fay, K., Kammerer, B., Gust, B., and Heide, L. (2006) A gene cluster for prenylated naphthoquinone and prenylated phenazine biosynthesis in *Streptomyces cinnamonensis* DSM 1042. *Chembiochem* **7**: 2016-2027.

Hardt, I.H., Jensen, P.R., and Fenical, W. (2000) Neomarinone, and new cytotoxic marinone derivatives, produced by a marine filamentous bacterium (actinomycetales). *Tetrahedron Lett.* **41**: 2073–2076.

Heide, L. (2009) Prenyl transfer to aromatic substrates: genetics and enzymology. *Curr. Opin. Chem. Biol.* **13**: 171–179.

Izumikawa, M., Khan, S.T., Takagi, M., and Shin-Ya, K. (2010) Sponge-Derived *Streptomyces* Producing Isoprenoids via the Mevalonate Pathway. *J. Nat. Prod.* **73**: 208–212.

Jensen, P.R. and Fenical, W. (2005) New natural-product diversity from marine actinomycetes. *Nat. Prod. Drug Discov. Ther. Med.* 315–328.

Kawasaki, T., Kuzuyama, T., Furihata, K., Itoh, N., Seto, H., and Dairi, T. (2003) A relationship between the mevalonate pathway and isoprenoid production in actinomycetes. *J. Antibiot. (Tokyo).* **56**: 957–966.

Khan, S.T., Izumikawa, M., Motohashi, K., Mukai, A., Takagi, M., and Shin-Ya, K. (2010) Distribution of the 3-hydroxyl-3-methylglutaryl coenzyme A reductase gene and isoprenoid production in marine-derived Actinobacteria. *FEMS Microbiol. Lett.* **304**: 89–96.

Kondratyuk, T.P., Park, E.-J., Yu, R., van Breemen, R.B., Asolkar, R.N., Murphy, B.T., Fenical, W., and Pezzuto, J.M. (2012) Novel marine phenazines as potential cancer chemopreventive and anti-inflammatory agents. *Mar. Drugs* **10**: 451–64.

Kuzuyama, T., Noel, J.P., and Richard, S.B. (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* **435**: 983–987.

Kuzuyama, T. and Seto, H. (2003) Diversity of the biosynthesis of the isoprene units. *Nat. Prod. Rep.* **20**: 171–183.

Kwon, H.C., Espindola, A.P.D.M., Park, J.-S., Prieto-Davo, A., Rose, M., Jensen, P.R., and Fenical, W. (2010) Nitropyrrolins A-E, Cytotoxic Farnesyl-alpha-nitropyrroles from a Marine-Derived Bacterium within the Actinomycete Family Streptomycetaceae. *J. Nat. Prod.* **73**: 2047–2052.

Leipoldt, F., Zeyhle, P., Kalinowski, J., Heide, L., and Kaysser, L. Diversity of ABBA prenyltransferases in marine *Streptomyces* sp. CNQ-509: Promiscuous enzymes for the biosynthesis of mixed terpenoid compounds. *Prep.*

Maddison, W.P. and Maddison, D.R. (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75.

Medema, M.H., Cimermancic, P., Sali, A., Takano, E., and Fischbach, M.A. (2014) A Systematic Computational Analysis of Biosynthetic Gene Cluster Evolution: Lessons for Engineering Biosynthesis. *PLoS Comput. Biol.* **10**: e1004016.

Medema, M.H., Takano, E., and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Mol. Biol. Evol.* **30**: 1218–23.

Pathirana, C., Jensen, P.R., and Fenical, W. (1992) Marinone and debromomarinone - antibiotic sesquiterpenoid naphthoquinones of a new structure class from a marine bacterium. *Tetrahedron Lett.* **33**: 7663–7666.

Penn, K., Jenkins, C., Nett, M., Udwary, D.W., Gontang, E.A., McGlinchey, R.P., Foster, B., Lapidus, A., Podell, S., Allen, E.E., Moore, B.S., and Jensen, P.R. (2009) Genomic islands

link secondary metabolism to functional adaptation in marine Actinobacteria. *ISME J.* **3**: 1193–1203.

Saleh, O., Haagen, Y., Seeger, K., and Heide, L. (2009) Prenyl transfer to aromatic substrates in the biosynthesis of aminocoumarins, meroterpenoids and phenazines: The ABBA prenyltransferase family. *Phytochemistry* **70**: 1728–1738.

Schultz, A.W., Lewis, C.A., Luzung, M.R., Baran, P.S., and Moore, B.S. (2010) Functional Characterization of the Cyclomarin/Cyclomarazine Prenyltransferase CymD Directs the Biosynthesis of Unnatural Cyclic Peptides. *J. Nat. Prod.* **73**: 373–377.

Seipke, R.F., Kaltenpoth, M., and Hutchings, M.I. (2012) *Streptomyces* as symbionts: an emerging and widespread theme? *Fems Microbiol. Rev.* **36**: 862–876.

Silvestro, D. and Michalak, I. (2011) raxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* **12**: 335-337.

Swofford, D.L. PAUP*: phylogenetic analysis using parsimony, version 4.0b10. 2003.

Watve, M.G., Tickoo, R., Jog, M.M., and Bhole, B.D. (2001) How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* **176**: 386–390.

Winter, J.M., Jansma, A.L., Handel, T.M., and Moore, B.S. (2009) Formation of the Pyridazine Natural Product Azamerone by Biosynthetic Rearrangement of an Aryl Diazoketone. *Angew. Chemie* **121**: 781–784.

Winter, J.M., Moffitt, M.C., Zazopoulos, E., McAlpine, J.B., Dorrestein, P.C., and Moore, B.S. (2007) Molecular basis for chloronium-mediated meroterpene cyclization - Cloning, sequencing, and heterologous expression of the napyradiomycin biosynthetic gene cluster. *J. Biol. Chem.* **282**: 16362–16368.

Zeyhle, P., Bauer, J.S., Steimle, M., Leipoldt, F., Rösch, M., Kalinowski, J., Gross, H., and Heide, L. (2014) A Membrane-Bound Prenyltransferase Catalyzes the O-Prenylation of 1,6-Dihydroxyphenazine in the Marine Bacterium *Streptomyces* sp. CNQ-509. *Chembiochem* **15**: 2385–92.

Ziemert, N., Lechner, A., Wietz, M., Millán-Aguiñaga, N., Chavarria, K.L., and Jensen, P.R. (2014) Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proc. Natl. Acad. Sci. U. S. A.* **111**: E1130–9.

Ziemert, N., Podell, S., Penn, K., Badger, J.H., Allen, E., and Jensen, P.R. (2012) The Natural Product Domain Seeker NaPDoS: A Phylogeny Based Bioinformatic Tool to Classify Secondary Metabolite Gene Diversity. *PLoS One* **7**: e34064.

Chapter 5: Microaerophilic conditions cue changes in secondary metabolite production in a marine-derived *Streptomyces* strain

5.1 Abstract

Members of the actinomycete genus *Streptomyces* are non-motile, filamentous bacteria that are generally considered obligate aerobes. They have primarily been isolated from highly heterogeneous environments, including soils and marine sediments, yet little is known about how these organisms grow or survive in microaerophilic conditions. Here we investigate the role of secondary metabolism in streptomycete adaptation to low oxygen environments by tracking secondary metabolite production by a marine *Streptomyces* strain, CNQ-525, in continuous culture as oxygen concentration was lowered. We found clear evidence for a shift in secondary metabolism that was linked to the napyradiomycin biosynthesis pathway, which under reduced oxygen produced a significantly higher amount of a predicted pathway intermediate, 8-amino-flaviolin. Napyradiomycin and 8-amino-flaviolin possess fundamentally different solubility and bioactivity, which suggests that depending on local environmental conditions, a single biosynthetic pathway can produce distinct products for different functions. Because molecules similar to 8-amino-flaviolin are known to function as endogenous electron shuttles, we tested the ability of strain CNQ-525 to reduce an insoluble electron acceptor. In a plate assay, this strain was able to reduce Mn(IV) via an indirect mechanism. Electrochemical studies of purified 8-amino-flaviolin confirm that this molecule is redox-active. The thermodynamic potential of the molecule, however, suggests that it cannot be reduced by NADH, as is the case for previously characterized electron shuttles. This study provides some of

the first evidence that secondary metabolism may play a role in *Streptomyces* adaptation to microaerophilic environments.

5.2 Introduction

The Gram-positive actinobacterial genus *Streptomyces* is comprised of nearly six hundred species (Gallagher et al., 2010) that have been cultured from diverse ecological niches. Streptomycetes can produce an incredible diversity of secondary metabolites, reflected in the fact that this genus has been one of the most important sources of natural products for the pharmaceutical industry (Watve et al., 2001; Berdy, 2005). Members of this genus are non-motile, spore-forming, and grow as branching hyphae that form a vegetative mycelium. They have largely been isolated from highly heterogeneous environments, such as soils and marine sediments, but are also known as plant endophytes, pathogens, and invertebrate mutualists (Seipke et al., 2012).

Recently, a largely marine-derived lineage within the streptomycetes, called the 'MAR4' clade, has been described (Gallagher et al., 2013). Members of this clade are characterized by the production of a suite of molecules termed hybrid isoprenoids (HIs). HIs are biosynthetic hybrids that are partially composed of a terpenoid moiety derived from five-carbon isoprene units. These molecules frequently contain moieties known to be redox-active, such as quinones or phenazines, that bear intriguing similarities to molecules that function in redox cycling ("electron shuttles") in some Gram-negative bacteria. Additionally, the

general structure of these compounds brings to mind the respiratory lipoquinones, HIs which serve as electron shuttling molecules in primary metabolism.

Electron shuttling via endogenous shuttles is thought to be a mechanism that bacteria use to adapt to low-oxygen or anaerobic conditions, and has been primarily studied in the Gram-negative genera *Shewanella* and *Pseudomonas* (Rabaey et al., 2007). This process allows bacteria to maintain intracellular redox homeostasis in low-oxygen conditions (Price-Whelan et al., 2006) or may even directly connect quinone pools with extracellular electron acceptors during respiration (Brutinel and Gralnick, 2012). In either case, redox cycling by small molecules will result in the reduction of insoluble extracellular electron acceptors, such as Mn(III,IV) oxides and ferric iron, both of which are prevalent in marine sediments.

Currently, the most well-studied shuttling molecules include the flavins produced by members of the genus *Shewanella* (Brutinel and Gralnick, 2012) and phenazines produced by the genus *Pseudomonas* (Wang et al., 2010; Glasser et al., 2014). Much less is known about Gram-positive electron shuttling (Rabaey et al., 2007), and to our knowledge there are currently no well-characterized endogenous electron shuttles produced by Gram-positive bacteria. Considering that the structures of many HIs produced by MAR4 possess probable redox-active moieties, one potential function for these compounds is as electron shuttles. The idea that streptomycete secondary metabolites are produced for this purpose has previously been proposed based on the finding

that mutant strains of *Streptomyces coelicolor* A3(2) unable to produce the pigmented secondary metabolites actinorhodin and undecylprodigiosin exhibited phenotypic changes (large and wrinkly colony morphology) identical to those observed in a phenazine knockout strain of *Pseudomonas aeruginosa*, suggesting a widely conserved role for redox-active secondary metabolites (Dietrich et al., 2008).

Streptomycetes in marine or terrestrial environments almost certainly encounter periods of microaerophilic or anaerobic conditions. Streptomycetes are typically assumed to be obligate aerobes, however some *Streptomyces* strains are known to survive periods of anaerobiosis during both vegetative growth and as spores (van Keulen et al., 2007). In spores of *S. coelicolor*, there is evidence that membrane potential is maintained in anaerobic conditions through nitrate respiration (Fischer et al., 2013). The mechanisms by which *Streptomyces* survive anaerobic events during vegetative growth, however, remain unknown. This is significant because in *Pseudomonas aeruginosa*, redox-cycling of pigmented phenazine molecules have been shown to play a role in membrane potential maintenance, which promotes survival, but not growth, in anaerobic conditions (Wang et al., 2010; Glasser et al., 2014). Electron shuttling via redox-active 'secondary' metabolites is therefore an untested mechanism by which streptomycetes may survive microaerophilic or anaerobic conditions.

In order to examine the role of secondary metabolism as oxygen decreases, we tracked compound production by the MAR4 strain *Streptomyces*

sp. CNQ-525 under aerobic and microaerophilic conditions. Strain CNQ-525 has previously been shown to produce several distinct classes of HIs, including a suite of prenylated napthoquinones called napyradiomycins (Cheng et al., 2013; Gallagher et al., 2013). We isolated and structurally characterized the major compound that was up-regulated in low-oxygen conditions, which was identified as 8-amino-2,5,7-trihydroxynapthalene-1,4-dione (8-amino-flaviolin). We also found that strain CNQ-525 is able to reduce MnO_2 in the absence of direct contact with the mineral, suggesting the involvement of a diffusible compound. Using cyclic voltammetry, we found that 8-amino-flaviolin is indeed redox-active, making it a candidate for the observed manganese reduction. Interestingly, 8-amino-flaviolin is an intermediate in the napyradiomycin biosynthesis pathway, the final products of which are potent antibiotics (8-amino-flaviolin is not bioactive). These results suggest that a single biosynthetic pathway yields products with different ecological functions depending upon environmental conditions.

5.3 Materials and methods

5.3.1 Chemostat culture

Streptomyces sp. CNQ-525 was originally isolated off the coast of La Jolla, California, USA (Soria-Mercado et al., 2005). Mycelial stocks of this strain are maintained in 5% glycerol stored at -80°C. Prior to chemostat inoculation, a CNQ-525 stock was inoculated into 25 mL of A1 medium (10 g soluble starch, 4

g yeast extract, 2 g peptone, 750 mL seawater, 250 mL deionized water) and cultured at 27°C with shaking at 230 rpm for seven days.

A 10 mL aliquot of the pre-culture was used to inoculate a BioFlo110 Fermentor (Eppendorf Inc., Enfield, CT, USA) containing 1 L total volume of one-sixth strength A1 medium (1.67 g soluble starch, 0.67 g yeast extract, 0.33 g peptone, 750 mL seawater, 250 mL deionized water). The pH was maintained throughout the experiment at 7 (adjusted with 1M NaOH and 0.2M H_2SO_4), while the agitation was set at 400 rpm, the temperature at 27°C, and the gas flow at 1.5 Lpm. Oxygen levels were controlled by blending N_2 into the gas mix in order to adjust the air saturation of the culture. The vessel was inoculated at a dO_2 of 20% air saturation. The culture was grown to stationary phase, indicated by a peak in oxygen consumption by the culture (approx. 48 h after inoculation), at which point fresh media was added at a dilution rate of 0.045 h^{-1} (where dilution rate = medium flow rate/culture volume).

Two 175 mL samples were taken at a dO_2 of 20% air saturation, after which the air saturation was lowered to a dO_2 of 5%. An additional two 175 mL samples were taken at a dO_2 of 5% air saturation. Samples were taken at least 24 hours apart, and at least 48 hours after a major change in culture conditions (i.e. beginning of continuous culturing, change in air saturation), in order to ensure that the culture had stabilized at the time of each sample. Dry weights were used to monitor biomass of the culture through the course of the experiment. Immediately after sampling, triplicate 3 mL sub-samples were filtered onto a 47

mm glass fiber filter (Pall Life Sciences), dried at 80°C in an oven overnight, and weighed. Approx. 10 μL of each sample was plated to test purity of the culture. The remainder of each sample was frozen and stored at -20°C, so that organic extractions of all samples could be performed simultaneously.

5.3.2 Secondary metabolite analysis

Each sample was extracted in duplicate, with 85 mL sub-samples extracted with 170 mL of ethyl acetate. The organic layer was separated and dried under vacuum. Extracts were analyzed by high performance liquid chromatography HPLC (Hewlett-Packard series 1100) using UV detection, a reversed-phase C_{18} column (4.6 mm x 100 mm; 5 μm pore size; Phenomenex Luna), and a solvent gradient from 10% to 100% CH_3CN containing 0.1% trifluoroacetic acid (TFA) and water containing 0.1% TFA. To measure the production of a purple pigment that was observed in the culture vessel, absorbance was monitored at 540 nm in addition to the standard 254 nm wavelength. UV absorbance spectra associated with each peak were evaluated by comparison to an in-house spectral library. Because strain CNQ-525 is known to produce a suite of molecules in the napyradiomycin class, and this class of molecules possesses a distinct UV absorbance profile, a peak was assigned to this class if its top match was to a napyradiomycin standard with a match score >950, calculated using the Agilent Technologies (Santa Clara, CA, USA) ChemStation software.

5.3.3 Compound purification and structure elucidation

One major peak was observed in the 540 nm LC trace that was predicted to represent the purple pigment that was observed in the culture and culture extract. In order to obtain sufficient material for structure elucidation and electrochemistry experiments, the entire volumes of three 1 L bioreactor runs, as well as the spent media reservoir (~7.5 L), were extracted with an equal volume of ethyl acetate at the end of the run. The combined crude extract was dissolved in CH_3OH and the pigmented molecule was purified by HPLC (35% CH_3CN in water, 0.1% TFA, 13 mL/min flow rate) using a Waters 600E multisolvent delivery system, with reversed-phase C_{18} column (5 μm; Phenomenex Luna). For smaller amounts of crude material, a 250 mm x 10 mm column was used (flow rate 3 mL/min). For larger amounts of material, a 250 mm x 21.2 mm column was used (flow rate 13 mL/min). A bright purple compound was isolated, confirming that the targeted peak represents the observed pigment. ^{1}H NMR spectra and 2D spectra (HMBC, HSQC) were recorded at 500 MHz in DMSO-d_6 (residual solvent referenced to 2.50 ppm) on a Varian Inova 500 MHz NMR spectrometer. ^{13}C NMR spectra were recorded at 125 MHz in DMSO-d_6 (referenced to 39.5 ppm) on a Varian VX 500 NMR spectrometer.

5.3.4 Quantification of relative compound production

Areas for peaks representing napyradiomycin compounds were calculated based on chromatograms where absorbance was measured at 254 nm while areas for peaks representing 8-amino-flaviolin were calculated based on

absorbance at 540 nm. Peak integration was calculated by the ChemStation software. Biomass measurements were then used to calculate peak area per gram biomass. A paired students t-test was implemented using the web tool available at http://www.graphpad.com/quickcalcs/ttest1.cfm.

5.3.5 Manganese reduction assay

Sterile MnO_2 was prepared according to published protocol (Tebo et al., 2007). 10 mM MnO_2 was added to molten A1 agar media (16 g agar per L) and 10 mL poured into 100 mm x 15 mm plates. A 90 mm 0.2 μm filter (Whatman Nuclepore track-etched membrane) was placed on top of the solidified MnO_2-containing agar plates. An additional 15 mL of MnO_2-free molten agar was then poured on top of the filter so that bacteria plated on top of the agar would be effectively separated from the MnO_2 below. Strain CNQ-525 and *E. coli* (negative control) were streaked onto plates and allowed to grow for several weeks. Manganese reduction was observed as of zones of clearing in the MnO_2 layer associated with bacterial growth.

5.3.6 Electrochemical measurements

Cyclic voltammetry (CV) was performed using a BASi Epsilon e2 potentiostat with a scan rate of 100 mV/s. A 3 mm diameter BASi stationary voltammetry glassy carbon electrode was used as the working electrode. The counter electrode was a platinum wire, and a Ag/AgCl wire was used as a pseudo-reference electrode. Electrochemical solutions were prepared as a 2 mM solution of 8-amino-flaviolin in a 1 M KNO_3 aqueous electrolyte solution, buffered

with 10 mM 4-morpholinepropanesulfonic acid (MOPS). The pH was adjusted to 7 using HCl or NaOH and the solution was sparged with N_2 prior to data collection. The redox potential was characterized by the half-wave potential and potassium ferricyanide ($E_{1/2}$ = 436 mV vs NHE) was used as an internal standard.

5.4 Results

In order to analyze the effect of oxygen on secondary metabolite production, *Streptomyces* sp. CNQ-525 was grown in a bioreactor where dissolved oxygen levels could be carefully controlled. Once in continuous culture in the bioreactor, conditions remain constant, including, crucially, bacterial growth rate (Hoskisson and Hobbs, 2005). This means that the bacterial culture no longer advances through the standard growth curve, and samples taken 24 hours apart were treated as replicates. This setup allowed us to take replicate samples and manipulate oxygen levels during a single bioreactor run. The results reported here are representative of four individual bioreactor runs.

5.4.1 Growth in aerobic and microaerophilic conditions

Upon reaching stationary phase following batch growth in high-oxygen conditions, fresh medium was added to the culture at a constant dilution rate. Two replicate samples were taken from the bioreactor at least 24 hours apart for each of the two oxygen conditions (20% and 5% air saturation). No significant difference (paired t-test, p=0.9175) was seen in biomass between the high- and low- oxygen conditions (Figure 5.1), suggesting that lowering oxygen did not negatively affect CNQ-525 growth.

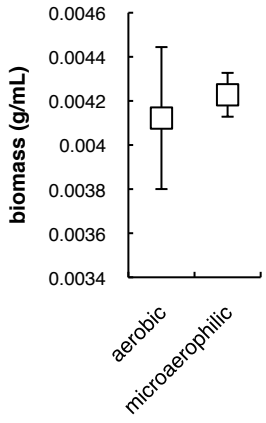


Figure 5.1 Biomass of strain CNQ-525 in aerobic versus microaerophilic conditions. Biomass is reported as the average of three sub-samples from two replicate samples taken 24 hours apart (p=0.9175, paired t-test).

5.4.2 Secondary metabolite production

During steady state conditions, strain CNQ-525 produced a purple-pigmented molecule that was visibly up-regulated in microaerophilic conditions (Figure 5.2). The ethyl acetate extracts, which clearly contained the pigment, were assessed by HPLC and one peak consistently increased in area between aerobic and microaerophilic conditions. A pink-purple compound is expected to absorb green-yellow wavelengths of light (~480-600 nm). Consistent with this, the absorption spectrum of the apparently up-regulated peak shows strong absorption from 500-600 nm (Figure 5.3), suggesting that it represents the target pigment. The only other consistent difference in secondary metabolite production between high- and low- oxygen conditions was a decrease in area for peaks representing a suite of molecules in the napyradiomycin class (Figure 5.3).

Napyradiomycins are prenylated napthoquinones that have previously been isolated from cultures of CNQ-525 (Winter et al., 2007; Cheng et al., 2013).

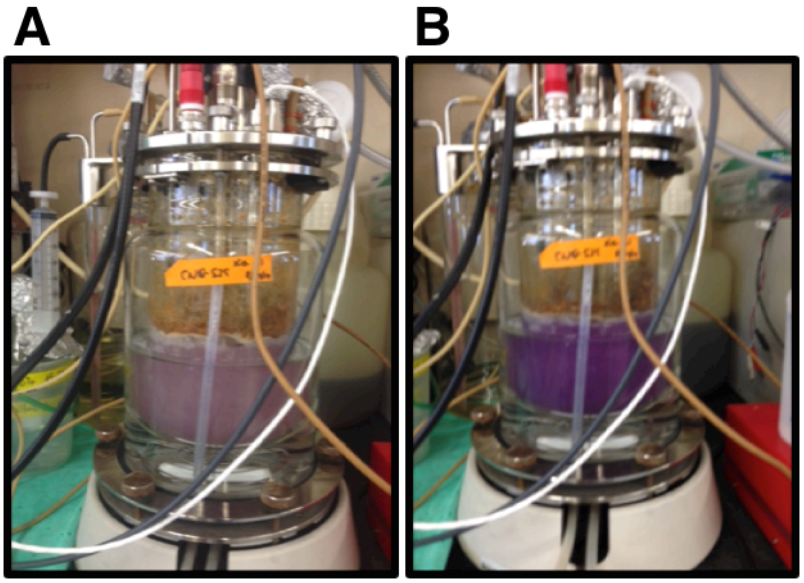


Figure 5.2 Photos of strain CNQ-525 culture grown in **(A)** aerobic and **(B)** microaerophilic conditions showing a visible increase in purple pigment production when oxygen levels are reduced.

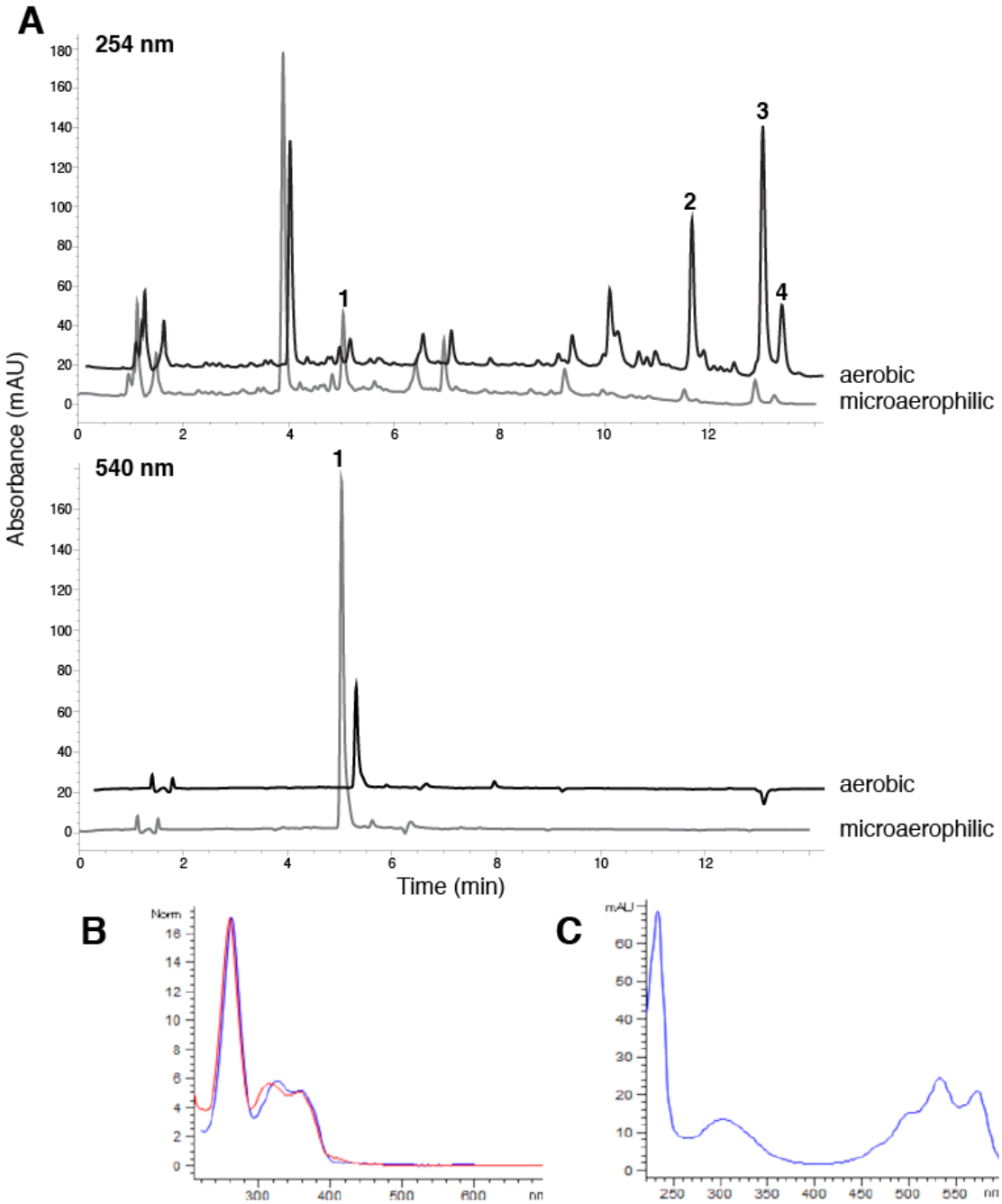


Figure 5.3 Secondary metabolite production in aerobic and microaerophilic conditions. **(A)** Representative HPLC UV traces for crude extracts (dissolved in equal amounts of solvent) of strain CNQ-525 growing in aerobic (gray) versus microaerophilic (black) conditions. To visualize all peaks, the two traces are offset by 1% in x and 10% in y. Peak 1 appears to be up-regulated in low-oxygen conditions, while peaks 2-4 are down-regulated. All other changes in peak area between microaerophilic and aerobic conditions were not consistent between individual bioreactor runs. **(B)** Absorption spectrum of peak **2** (red) compared with a napyradiomycin standard entry in an in-house library (blue). UV spectra of peaks **2**, **3**, and **4** all match very closely with known napyradiomycin derivatives, and so are predicted to represent molecules in this class. **(C)** Absorption spectrum of peak **1**, which is predicted to represent the visibly upregulated pigment.

5.4.3 Isolation and structure elucidation of 8-amino-flaviolin

Isolation and structure elucidation of the up-regulated pigment was carried out in collaboration with Prof. Chambers Hughes from Scripps Institution of Oceanography, who provided guidance on all analytical chemistry techniques. The target pigment was isolated as a purple solid by preparative HPLC. Mass and 2D NMR spectra of the purified compound were found to be consistent with that of 8-amino-2,5,7-trihydroxynapthalene-1,4-dione (8-amino-flaviolin) (Figure 5.4-5.9, Table 5.1). Residual TFA from the HPLC purification appears in the carbon NMR spectrum (Figure 5.5). We observed an *m/z* of 220.0252 (Figure 5.4) for the purified compound when measured in negative ion mode. The calculated *m/z* of 8-amino-flaviolin ($C_{10}H_7NO_5$) is consistent with this finding (*m/z* 220.0251) within the expected range of error (delta 0.5 ppm).

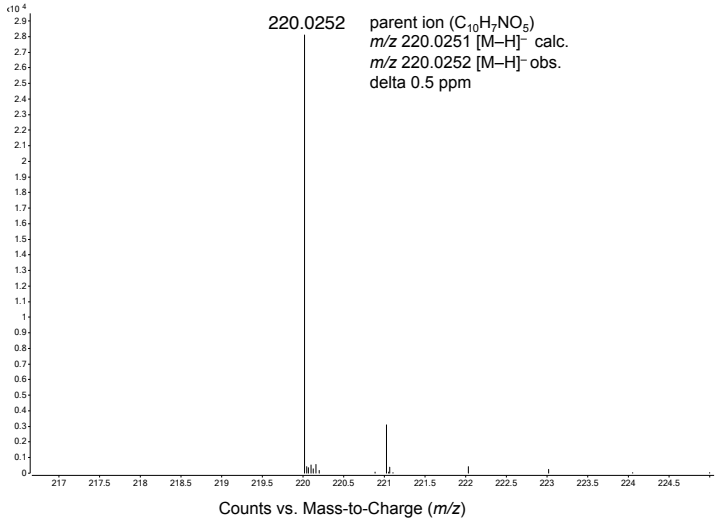


Figure 5.4 Mass spectrum of purified 8-amino-flaviolin in negative ion mode.

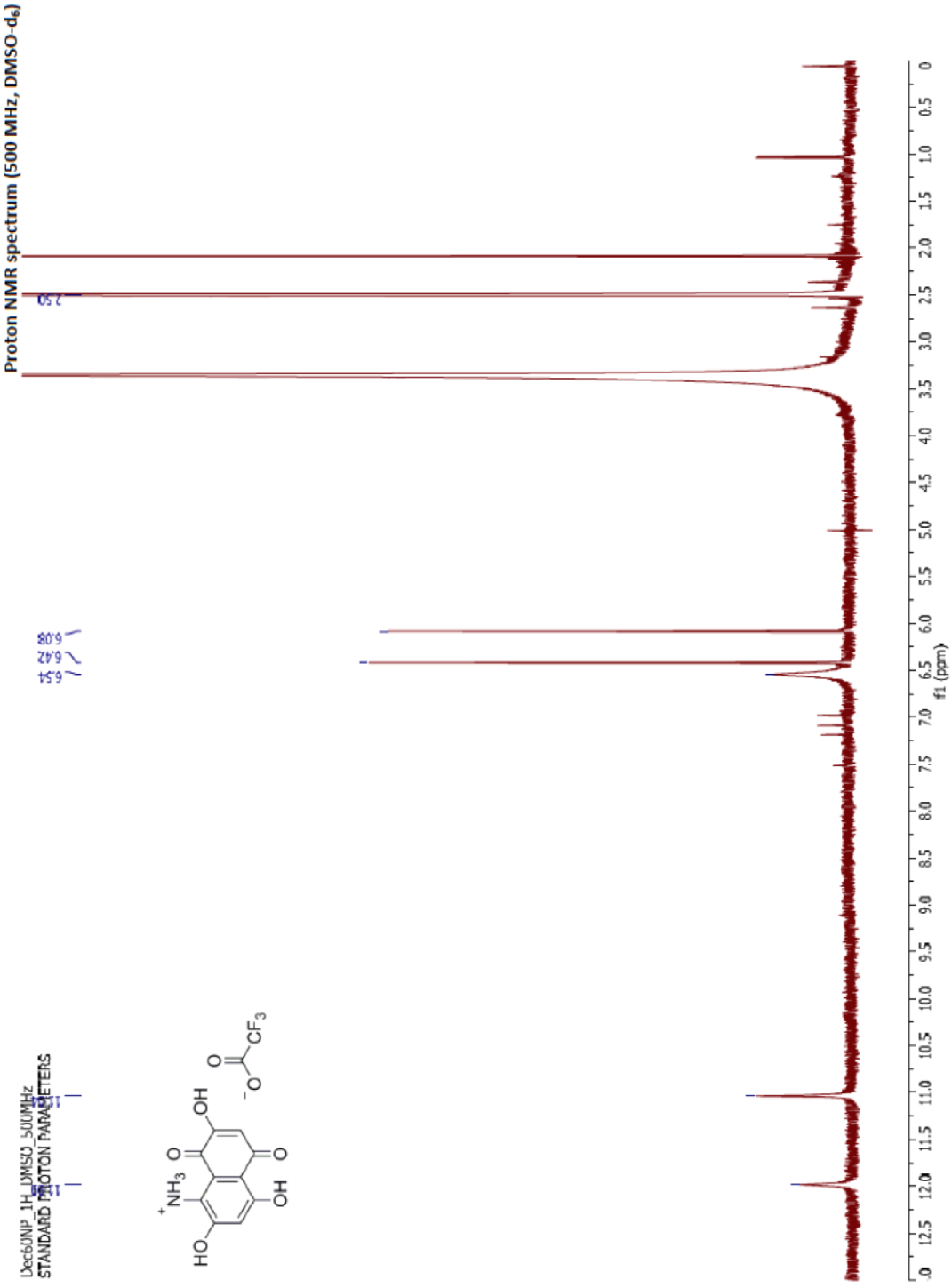


Figure 5.5 Proton NMR spectrum of 8-amino-flaviolin TFA salt. 500 MHz, DMSO-d_6. Structures depict 8-amino-flaviolin (left) and TFA (right).

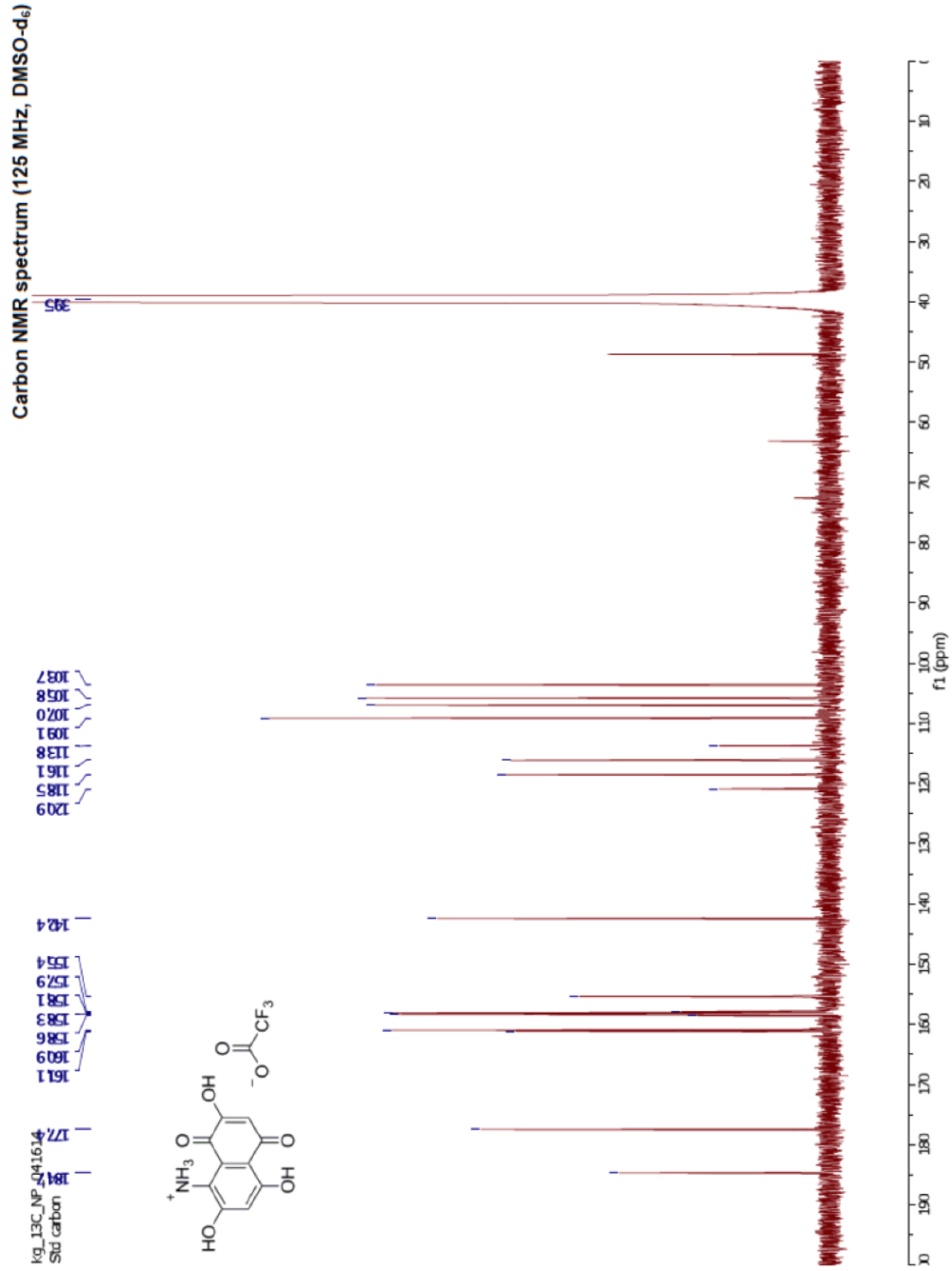


Figure 5.6 Carbon NMR spectrum of 8-amino-flaviolin TFA salt. 500MHz, DMSO-d_6. Structures depict 8-amino-flaviolin (left) and TFA (right).

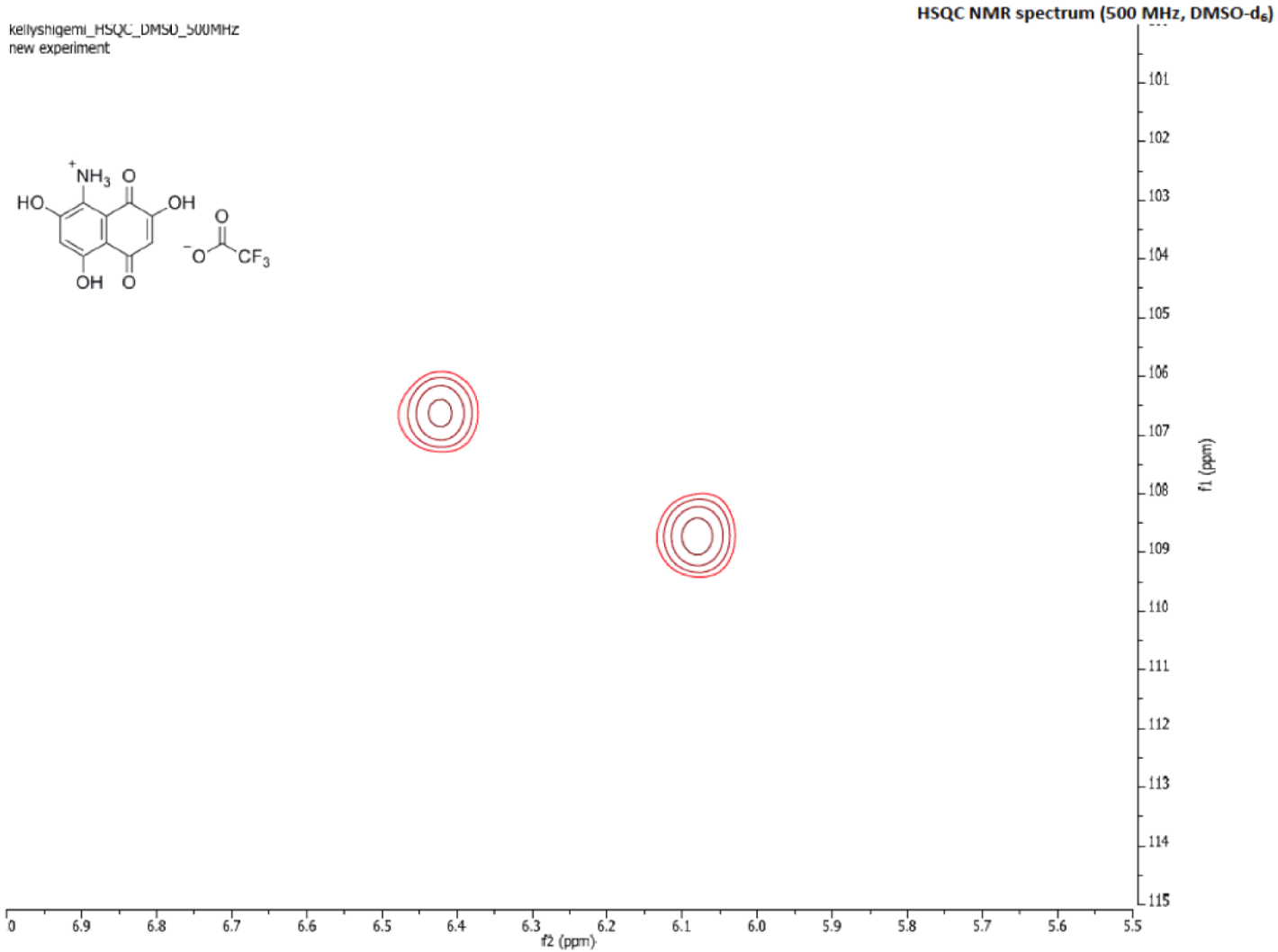


Figure 5.7 HSQC NMR spectrum of 8-amino-flaviolin TFA salt. 500 mHz, DMSO-d_6. Structures depict 8-amino-flaviolin (left) and TFA (right).

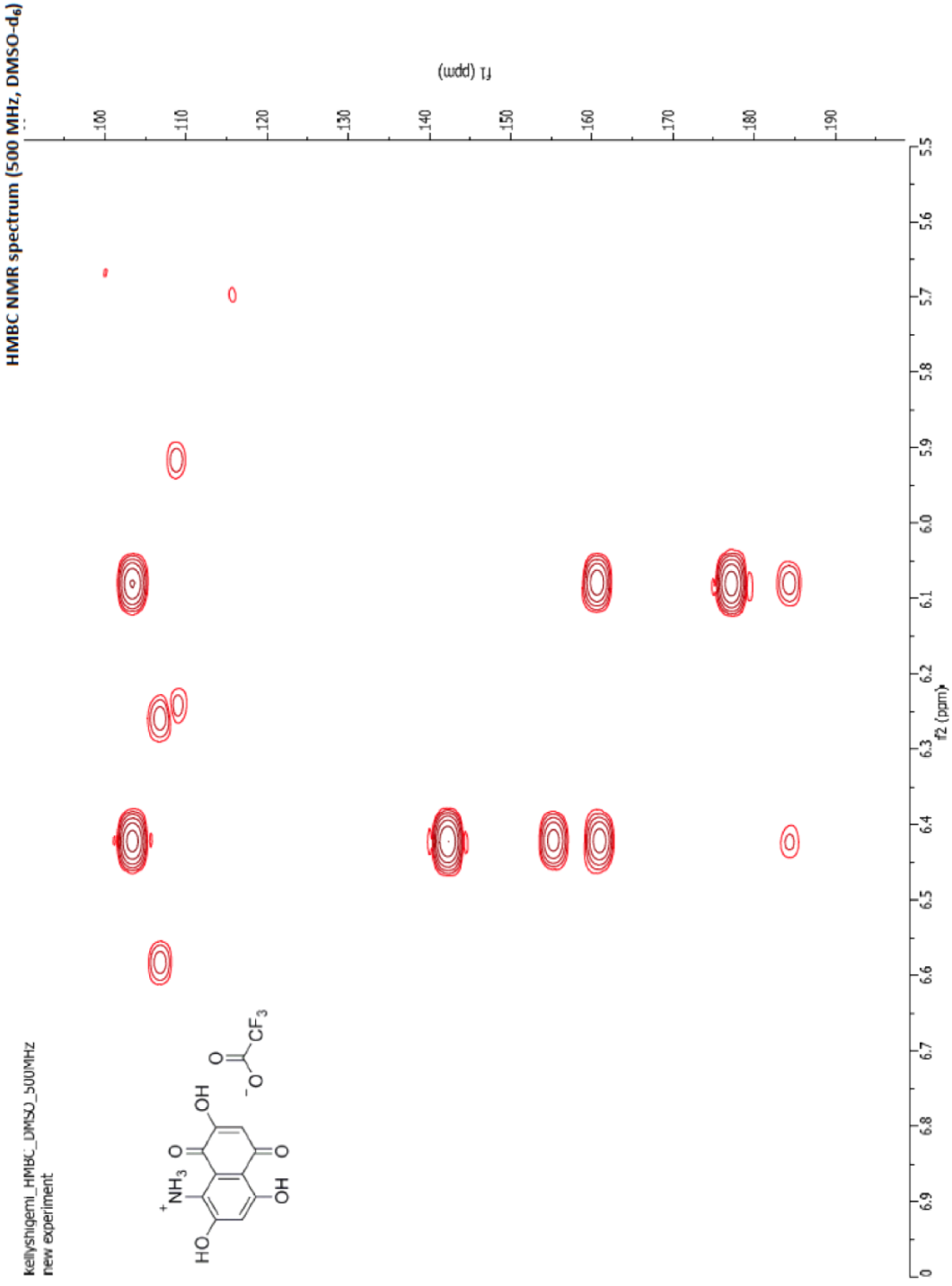


Figure 5.8 HMBC NMR spectrum of 8-amino-flaviolin TFA salt. 500 MHz, DMSO- d_6. Structures depict 8-amino-flaviolin (left) and TFA (right).

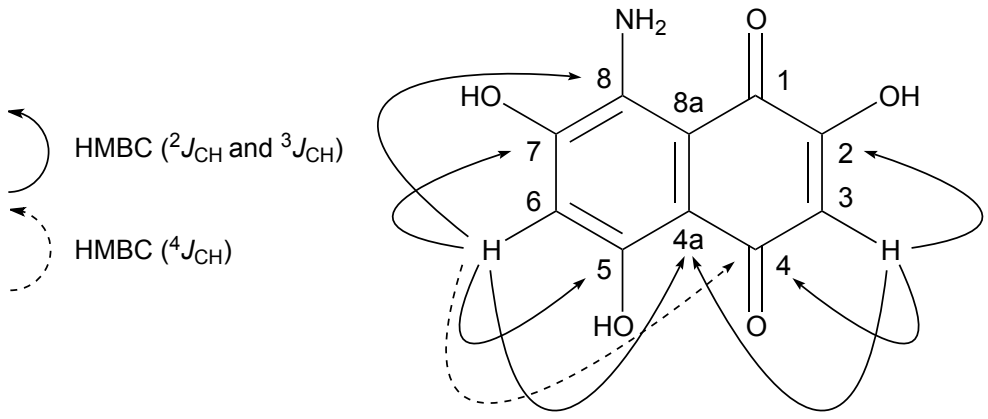


Figure 5.9 Structure of 8-amino-flaviolin, showing HMBC correlations.

Table 5.1 Tabulated NMR spectral data. s= singlet; br s = broad singlet.

position	δ_C[a]	δ_H, mult.[b]	HMBC[b]
1	177.4		
2	160.9		
3	109.1	6.08, s	1,2,4,4a
4	184.7		
4a	103.7		
5	161.1		
6	107.0	6.42, s	4,4a,5,7,8
7	155.4		
8	142.4		
8a	105.8		
5/6-OH, 8-NH_2		6.54 br s, 11.04 br s, 11.99 br s	

[a] 125MHz. [b] 500 MHz

Quantification of the relative amount of secondary metabolite production in the two conditions confirms a significant increase in the concentration of 8-amino-flaviolin under lower oxygen conditions (paired t-test, p=0.0008), and a significant decrease (paired t-test, p=0.0268) in molecules belonging to the napyradiomycin class (Figure 5.10).

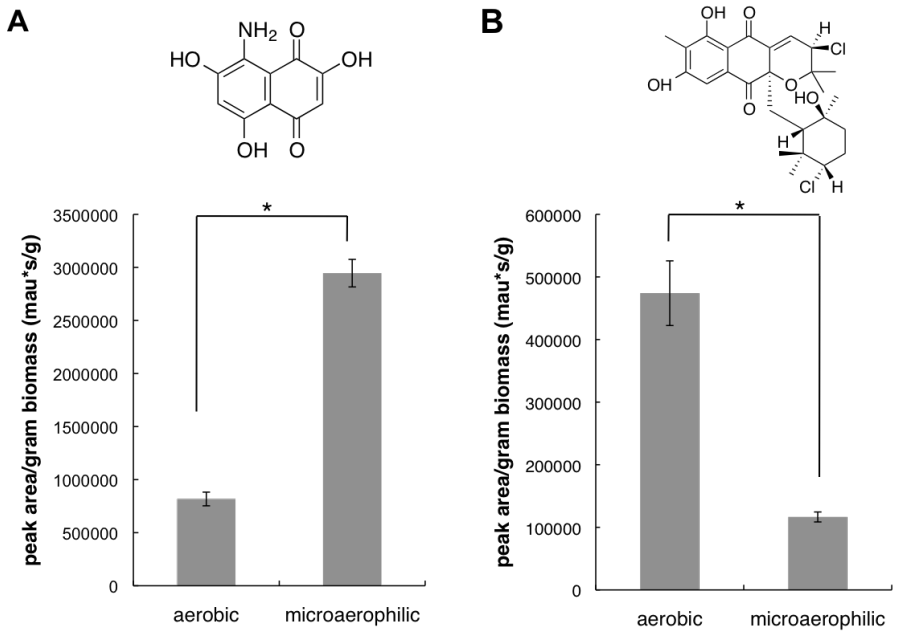


Figure 5.10 Summary of **(A)** 8-amino-flaviolin and **(B)** napyradiomycin production by strain CNQ-525 in aerobic versus microaerophilic conditions. Compound production for each condition is reported as the average of two samples taken 24 hours apart. The structure of 8-amino-flaviolin is depicted in A, and an example of a molecule in the napyradiomycin class is depicted in B. These results are representative of four individual bioreactor runs. p-values were calculated using a paired t-test. (*$p < 0.05$).

5.4.4 Biosynthesis of 8-amino-flaviolin and napyradiomycin

The structure of 8-amino-flaviolin was originally reported as a precursor molecule in the biosynthesis of furaquinocin (Isogai et al., 2012), a hybrid isoprenoid very similar to napyradiomycin compounds. The three genes that encode for the biosynthesis of 8-amino-flaviolin were identified as *fur1*, *fur2*, and *fur3* (Isogai et al., 2012). These three genes encode for a type III polyketide synthase, a monooxygenase, and an aminotransferase, respectively. The napyradiomycin (*nap*) pathway contains *napB1*, *B2*, and B3, which are homologs of *fur1*, *fur2*, and *fur3* (Figure 5.11). A MultiGeneBlast (Medema et al., 2013)

search of the CNQ-525 genome revealed that the napyradiomycin pathway was indeed the only place in the genome where homologs of these three genes are found. This suggests that under microaerophilic conditions, the *nap* pathway shifts compound production towards the 8-amino-flaviolin precursor (Figure 5.11).

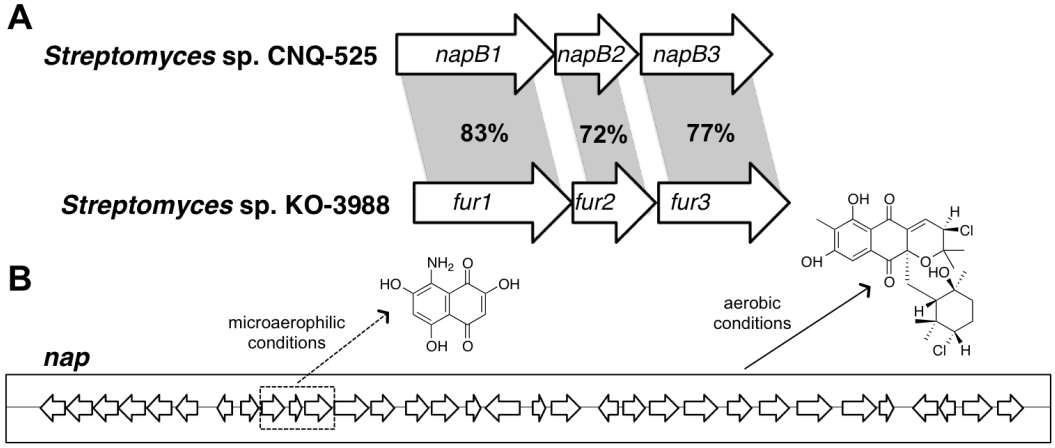


Figure 5.11 (A) Comparison of genes in the napyradiomycin (*nap*) pathway of *Streptomyces* sp. CNQ-525 to genes in the furaquinocin biosynthetic pathway of *Streptomyces* sp. KO-3988 that are responsible for the biosynthesis of 8-amino-flaviolin. Percent identities between homologous genes are indicated. **(B)** Entire *nap* pathway, adapted from Winter et al., 2007. Under microaerophilic conditions, the major product of the pathway is 8-amino-flaviolin, which is biosynthesized by NapB1, B2, and B3 (outlined in dashed box). In aerobic conditions, the major pathway product is a suite of napyradiomycin derivatives (example structure shown).

5.4.5 Bioactivity of 8-amino-flaviolin

We measured the bioactivity of 8-amino-flaviolin in two bioassays and compared the results to a previous study of napyradiomycins isolated from the same strain (Cheng et al., 2013). In the previous report, all but one napyradiomycin derivative possessed measureable cytotoxicity against the human colon carcinoma cell line HCT-116 and/or was inhibitory against methicillin-resistant *Staphylococcus aureus* (MRSA). In contrast, we found that 8-

amino-flaviolin possesses no cytotoxicity or antimicrobial activities in the same assays (data not shown).

5.4.6 CNQ-525 manganese (IV) reduction

We next tested if strain CNQ-525 could reduce an extracellular oxidant in the absence of physical contact. Strain CNQ-525 was inoculated onto petri plates physically separated from a layer of agar media containing 50 mM MnO_2. Oxidized manganese is insoluble, appears as a black solid, and upon reduction becomes soluble and clear. Strain CNQ-525 was able to reduce MnO_2 as evidenced by zones of clearing on the plates. The assay was repeated with *E. coli* as a negative control (Figure 5.12). The fact that the culture was physically separated from the MnO_2 indicates that strain CNQ-525 can reduce MnO_2 via a diffusible redox-active molecule.

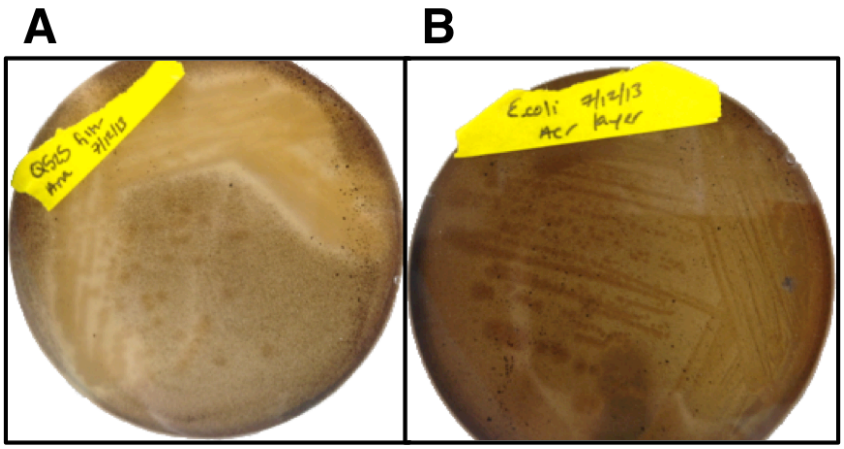


Figure 5.12 Manganese reduction assay. **(A)** Strain CNQ-525 reduces MnO_2 resulting in a clearing zone around the growing bacteria. **(B)** *E. coli* (negative control) shows no evidence of MnO_2 reduction.

5.4.7 Thermodynamic potential of 8-amino-flaviolin

Electrochemical experiments were performed in collaboration with Jane Henderson and Mark Llorente from the Kubiak Research Group in the Department of Chemistry and Biochemistry at the University of California, San Diego. Cyclic voltammetry was used to determine the redox activity of 8-amino-flaviolin. Using a potentiostat, the potential of an electrode was swept linearly with time in repeated cycles while current was recorded. The electrode was submerged in an electrolyte solution containing purified, oxidized 8-amino-flaviolin. During the sweep from more positive to more negative potentials, a peak in current reflects reduction of the compound. If the reduction is reversible, a corresponding peak will be observed during the opposite sweep reflecting the re-oxidation of the compound. Reduction and re-oxidation peaks were observed in aqueous solution at pH 7 (Figure 5.13), confirming that the compound is reversibly redox-active. The re-oxidation peak is slightly smaller in magnitude than the reduction peak. The midpoint between the reduction and re-oxidation peaks (the $E_{1/2}$ of the molecule), which is approximately equal to the formal reduction potential, was found to be –474.5 mV when standardized versus the normal hydrogen electrode (NHE) (Figure 5.13).

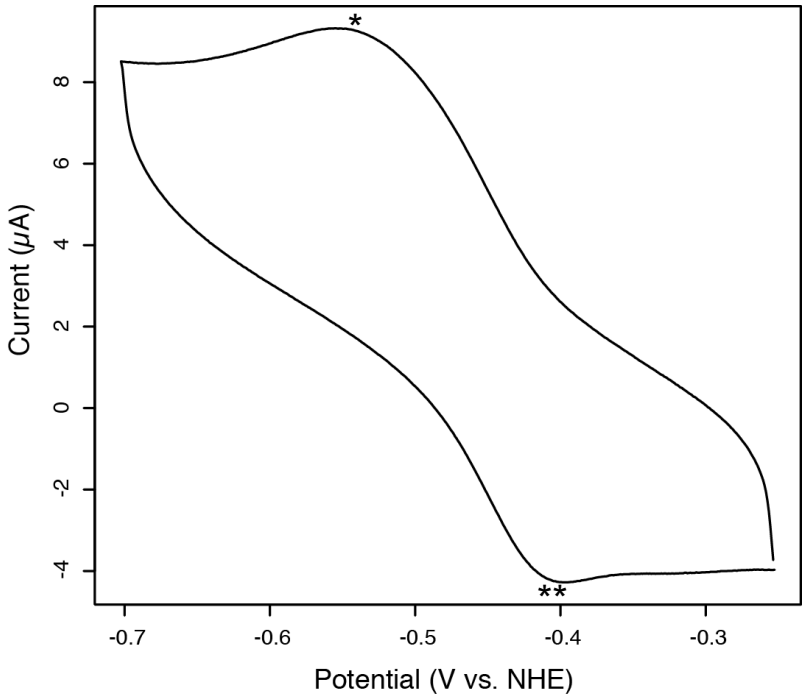


Figure 5.13 Cyclic voltammogram of 8-amino-flaviolin. The compound was dissolved in an electrolyte solution at pH 7. The $E_{1/2}$ vs. NHE was calculated using potassium ferricyanide (E°' = 436 mV vs. NHE at pH 7 (Clark, 1960)) as an internal standard. *reduction peak; **re-oxidation peak

5.5 Discussion

While hundreds of secondary metabolites have been isolated from *Streptomyces* spp. (Watve et al., 2001), it is rare for compounds to be connected to their ecological functions. This is due, in part, to the fact that most interest in secondary metabolites is related to their biomedical or industrial applications while little investment has been made in the development of methods to study functions in nature. Outside of iron acquisition and quorum sensing, very little is known about the role of secondary metabolites in surviving environmental change. This chapter addressed the hypothesis that some endogenous small

molecules can act as redox shuttles. While this has previously been explored in some Gram-negative bacteria (Rabaey et al., 2007), it has not yet been shown in streptomycetes. The results presented here provide evidence that the production of a redox-active compound by a *Streptomyces* strain increases in response to low oxygen conditions. We also show that a single biosynthetic pathway can yield compounds with fundamentally different biological activities depending on culture conditions. This suggests that the products of the pathway are tailored for a specific set of environmental conditions.

The presence of three contiguous homologs of the genes required for 8-amino-flaviolin production in a very similar biosynthetic pathway strongly suggest that NapB1-3 are responsible for 8-amino-flaviolin production, though confirmation of this would require knock-out mutagenesis in strain CNQ-525. In microaerophilic conditions, the observed decrease in napyradiomycin production at the same time as increase in 8-amino-flaviolin production (Figure 5.10) suggests that the *nap* pathway shifts production towards this intermediate, which may be a functional product of the pathway in addition to being a precursor in napyradiomycin biosynthesis. It would be interesting to test if the pathway plasticity in low-oxygen environments reported here occurs for other napthoquinone pathways containing *fur1-3* homologs, which would suggest a conserved role for this pigment in distinct biosynthetic pathways.

Almost every napyradiomycin derivative tested has proven to be bioactive (Cheng et al., 2013), either in a cytotoxicity or antimicrobial assay. We found that

8-amino-flaviolin shows no activity in the same assays. Additionally, we were able to test the electrochemical properties of 8-amino-flaviolin in aqueous conditions, but were unable to test any napyradiomycin derivatives in the same aqueous solutions because these molecules are insoluble in water. The fact that the two major products of the *nap* pathway have measurably different properties is consistent with the hypothesis that they have different ecological functions.

It is well-known that environmental conditions can dramatically influence the production of secondary metabolites, and screening programs frequently grow strains in diverse culture conditions in the hopes of inducing cryptic secondary metabolite production (Bode et al., 2002). For example, regulation of the iron-chelating desferrioxamines in streptomycetes is under the control of an iron-dependent repressor (Flores and Martín, 2004), which ensures that these siderophores are only produced under iron-limited conditions. What is unique about this study is that it suggests that environmental cues trigger the production of distinct compounds from a single biosynthetic pathway. Despite the knowledge that biosynthetic pathways can produce diverse products, environmental controls on product variability are rarely explored. In fact, the *nap* pathway has been shown using stable isotope tracer experiments in the MAR4 strain *Streptomyces* sp. CNQ-766 to be responsible for the production of the pyridazine molecule azamerone (Winter et al., 2009) in addition to the napyradiomycins and 8-amino-flaviolin. The production of azamerone by strain CNQ-525 was not detected in

the present study, though it would be interesting to test further environmental conditions to establish when each of the *nap* pathway products are produced.

It remains unclear if the apparent truncation of the *nap* pathway is the result of differential regulation in low oxygen conditions or because some of the enzymes required for napyradiomycin biosynthesis are not functional in low oxygen conditions. No characterized enzymes downstream of NapB1-B3 have a clear requirement for oxygen, but the possibility that the truncation of the pathway arises because of enzymatic impairment cannot be ruled out. Fur2 (NapB2), the monooxygenase required for 8-amino-flaviolin production, requires molecular oxygen (Funa et al., 2005). The up-regulation of a molecule in microaerophilic conditions despite the requirement of molecular oxygen for one of its key biosynthetic enzymes was an unexpected finding. The most logical conclusion from this observation is that the microaerophilic conditions tested provided enough oxygen so as to not impair this enzyme. Alternatively, an additional oxygen-sequestering mechanism may be used to supply the monooxygenase with oxygen. One uncharacterized enzyme in the pathway, NapU4 (Winter et al., 2007), contains a putative hemerythrin-like domain, which is typically associated with oxygen binding in bacteria and eukaryotes (French et al., 2008). In one case, a hemerythrin protein has been hypothesized to provide oxygen for a methane monooxygenase (Chen et al., 2012). Thus it is possible that the *napU4* product and could be serving a similar function for the monooxygenase of the *nap* pathway. No regulatory elements that are clearly related to responding to low

oxygen exist in the *nap* pathway, but transcriptional analysis of the *nap* pathway under high and low oxygen conditions could be used in the future to unravel the specific regulatory factors that may influence product variability.

Bacteria can reduce extracellular substrates via a variety of mechanisms including direct contact (Hernandez and Newman, 2001). *Streptomyces* strains have previously been reported to reduce Mn(IV) (Gomah et al., 1980; Nogueira et al., 2007), but the mechanism of extracellular electron transfer by this genus has not previously been explored. In an effort to determine if strain CNQ-525 is producing a diffusible electron shuttle, an assay was employed that physically separated the growing cells from the oxidant. We found that this strain was able to reduce MnO_2, even when physically separated from the mineral (Figure 5.12), suggesting the involvement of a diffusible small molecule.

Because 8-amino-flaviolin is water-soluble and up-regulated in low-oxygen environments, it is a candidate for the observed MnO_2 reduction. To assess this possibility, we used cyclic voltammetry to determine if 8-amino-flaviolin is redox-active. The presence of reduction and re-oxidation peaks in the cyclic voltammogram for 8-amino-flaviolin confirm that the molecule is redox-active (Figure 5.13). The re-oxidation was observed to be slightly smaller in magnitude, suggesting that the reduction is quasi-reversible in the conditions tested. This means that a portion of the compound in solution could not be re-oxidized following reduction. The reduction potential of the molecule is at -474.5 mV vs. NHE. This midpoint potential means that the reduced form of 8-amino-flaviolin

could reduce oxidized manganese. It is not very likely, however, that the oxidized form of 8-amino-flaviolin could be reduced by NADH ($E^{\circ\prime}$= -320 mV vs. NHE), which is responsible for the reduction of phenazine compounds that act as electron shuttles in *Pseudomonas aeruginosa* (Glasser et al., 2014). It is therefore unlikely that 8-amino-flaviolin acts as a shuttle by the same mechanism as these previously characterized compounds. Though it is clear that the production of 8-amino-flaviolin is up-regulated under low oxygen conditions, further studies are needed to elucidate the specific role of this molecule in these conditions.

Though the functions of the vast majority of secondary metabolites remain unknown, bacterially-produced small molecules are increasingly being shown to play diverse ecological roles (Davies et al., 2006; Price-Whelan et al., 2006; Romero et al., 2011; Bernier and Surette, 2013). Future studies on the products of the *nap* pathway could consider additional functions for 8-amino-flaviolin, including iron chelation or signaling. The present study provides a link between an environmental variable and plasticity in the products of a secondary metabolic biosynthetic pathway. Careful consideration of how the environment impacts secondary metabolite production will be an essential step towards the goal of understanding of how microbes interact with their communities and environments via small molecules.

5.6 Acknowledgements

Thanks to Dianne Newman, Nicholas Shikuma, and David Lacy for helpful discussions and assistance with the initial electrochemistry experiments. I also acknowledge the J. Craig Venter Institute for the use of the chemostat equipment, and Greg Wanger for training and advising on chemostat culturing. Thanks to Chambers Hughes, who provided guidance with the structure elucidation components of this chapter. Finally, Jane Henderson and Mark Llorente are gratefully acknowledged for their guidance on cyclic voltammetry experiments.

Chapter 5 is currently being formulated into a manuscript that will be submitted for publication in 2015. Kelley Gallagher, Greg Wanger, Chambers Hughes, Jane Henderson, Mark Llorente, and Paul Jensen. The dissertation author was the primary investigator on these studies.

5.7 References

Berdy, J. (2005) Bioactive microbial metabolites - A personal view. *J. Antibiot. (Tokyo).* **58**: 1–26.

Bernier, S.P. and Surette, M.G. (2013) Concentration-dependent activity of antibiotics in natural environments. *Front. Microbiol.* **4**: 20.

Bode, H.B., Bethe, B., Höfs, R., and Zeeck, A. (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. *Chembiochem* **3**: 619–27.

Brutinel, E.D. and Gralnick, J.A. (2012) Shuttling happens: soluble flavin mediators of extracellular electron transfer in *Shewanella*. *Appl. Microbiol. Biotechnol.* **93**: 41–48.

Chen, K.H.-C., Wu, H.-H., Ke, S.-F., Rao, Y.-T., Tu, C.-M., Chen, Y.-P., Kuei, K.-H., Chen, Y.-S., Wang, V.C.-C., Kao, W.-C., and Chan, S.I. (2012) Bacteriohemerythrin bolsters the activity of the particulate methane monooxygenase (pMMO) in *Methylococcus capsulatus* (Bath). *J. Inorg. Biochem.* **111**: 10–7.

Cheng, Y.-B., Jensen, P.R., and Fenical, W. (2013) Cytotoxic and Antimicrobial Napyradiomycins from Two Marine-Derived, MAR4 *Streptomyces* Strains. *European J. Org. Chem.* **2013**: 3751-3757.

Clark, M.W. (1960) Oxidation-Reduction Potentials of Organic Systems. The Williams & Wilkins Co.

Davies, J., Spiegelman, G.B., and Yim, G. (2006) The world of subinhibitory antibiotic concentrations. *Curr. Opin. Microbiol.* **9**: 445-453.

Dietrich, L.E.P., Teal, T.K., Price-Whelan, A., and Newman, D.K. (2008) Redox-active antibiotics control gene expression and community behavior in divergent bacteria. *Science (80-.).* **321**: 1203–1206.

Fischer, M., Falke, D., and Sawers, R.G. (2013) A respiratory nitrate reductase active exclusively in resting spores of the obligate aerobe *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **89**: 1259–1273.

Flores, F.J. and Martín, J.F. (2004) Iron-regulatory proteins DmdR1 and DmdR2 of *Streptomyces coelicolor* form two different DNA-protein complexes with iron boxes. *Biochem. J.* **380**: 497–503.

French, C.E., Bell, J.M.L., and Ward, F.B. (2008) Diversity and distribution of hemerythrin-like proteins in prokaryotes. *FEMS Microbiol. Lett.* **279**: 131–45.

Funa, N., Funabashi, M., Yoshimura, E., and Horinouchi, S. (2005) A novel quinone-forming monooxygenase family involved in modification of aromatic polyketides. *J. Biol. Chem.* **280**: 14514–23.

Gallagher, K.A., Fenical, W., and Jensen, P.R. (2010) Hybrid isoprenoid secondary metabolite production in terrestrial and marine actinomycetes. *Curr. Opin. Biotechnol.* **21**: 794–800.

Gallagher, K.A., Rauscher, K., Pavan Ioca, L., and Jensen, P.R. (2013) Phylogenetic and Chemical Diversity of a Hybrid Isoprenoid-producing Streptomycete Lineage. *Appl. Environ. Microbiol.* **79**: 6894–902.

Glasser, N.R., Kern, S.E., and Newman, D.K. (2014) Phenazine redox cycling enhances anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a proton-motive force. *Mol. Microbiol.* **92**: 399–412.

Gomah, A.M., Soliman, M.S.A., and Abdel-Ghaffar, A.S. (1980) Manganese Mobility in Egyptian Soils as affected by Inoculation with Manganese Reducing Organisms. *Zeitschrift für Pflanzenernährung und Bodenkd.* **143**: 274–282.

Hernandez, M.E. and Newman, D.K. (2001) Extracellular electron transfer. *Cell. Mol. Life Sci.* **58**: 1562–1571.

Hoskisson, P.A. and Hobbs, G. (2005) Continuous culture--making a comeback? *Microbiology* **151**: 3153–3159.

Isogai, S., Nishiyama, M., and Kuzuyama, T. (2012) Identification of 8-amino-2,5,7-trihydroxynaphthalene-1,4-dione, a novel intermediate in the biosynthesis of *Streptomyces* meroterpenoids. *Bioorg. Med. Chem. Lett.* **22**: 5823–6.

Van Keulen, G., Alderson, J., White, J., and Sawers, R.G. (2007) The obligate aerobic actinomycete *Streptomyces coelicolor* A3(2) survives extended periods of anaerobic stress. *Environ. Microbiol.* **9**: 3143–3149.

Medema, M.H., Takano, E., and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Mol. Biol. Evol.* **30**: 1218–1223.

Nogueira, M.A., Nehls, U., Hampp, R., Poralla, K., and Cardoso, E.J.B.N. (2007) Mycorrhiza and soil bacteria influence extractable iron and manganese in soil and uptake by soybean. *Plant Soil* **298**: 273–284.

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2006) Rethinking “secondary” metabolism: physiological roles for phenazine antibiotics (vol 2, pg 71, 2006). *Nat. Chem. Biol.* **2**: 221.

Rabaey, K., Rodriguez, J., Blackall, L.L., Keller, J., Gross, P., Batstone, D., Verstraete, W., and Nealson, K.H. (2007) Microbial ecology meets electrochemistry: electricity-driven and driving communities. *ISME J.* **1**: 9–18.

Romero, D., Traxler, M.F., López, D., Kolter, R., and Lopez, D. (2011) Antibiotics as Signal Molecules. *Chem. Rev.* **111**: 5492–505.

Seipke, R.F., Kaltenpoth, M., and Hutchings, M.I. (2012) *Streptomyces* as symbionts: an emerging and widespread theme? *Fems Microbiol. Rev.* **36**: 862–876.

Soria-Mercado, I.E., Prieto-Davo, A., Jensen, P.R., and Fenical, W. (2005) Antibiotic terpenoid chloro-dihydroquinones from a new marine actinomycete. *J. Nat. Prod.* **68**: 904–910.

Tebo, B.M., Clement, B.G., and Dick, G.J. (2007) Biotransformations of manganese. 1223–1238.

Wang, Y., Kern, S.E., and Newman, D.K. (2010) Endogenous Phenazine Antibiotics Promote Anaerobic Survival of Pseudomonas aeruginosa via Extracellular Electron Transfer. *J. Bacteriol.* **192**: 365–369.

Watve, M.G., Tickoo, R., Jog, M.M., and Bhole, B.D. (2001) How many antibiotics are produced by the genus *Streptomyces*? *Arch. Microbiol.* **176**: 386–390.

Winter, J.M., Jansma, A.L., Handel, T.M., and Moore, B.S. (2009) Formation of the Pyridazine Natural Product Azamerone by Biosynthetic Rearrangement of an Aryl Diazoketone. *Angew. Chemie* **121**: 781–784.

Winter, J.M., Moffitt, M.C., Zazopoulos, E., McAlpine, J.B., Dorrestein, P.C., and Moore, B.S. (2007) Molecular basis for chloronium-mediated meroterpene cyclization - Cloning, sequencing, and heterologous expression of the napyradiomycin biosynthetic gene cluster. *J. Biol. Chem.* **282**: 16362–16368.

Chapter 6: Concluding remarks

The incredible diversity of secondary metabolites that are produced by *Streptomyces* spp. makes this an ideal genus for studying the relationships between secondary metabolite biosynthetic gene cluster evolution, structural diversity, and function. The work presented in this dissertation specifically addresses these issues for HI secondary metabolites by examining the phylogenetic distribution of HI production (Chapter 2), the diversity of closely related HI producers (Chapter 3), the phylogeny of a key HI biosynthesis gene (Chapter 4), and finally by testing a functional hypothesis related to these molecules (Chapter 5). Though there remains much to be learned about the HI-enriched MAR4 clade, this work provides a foundation for further studies of these interesting molecules and the bacteria that produce them. This final chapter will discuss future directions for studies of the MAR4 clade in light of recently developed microbiological techniques.

Over the last decade, affordable genome sequencing has greatly facilitated large-scale studies of secondary metabolite gene cluster evolution (Doroghazi et al., 2014; Medema et al., 2014; Ziemert et al., 2014). Combined with knowledge of the enzymology of small molecule biosynthesis, the genetic potential of bacterial isolates for secondary metabolite production can be estimated prior to chemical analysis of the strains in a process frequently referred to as “genome mining” (Challis, 2008; Winter et al., 2011). Recently, several sophisticated tools have been developed that facilitate the identification of biosynthetic gene clusters in microbial genomes, including antiSMASH (Blin et

al., 2013), NaPDos (Ziemert et al., 2012), and MultiGeneBlast (Medema et al., 2013). Applying these genome mining techniques has led to a much greater understanding of the astounding diversity of secondary metabolite biosynthetic gene clusters that exist in nature, which for the actinobacteria alone was recently estimated at 17,350 gene cluster families (Doroghazi et al., 2014).

Genome mining has also significantly improved the techniques available for connecting biosynthetic gene clusters to the molecules they produce. For example, it is now possible to target compound isolation from chemical extracts based on bioinformatic predictions (Gross, 2007) or express secondary metabolite clusters in a heterologous host (Bonet et al., 2014). In Chapter 4 of this dissertation, twelve putative HI gene clusters were identified that do not have a known product. This analysis could be used in the future to connect known HI molecules produced by MAR4 strains to a biosynthetic gene cluster, and to guide the identification of novel HIs. Additionally, many HIs are bioactive, and thus the isolation of novel molecules in this structural class is of interest to the pharmaceutical industry (Botta et al., 2005).

The availability of affordable sequence data has also led to the development of metagenomics as a means to study the phylogenetic and biosynthetic diversity present in the environment (Milshteyn et al., 2014). The selective amplification and subsequent sequencing of secondary metabolite biosynthetic genes directly from environmental DNA extracts makes it possible to examine how the presence of these genes varies with environmental conditions

and community composition, without the limitation of working with bacterial isolates in culture. A recent metagenomic survey of terrestrial soils observed a correlation between soil type and biosynthetic gene content (Charlop-Powers et al., 2014), which supports the idea that secondary metabolism is involved in niche adaptation to specific environments.

Building on the work presented in this dissertation, it would be interesting to apply metagenomic techniques to study the distribution of ABBA PTases in the environment, which could provide insights into the ecological functions of HIs by identifying environments in which these biosynthesis genes are enriched. A further step would be to examine the expression of biosynthesis genes in different sample types in order to determine when biosynthetic genes are transcribed in nature. Additionally, to further test the hypothesis presented in Chapter 5 that 8-amino-flaviolin is important in low-oxygen environments, it would be interesting to assess the distribution of genes for 8-amino-flaviolin production in samples with varying oxygen concentrations. In 2012, I collected a set of marine sediment samples that could potentially be used for future metagenomic analyses. The samples were collected using a sediment multicore along a transect through an Oxygen Minimum Zone (OMZ) off the coast of San Diego, California. The same transect was visited twice, once in the summer when the OMZ is at its peak, and once in the winter when oxygen levels are relatively high. The sediment cores were sectioned by centimeter, flash frozen and stored at -80^{o}C. Additionally, through collaboration with the Orphan Laboratory at the

California Institute of Technology, geochemical data will be available for many of the core samples. This provides the opportunity to examine correlations between sediment composition and biosynthetic gene distribution/expression. The availability of metadata along with the temporal and spatial elements of the sample collection make this set of marine sediment samples ideal for future studies concerning HI ecology.

Although next-generation sequencing has greatly improved knowledge of secondary metabolites at the level of biosynthetic genes, there remain considerable obstacles to elucidating the ecological functions of secondary metabolites. This is in part due to the methodological challenges associated with studies of the highly complex microbial communities where secondary metabolites are most relevant. Few compounds have been examined in detail from a functional perspective, but are likely to have multifaceted impacts on microbial communities. For example, phenazine molecules have been linked with several distinct functions including iron acquisition, serving as antibiotics, as signaling molecules, and in redox cycling (Price-Whelan et al., 2006; Glasser et al., 2014). In the future, it will be important to test multiple functional hypotheses in order to fully appreciate the role of specific secondary metabolites in nature.

As the work presented in Chapter 5 demonstrates, a single biosynthetic pathway may produce distinct molecules depending on culture conditions. Though Chapter 5 clearly showed that the production of 8-amino-flaviolin is related to oxygen concentration, careful molecular studies will be required to fully

elucidate the function of this molecule. For example, comparing transcription of the *nap* pathway by MAR4 strains cultured in high and low oxygen could be used to determine if the pathway is differentially regulated in microaerophilic conditions. Comparing global transcription patterns with variable oxygen concentrations could provide insights into additional ways that these organisms respond to limited oxygen. It will also be important to connect 8-amino-flaviolin production to fitness benefits in low oxygen conditions, which could be accomplished by mutational analysis.

New methodologies that are currently being developed to improve the fine-scale detection of small molecules have the potential to greatly facilitate future studies of secondary metabolism. Techniques such as imaging mass spectrometry (Esquenazi et al., 2009) and Raman microspectroscopy (Wagner, 2009) are making it possible to decipher the chemical dynamics that occur during microbial interactions. With advances in the sensitivity of these techniques, it may even become possible to study these dynamics *in situ*. Combining these technologies with already well-developed sequencing methods will provide a powerful set of tools for future studies in microbial chemical ecology. The combination of phylogenetics, genomics, and culture-based secondary metabolite analyses employed in this thesis demonstrate the importance of an interdisciplinary approach to studies focused on specific compounds. As the HI example suggests, future research concerning secondary metabolites will likely

incorporate diverse techniques to achieve a detailed understanding of small molecule-based interactions between microbes.

References

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013) antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Res.* **41**: W204–W212.

Bonet, B., Teufel, R., Crüsemann, M., Ziemert, N., and Moore, B.S. (2014) Direct Capture and Heterologous Expression of *Salinispora* Natural Product Genes for the Biosynthesis of Enterocin. *J. Nat. Prod.*

Botta, B., Vitali, A., Menendez, P., Misiti, D., and Delle Monache, G. (2005) Prenylated flavonoids: Pharmacology and biotechnology. *Curr. Med. Chem.* **12**: 713–739.

Challis, G.L. (2008) Mining microbial genomes for new natural products and biosynthetic pathways. *Microbiology-Sgm.* **154**: 1555–1569.

Charlop-Powers, Z., Owen, J.G., Reddy, B.V.B., Ternei, M.A., and Brady, S.F. (2014) Chemical-biogeographic survey of secondary metabolism in soil. *Proc. Natl. Acad. Sci. U. S. A.* **111**: 3757–62.

Doroghazi, J.R., Albright, J.C., Goering, A.W., Ju, K.-S., Haines, R.R., Tchalukov, K.A., Labeda, D.P., Kelleher, N.L., and Metcalf, W.W. (2014) A roadmap for natural product discovery based on large-scale genomics and metabolomics. *Nat. Chem. Biol.* **10**: 963–8.

Esquenazi, E., Dorrestein, P.C., and Gerwick, W.H. (2009) Probing marine natural product defenses with DESI-imaging mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **106**: 7269–70.

Glasser, N.R., Kern, S.E., and Newman, D.K. (2014) Phenazine redox cycling enhances anaerobic survival in *Pseudomonas aeruginosa* by facilitating generation of ATP and a proton-motive force. *Mol. Microbiol.* **92**: 399–412.

Gross, H. (2007) Strategies to unravel the function of orphan biosynthesis pathways: recent examples and future prospects. *Appl. Microbiol. Biotechnol.* **75**: 267–277.

Medema, M.H., Cimermancic, P., Sali, A., Takano, E., and Fischbach, M.A. (2014) A Systematic Computational Analysis of Biosynthetic Gene Cluster Evolution: Lessons for Engineering Biosynthesis. *PLoS Comput. Biol.* **10**: e1004016.

Medema, M.H., Takano, E., and Breitling, R. (2013) Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Mol. Biol. Evol.* **30**: 1218–23.

Milshteyn, A., Schneider, J.S., and Brady, S.F. (2014) Mining the metabiome: identifying novel natural products from microbial communities. *Chem. Biol.* **21**: 1211–23.

Price-Whelan, A., Dietrich, L.E.P., and Newman, D.K. (2006) Rethinking “secondary” metabolism: physiological roles for phenazine antibiotics (vol 2, pg 71, 2006). *Nat. Chem. Biol.* **2**: 221.

Wagner, M. (2009) Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging. *Annu. Rev. Microbiol.* **63**: 411–29.

Winter, J.M., Behnken, S., and Hertweck, C. (2011) Genomics-inspired discovery of natural products. *Curr. Opin. Chem. Biol.* **15**: 22–31.

Ziemert, N., Lechner, A., Wietz, M., Millán-Aguiñaga, N., Chavarria, K.L., and Jensen, P.R. (2014) Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proc. Natl. Acad. Sci. U. S. A.* **111**: E1130–9.

Ziemert, N., Podell, S., Penn, K., Badger, J.H., Allen, E., and Jensen, P.R. (2012) The Natural Product Domain Seeker NaPDoS: A Phylogeny Based Bioinformatic Tool to Classify Secondary Metabolite Gene Diversity. *PLoS One* **7**: (3): e34064.