UNIVERSITY OF CALIFORNIA, SAN DIEGO

MefA-Mediated Macrolide Resistance in Group A Streptococci: The Search for Efflux Pump Inhibitors from Marine Natural Products

A Dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Oceanography

by

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The Dissertation of Nicole Yamoah Turkson is approved, and it is

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Chair

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2007

DEDICATION

To my father, the most dedicated scholar I've ever known,
who led by example and gave me the tools to follow my dreams.

*****

To my mother, who always told me that
"Anything boys can do, girls can do- better."

*****

To my grandfather, who believed that there is no goal that
I can not attain, no summit that I can not reach
and nothing in this world that I can not achieve.

EPIGRAPH

The History of Medicine

2000 B.C.- Here, eat this root.
1000 A.D.- That root is heathen. Here, say this prayer.
1850 A.D.- That prayer is superstition. Here, drink this potion.
1920 A.D.- That potion is snake oil. Here, swallow this pill.
1945 A.D.- That pill is ineffective. Here, take this penicillin.
1955 A.D.- Oops....bugs mutated. Here, take this tetracycline.
1960-1999- 39 more "oops"....Here, take this more powerful antibiotic.
2000 A.D.- The bugs have won! Here, eat this root.

-Anonymous

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ARF	acute rheumatic fever
ATP	adenosine triphosphate
Bp	base pairs
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
CFU	colony-forming unit
CM	chloramphenicol
DMSO	dimethyl sulfoxide
DNP	dinitrophenol
ENSO	El Niño Southern Oscillation
EPI	efflux pump inhibitor
Erm	erythromycin
EtOAc	ethyl acetate
EtOH	ethanol
GAS	group A streptococci
GBS	group B streptococci
HCT	human colon tumor
HEp-2	human epithelial pharyngeal cell line
HIV/AIDS	human immunodeficiency virus/ acquired immune deficiency syndrome

HPLC	high pressure liquid chromatography
Kb	kilo bases
LA	Luria Bertani agar
LB	Luria Bertani broth
LC/MS	liquid chromatography/mass spectrometry
MATE	multi-drug and toxic efflux
MDR	multi-drug resistance
MeOH	methanol
MIC	minimum inhibitory concentration
MF	major facilitator
MRSA	methicillin-resistant *Staphylococcus aureus*
MXR	multi-xenobiotic resistance
NMR	nuclear magnetic resonance
OD	optical density
O/N	overnight
PBS	phosphate-buffered saline
P-gp	P-glycoprotein
PMF	proton motive force
RND	resistance nodulation division
rpm	rotations per minute
SARS	severe acute respiratory syndrome
SMR	small multi-drug resistance

TB	tuberculosis
THA	Todd Hewitt Agar
THB	Todd Hewitt Broth
TMS	trans-membrane segments
UV	ultra-violet
VRE	vancomycin-resistant enterococcus
VRSA	vancomycin-resistant *Staphylococcus aureus*

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ACKNOWLEDGEMENTS

There are so many people who have contributed to my growth and education; it is difficult to know where to begin. First, to my parents I wish to say 'thank you' for which words can not truly suffice. You have supported me, both financially and emotionally through thirty years of education, well past the point at which I should have been finished with school and out in the 'real world'. You have instilled in me a love of knowledge through which I find fascination and joy in life, and a belief in myself which is my compass as I navigate through life on my own terms.

In the life of every student there is one teacher who stands out above all others by listening, guiding, and inspiring while instilling not only knowledge, but also the desire and the enthusiasm to learn. I have been fortunate enough to have had several- in science, literature, history and music, from elementary through graduate school, and for what each of them has given me I am truly thankful.

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changes, you have been beside me, despite the distances that separate us, and I am blessed and proud to be your friend. My peeps- you know I love you best!

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ABSTRACT OF THE DISSERTATION

MefA-Mediated Macrolide Resistance in Group A Streptococci:

The Search for Efflux Pump Inhibitors from Marine Natural Products

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Nicole Yamoah Turkson

Doctor of Philosophy in Oceanography

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Professor William H. Fenical, Chair

Efflux pumps are a detoxification mechanism widely distributed in biological systems. Their ability to extrude a broad range of pharmaceutical agents intended to treat infectious diseases makes them a formidable obstacle to effective treatment. Overuse, misuse and abuse of antibiotics throughout much of the world have led to the emergence of bacterial strains against which current therapies are becoming increasingly ineffective. The MefA pump is encoded by the *mefA* gene and confers resistance to 14- and 15-membered macrolide antibiotics (e.g.

erythromycin, azithromycin) in *Streptococcus pyogenes* (Group A *Streptococcus*) and *Streptococcus pneumoniae*. A compound that blocks, or inhibits the action of this efflux pump could restore sensitivity of these bacteria to this important family of antibiotics. In this study, attention is turned toward secondary metabolites of marine origin as sources of novel bioactive compounds. An assay was developed to screen both crude extracts and pure compound samples for their ability to restore sensitivity of a *mefA*(+) strain of GAS to erythromycin. Parallel screens were performed using a serotype matched *mefA*(-) strain to control for independent antimicrobial activity. Several extracts were identified that reversed MefA-mediated erythromycin resistance, and these were fractionated using HPLC and subjected to various analytical techniques including mass spectrometry, UV spectroscopy and NMR spectroscopy to isolate the bioactive compounds and determine their chemical structures. Identification of a specific MefA inhibitor can serve as a lead point for rational design of novel antimicrobial agents to combat bacterial resistance mediated by drug efflux pumps. Additional molecular studies involve targeted mutagenesis and heterologous expression of *mef*A to determine its contribution to GAS host cell interactions, invasiveness and intracellular survival.

CHAPTER 1

INTRODUCTION TO MARINE NATURAL PRODUCTS DRUG DISCOVERY

Introduction

Antibiotic drug discovery, while once the foundation of the pharmaceutical industry, has in recent years been abandoned by most large pharmaceutical companies. Today, the rate at which new antibiotic compounds are discovered has fallen dramatically. Due to the rise of antibiotic resistance, there exists an urgent need not only to discover new antibiotics, but also to identify new sources of bioactive compounds having pharmaceutical potential.

Traditionally, drug discovery has taken place in the terrestrial realm. However, as the bounty of terrestrial sources is waning, we are finding that it is essential to begin to look elsewhere. The marine environment presents a vast and largely unexplored reservoir of chemical compounds having pharmaceutical potential. Marine organisms, both multi-and single-celled, are becoming recognized as a unique source of chemical diversity not found on land. The relatively new field of marine natural products drug discovery has already led to the isolation of a number of compounds with pharmaceutical value and promises to yield many more promising future discoveries as the secrets of the marine realm are revealed.

The Golden Age of Antibiotic Drug Discovery

In 1928, Sir Alexander Fleming noted that a mold growing on an agar plate with *Staphylococcus aureus* appeared to inhibit the growth of this bacterium. The result of Fleming's observation was the discovery of the world's first antibiotic produced by the fungus *Penicillium notatum*. The isolation and production of penicillin in the early 1940's led to the rapid development of a whole family of penicillin-based antibiotics. The subsequent discoveries of actinomycin, streptomycin, cephalosporins, quinolones, tetracycline, and macrolide antibiotics, as well as antifungals, antiparasitics and later antivirals have saved millions and vastly improved the quality of human life.

Fleming's discovery heralded what was to become the "Golden Age" of antibiotic drug discovery. During the coming decades, virtually all of the classes of compounds were discovered that continue to provide the basic scaffold for antibiotic development to this day. Medicinal chemistry modifications to improve upon known structures have provided generation after generation of analogs having improved efficacy and fewer side effects than their parent compounds.

In the early days of antibiotic drug discovery, pharmaceutical companies were able to exploit the finding that many microbes produce secondary metabolites, and that these compounds often have antibiotic activity. Systematic screening of soil and other environmental samples provided vast natural product libraries. Actinomycete bacteria isolated from soil, in particular, have provided an immense

array of antibiotic compounds, beginning with the isolation of actinomycin from the actinomycete, *Actinomyces antibioticus* by Russian émigré Selman Waksman in the early 1940's. Shortly thereafter, Waksman isolated streptomycin from *Streptomyces griseus*. This important first aminoglycoside antibiotic revolutionized the treatment of tuberculosis and succeeded in holding at bay one of the most significant diseases ever to threaten human health.

During the 1940's and 1950's, antibiotic drug discovery was at the core of every large pharmaceutical company. However, as generation after generation of antibiotics reached the market, the consensus of the medical community became that the threat of infectious diseases had been effectively eliminated, and was therefore no longer a human health priority. So wide-spread was this belief that during the 1960's, U. S. Surgeon General William H. Stewart stated that "We have essentially defeated infectious diseases and can close the book on them." Subsequently, pharmaceutical companies turned their attention toward more profitable chronic disease therapies such as pain management, cardiovascular drugs, arthritis and cholesterol-lowering agents, not to mention therapies for non-debilitating conditions such as erectile dysfunction and hair loss. [1]

Although there are in the neighborhood of two hundred antibiotics currently available on North American markets, most are the synthetic or semi-synthetic derivatives of only about fifteen base compounds discovered during the "Golden Age" referred to above. A few structural classes, including the quinolones, sulfonamides, β-lactams, macrolides, aminoglycosides, tetracyclines, lincosamides

and glycopeptides have provided the base for second and third generation antibiotics in use today. These newer versions of early antibiotics often have enhanced oral bioavailability, broadened antimicrobial activity, and improved toxicological and pharmacokinetic profiles. Unfortunately, the large number of antibiotic agents currently in use share the same small number of bacterial targets within the cell. The past thirty years have seen the introduction of only two truly novel agents, linezolid (Pharmacia & Pfizer) and daptomycin (Cubist). [1] It is estimated that each new anti-infective drug that makes it to market represents US $500 million and 10 – 20 years of research and development. [2]

Further compounding the near cessation of novel antibacterial compound discovery has been a decline in the number of improved analogs of existing compounds being generated. Because many of the antibacterial agents on the market today act against the same targets within the cell, an organism resistant to one agent may be able to withstand others as well. The rise of drug resistance has grown to become one of the most significant threats to global health, and at its current rate threatens to return mankind to a pre-antibiotic state in which a majority of infectious diseases are simply untreatable.

The Marine Environment

Evidence suggests that life originated in the sea. Indeed, more than 70 percent of the surface of the "Blue Planet" is covered by oceans having an average depth of 4 kilometers. It is often said that we know more about the surface of the

moon than we do about the depths of our oceans. With this in mind, it seems only natural that we should wish to explore the marine environment. After all, new species are identified with every expedition into the deep. Occasionally surprising discoveries are made, such as the finding of a living coelocanth, a prehistoric fish believed to be extinct, and the discovery of the true giant squid, long thought to be a creature of myth and sea-faring legend.

Arguably among the most species-rich marine ecosystems are coral reef habitats. Some experts estimate that the biological diversity found on coral reefs may be higher than that of rainforests.[3] Space in these crowded ecosystems is at a premium and location here is indeed everything. Competition is fierce, and thus many of the sessile, soft-bodied invertebrate organisms which inhabit coral reefs have developed chemical means by which to ensure their survival. Bioactive, often toxic secondary metabolites are produced to deter predation, attract mates, prevent fouling, or even inhibit the growth or reproduction of competing species. Many reef species have co-evolved for millennia, resulting in highly developed forms of chemical warfare. And because once released into the aqueous environment, chemical compounds are rapidly diluted, the bioactive secondary metabolites produced by marine organisms must be highly concentrated in order to maintain effective potency.

All of this translates into very good news for the marine natural products chemist looking to discover new bioactive molecules. If there is any doubt, one can look to those compounds already identified for assurance of the riches contained

within the marine realm. As early as the 1950's compounds were extracted from a marine sponge which led to the development of the antiviral drug Acyclovir (Zovirax®) and that of Cytarabine (Cytosar®), a treatment for non-Hodgkin's lymphoma. Another surprising find was the discovery of cephalosporin antibiotics in a sample from the Mediterranean Sea.[4] More recent efforts have seen the discovery of the anticancer compounds discodermolide, ecteinascidin (ET 743) and halichondrin B, the anti-inflammatory agent pseudopterosin and the cone snail venoms.[4]

The Actinomycetes

Terrestrial bacteria of the order *Actinomycetales* have long been recognized as prolific producers of antibiotic and other highly biologically active secondary metabolites. These aerobic, Gram-positive, fungus-like filamentous microbes are an important component of organic decomposition pathways in soil and, interestingly, have given rise to some of our most important antibiotics, including actinomycin, streptomycin, neomycin and grisein. The laboratory of Selman Waksman at Rutgers University was instrumental in the early development of antibiotics from this important group of microbes. Selman Waksman himself coined the term 'antibiotic' during the 1940's, which he defined as "A chemical substance produced by a microbe which has the capacity to inhibit the growth of and even to destroy other microbes." [5] Waksman and his colleagues isolated more than twenty actinomycete-derived antibiotic compounds over the course of his

career. Waksman himself was awarded the Nobel Prize in 1952 for his discovery of streptomycin and the subsequent impact it had on the treatment of tuberculosis.

Actinomycetes may be considered to be the single most important source of prescription drugs, particularly antibiotics. It has been estimated that by 1988, two-thirds of all naturally occurring antibiotics were derived from actinomycete bacteria.[1] However, as the pharmaceutical industry continued to exploit this rich source of bioactive metabolites, it became increasingly common to repeatedly isolate the same actinomycete species, and thus rediscover the same known compounds. Because de-replication of compounds can be costly and time-consuming, interest in natural product discovery from actinomycete bacteria began to decline in favor of synthetic methods such as combinatorial chemistry coupled with high-throughput screening.

However, there are those who would argue that nature is still the ultimate engineer, and that the diversity of chemical compounds found in living organisms can never be rivaled by human technology. Although they are not essential to the primary metabolic functioning of the producing organism, secondary metabolites evolved with specific functions, and it is these functions, whether known or unknown to us, that are responsible for the bioactivity so often displayed by these types of compounds. And because they evolved to get into cells and bind to receptors, natural products are more likely to have favorable pharmacokinetic profiles than synthetic ones.[6]

In an effort to discover new sources of bioactive natural compounds having pharmaceutical potential, the research group of Dr. William Fenical at UCSD's Scripps Institution of Oceanography turned to actinomycetes found in marine sediments. The reasoning behind this was that if actinomycetes on land are producers of valuable secondary metabolites, then those occurring in the ocean may also prove to be chemically prolific. They reasoned further that marine actinomycetes could prove to be a source of unique secondary metabolites, particularly if they have been isolated long enough from their terrestrial counterparts for evolutionary divergence to have occurred.

The pharmaceutical industry was initially reluctant to embrace the idea of obligate marine actinomycetes. Actinomycetes are able to form highly resistant spores which may persist in a dormant state for very long periods of time, and the long-held idea that actinomycetes found in ocean sediments originated from terrestrial run-off was not firmly dispelled until Dr. Fenical and colleagues identified the first true marine actinomycete genus, the *Salinospora* in 2001. Since this discovery, the Fenical lab has continued to discover in tropical and subtropical sediments new actinomycete genera from all of the major actinomycete families found on land, as well as the MAR 1, a major new taxon of obligate marine actinomycete.[7]

As suspected, marine actinomycetes have proven to be prolific producers of novel bioactive metabolites, including agents having antimicrobial, anti-inflammatory and anticancer properties. The most significant discovery by the

Fenical group to date has been that of Salinosporamide A, a potent proteasome inhibitor from the new marine actinomycete genus *Salinospora*, which is currently in Phase I clinical trials for the treatment of multiple myeloma.[8] With the establishment of a new collaborative effort between Scripps Institution of Oceanography, the School of Medicine and the Skaggs School of Pharmacy and Pharmaceutical Sciences at UCSD dedicated to the discovery of new antibiotic therapies, the potential for making a similarly profound discovery in the antibiotic realm is likely to become a reality in the very near future.

A Challenging Future

Like all pharmaceutical endeavors, marine natural products drug discovery is not without significant difficulties. It has even been said that "virtually every aspect of pharmacological research from ocean sources is more difficult and intrinsically slower than land-based research." [9] Once a potentially useful compound has been identified, there must exist a continually reliable source from which it can be obtained in order to provide sufficient material for its development, at least until its successful synthesis can be achieved. In the case of macroorganisms, it is simply not feasible to return repeatedly to an underwater location to harvest the producing organism without eventually depleting it, particularly when yield of the desired compound is extremely low. That for the anticancer agent, Ecteinascidin (ET743) is one precious gram per ton of the tunicate which produces it.[9] There has been some success with aquaculture, as with the

bryozoan, *Bugula neritina*, which produces the bryostatins, but this, too, requires large quantities of the organism to be produced.

A greater difficulty arises when the identity of the producing organism itself is unclear. In the case of the dolastatins, anticancer agents isolated from *Dolabella auricularia*, it was found that the sea hare is able to obtain and sequester these compounds from a cyanobacterial dietary source.[10] Additionally, many marine invertebrates harbor symbiotic microbes within their tissues. Symbionts can make up greater than half of the mass of an organism, particularly that of a sponge. Frequently, the structure of bioactive metabolites isolated from marine macrobes resemble those known from microbial sources, lending support to the theory that symbiotic microbes are the true producers of the observed bioactivity.[11]

Marine microbes are notoriously difficult to culture under laboratory conditions, particularly when their natural environment is within the tissues of a macroorganism which is itself difficult to culture. The species of symbionts which may inhabit the tissues of a marine invertebrate also may vary geographically, temporally and with any number of other factors including depth, temperature, sunlight and the presence or absence of competing species. Taken together, these factors make it extremely difficult to reproduce the bioactive molecules which are observed from the natural environment.

However, new technologies which enable the cloning and expression of biosynthetic gene clusters into backgrounds more amenable to laboratory culturing, such as the easily cultured bacterium, *Escherichia coli*, are creating exciting new

possibilities in the realm of natural products drug discovery. The burgeoning field of metagenomics seeks to sequence the genetic content of whole environmental samples while eliminating the need to ever culture the individual organisms which compose them.

These and other technologies will no doubt prove to be the future of marine natural products drug discovery. And as research into the potential of marine organisms to offer up new and effective treatments for the diseases which ail us continues, we are likely to discover that Davey Jones' Locker may turn out to be Earth's ultimate medicine chest.

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CHAPTER 2

THE CURRENT STATE OF ANTIMICROBIAL DRUG RESISTANCE

Introduction

Since the introduction of penicillin in the early 1940's heralded the "Antibiotic Era," the lives of millions of people have been saved who might otherwise have died of infections. Throughout much of history, mankind has suffered the devastating effects of periodic epidemics of diseases such as typhoid fever, influenza, cholera and plague. The "Black Death" of the 15th century cut the population of Europe in half. More recently, the influenza pandemic which swept through much of the world during the fall of 1918 killed an estimated 30 million people. The average human life expectancy in Europe and North America through the 19th Century was a mere 50 years, largely due to the high probability of dying prematurely from infectious disease. Even seemingly minor conditions such as eye, ear or bladder infections could lead to lifelong blindness, deafness or kidney failure and death. [1]

The availability of antimicrobial drugs, in addition to effective vaccines in the developed world has in large part relieved human populations from the oppression of infectious diseases. However, conditions in developing nations

remain much as they were before the advent of penicillin. Diseases of poverty, such as diarrhea, tuberculosis, malaria and HIV/AIDS continue to cause disproportionately high mortality and morbidity in populations without access to adequate healthcare. Small pox, yellow fever, typhoid fever, measles and polio are only some of the diseases which have been eradicated in developed nations, but remain an ever present threat to peoples in the developing world.

It is largely due to economic and political factors that the benefit of antimicrobial therapy has been disproportionately directed toward developed nations, while the developing world has continued to suffer the burden of infectious diseases. Extreme poverty, civil unrest, displacement of populations, poor sanitation and limited access to health care are conditions which contribute to the burden of disease. This burden consists not only of morbidity itself, but extends to promote the cycle of lost wages, lost productivity and increased poverty, all of which serve to hinder the economic and social development of a nation and its peoples.

Developed nations have too often geared their efforts toward eliminating disease within their own borders. These efforts have been largely successful, but have failed to take into account the magnitude and speed of modern transportation and global travel. In the modern world, mutations which occur in disease-causing microbes, particularly those which result in the pathogen's ability to survive conventional therapy, can be rapidly disseminated throughout the world. The result has been the acceleration of antimicrobial drug resistance, which has become a

public health concern of global proportions affecting diseases from head lice to HIV.

In recent years, the world has seen the emergence of drug-resistant pneumonia, tuberculosis, viral hepatitis and HIV. The rise of antibiotic resistant bacterial pathogens is of particular concern and is the subject of this study. Our arsenal of effective antimicrobial drugs is diminishing at a rate faster than new agents are being discovered and it is feared that if this disturbing trend continues, the world will once again find itself in a pre-antimicrobial state in which treatments do not exist to fight diseases which were once thought to be under control.

The Rise of Resistance

While genetic changes in macroorganisms evolve slowly over time, bacterial mutations can manifest themselves quickly due to the much shorter generation times of prokaryotes. A mutation which allows a bacterium to survive in the presence of a toxic agent can spread rapidly, giving rise to new strains immune to that agent. Bacteria possess a wide range of mechanisms to promote their survival in the presence of toxic compounds. The modification of a drug binding site, sequestration of the compound through protein binding, enzymatic inactivation of the compound, reduced uptake and active efflux are only some of the ways in which bacteria are able survive in the presence of current antibiotic therapies. [1]

Unfortunately, the evolution of resistance to antibacterial agents is inherent in the use of these medicines. The ability of bacteria to naturally select for

survivors under selective drug pressure in a population prone to spontaneous mutation leads to the survival of a small number of bacterial cells which are then able to divide and disseminate their resistance genes throughout a whole new population. [2] There is evidence that the presence of an antimicrobial agent may actually accelerate mutation and recombination rates, hastening the emergence of resistance to that antimicrobial agent. [3] Due to the comparatively small number of genes contained within a microbial genome, a single mutation may result in profound changes in the organism's ability to survive and invade.[4]

Compounding this problem is the fact that many resistance determinants are located on transposable genetic elements which are able to pass from bacterium to bacterium and even between species to create newly resistant strains. In addition, a plasmid or transposon may carry more than one resistance gene, such that acquisition of the genetic element is able to confer resistance to multiple types of antibiotics. [5]

Although resistance is the inevitable result of antimicrobial drug use, its magnitude has been greatly increased through human activity. [6] Too often, health care workers are prone to indiscriminate dispensation of drugs to their patients due to patient demands or constraints of cost or time, when the population would be better served by taking the time to ensure a proper diagnosis and to identify the most appropriate course of treatment on a case-by-case basis. Additionally, patients who are prescribed antibiotics do not always follow proper dosing instructions or

complete the course of therapy, thereby increasing the likelihood that resistant bacteria will survive and spread.

While developed nations are guilty of overuse of antimicrobial drug therapies, the picture in developing nations is quite different. Not only do poor nations have limited access to much needed antimicrobial drugs, but often times the drugs that are available to them are of poor quality or even counterfeit, having little or no efficacy toward the diseases they are expected to cure. [7] This situation further contributes to the emergence and spread of resistant pathogens throughout the rest of the world.

We are now beginning to realize that the imbalance in access to antimicrobial therapies in developing versus developed nations has resulted in an unexpected paradox. The overuse and misuse of antimicrobial drugs in the developed world, coupled with their underuse in the developing world has led to the decreased efficacy of these drugs for all nations. [6] Alarmingly, increased resistance of pathogens to our arsenal of drugs ultimately increases our risk of acquiring infection. [7]

Also of concern is the agricultural use of antimicrobial agents. Antibiotics are routinely added to feed and water to promote growth of livestock, prevent and treat infection in agricultural animals and to eliminate hazardous pests which threaten "cultivated foodstuffs." Agricultural use accounts for sixty to eighty percent of antibiotic production in the U. S.[8] We use an estimated 24.6 million pounds of antibiotics in healthy animals each year, with an additional 2 million

pounds directed toward treating sick livestock. [9] The steady and prolonged use of these powerful antimicrobial agents by the agricultural industry has led to the inevitable development of resistance. It is believed that resistance genes harbored by pathogens affecting livestock may pass to those affecting humans, as is believed to have been the origin of vancomycin resistant enterococci (VRE). [6]

Resistance to antibiotics is most likely to emerge where the overuse, misuse and abuse of these powerful drugs are rife. The World Health Organization (WHO) has estimated that medical practitioners in the United States and Canada routinely over-prescribe antibiotics by as much as 50%. [6] Health care workers' failure to identify the causative organism of infection, indiscriminate prescription practices, use of poor quality or counterfeit drugs, poor patient compliance and agricultural use of antibiotics are all factors which contribute to the rise and spread of antimicrobial resistance. [7] Ironically, even in this modern era of potent antimicrobial drugs, infectious diseases maintain their position as the most significant threat to human life. [6]

Nosocomial Infections

Of particular concern in recent years has been the hospital setting as a breeding ground for resistant strains of pathogenic bacteria. The close proximity of patients to each other, coupled with the broad use of antibiotics provides an ideal setting for resistance to develop. Infections caused by resistant microbes which developed inside the hospital setting may subsequently spread to outside

communities. Nearly 2 million hospitalized patients acquire nosocomial infections in the United States each year, of which an estimated 90,000 result in fatalities- a near 7-fold increase since 1992. Patients with compromised immune systems, such as from severe illness or organ transplantation have greater difficulty in fighting off infection and are frequently given heavy doses of antibiotics over extended periods of time. This then further contributes to the emergence of drug resistant pathogens and ultimately presents an even greater threat to immunocompromised patients. [7]

It has been found that some seventy percent of bacteria implicated in hospital acquired infections (HAI's) are resistant to at least one of the antibiotics that are commonly prescribed to treat them. Therefore, hospital physicians are having to rely increasingly on the small number of second- and third-line antibiotics, such as vancomycin and teicoplanin, that remain effective against these infections. Unfortunately, even these drugs are now beginning to fail.

Two of the most prevalent HAI's are caused by VRE and methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance of *S. aureus* to penicillin due to deactivation by β-lactamase enzymes arose within four short years of the introduction of penicillin in the 1940's. [7] Resistance of some *S. aureus* strains to erythromycin, chloramphenicol and the tetracyclines followed in the 1950's. The development of β-lactamase-resistant semisythetic penicillins, including methicillin in the early 1960's provided effective therapies for the next decade, but culminated in the emergence of *S. aureus* strains resistant to methicillin. [10] While MRSA was initially endemic to the hospital setting, it soon made its way into the community. [7]

Strains of MRSA subsequently emerged which were resistant to all classes of antibiotics, with the exception of the glycopeptide antibiotics vancomycin and teicoplanin. [10]

Infections caused by MRSA have remained primarily susceptible to vancomycin, a drug which has been used sparingly in order to preserve its efficacy and prevent the development of resistance to this last line of defense. However, increasing dependence on vancomycin in hospitals has led to the emergence of MRSA strains with reduced susceptibility to vancomycin. A Japanese hospital reported the isolation of the first such strain in 1997, followed shortly by two cases in the United States. [11, 12] In 2002, the United States Centers for Disease Control and Prevention (CDC) reported the occurrence of a fully vancomycin-resistant strain of *S. aureus* (VRSA) in a Michigan hospital.[13] A second case followed later that year in Pennsylvania, followed by a third in New York in 2004.[14, 15] Further cases continue to occur.

The Macrolides

Although cases of MRSA and VRE frequently make the news when they occur, resistance of bacterial pathogens has emerged to affect all classes of existing antibiotics. The penicillins, cephalosporins, fluoroquinolones, tetracyclines and macrolides are only some of the antibiotic classes which have lost much of their former efficacy in the treatment of bacterial infections. This study focuses on the class of antibiotics known as the macrolides.

Macrolide antibiotics belong to the polyketide class of natural products and are commonly synthesized by bacterial Type I modular PKS enzymes. They are characterized by a macrocyclic lactone ring, typically with a ring size of 14, 15 or 16 atoms. Macrolides generally carry two or more 6-deoxy sugar units attached via glycoside linkages, at least one of which is an amino sugar. These compounds may have extensive branching via methyl substituents. Antibiotics of the macrolide class are primarily active against Gram-positive pathogens. They tend to have a spectrum of antibacterial activity which is similar to that of penicillin, making these antibiotics clinically useful in cases of penicillin allergy. [16]

Currently, the most widely prescribed macrolide antibiotic in the U.S. is erythromycin (Figure 2.1). [16] Isolated in 1952 from a soil sample collected in the Philippine Archipelago, the erythromycins are biosynthesized by the soil actinomycete, *Saccharopolyspora erythrea* (formerly *Streptomyces erythreus*) through the well-characterized 6-deoxyerythronolide B synthase (DEBS) path-way. [17] Propionate units exclusively serve as both starter unit and extension units in erythromycin biosynthesis via methylmalonyl-Coenzyme A. This 14-membered macrolide has as its sugar units L-cladinose and D-desosamine.

Commercial preparation of erythromycin is a mixture comprised primarily of erythromycin A and contains small amounts of erythromycins B and C (Figure 2.1). The activity of erythromycin varies between bactericidal or bacteriostatic in different microorganisms and conditions including concentration and pH. It is active mainly against Gram-positive microorganisms, including cocci

such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*, and bacilli including *Corynebacterium diphtheriae* and *Listeria monocytogenes*. Erythromycin is also frequently the drug of choice in treating infections of *Legionella pneumophila*, the causative organism in Legionnaire's disease, and in cases of staphylococcal infections resistant to penicillin, although resistance to erythromycin limits its therapeutic utility against many staphylococcal strains. [16]

Erythromycin and other macrolide antibiotics act as inhibitors of protein synthesis in prokaryotes. Erythromycin prevents assembly of the bacterial ribosome early in protein synthesis. [18] The bacterial ribosome is composed of a small 30S subunit and a large 50S subunit. The 50S subunit is made up of 23S rRNA and a minimum of thirty proteins. Prokaryotic 23S rRNA has a secondary structure such that it is folded to form six domains, numbered I-VI. [19] The binding site of erythromycin A is located at the site of polypeptide chain synthesis in the peptidyl transferase center near domain V. [20] Rather than inhibit the action of peptidyl transferase, the reversible binding of erythromycin A acts to prevent extension of the polypeptide chain and stimulates early release of peptidyl tRNA. [21] Protein synthesis is essential for the growth and survival of the bacterial cell.

Semisynthetic analogues of erythromycin, including azithromycin, clarithromycin and roxithromycin are also in use (Figure 2.1), which have enhanced activity compared to that of erythromycin. Erythromycin has given rise to a separate class of semisynthetic antibiotic drugs known as the ketolides, only one of which has gained approval by the U. S. Food and Drug Administration.

Telithromycin, approved in April, 2004, is a broad-spectrum antibiotic in current use against respiratory tract infections and is valuable for its effectiveness against Gram-positive microorganisms which are resistant to older macrolide antibiotics.

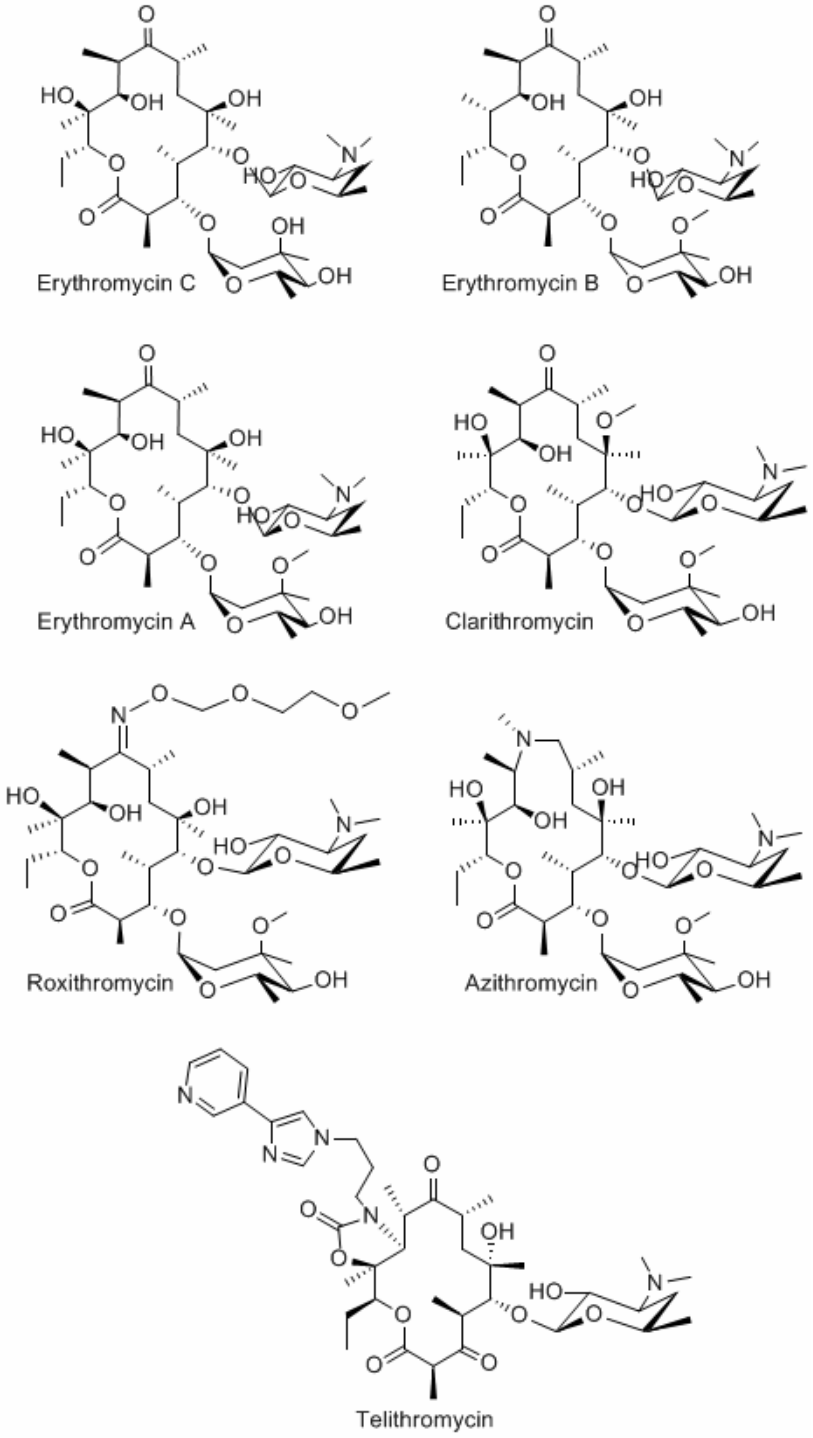

Figure 2.1. Chemical structures of common macrolide antibiotics

Group A Streptococci

Group A Streptococci (GAS), or *Streptococcus pyogenes*, are Gram-positive, non-motile, non-sporeforming bacteria that are responsible for causing a number of diseases in humans. GAS can infect almost any part of the body. Primary illnesses include pharyngititis (strep throat), impetigo, cellulitis, and necrotizing fasciitis, as well as an array of other infections ranging from mild to severe. [22] In addition, GAS infection can be responsible for several life-threatening post-infective sequelae, including scarlet fever and acute rheumatic fever (ARF). [23] GAS are characterized according to serotyping of the anti-phagocytic cell-surface *emm* (M) protein. [22] Over 130 M-serotypes have been identified and numerous studies have indicated significant correlations between M-type and the type and severity of infection caused. [24, 25]

GAS possess a number of virulence factors, which can allow them to evade the body's immune system. The infecting bacteria are able to release toxins into the bloodstream, which can lead to severe complications such as sepsis and toxic shock. The development of acute rheumatic fever following GAS pharyngitis is a complication of particular concern in children. ARF is a serious condition affecting the heart, and is the leading cause of acquired heart disease in children (Will Keough, personal communication).

The drug preferred to treat GAS infections of the upper respiratory tract is penicillin V. [26] In cases of penicillin allergy, alternative treatments belonging to the

macrolide class of antibiotics are often prescribed. [27] Although it is not recommended as a first-line treatment for streptococcal pharyngitis, azithromycin, which is available in a single daily dose for five days of treatment has become an increasingly popular choice by both prescribing physicians and their patients. [28, 29] Evidence now suggests that the increasing emergence of macrolide-resistant GAS is correlated to the rise in macrolide use, particularly that of the once-daily dosed azithromycin. [29] In fact, a reduction in consumption of macrolide antibiotics in Finland in 1991 due to the prevalence of resistance was followed by a reduction in resistance rates of more than 50% in the years that followed. [30] A similar outcome was reported in Japan in 1994. [31]

Through monitoring and surveillance programs, a number of European and Asian countries have reported a rise in macrolide resistance among GAS isolates during the past 15-20 years. [32-37] By comparison, macrolide resistance in isolates of GAS in the United States had remained below 5% until recently. [38, 39] In a 2002 article published in the New England Journal of Medicine, researchers in Pittsburgh, PA reported an outbreak of erythromycin-resistant GAS pharyngitis among school-aged children during the 2000-2001 school year, in which 48% of isolates demonstrated resistance to erythromycin against a previous resistance level of zero. [40]

Macrolide Resistance in Group A Streptococci

The world's first documented case of macrolide resistance in GAS was reported in Great Britain in 1959. [41] Macrolide-resistant GAS was identified in the United States seven years following. [42] The cause of this resistance was unknown at the time, but three separate genetic mechanisms of macrolide resistance have now been characterized in pathogenic Gram-positive bacteria. The first involves the base-specific post-transcriptional dimethylation of bacterial 23S ribosomal RNA by erythromycin-resistant methylases encoded by the *erm* genes A,B and subgroup TR. Methylation takes place in the region of the macrolide attachment site on the ribosome and prevents the binding of antibiotics of the macrolide, lincosamide and streptogramin B chemical classes. For this reason, this phenotype is designated MLS_B. [43] Expression of the *erm* genes may confer either low (MIC 2-8 μg/mL) or high (16-64 μg/mL) resistance to erythromycin. [44] The second mechanism involves point mutations within a gene encoding a ribosomal protein and is extremely rare. [45]

The final known mechanism of macrolide resistance, and the subject of this study, employs an energy dependent trans-membrane efflux protein homologue encoded by the *mefA* gene, so named for its function in macrolide efflux. The MefA efflux pump displays 90% homology to the MefE efflux pump which has been shown to mediate macrolide resistance in *S. pneumoniae*. [46] The action of MefA keeps intracellular levels of 14-and 15-membered macrolides below therapeutic levels by expelling these compounds from within the bacterial cell, thus allowing it to survive within the body.

Expression of the *mefA* gene confers resistance to 14- and 15-membered macrolides, but not to 16-membered macrolides, lincosamides or streptogramin B, and for this reason has been designated the phenotype M. [43] MIC's of erythromycin in Gram-positive bacteria expressing *mefA* have been reported at 16-64 μg/mL. [47]

Mef A was first identified in *Streptococcus pyogenes* in 1996, and has been described in a number of Gram-positive and Gram-negative bacteria, including group C and group G streptococci, *S. pneumoniae* and many phylogenetically unrelated strains of GAS. [40, 43] Wide distribution of this resistance determinant suggests that the *mefA* gene may be acquired by bacteria through lateral gene transfer of a mobile genetic element. [48] Banks et al. have identified the element which carries the *mefA* gene as a 7.2 kb contig transposon which includes DNA having high sequence homology to Tn1207.1, a transposable element believed to encode the *mefA* gene in macrolide-resistant pneumococci. [40, 49] The same researchers have also shown evidence that phage-mediated transduction may play an important role in the horizontal transfer of the *mefA* gene between GAS strains, thus contributing to their evolution.

The Pittsburgh Study

The research which is the topic of this dissertation is the result of a collaboration between the University of Pittsburgh School of Medicine, the University of California at San Diego School of Medicine and Scripps Institution of Oceanography at UCSD. The four strains of GAS which I used to conduct my

doctoral research were graciously donated by Dr. Timothy Mietzner of Children's Hospital in Pittsburgh, PA. The clinical isolates were acquired from school-aged children during an outbreak of macrolide-resistant GAS pharyngitis described below.

As part of a longitudinal study conducted by Martin et al. at the University of Pittsburgh School of Medicine, pharyngeal isolates of GAS were collected from students attending a private elementary school (grades K-8) in Pittsburgh, PA beginning in 1998. Throat swabs were obtained twice a month from participating children from October to May during the academic year and plated onto sheep's blood agar plates for evaluation of antibiotic sensitivity in the laboratory. [29]

During the first two years of this study, GAS isolates were found to be 100% susceptible to erythromycin. However, during the third year of the study (2000-2001), an outbreak of GAS pharyngitis occurred among the school children which displayed an erythromycin resistance level of 48%. Isolates from children classified as resistant to erythromycin or to the lincosamide antibiotic clindamycin (MIC $\geq$ 1 μg/mL) were further evaluated to determine the resistance phenotype of MLS_B versus M. The presence of *erm* and *mefA* genes was evaluated using polymerase chain reaction.

The Pittsburgh outbreak was traced by the CDC to a single clone of serotype M6. All isolates were sensitive to clindamycin and no presence of *erm* genes was detected. Those isolates which were resistant to erythromycin remained sensitive to clindamycin and lacked the MLS_B phenotype. Consistent with the M phenotype,

expression of *mefA* was confirmed in these isolates, suggesting that the causative factor for erythromycin resistance of GAS in this population is the *mefA* gene. [29]

The 2000-2001 GAS pharyngitis outbreak also extended to the wider community within Pittsburgh. That the prevalence of erythromycin resistance in this same population had been reported at zero in the year prior to this outbreak in conjunction with strong evidence of phage-mediated gene transfer of the transposable genetic element bearing the *mefA* gene suggests that GAS are able to acquire *mefA*-mediated macrolide resistance. [40]

The emergence of macrolide resistance in the Pittsburgh population may very well have been influenced by the steady increase in prescription rates of macrolide antibiotics in the Pittsburgh area from 1998 – 2001, which mirrors a nation-wide trend in the treatment of upper respiratory infections, particularly through use of short courses of azithromycin. [29, 50-52] This, together with similar scenarios occurring all over the world should be taken as evidence of the urgent need for more responsible prescribing practices if we are to maintain effective defenses against the microbial pathogens which threaten us.

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CHAPTER 3

TRANS-MEMBRANE EFFLUX PUMPS:

SCREENING FOR MEFA INHIBITORS

The primary aim of this research is to identify compounds which are able to inhibit the *mefA* efflux pump from a library of marine natural products extracts. Blocking the *mefA* pump will allow erythromycin to accumulate inside the GAS bacterial cell, thus restoring sensitivity of the organism to this important antibiotic. Discovery of such a compound could lead to the combined therapy of erythromycin with an efflux pump inhibitor to treat infections of GAS and other pathogens that exhibit this resistance mechanism. In order to screen large numbers of crude extracts from marine microbes for their potential to block the *mefA* efflux pump, an assay was developed and conducted at Scripps Institution of Oceanography. Two GAS strains were utilized in this assay, a *mefA* negative, erythromycin sensitive strain and a *mefA* positive, erythromycin resistant strain. The assay was used to screen 1.) marine microbial crude extracts in which a certain level of bioactivity has been previously identified through preliminary cytotoxicity testing using a human colon tumor (HCT) cell line and 2.) pure compounds previously isolated at Scripps Institution of Oceanography from marine invertebrate macroorganisms.

Introduction

The extrusion of medicinal compounds from within target cells through the action of trans-membrane efflux pumps is one of the most common strategies of survival employed by drug resistant bacteria.[1] In fact, it is estimated that 5-10% of all bacterial genes are relegated toward transport, with a large proportion of these genes encoding efflux proteins.[2, 3] The percentage of genes encoding efflux pumps is even higher in some microbial genomes. For instance, that of *E. coli* has been calculated to be 15-20%.[4] In the neighborhood of three hundred gene products which transport known substrates have been described, and of these at least thirty specifically transport antibiotics and other drugs.[5] These transporter proteins protect both Gram positive and Gram negative bacteria from harmful effects by pumping toxic compounds, including antibiotic agents, out of the cytoplasm[6]. Pharmaceutical agents that are perceived as a threat to the cell are in this way prevented from reaching effective therapeutic levels within the cytoplasm.

Efflux as a resistance mechanism was first described in tetracycline resistance in *E. coli* during the early 1980's and has subsequently been described in a wide array of host backgrounds involving a broad range of substrate classes.[7-13] However, this mechanism is not limited to bacterial cells undergoing antimicrobial therapy. It is also a cause of resistance in eukaryotic cells, including fungal pathogens, protozoan parasites such as malaria and human cancer cells undergoing chemotherapy.[14] Efflux pumps are commonly found in the cells of macroorganisms, from marine invertebrates to human beings.[15, 16] The expression

of efflux pumps conferring multixenobiotic resistance (MXR) in many aquatic and marine organisms can even serve as a useful ecological indicator of the level of pollution in the environment in which they live, and has been found to be inducible by the presence of various chemicals.[17] Similarly, efflux-mediated resistance to an antibiotic agent in bacteria is often initiated by exposure of the bacterium to that or some other antibiotic compound.[4]

Genes that encode for efflux proteins may be chromosomally located, having implications for intrinsic resistance, or they may be located on transposable genetic elements and therefore may be acquired by the cell through bacterial conjugation, leading to acquired resistance.[18] Efflux genes located on chromosomes may receive signals for upregulation by exposure of the cell to environmental cues, such as an antimicrobial agent. It is believed that this mechanism is the cause behind the intrinsic resistance of many Gram negative bacteria to antibiotic therapies, relative to Gram positive bacterial pathogens[6]. Even low-level intrinsic resistance due to an efflux system can confer enhanced survival to bacterial cells, allowing them to persist in the face of chemotherapy. This may favor the occurrence of mutations involving drug target binding sites which may well lead to the emergence of highly resistant bacterial strains.[6, 18, 19]

Trans-membrane efflux pumps generally fall into two categories: those which act upon a specific type of compound, such as the Tet-pumps responsible for tetracycline resistance in both Gram-negative and Gram-positive bacteria, or they may act upon a broad array of structurally and mechanistically unrelated

compounds. The P-glycoprotein pump which confers mulditrug resistance (MDR) in human cancer cells and the *mex AB* system in *P. aeruginosa* which confers resistance to a range of antibiotics, including fluoroquinolones, chloramphenicol, and β-lacams, in addition to the household biocide triclosan are examples of MDR pumps. [20, 21]

Genes encoding for many MDR pumps have been found to be normal constituents of bacterial chromosomes, while those encoding for antibiotic-specific efflux pumps are often located on plasmids or transposons which are transmissible across bacterial species.[19] It should be noted also that a given antibiotic may be transported by different pumps and that a given bacterium may express more than one type of efflux pump.[4] In the case of efflux-mediated fluoroquinolone resistance in *P. aeruginosa*, a single bacterium may express multiple pumps which share the same substrate, the effects of which may be multiplicative or additive.[19, 22]

Membrane transport proteins are not limited to the transport of pharmaceutical agents. They may transport a wide range of cellular and other products. There are five main groups, or superfamilies of efflux transporters which are known to function in prokaryotes.[2] These are the major facilitator (MF) superfamily, the small multidrug resistance (SMR) superfamily, the resistance-nodulation-division (RND) superfamily, ATP binding cassette (ABC) transporters and the multidrug and toxic efflux (MATE) superfamily.[5, 6, 23-25] All of these, with the exception of the ATP-binding cassette, utilize proton motive force (PMF) as an

energy source to drive efflux across cell membranes. ABC transporters alone utilize ATP hydrolysis as their energy source.[6]

In prokaryotes, the vast majority of efflux systems are proton-driven. *E. coli*, for instance, has been characterized as having seventeen MF, three SMR and six RND drug transporters, while only having three belonging to the ABC superfamily.[1] ABC transporters are wide-spread in eukaryotes, however, as is exemplified by the P-gp pump which has homologs across a wide range of species.[16]

Transporters of the MF superfamily, such as the TetA(K) pump which mediates tetracycline resistance in *S. aureus*, generally consist of around four hundred amino acids which are proposed to form twelve membrane-spanning helices having a large cytoplasmic loop between helices six and seven.[1] This loop putatively links the two halves of the protein, which are usually similar in sequence. Thus, the theory has arisen that the structure of these transporters arose through gene duplication. Members of this family are also known which have fourteen trans-membrane segments (TMS), such as the TetA(B) pump in *E. coli*. The work of Marger and Saier has identified five distinct clusters of transport proteins within the MF superfamily based on their substrate and function, those involved in: (i) drug resistance, (ii) sugar uptake, (iii) Krebs cycle intermediate uptake, (iv) phosphate/phosphate-ester antiport, and (v) oligosaccharide uptake.[26] Cluster i is composed of PMF-mediated drug-specific efflux and multidrug efflux proteins, such as the MefA pump in *S. pyogenes* which is the subject of this study.[27]

Inhibition of Efflux Pumps

The growing problem of bacterial resistance to antibiotics has become a significant concern to the medical community. Although it is crucial to continue the search for new classes of antibiotics to fight infection, attention is now being turned to the possibility of circumventing antibacterial resistance. Two approaches to addressing the problem of antibiotic efflux are being taken. The first approach involves the modification of existing antibiotic structures to decrease their affinity as substrates of efflux pumps. This has been successfully achieved through the development of new classes of semi-synthetic tetracyclines, fluoroquinolones and macrolides/ketolides.[28-34]

The second approach, and the topic of this dissertation, is the circumvention of efflux-mediated antibiotic resistance through the identification of a compound which blocks, or inhibits an efflux pump in order to restore sensitivity of the bacteria to drugs it was previously resistant to (Figure 3.1). Administered in combination with an antibiotic, an efflux pump inhibitor (EPI) could allow continued use of existing antibacterial agents.[1] A precedent for combination therapy has been seen in the use of β-lactamase inhibitors in conjunction with β-lactam antibiotics, such as with the oral therapy marketed under the commercial name Augmentin®.[35]

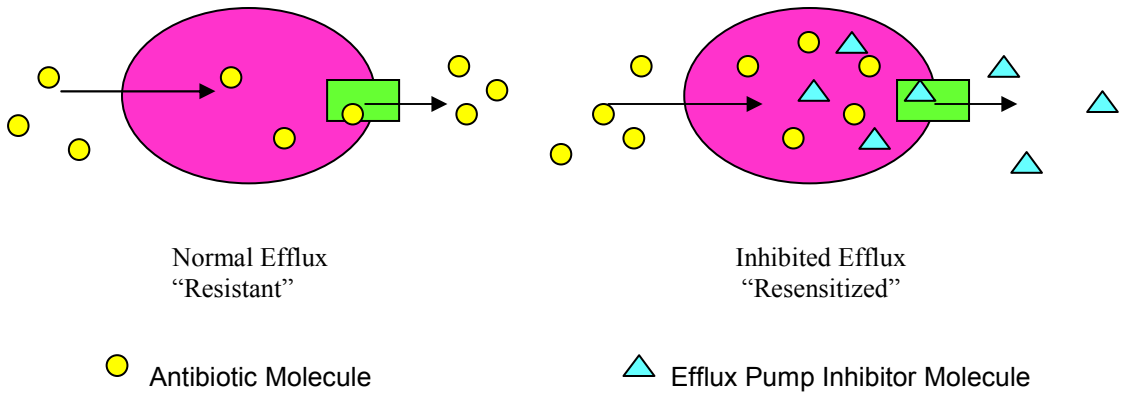

Figure 3.1. Trans-membrane efflux pump schematic. Normal efflux in a resistant bacterial cell due to the action of an antibiotic efflux pump is depicted on the left. Inhibited efflux through the application of a specific efflux pump inhibitor, shown on the right, would re-sensitize a resistant bacterial cell to antibiotics it was previously resistant to, thereby allowing their continued use.

The efflux inhibition strategy has been employed against the Mex pumps which confer fluoroquinolone resistance in *P. aeruginosa*.[2] Lomovskaya et al. have identified a compound which significantly decreased the level of intrinsic resistance, reversed acquired resistance and effectively lowered the frequency with which highly resistant strains emerged.[2] Studies using other bacterial species, including *S. pneumoniae*, have also suggested that the inhibition of efflux systems may reduce the frequency of emergence of highly resistant strains.[36, 37]

Particularly attractive is the possibility that, due to the high degree of structural homology among efflux pumps, a single EPI may be effective against a range of efflux pumps occurring in different bacterial types.[6] However, many challenges to the development of combination inhibitor and antibiotic therapies exist. Like any other drug, an inhibitory compound may carry its own toxicity concerns in eukaryotic cell systems. Several inhibitors of bacterial efflux pumps are known, but are useless in the clinical setting due to their strong pharmacological activities.[4] Like the antibiotic itself, the EPI must have appropriate potency and spectrum of activity, clearance and bioavailability. In addition to these requirements, it must significantly improve upon the efficacy of the partner antibiotic in clinical trials.[19] Very few compounds with clinical potential have yet been identified, but include inhibitors of the Tet transporters, the NorA transporter of *S. aureus* and the RND transporters of *P. aeruginosa* that are responsible for the efflux of macrolides, quinolones, chloramphenicol and tetracycline.[2, 38-41]

The initial discovery phase of EPI's presents its own particular challenges. In their work involving the inhibition of levofloxacin efflux in *P. aeruginosa*, Lomovskaya et al. have developed six criteria that a hit compound must meet. In order to be classified as a true EPI, a compound: (i) must enhance the activities of (levofloxacin) and other antibiotics that are effluxed in strains containing functioning pumps, (ii) must not significantly potentiate the activities of antibiotics in a strain that lacks efflux pumps, (iii) must not potentiate the activities of antibiotics that are not effluxed, (iv) must increase the level accumulation and decrease the level of extrusion of efflux pump substrates and (v) must not affect the proton gradient across the inner membrane.[2] These criteria may be applied to the inhibition of other efflux pump systems and their substrates, and have been adapted to the work described in this study.

Screening Marine Natural Products for Inhibitors of MefA in GAS

The primary aim of the research presented in this dissertation was to develop a system which could be used to identify agents which are able to inhibit the *mefA* efflux pump from a library of marine natural products extracts and known compounds, concentrating on those originating from marine microbial, primarily actinomycete sources. Potent and specific inhibitors of the MexAB-OprM efflux pump in *P. aeruginosa* have been previously isolated from terrestrial actinomycete fermentation products by Lee et al., suggesting that the investigation of marine actinomycetes for their potential to produce EPI's may also prove valuable.[42]

Blocking the *mefA* pump would allow erythromycin to accumulate inside the GAS bacterial cell, thus restoring sensitivity of the organism to this clinically important antibiotic. Identification of such a compound could lead to the combined therapy of erythromycin with an inhibitor of the MefA efflux pump to treat infections of GAS and other pathogens that exhibit this resistance mechanism.

In pursuance of this goal, a collaborative effort was established between Scripps Institution of Oceanography, UCSD School of Medicine and the University of Pittsburgh Children's Hospital. Four strains of clinical isolates obtained during the Pittsburgh GAS outbreak were obtained for use in conducting this research. These M6 strains were previously reported to display a range of erythromycin resistance as follows:

DF1- *mefA* negative and erythromycin sensitive MIC = <1 μg/mL

DC7- *mefA* positive and erythromycin resistant MIC = 32 μg/mL

DC2- *mefA* positive and erythromycin sensitive MIC = <1 μg/mL

KC9- *mefA* positive and erythromycin resistant MIC = 64 μg/mL

Strains DC7 and DC2 were obtained from the same child nine months apart. Isolates from this child displayed erythromycin resistance and sensitivity sequentially, suggesting the occurrence of a mutation within *mefA*, which caused it to become re-sensitized to erythromycin. MIC testing of these four strains was performed upon their receipt and the results differed from those reported by the

Pittsburgh researchers. In my hands, the erythromycin MIC's of the four strains were as follows:

DF1- *mefA* negative and erythromycin sensitive MIC = <1 μg/mL

DC7- *mefA* positive and erythromycin resistant MIC = ≤5 μg/mL

DC2- *mefA* positive and erythromycin sensitive MIC = <1 μg/mL

KC9- *mefA* positive and erythromycin resistant MIC = ≤5 μg/mL

Experiments utilizing these bacterial strains were two-fold: those focusing on the screening of marine natural products to identify potential pharmaceutical candidates described below, and those focusing on *mefA* and its behavior within the bacterial cell, described in Chapter 4 of this dissertation.

Methods

In order to screen large numbers of crude extracts from marine microbes for their potential to block the *mefA* efflux pump, an assay was developed and conducted at Scripps Institution of Oceanography. The two bacterial strains utilized in this assay are the *mefA* negative, erythromycin sensitive DF1 and the *mefA* positive, erythromycin resistant KC9. Marine natural products screened at Scripps Institution of Oceanography were of two varieties: 1.) microbial crude extracts from a library in which a certain level of bioactivity has been identified through preliminary cytotoxicity testing using a human colon tumor (HCT) cell line

in the laboratory of Dr. William H. Fenical and 2.) pure compounds isolated from marine invertebrate macroorganisms by the research group of Dr. D. John Faulkner. All extracts and pure compounds were stored frozen at -20° C in 96-well boxes with a well volume of 1 mL. Crude extracts were stored at a concentration of 25 mg/mL in DMSO. Pure compounds were stored at concentrations of 2 mg/mL and 5 mg/mL in DMSO.

Experimental Design: Assay Development

Briefly, strain KC9 was grown in 96-well plates in growth medium containing erythromycin and extract in a range of concentrations to identify those wells that do not contain bacterial growth. This indicates a reversal of resistance and the return of erythromycin sensitivity. In order to distinguish those extracts that inhibited the MefA efflux pump from those having novel antibiotic activity, strain DF1 was screened in parallel with KC9. These sensitive bacteria were grown in 96-well plates in medium containing the same extracts, but in the absence of erythromycin. Growth inhibition of these bacteria suggested independent antibiotic activity of the extract.

Primary Screening of Crude Extracts

Bacterial strains DF1 and KC9 were inoculated into THB medium (Appendix B) and THB+2 μg/mL erythromycin, respectively, at 10-25 mL and grown overnight at 37° C in tightly capped centrifuge tubes. The following day,

each strain was removed from incubation, and an optical density reading was taken. Dilutions were then made using THB and the cultures were allowed to obtain a final OD reading that measured between 0.04 and 0.06. A dilution plate was made using a 96-well plate by adding 195 µL sterile THB medium to wells A1-H10 plus 5 µL crude marine extract transferred from the 96-well extract box to achieve a total volume of 200 µL at a concentration of 625µg/mL in each of 80 sample wells. Two assay plates, one for each GAS strain, were set up for each assay using 96-well plates. For strain DF1, pure cell suspension was used, whereas for strain KC9, erythromycin was added to the cell suspension to a concentration of 2µg/mL. For each plate, 100 µL cell suspension were added to wells A1-H10. Using a multi-channel pipettor, 9 µL sample were transferred from the dilution plate to the assay plate and gently pipetted up and down to mix. The final concentration of extract in each sample well was 51.6 µg/mL. As an antibiotic standard for both plates, 195µL cell suspension without erythromycin were added to A12 and 100µL to the rest of column 12 (B12-H12). 5 µL erythromycin at 4 mg/mL were added to A12, and serial dilutions were made down the column, discarding the last 100 µL from H12 to obtain erythromycin concentrations of 100 µg/mL-0.78 µg/mL. G11 and H11 served as blanks, containing only 100 µL of sterile THB. The remaining wells of column 11 contained 9 µL DMSO in the absence of extract. Plates were covered and incubated overnight at 37° C and read the following morning using an Emax plate reader with SoftMaxPro software at a wavelength of 600 nm. The absorbance values were converted to percent survival, and those samples with a percent

survival of >90% in strain DF1 and <10% in strain KC9 were considered preliminarily active and chosen for secondary screening.

Secondary Screening of Crude Extracts

Two assay plates, one for each strain, were set up for each assay using 96-well plates (Figure 3.2). For strain DF1, pure cell suspension was used, whereas for strain KC9, erythromycin was again added to the cell suspension to a concentration of 2μg/mL. For strain DF1, 195 μL cell suspension were added to row A and 100 μL to rows B-H, leaving G11 and H11 for use as blanks. 5 μL of each of 10 samples was then added to A1-A10. 5 μL of DMSO were added to A11as a negative control and 5 μL of erythromycin at 4 mg/mL were added to A12 to serve as an antibiotic standard and positive control for each strain. Serial dilutions were then made down the entire plate using a multi-channel pipettor, with the exception of G11 and G12, which contained 100 μL of sterile THB medium. The same procedure was repeated for strain KC9, except that all sample wells also contained 2 mg/mL erythromycin. Assay plates were incubated and read as above. Using the same selection criteria, extracts were chosen for further evaluation using n = 3-5 and repeated 2-4 times.

	Ext. 1	Ext. 2	Ext. 3	Ext. 4	Ext. 5	Ext. 6	Ext. 7	Ext. 8	Ext. 9	Ext. 10	DMSO	Erm. Stnd.
A	625 µg/mL											100 µg/mL
B	312 µg/mL											50.0 µg/mL
C	156 µg/mL											25.0 µg/mL
D	78.1 µg/mL											12.5 µg/mL
E	39.1 µg/mL											6.25 µg/mL
F	19.5 µg/mL											3.13 µg/mL
G	9.77 µg/mL										Blank	1.56 µg/mL
H	4.88 µg/mL										Blank	0.78 µg/mL

Figure 3.2. Schematic of the efflux pump inhibitor assay conducted in 96-well plate format. Ten extracts are tested at 8 concentrations per plate. Extracts are screened in parallel using GAS strains KC9 and DF1 to distinguish between EPI and independent antibiotic activity.

Re-growth and Extraction of Actinomycete Cultures

Twenty-seven actinomycete strains demonstrating EPI activity based on the above assay were re-grown to test for the continued presence of activity. The actinomycete cultures were started from freezer stock (stored at -80° C) in 25 mL of A1 medium (Appendix B) in 125 mL flasks and allowed to shake continuously at 28° C. When the cultures were abundant (10 days-2 weeks), 10 mL were transferred into 100 mL of the medium appropriate to the strain in 500 mL flasks and returned to the 28° C shakers. XAD-7 dry resin was then added to the cultures on the shakers at least 1 hour prior to extraction with acetone. Samples were dried in a Savant Speed Vacuum overnight and re-suspended in a 1:1:1 solution of ethyl acetate/methanol/acetone. These extractions were frozen at -20° C for 1-2 days, then removed from the freezer and placed on a shaker for approximately 2 hours. The extracts were filtered through cotton-packed Pasteur pipets into pre-weighed tubes and again dried in a Savant Speed Vacuum for approximately 3 hours. The dry mass of the extracts were recorded, and the appropriate volume of DMSO was added to each dried extract to yield a final concentration of 25 mg/mL. Extracts were then assayed using GAS strains DF1 and KC9 as described above. Those demonstrating an active result were separated using HPLC. The fractions were tested for EPI activity in the same manner as the crude extracts. Based on the outcome of these assays, six strains were again re-grown (5 having EPI and 1 having antibiotic activity), this time in culture volumes of 100 mL and 1 L and extracted as above. These extracts were then assayed to test for continued activity.

CNQ719

An additional actinomycete strain, CNQ719 was selected for re-growth with a time-course study to determine if the observed EPI activity could be re-produced, and if so, whether the compound(s) responsible for the activity reach peak concentration at certain times, or is produced continuously in culture. The culture were started as above and scaled up to a volume of 1 L. Once the culture reached abundance, samples were taken and extracted on each of seven days. For each extraction, 25 mL of culture were removed and transferred to a glass separation funnel. 25 mL of EtOAc were added and the flask swirled and allowed to stand for 10-15 minutes. The aqueous and organic fractions were separated, and the procedure was repeated two more times by adding 25 mL of fresh EtOAc to the aqueous fraction. The organic fractions of each extraction were combined and dried using rotary evaporation. The resulting residues were dissolved in 2 mL of EtOAc and transferred to pre-weighed glass vials. The samples were dried under a N_2 stream to evaporate, and then placed in a freeze drier overnight. A final mass was determined for each sample before dissolving in DMSO to obtain a final concentration of 25 mg/mL. Time-course samples were then assayed for EPI activity.

A second 1 L culture of CNQ719 was extracted for separation using HPLC. 6 fractions were obtained and screened using the assay. Based on these results, CNQ719 was grown in a culture of volume of 5 L, followed by 30 L in order to

obtain more sample. The 1 L, 5 L and 30 L extractions were subjected to HPLC separation, and the resulting fractions were screened for EPI activity. Mass spectrometry data was obtained for the 5 L extraction. Mass spectrometry and NMR spectroscopy data was obtained for the 30 L fraction.

Screening of Pure Compounds

Assay plates to screen pure compounds were set up using the same method described above for the secondary screening of crude extracts. For pure compounds in the Faulkner Pure Compound Collection having a concentration of 2 mg/mL DMSO, 10 µL of sample were added to wells in row A containing 190 µL of cell suspension for an initial concentration of 100 µg/mL. Serial dilutions were made down the plate to achieve a range of concentrations from 100 µg/mL- 0.78 µg/mL. For those compounds having a concentration of 5 mg/mL DMSO, 5 µL of sample were added to wells in row A containing 195 µL of cell suspension for an initial concentration of 125 µg/mL. Serial dilutions were made down the plate to achieve a range of concentrations from 125 µg/mL- 0.98 µg/mL. Plates were incubated and read as above. Samples were evaluated based on the same criteria described above, and assays using those samples which were considered active were repeated 2-4 times with n = 2-5. Compounds which strongly inhibited the growth of both GAS strains were also tested for antimicrobial activity against strains of MRSA and VRE. In addition to those compounds contained within the Faulkner Pure

Compound Collection, a number of unknown compounds recently isolated in the Fenical lab were also screened for EPI activity.

Results

Screening of Crude Extracts

Between March, 2004 and August, 2005, a total of 1,760 marine actinomycete crude extracts (22 boxes) were screened, yielding 61 initial positive hits, resulting in a hit rate of 3.5%. Upon multiple repetitions of the assay, 31 samples consistently yielded positive results. Twenty-seven of these (4 were missing from collection) were re-grown to test for the continued presence of activity. Of these, 6 maintained activity and were subjected to separation using HPLC. However, testing of the resulting fractions yielded no active results. These strains were again re-grown and extracted in volumes of 100 mL and 1 L. Upon testing these extracts, 4 were found to initially demonstrate moderate EPI activity (Table 3.1a). However, upon repeating the assay the following day, this activity was no longer seen in all but one sample. The 100 mL extract of CNQ036 was the only sample to maintain EPI activity upon retest. Because insufficient material was obtained to allow LC/MS analysis, this culture was re-grown. However, upon testing of the extract from the re-grow, no EPI activity was observed.

Four samples were found to exhibit antibiotic activity toward both GAS strains (Table 3.1b). LC/MS analysis of the 1 L CNQ036 extract and the 100 mL CNP066 extract revealed the presence of piericidin, a known antibiotic from

Streptomyces sp. (Figure 3.3), and possibly new derivatives thereof. However, when pure piericidin A was tested, no activity against GAS was found. LCMS analysis of the CNQ066 1 L extract revealed the presence of agromycin, a known wide spectrum tetracycline antibiotic for agricultural use. LCMS analyses of the remaining extracts were unremarkable.

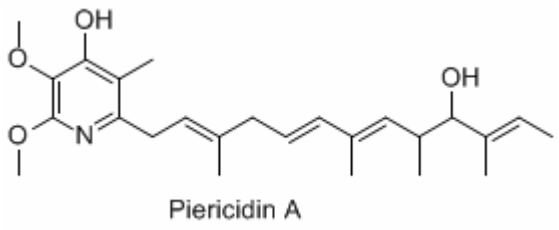

Figure 3.3. Chemical structure of Piericidin A

Table 3.1a. Actinomycete crude extracts demonstrating EPI activity. MIC's are given in µg/mL. NA= no activity.

Culture Extract	Medium	Location	MIC	DF1/KC9 %Surv. 1	2
CNQ026-100 mL	A1BFe+C	San Diego, CA	9.77	83/16	NA
CNQ026-1L	A1BFe+C	San Diego, CA	39.1	90/12	NA
CNQ036-100 mL	A1BFe+C	San Diego, CA	312	80/28	69/6
CNP066-100 mL	A1BFe+C	SanClemente Island, CA	39.1	80/10	4/11

Table 3.1b. Actinomycete crude extracts demonstrating antimicrobial activity. MIC's are given in µg/mL. NA= no activity.

Culture Extract	Medium	Location	MIC	DF1/KC9 %Surv. 1	2
CNP081-100 mL	TCG	San Clemente Island	78.1	4/6	3/18
CNP081-1 L	TCG	San Clemente Island	9.77	2/8	3/18
CNQ036- 1 L	A1BFe+C	San Diego	156	10/9	11/10
CNP066-1 L	A1BFe+C	San Clemente Island	39.1	7/12	5/15

CNQ719

CNQ719, a marine actinomycete belonging to the MAR 3 group which was isolated from organic-rich sediments collected in Guam in 2002, fell within the selection criteria (DF1 survival > 90%, KC9 survival < 10%) of the efflux pump inhibitor assay developed for this study in the primary screen at a concentration of

51.6 µg/mL. When put through secondary screening, CNQ719 again fell within the selection criteria in three out of three assay repetitions at a concentration of 78.1µg/mL using the original sample contained within the Fenical Crude Extract Collection. The time-course study revealed activity in the extractions of days 3 and 5, with DF1/KC9 survival percentages of 114/10 and 116/17, respectively, at an extract concentration of 6.25 µg/mL.

HPLC separation of the 1 L culture yielded 6 fractions. Fractions 1-4 showed no significant activity, while fractions 5 and 6, which fell within the 50-60% acetonitrile range, did reveal EPI activity. Fraction 5 showed the highest activity, with DF1/KC9 survival percentages of 121/8 at a sample concentration of 12.5 µg/mL. DF1/KC9 survival percentages for fraction 6 were 95/13 at a sample concentration of 50µg/mL.

Upon mass spectrometry analysis, the 50-60% acetonitrile fractions obtained from the 5 L re-grow revealed a series of compounds having molecular weights of approximately 900, but that appeared to be in complexation with other compounds. The UV profile was distinct and did not correspond to any previously known compounds. Insufficient material was obtained from this extraction for NMR analysis.

Additional material obtained from the 30 L re-grow again revealed EPI activity in the 50-60% acetonitrile range. The 900 MW compounds were again found to be present through MS, and again appeared to be part of a complex mixture of compounds, including those of MW 703-740. Compounds of MW 719

were purified, but were found to be inactive in the EPI assay. The unknown compounds having molecular weights of approximately 900 could not be successfully purified due to their state of complexation. NMR spectra of these samples confirmed that they contained a complex of compounds.

Screening of Pure Compounds

One-hundred seventy-three previously isolated compounds contained in the library of Dr. D. John Faulkner were screened for EPI activity between September, 2005 and December, 2005. Halisulfate-1, a sulfated sesterterpene hydroquinone previously shown to demonstrate anti-inflammatory activity by inhibiting phospholipase 2, anti-proliferative activity against fertilized sea urchin eggs and antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* emerged as the only sample within the collection which showed consistent EPI activity (Figure 3.5).[43] This compound was isolated from a marine sponge of the genus *Halichondria*, collected from Scripps Canyon, La Jolla, CA at a depth of 30 m. in 1987. However, NMR and LCMS analysis indicated that degradation of this sample had taken place, such that it contained a number of degradation products. HPLC separation of the sample was performed in an attempt to isolate the observed EPI activity to a single fraction. However, EPI assay of the resulting fractions yielded no significant activity.

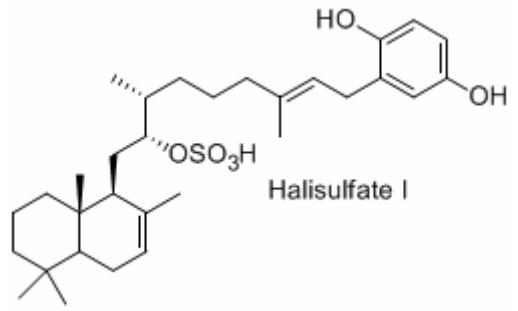

Figure 3.5. Chemical structure of Halisulfate I

A number of the Faulkner compounds tested demonstrated antimicrobial activity toward both strains DF1 and KC9. Some of these are known disinfectants, while others demonstrate a variety of activities (Table 3.2).

Table 3.2. Pure compounds having antibiotic activity. MIC's given are in µg/mL. NSA = no significant activity.

Compound	Type	Known Activity	GAS MIC	MRSA/VRE MIC
Halistanol	Steroidal sulfate	Anti-inflammatory	7.81	1.8/3.91
Haliclonacyclamine			3.91	62.5/62.5
Heteronemin	Sester-terpene	Anti-tuberculosis	15.6	NSA
Episclaradial		Anti-inflammatory	7.81	15.6/15.6
Plakotenin	Carboxylic Acid	Anti-proliferative	3.13	3.13/3.13
Homoplakotenin	Carboxylic Acid	Anti-proliferative	3.91	3.13/6.25
Agelasine	Diterpene	Anti-microbial	1.95	1.95/7.81
Arenarol	Sesqui-terpene	Anti-cancer	3.91	1.95/1.95

An additional unknown compound from actinomycete strain CNQ049 demonstrated strong antibiotic activity (Figure 3.6). Structure determination suggested fusidic acid, a known antimicrobial agent isolated from the fungi, *Fusidium coccineum* and *Calcarisporium antibioticum*. This fusidane is notably active against staphylococci, including those which are methicillin resistant.[44, 45] It has been investigated in vitro for the treatment of penicillin-resistant pneumococci in combination with the fluoroquinolone antibiotic, levofloxacin, but did not appear to potentiate the intermediate activity of levofloxacin in this pathogen.[45]

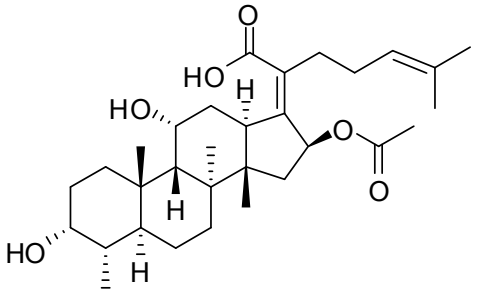

Figure 3.6. Proposed structure for CNQ049.516

Discussion

The reason for the disappearance of observed activity in the crude marine actinomycete extracts which initially tested positive in the EPI assay is most likely one of secondary metabolite production. In cases where activity which was observed in the original sample was no longer present after the strain was re-grown, it is likely that the active compound could not be produced under our laboratory conditions. The physical and chemical factors which regulate secondary metabolite production in marine actinomycetes are numerous. Efforts can be made to reproduce such factors as temperature, light, wave action and nutrient availability, but other factors may be at work in the natural marine environment which are unknown to us.

For example, certain secondary metabolites may be produced only in the presence of a particular stress factor, such as a toxic environmental contaminant or the presence of a competing species. Or they may display seasonal variations of which we are unaware. The absence of a desired secondary metabolite upon re-growth of the producing strain is quite common and presents a substantial limitation to the development of marine natural products for medicinal interest. As with any natural product, whether terrestrial or marine, the reliable availability of the compound of interest is an absolute requirement for further pharmaceutical development.

The identification of independent antibiotic activity is a benefit of the EPI assay developed here. As expected, the overall number of samples throughout the

screening process which displayed antimicrobial activity toward both strains DF1 and KC9 were by far more numerous than those which displayed EPI activity. It should be noted that the assay results in most cases suggested that strain KC9 is inhibited at somewhat lower concentrations than strain DF1 by the same sample. This may be due to the metabolic cost of maintaining a functioning efflux pump in strain KC9. The action of the pump confers a survival benefit to the bacterium in the presence of an antibiotic which is a substrate of the pump, but may come at the cost of decreased survival in the presence of other harmful agents which are not expelled by the pump.

Of the 1760 crude marine actinomycete extracts which were screened for EPI activity during the course of this research, the most promising results emerged from CNP719. This actinomycete strain showed consistent EPI activity with every re-grow of culture. LC/MS and UV analysis suggest the presence of unknown compounds of MW ~900 in a state of complexation with other compounds. This presented significant difficulty in separating and purifying these unknown compounds. The NMR spectra which were obtained confirmed this difficulty. Better methods are required to achieve the isolation of these potential EPI's.

A number of the crude extracts obtained from re-grow cultures demonstrated EPI activity which was no longer present upon separation with HPLC. The most probable explanation for this effect is that the observed EPI activity in these samples is due to the synergistic interaction of two or more compounds within the crude extract. A crude extraction may contain a large

number of compounds of varying polarity, which may be isolated to different fractions upon separation by HPLC. To further test this theory, the separate fractions should be recombined and screened for the reappearance of EPI activity. However, EPI activity due to the combined effect of multiple compounds presents obvious difficulties for pharmaceutical development, as each compound must meet the parameters of pharmacokinetic activity that a potential pharmaceutical agent is subject to in the clinical setting.

The search for a compound which will effectively inhibit the action of a drug-specific efflux pump such as MefA is an extremely challenging undertaking. Particularly compounding is the large number of these types of pumps possessed by pathogenic bacteria. Although the high degree of structural homology between these types of efflux proteins gives hope to the idea that a compound which inhibits one pump may also effectively inhibit others, each drug pump is essentially unique. Screening for an inhibitor of a particular pump involves searching for highly specific activity. Vast numbers of samples may be tested without the emergence of a single promising lead. Although the search for a 'needle in a haystack' may prove discouraging, it should be kept in mind that, when found, what a valuable needle it will be.

Conclusions

The incidence of antibiotic resistance is on the rise and presents a substantial risk to public health. Although the need for new antibiotics is ever present, there exists an urgent need to explore new approaches to combating bacterial pathogens if we are to maintain an effective arsenal of antibiotic therapies and continue to enjoy the benefits of the modern antibiotic era. One such approach is the inhibition of efflux pumps in order to re-sensitize bacterial pathogens to antibiotics which they have developed resistant to. The application of efflux pump inhibitors in combination with antibiotics would allow the continued use of existing medicinal agents, and give new life to proven therapies. Further more, the marine realm offers an exciting and diverse storehouse containing a wealth of compounds previously unexplored for their medicinal potential.

Although an EPI of the MefA efflux pump of *S. pyogenes* was not isolated and identified during the course of this work, the goal of this project was ultimately met in the successful design and establishment of a working system to enable the screening of large numbers of both crude extracts and pure compounds for their potential to inhibit this and other antibiotic efflux pumps. This assay allowed for the identification of both crude marine extracts and previously isolated compounds which demonstrated EPI activity. Utilization of this assay in conjunction with bioassay-guided fractionation may in future lead to the isolation and identification of a pure compound, whether novel or known, which may successfully inhibit the action of one or more of the multitude of trans-membrane efflux proteins which

mediate antibiotic resistance in the microbial pathogens of greatest concern to human health.

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CHAPTER 4

MOLECULAR STUDIES

In order to investigate the role of *mefA*, several genetic studies were undertaken. First, the *mefA* gene from two strains of erythromycin resistant group A streptococci (GAS) was complemented into a number of *mefA*-negative bacterial strains, including *Streptococcus agalactiae* (GBS), and various GAS strains, including the erythromycin sensitive, *mefA* negative M6 GAS wild-type strain DF1 and the highly resistant, *mefA*-positive GAS M6 strain KC9. MIC values for erythromycin in these recombinant strains varied from 0.01 μg/mL to 5.0 μg/mL. Efforts were also made to generate a *mefA* negative "knock-out" strain from the highly erythromycin resistant, *mefA*-positive KC9 parent strain using precise in-frame allelic exchange mutagenesis.[1] The resulting strain carried the expected phenotype of a *mefA* knock-out mutant but retained the genotype of the parent strain. In addition, the *mefA* gene was cloned and sequenced from a *mefA*-positive M6 strain which had reverted to erythromycin sensitivity. Sequencing indicated that this strain had undergone a mutation which resulted in deletion of approximately two-thirds of the *mefA* gene. Finally, a study was performed to compare the ability of *mefA*-positive GAS strains to invade host cells with that of a *mefA*-negative strain, the results of which indicated that these *mefA*-expressing

strains have variable levels of invasion and that mutation of *mefA* does not lead to reduced invasion.

Introduction

Streptococcus pyogenes, or Group A streptococci are pathogens responsible for a variety of human diseases, ranging from mild infections such as impetigo to life-threatening conditions such as necrotizing fasciitis. *S. pyogenes* is the leading cause of bacterial pharyngitis in the pediatric population. The drug most suited to treat infections of GAS, and that recommended by Infectious Disease Society of America guidelines is penicillin V.[2] For patients having allergy to penicillin, erythromycin and other macrolides are recommended. Penicillin resistance and cross-resistance to macrolides has been reported in *Streptococcus pneumnoniae*, but so far has not been observed in *S. pyogenes*.[2, 3] In cases of macrolide resistance, clindamycin is the next line of defense, and in cases of recurrent infection, the new ketolide antibiotic, telithromycin is recommended.[3]

Three mechanisms by which macrolide resistance may occur have been characterized: (i) by post-transcriptional modification of the 23S rRNA subunit through the addition of methyl groups by methyl transferase enzymes encoded by the *erm* genes; (ii) by active transport of the antibiotic out of the cell through the action of a trans-membrane efflux pump encoded by the *mefA* gene; and (iii) by rare mutation in the ribosomal binding site.[4] The presence of *erm* genes is characterized by the MLS_B resistance phenotype in which the isolate is resistant to

macrolides, lincosamides and streptogramin B, whereas those resistant through the action of the MefA efflux pump commonly display the M phenotype of resistance to 14- and 15-membered macrolides only.[5] However, more than one resistance gene may occur in a single strain.[2]

The prevalence of resistance of *S. pyogenes* to macrolide antibiotics has been on the rise worldwide in recent years.[6] A number of European countries have reported a change in the prevailing macrolide resistance phenotype occurring along with an increase in macrolide resistance in GAS.[7] Silva-Costa et al. reported a shift in macrolide resistance phenotype of *S. pyogenes* isolates in Portugal from 80% expressing the MLS_B phenotype in 1998 to 76.6% expressing the M phenotype in 2002.[8] This could very well be due to the introduction of highly successful clones from other parts of the world, as resistance determinants have been shown to display a strong geographic component.[3] Incidentally, *mefA* has been identified as the prevalent macrolide resistance gene in neighboring Spain.[9]

The *mefA* gene has also emerged as the predominant macrolide resistance determinant in a number of other European countries.[10-12] Since the 2000-2001 occurrence of erythromycin-resistant GAS pharyngitis in Pittsburgh, PA reported by Martin et al., *mefA* has also become the predominant mechanism of macrolide resistance in the U.S., and subsequently a cause for growing concern.[13, 14]

Methods

Chromosomal DNA Isolation from GAS

Initial isolation of DNA was done using a traditional protocol, rather than a DNA isolation kit. For this procedure, 5 mL O/N cultures of each GAS strain were pelleted using a Jouan centrifuge at 4000 rpm for 5 minutes, re-suspended in 200 µL of a lysis buffer consisting of 6.7% sucrose/50 mM Tris 7.0/1 mM EDTA and transferred to microcentrifuge tubes which contained 25 µL mutanolysin (10,000 u/mL in lysis buffer). 1 µL RNAse was added to each tube and the samples were mixed and incubated for 15 minutes at 37° C. After the samples were removed from incubation, 8 µL Proteinase K (20 mg/mL) and 10 µL SDS were added to each. The samples were mixed and placed in a 55° C incubator for 45 minutes. Following this incubation, 250 µL water and 250 µL phenol (neutral pH) were added to each and the samples were vortexed and then spun briefly. 250 µL chloroform/isoamyl alcohol were added and the samples were vortexed and spun for 5 minutes at maximum speed in a table-top microcentrifuge. The supernatants were removed and transferred to clean tubes containing 500 µL phenol/chloroform/isoamyl alcohol. The samples were again vortexed and spun for 5 minutes. The supernatants were transferred to clean tubes containing 500 µL chloroform/isoamyl alcohol. The samples were again vortexed and spun for 5 minutes. The resulting supernatants were then transferred to clean tubes containing

1/10th their volume of 3 M NaAc (pH 5.2) and 2 volumes of ethanol, mixed and allowed to incubate for 5 minutes at room temperature. The samples were spun for 5 minutes and the supernatant drained from the resulting pellets. The pellets were then washed with 70% ethanol to remove salts and dried using a speed vacuum. The pellets were re-suspended in 50 µL tris-chloride and measurements of DNA concentration were taken.

PCR Confirmation of *mefA*

PCR analysis was performed using the DNA extracted above to confirm the presence of the *mefA* gene in the 4 M6 GAS clinical isolate strains obtained from the University of Pittsburgh Children's' Hospital. The "external" primers (Appendix A) used were also obtained from Pittsburgh and were designed to amplify the entire 1220 base pair *mefA* sequence. Primers were added to PCR tubes containing approximately 200 µg of DNA in Platinum Supermix® (Invitrogen) to a total sample volume of 50 µL for each reaction. Samples were cycled and loaded onto 1% agarose gel for visualization. A second set of "internal primers" (Appendix A) designed to amplify a smaller portion within the *mefA* gene was also used.

Creating the *mefA* Expression Vector

The 1220 bp *mefA* gene, along with upstream and downstream regions of approximately 320 bp was amplified by PCR from chromosomal DNA isolated

from strains DC7 and KC9 using primers specifically designed for that purpose (Appendix A). The upstream primer was engineered to contain the restriction site *Xba* I; the downstream primer contained *Bgl* II. The PCR product contained T-overhangs to allow TOPO TA Cloning® according to the Invitrogen protocol. The cloning reaction mixture contained 2 µL fresh PCR product, 1 µL salt solution, 2 µL sterile water and 1 µL pCR 2.1 TOPO® vector with a final volume of 6 µL. The cloning reaction was mixed gently, incubated for 5 minutes at room temperature and placed on ice.

The cloning reaction was transformed into chemically competent One Shot® Top 10 *E. coli* by adding 2 µL of the reaction per vial of cells and incubating the samples on ice for 5 minutes. The samples were heat shocked for 30 seconds at 42° C and immediately placed on ice. 250 µL of SOC medium was added to each sample and the vials were capped and shaken horizontally (200 rpm) at 37° C for 1 hour. Because TOPO® vector contains a kanamycin resistance cassette, the recovered transformations were plated in volumes of 25 and 50 µL onto LB plates containing 50 µg/mL kanamycin to allow for antibiotic selection. Strain KC9 plated onto THA + kanamycin and transformation plated onto a kanamycin-free THA plate were used as controls. The plates were placed in a 37° C incubator O/N and monitored for colony growth beginning the following day.

To confirm the vector construct, plasmid preps of 10 transformant colonies (5 transformed with DC7 *mefA* and 5 with KC9 *mefA*) were performed according to the Qiagen QIAprep Mini Prep Kit® protocol using O/N cultures. Restriction

enzyme digests were performed according to New England Biolabs® protocol using these plasmid preps with enzyme *Hin*d III. This restriction site is contained within both TOPO vector and within the *mefA* gene. Each reaction mixture contained 1 µg plasmid DNA, 2 µL 10X N. E. Buffer 2 (NEB 2), 1 µL restriction enzyme and 7 µL sterile water to a total volume of 20 µL. The samples were incubated at 37° C O/N and run out in 1% agarose gel for 80 minutes at 100 V the following morning. 1 kb DNA ladder was used for reference. The gel was visualized and photographed using UV.

To cut the *mefA* insert from pCR 2.1 TOPO® vector, double restriction digest was performed using restriction enzymes *Xba* I and *Bgl* II. The reaction mixture contained 1 µg plasmid DNA, 3 µL N. E. B. 2, 0.5 µL each of *Xba* I and *Bgl* II enzymes and sterile water to a total volume of 30 µL. Double restriction enzyme digest to linearize plasmid pDC123, which contains a chloramphenicol acetyl transferase determinant (*cat*) to confer chloramphenicol resistance, was also performed using enzymes *Xba* I and *Bgl* II. This reaction mixture contained 1 µg plasmid DNA, 3 µL N. E. Buffer *Bam* HI, 0.5 µL each of enzymes *Xba* I and *Bam* HI and sterile water to a total volume of 30 µL. The reactions were incubated at 37° C O/N and run out 1% agarose gel for 1 hour at 100 V. 1 kb DNA ladder was used for reference. The gel was visualized and photographed using UV and the desired bands were cut from the gel and purified using the Qiagen QIAquick Gel Extraction Kit® protocol.

Ligation reaction was then performed using the products of the double enzyme restriction digests. The reaction mixture contained *mefA*+flanking regions from either strain DC7 or KC9 and linearized pDC123 in a ratio of 3:1 (insert:vector), 4 µL 5X ligase reaction buffer, 1 µL T4 DNA ligase and autoclaved, distilled water to a total volume of 65 µL. The samples were gently mixed, centrifuged briefly in a table-top microcentrifuge and incubated at room temperature for 5 minutes. 2 µL of the ligation reaction were then used for each chemical transformation of One Shot® Top 10 *E. coli* as described above. Electroporation using electrocompetent E. coli strain MC1061 was also performed using 2 µL of ligation reaction per vial of cells at 1500 V. The transformations were recovered for 90 minutes at 37° C with shaking and spread onto LA plates containing 15 µg/mL chloramphenicol in volumes of 50-200 µL to allow for antibiotic selection. The plates were placed in a 37° C incubator O/N and monitored for colony growth beginning the next day.

Resulting transformant colonies were grown in LB (Appendix B) O/N with 15 mg/mL chloramphenicol at 37° C with shaking. Plasmid preps were performed using the Qiagen QIAprep Mini Prep Kit® protocol. The resulting plasmid DNA was digested with restriction enzyme Hindi III in order to confirm the correct plasmid. The reaction mixture was the same as describe above. Samples were incubated at 37° C for 2.5 hours and run out in 1% agarose gel with 1 kb DNA ladder for 2 hours at 100 V. The gel was visualized using UV.

Creating *mefA* Expression Systems

pDC123Δ(KC9) *mefA* plasmid DNA from resulting *E.coli* transformants was transformed into an electrocompetent GBS strain, COH1, GAS M6 strain DF1, GAS M1 strain M15448 and GAS M49 strain NZ131 using electoporation. Transformations were recovered for 1.5 hours at 37° C and plated onto THA containing 2 or 5 µg/mL chloramphenicol and THA without antibiotic as a control. All plates were placed at 37° C O/N and monitored for colonies beginning the next day. Plasmid DNA extractions from the resulting transformant colonies were subjected to PCR analysis in order to confirm the presence of pDC123Δ*mefA*. Erythromycin MIC testing of confirmed transformants was performed to explore their level of *mefA* function. Transformants were also passed into medium containing gradually increasing erythromycin concentrations in order to discover the highest level of resistance *mefA* is able to mediate in these various bacterial systems.

DC2 Mutagenesis

In order to determine the difference between the functioning *mefA* gene of strain DC7 and the non-functioning *mefA* gene of strain DC2, *mefA* DNA from DC2 was amplified using Fail Safe™ PCR with the primers designed to amplify from the 320 bp upstream and downstream regions of the *mefA* gene (Appendix A). Gel electrophoresis revealed 4 bands present in reaction F. This PCR product was cloned into One Shot® Top 10 *E. coli* using the Invitrogen TOPO TA Cloning®

protocol. Plasmid DNA was extracted from resulting colonies and TOPO®ΔDC2*mefA* was confirmed through PCR methods. Restriction enzyme digest using Eco RI was performed to determine the size of the insert. The TOPO®ΔDC2*mefA* vector was submitted for DNA sequencing.

mefA Allelic Exchange Mutagenesis

For this procedure, flanking regions of approximately 320 bp and 328 bp immediately upstream and downstream of *mefA* from strain KC9 were amplified using PCR with internal primers overlapping the ends of the chloramphenicol acetyl transferase (*cat*) gene (Appendix A). The flanking regions were then linked to the *cat* gene via fusion PCR with all three DNA fragments and cloned into pCR 2.1-TOPO® vector, which contains specific restriction sites. This vector construct was then chemically cloned into One Shot® Top 10 *E. coli*.

The constructed sequence was cut out of pCR 2.1-TOPO® vector using the restriction sites Bgl II and Xba I. The temperature sensitive vector, pHY304 was digested using Bam HI and Xba I. These two products were then ligated together to generate a "knock-out" vector, pHY304Δ*mef/cat/mef*. In this vector, the 1200 bp *mefA* gene has been replaced precisely with the 650 bp *cat* gene, while the upstream and downstream flanking regions of *mefA* remain intact. These flanking regions should serve as sites for homologous recombination with the GAS chromosome when transformed into the erythromycin resistant, *mefA* positive KC9 strain. The knock-out plasmid was transformed into electro-competent *E. coli* strain MC1061.

The transformation was recovered in SOC medium at 30° C with shaking for 3 hours, then plated onto LA containing 10 µg/mL chloramphenicol. Plates were incubated at 30° C O/N and monitored for colony growth beginning the following day. Plasmid DNA extracted from resulting colonies was subjected to PCR analysis to confirm transformants expressing the knock-out plasmid. Plasmid DNA from a confirmed MC1061 transformant was transformed into GAS strain KC9 using the electroporation method and grown on THA plates containing chloramphenicol as the selective agent at the permissive temperature for plasmid replication (30°C). GAS transformed with the plasmid were then shifted to a temperature at which the plasmid can no longer replicate (37°C), and is forced to integrate into the chromosome in order to persist. Resistance to both erythromycin and chloramphenicol at 37°C identified these single crossovers. Single crossovers were then relaxed again to 30°C to promote plasmid excision, and were screened for double crossover events in which the cat gene has precisely replaced *mefA* within the chromosome, thus generating a *mefA* "knock-out" mutant. This mutant exhibits restored sensitivity to erythromycin while maintaining resistance to chloramphenicol. A mutant was generated which displays this phenotype, and PCR reactions were performed to confirm the *mefA* knock-out mutant.

Invasion Assay

To compare the ability of *mefA*-positive GAS to invade host cells with that of *mefA*-negative GAS, an invasion assay was performed using the *mefA*-positive erythromycin-resistant M6 strains DC7 and KC9 and the *mefA*-positive, but erythromycin-sensitive strain DC2. The assay was performed in a 24-well plate format using HEp-2 (human epithelial pharyngeal) cells. All three bacterial strains were grown O/N at 37° C in THB medium (Appendix B), the two former strains with 1 μg/mL erythromycin added. The following morning, the cultures were diluted 1:10 and allowed to grow to an OD of 0.4, corresponding to a concentration of 10^8 bacterial cells per mL. 100 μL of each culture were added to 1.7 mL tubes containing 900 μL of PBS and vortexed briefly 3 times. This 1:10 dilution was repeated 5 times in total to result in 5 tubes containing bacterial concentrations of 10^7, 10^6, 10^5, 10^4 and 10^3. 50 μL of 10^3 were plated on to THB and incubated at 37° C O/N for quantification. For the invasion assay, 100 μL of 10^6 CFU/mL of each strain were added to each of 4 wells in a 24-well plate containing a confluent mono-layer of HEp-2 cells in 500 μL of fresh RPMI media + 10%FBS and containing nonessential amino acids and L-glutamine. The plates were spun down at 1500 rpm for 5 minutes and incubated at 37° C + 5% CO_2 for 2 hours. Following this incubation, the medium was removed from the wells and the mono-layers were washed 3 times in PBS. 500 μL of fresh RPMI containing 100 μg/mL gentamycin and 5 μg/mL penicillin were added to each well and the plates were returned to incubation for an additional 2 hours. Following this incubation, the medium was

removed and the mono-layers were trypsinized. 400 µL of 0.025% Triton-X were added to each well and the samples were plated at eight different dilutions onto fresh THA plates and incubated O/N at 37° C. CFU were counted the following day.

Results

PCR Confirmation of *mefA*

PCR analysis of DNA extracted from the four M6 GAS clinical isolate strains using external *mefA* primers yielded the expected results. Bands of approximately 1200 bp were seen for the two *mefA* positive strains KC9 and DC7, but were absent for the *mefA* negative strains DF1 and DC2 (Figure 4.1). PCR analysis using the internal primers revealed bands of approximately 350 bp for strains KC9, DC7 and DC2, indicating that a portion of the *mefA* gene remains present in the erythromycin-sensitive strain DC2.

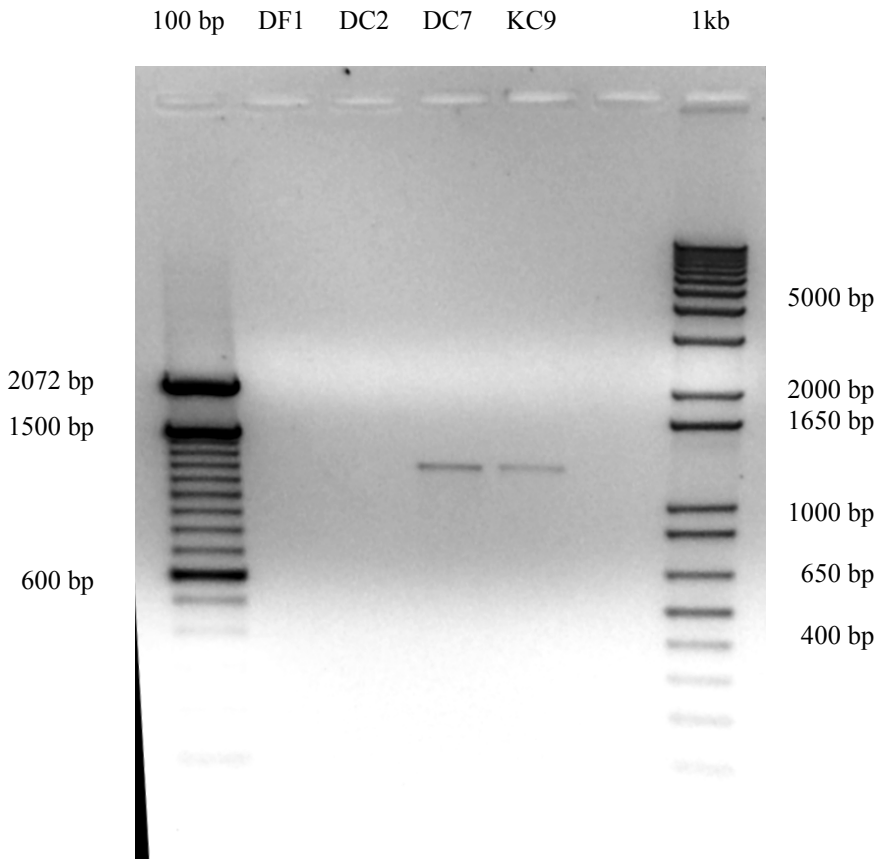

Figure 4.1. PCR confirmation of the *mefA* gene present in 4 GAS clinical isolate strains. The *mefA* gene is present in strains DC7 and KC9 and absent in strains DF1 and DC2.

Creating the *mefA* Expression Vector

Two pCR 2.1 TOPO®Δ*mefA E. coli* transformants were confirmed by restriction enzyme digest with Hindi III, one from strain DC7 and one from strain KC9. Expected band sizes were seen at approximately 940 bp and 4200 bp. pDC123Δ*mefA E. coli* transformant colonies were obtained from both electroporation and chemical transformation using the Qiagen® TOPO TA protocol. Four transformants were confirmed through restriction enzyme digest using *Hin*d III. Expected bands for these transformants were seen at 5700 bp. All those confirmed were from electoporation.

Colonies obtained from transformation of pDC123Δ*mefA* transformants into the varying bacterial backgrounds were confirmed through PCR methods. MIC testing of these expression systems to investigate whether introduction of the *mefA* gene is sufficient to impart resistance to erythromycin revealed varying levels of erythromycin resistance (Figure 4.2). These mutants initially displayed resistance only toward the selecting antibiotic, chloramphenicol. Erythromycin resistance was induced through exposure to step-wise increasing concentrations of erythromycin. GAS strain M15448 reached the highest level, exhibiting an MIC of 5 μg/mL erythromycin. GAS strains DF1 and NZ131 showed no resistance to erythromycin (MIC's of 0.08 μg/mL and 0.075 μg/mL, respectively), having MIC values comparable to the wild-types. Resistance to erythromycin in these strains was not inducible, even upon repeated exposure of extremely low levels of the antibiotic.

The GBS strain, COHI showed intermediate resistance with an MIC of 2 µg/mL erythromycin. In addition, an early experiment in which the *mefA* expression vector was transformed into GAS M1 strain DC7 suggested a significant increase in erythromycin resistance in this *mefA*-positive strain.

The *mefA*-positive GAS clinical isolate strains KC9 and DC7 were also exposed to step-wise increasing concentrations of erythromycin. Both of these strains showed increased levels of erythromycin resistance following increased exposure. The previous MIC's of 2 µg/mL were raised to 5 µg/mL in DC7 and to 10 µg/mL in KC9 (Figure 4.3).

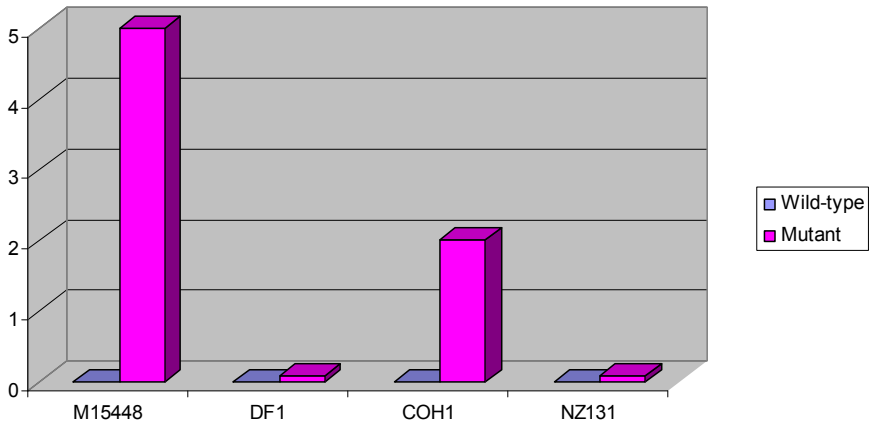

Figure 4.2. Erythromycin MIC's for *mefA* expression mutants after exposure to step-wise increasing concentrations of erythromycin. MIC's are shown in µg/mL.

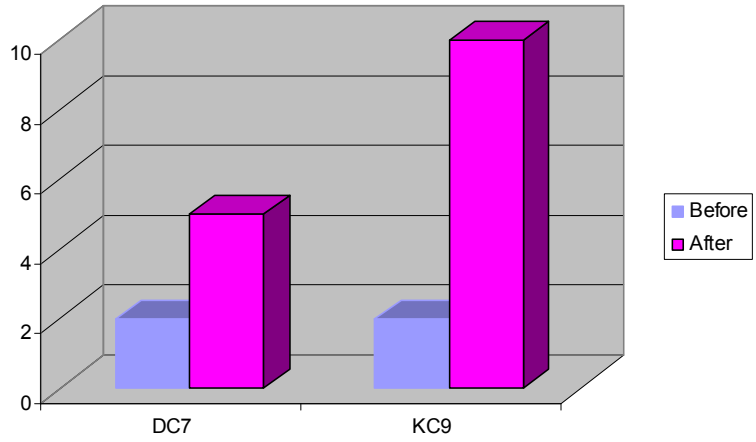

Figure 4.3. Erythromycin MIC's for *mefA*-positive clinical isolate strains following exposure to step-wise increasing concentrations of erythromycin. MIC's are shown in µg/mL.

DC2 Mutagenesis

Restriction enzyme digest of the vector, TOPO®ΔDC2*mefA* with Eco RI, revealed the size of the DC2 *mefA* insert to be approximately 850 bp. This piece of DNA included the 320 bp upstream region, plus and additional 11 bp, and 163 bp of the 328 bp downstream region, indicating that the size of the *mefA* gene in strain DC2 is approximately 356 bp. It appears, therefore, that the mutation which occurred in strain DC2 deleted at least two-thirds of the *mefA* gene (Figure 4.4). DNA sequencing confirmed this finding.

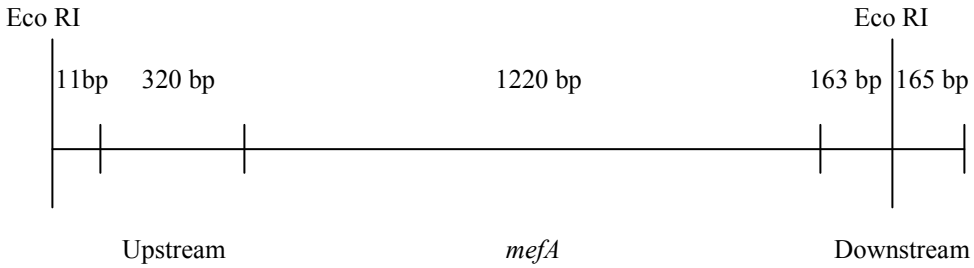

Figure 4.4. Map showing location of Eco RI restriction sites flanking the *mefA* gene on the chromosome. The size of the native *mefA* gene is approximately 1220 bp. In strain DC2, the size of the *mefA* gene is reduced to approximately 360 bp.

mefA Allelic Exchange Mutagenesis

Single cross-over mutants containing the *cat* gene integrated into the GAS bacterial chromosome following temperature shifting from the permissive temperature of 30° C for pHY304 replication to the non-permissive temperature of 37° C to promote plasmid integration were not confirmed through PCR. A mutant was obtained which displayed the expected single cross-over phenotype of resistance to both erythromycin and chloramphenicol. PCR analysis indicated the presence of the *mefA* gene in strain KC9, but failed to confirm that of the *cat* gene. After relaxation to 30° C to promote plasmid excision from the chromosome, a mutant was obtained which displayed the double cross-over phenotype of resistance to chloramphenicol and sensitivity to erythromycin. However, once again PCR analysis of this mutant indicated the presence of the *mefA* gene and failed to confirm the presence of the *cat* gene, despite the observed phenotype of the expected allelic exchange mutant.

Invasion Assay

In this study, the *mefA*-positive strain KC9 demonstrated the greatest ability to invade HEp-2 cells (Figure 4.5b). The *mefA*-negative strain DC2, however, showed higher invasiveness than its *mefA*-positive counterpart, strain DC7, suggesting that in these two strains, the expression of a functioning *mefA* gene does not impart a greater ability to invade host cells.

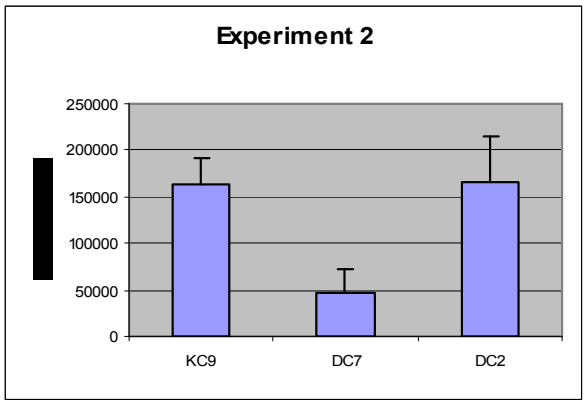

Figure 4.5a. CFU's of two *mefA*+ (KC9 and DC7) and one *mefA*- (DC2) GAS strains recovered from HEp-2 cells following invasion protocol.

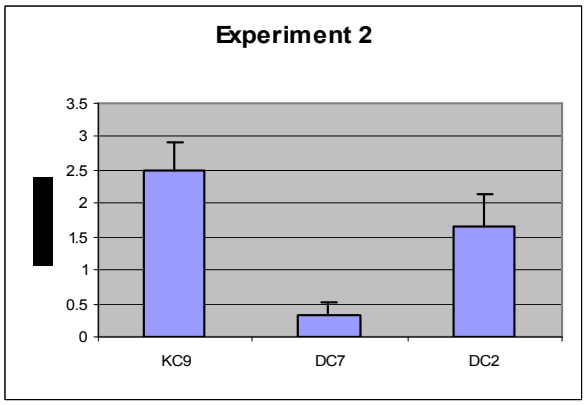

Figure 4.5b. Percent invasion of HEp-2 cells by GAS strains following invasion protocol.

Discussion

Transformation of the expression vector, pDC123Δ*mefA* into a variety of bacterial backgrounds had varying results, suggesting that *mefA* function may differ between types of bacteria. GAS strain M15448 is a highly virulent strain which has been implicated in invasive disease. Of all of the expression systems tested for erythromycin resistance, this strain reached the highest level, exhibiting an MIC of 5 μg/mL erythromycin. The relatively benign GAS strains DF1 and NZ131 showed almost no resistance to erythromycin, even upon repeated exposure of extremely low levels of the antibiotic in an attempt to induce resistance. The GBS strain, COHI showed intermediate resistance with an MIC of 2 μg/mL erythromycin. These results may have implications for a linkage between antibiotic resistance and virulence in pathogenic bacteria.

The homologous recombination procedure designed to replace the chromosomal *mefA* gene with the *cat* gene failed in GAS strain KC9. Although the chloramphenicol-resistant, erythromycin-sensitive phenotype of a *mefA* knock-out was achieved, PCR analysis indicated that the native *mefA* gene remained and that the *cat* gene was absent. This creates a very interesting possibility. Like erythromycin and other macrolide antibiotics, chloramphenicol is an inhibitor of protein synthesis in prokaryotes. Since the binding site of chloramphenicol is very similar to that of erythromycin on the bacterial ribosome, it may be possible that these antibiotics share similar binding sites on the *mefA* protein as well. If *mefA* is

able to efflux chloramphenicol with a greater affinity than that of erythromycin, this would explain the apparent shift from erythromycin resistance to chloramphenicol resistance.

To investigate this further, attempts were made to induce chloramphenicol resistance in the *mefA*-positive wild-type KC9. This resistance could not be induced, and KC9 remained sensitive to chloramphenicol with an MIC of 1 µg/mL. The MIC in the phenotypic knock-out created in this study was observed to be 5 µg/mL, although it lacks the *cat* gene. It is known that expression of CAT is generally inducible in gram-positive bacteria by sub-inhibitory concentrations of chloramphenicol.[15, 16] The failure to induce resistance to this antibiotic in KC9 further supports the absence of a *cat* gene in this strain.

This disparity between MIC values in two instances of the same GAS strain could be explained as follows. The KC9+pHY304Δ*mef/cat/mef* transformant was confirmed through PCR methods. This transformant carried the *cat*-expressing plasmid which replicated only at 30° C, imparting chloramphenicol resistance at this temperature. It is possible that this gave the bacterium the advantage of surviving in the presence of chloramphenicol long enough for a mutation in *mefA* to take place which allows the efflux pump to transport chloramphenicol to the exclusion of erythromycin. The *cat* gene would have disappeared with a temperature shift to 37° C. However, this resistance gene would no longer have been necessary for the bacterium to maintain chloramphenicol resistance if *mefA* were now able to efflux this antibiotic.

There remain, of course, any number of possibilities not considered here to explain chloramphenicol resistance in the *cat*-negative strain. Interestingly, a broad-host-range self-transferable plasmid, pIP501, is known from streptococci and enterococci which encodes resistance to both chloramphenicol and to erythromycin.[17, 18] Isolates expressing this plasmid demonstrate the MLS_B phenotype associated with the *erm* genes rather than the M phenotype associated with *mefA*. It has also been shown that the *cat* determinant from pIP501 is widely distributed on other plasmids and also present on the chromosomes of GAS, GBS and GGS.[19]

Results of the invasion assay indicate that no mechanistic correlation can in this instance be drawn between the *mefA* gene and increased invasion, despite the report of Fascinelli et al.[20] Invasion in this study appears to vary between strains, as is evidenced by the variation observed between strains KC9 and DC7, both *mefA* positive. Strains DC7 and DC2 are clinical isolates taken from the same child nine months apart, respectively displaying erythromycin resistance and restored sensitivity. Sequencing of DC2 indicated that a portion of the *mefA* gene has been deleted, rendering the MefA pump inactive. Inactivation of the *mefA* did not, in this instance, reduce invasiveness, but rather appears to have had the opposite effect, suggesting that *mefA* is not here a factor in the ability to invade.

Further Study

Further experiments should be carried out to evaluate the ability of the MefA efflux pump to efflux chloramphenicol. This could be achieved through efflux/accumulation assays using radio- or fluorescently-labeled chloramphenicol in the presence and absence of a known MF pump inhibitor, such as reserpine or a proton-gradient disruptor such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or dinitrophenol (DNP).[21] Sequencing of *mefA* in the phenotypic knock-out would reveal any mutation which may have taken place to cause the observed shift from erythromycin resistance to chloramphenicol resistance.

Once *mefA* has been knocked-out, it can be complemented back in to KC9 using a similar procedure with a plasmid bearing *mefA* along with its flanking regions. A return to the initial erythromycin resistant, chloramphenicol sensitive state of KC9 will be taken as evidence of the role of *mefA* in conferring resistance to erythromycin in this clinical isolate from the Pittsburgh outbreak.

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CHAPTER 5

CONCLUSIONS AND FUTURE OUTLOOK: INFECTIOUS DISEASE AND THE ENVIRONMENT

The primary contribution of this project has been the development of an assay with which to screen extracts of natural products or pure compounds for their potential to inhibit the action of a bacterial efflux pump and therefore to restore sensitivity of a bacterium to an antibiotic it was previously resistant to. Near to two thousand samples of marine origin were screened using this assay during the course of this study, but the protocol now exists for the screening of countless future samples. The work contained within this dissertation is truly only the tip of the proverbial iceberg.

I came to Scripps Institution of Oceanography in September of 2001 with an interest in trans-membrane efflux pumps and drug resistance. I had recently completed a master's degree for which my research, conducted within the Division of Biomedical Marine Research at Harbor Branch Oceanographic Institution, involved investigation of the P-glycoprotein pump in human uterine carcinoma cells and its role in the development of resistance to the newly discovered anticancer agent discodermolide. I wished also to be involved in the process of marine-based drug discovery, so when the opportunity arose to develop the *mefA*

project in collaboration with Dr. Nizet, I leapt at the opportunity. The result has been a growing experience, both personally and professionally.

The choice of my career path has been a source of great difficulty to me since childhood. Whether to study medicine or marine ecology has been a decision that I have wrestled with at every step along the way. I wished to find some way to combine the two in a way which has relevance to human populations on a global scale, and I believed that I had finally discovered it in the field of marine natural products drug discovery. However, while working at Harbor Branch I quickly discovered that the marine component was largely lacking. I worked in the tumor biology and immunology laboratory and although it was literally outside my door, I seldom even saw the ocean.

This all changed when I came to SIO. I was given knowledge not only of human pathogens and infectious diseases, but I was also able to gain exposure to the entire process of marine natural products drug discovery, from sample collection and extraction to fermentation and from bioassay-guided fractionation to isolation and structure elucidation. I studied everything from chemical ecology to pharmacology and gained a broader appreciation of the seldom recognized yet intricate relationship between mankind and the marine environment.

It is an understanding of this relationship that has given me the insight which has led me to ask the important questions which will guide my career path from this point onward. If we are learning that mankind can gain unknown life-saving remedies from the sea, then what other profound relationships between

human health and our environment have we hitherto ignored? As humans, we tend to view ourselves as separate from our environment. We in the developed world in particular sequester ourselves within man-made concrete habitats, seemingly eons away from the hazards of life in the natural world which were the everyday concerns of our ancestors.

We tend indeed to forget that in evolutionary terms, those ancestors lived not ambiguous ages ago, but rather closer to yesterday. Despite our hallmarks of civilization, the factors which shaped their health and well-being still shape ours today. From the first hominid to stand and walk upright to the nuclear age, factors such as climate change, biodiversity, wildlife population dynamics, food animal consumption and natural disaster determine the quality of our lives lived not in isolation, but in intimate association with the biosphere.

The boon of bioactive secondary metabolites upon which we have relied to provide medicines for what ails us is a primary example of how the natural world can benefit humankind. But what of the ways in which it can harm us? The hazards of the natural world to mankind are many, but upon even cursory examination it becomes evident that many of these dangers result from the hazards presented to the natural world by mankind, however inadvertent they may be. Perhaps in addition to asking where emerging infectious diseases such as SARS and avian influenza come from, we should be asking what we are doing to cause them to emerge.

A prime example is the potential effects of deforestation on human health. Forests, particularly tropical forests, are known to be foci of disease. The Amazonian forests alone harbor one-hundred and eighty-two known apoviruses, and are being cut down at a rate of ten thousand square kilometers per year, driven primarily by cattle ranching.[1] Such changes in land cover result in habitat loss for the species which inhabit them and bring humans into closer contact with wild animals and the diseases which find a reservoir within them. Environments where high diversity of wildlife meets growing human populations and changes in land cover are ideal birthplaces for new zoonoses. In light of this, it is not surprising that three-quarters of the world's emerging infectious diseases are zoonotic.[2] This is compounded by the loss of biodiversity which results from deforestation, forcing disease vectors such as mosquitoes, fleas and bats to resort to alternate habitats and host organisms.

A thought-provoking example of such a scenario is illustrated in the case of Nipah Virus in Malaysia. This paramyxovirus is a known component of the natural assemblage of fruit bats, which prefer to roost in trees of rainforests. With the conversion of forest to farmland to accommodate a growing population, bats have been forced to roost in fruit trees which are often planted above pig sties. Bat droppings infect pigs, which develop respiratory syndrome and infect both other pigs and humans, who may then develop viral encephalitis.[3] Multiple farms located in close proximity to each other allow transmission between farms and

increases the risk of mutation and of separate strains merging into new strains with increased virulence.

Mutation of pathogenic organisms is cause for great concern, as it can facilitate the leap of pathogens which infect animal species into human hosts, as appears to have been the case with SARS.[4] Animals of different species being kept in squalid conditions in close proximity to each other and to their human keepers, such as in crowded market places and rural farms creates dangerous breeding grounds for the emergence of new human pathogens. Avian influenza strain H5N1 is able to infect a variety of bird species and has made the leap to human beings with a case fatality of fifty-nine percent.[5] The specter of recombination with strains of human flu which will allow human-to-human transmission and spur a global pandemic is a terrifying prospect which is fueling the race to develop a vaccine and exemplifies the worst-case scenario in the field of emerging infectious diseases.

The emergence and spread of infectious diseases can be severely exacerbated by the facets of modern civilization, particularly when it comes to vector-borne diseases. Malaria, Yellow Fever, Dengue Hemorrhagic Fever and West Nile Virus are all spread by the bite of infected mosquitoes. Infected mosquitoes can spread these viruses to new locations through both natural and anthropogenic means. Human travel by boat and airplane and the transport of pets and livestock are perhaps even more effective than wind or bird migration in disseminating mosquitoes and the diseases they carry. In addition, increased rainfall resulting from anthropogenic changes to the atmosphere coupled with

deforestation creates more extensive wet habitat for mosquitoes to breed, thereby altering population densities of mosquitoes and the species which feed on them, which is manifested in changing patterns of disease.

The impacts of anthropogenic activity are not limited to alteration of local environments. The larger consequences of industrialization are becoming increasingly apparent through climate changes attributable to global warming. Increasing El Niño Southern Oscillation (ENSO) events can have profound effects on the spread of infectious diseases. Cases of Hanta Virus which recently occurred in the American southwest have been attributed to increased rainfall linked to ENSO.[6] Increased rainfall resulted in greater food availability for vector deer mice, which in turn led to increased rodent density and subsequent transmission of the virus to humans.

But what about the world's oceans? Man-induced changes due to erosion, sedimentation, coastal pollution, over fishing and recreational water sports are all having effects on the marine environment. Certain indicator species, such as crustaceans, which are extremely sensitive to pesticide pollution can be used to gage the health of marine ecosystems, particularly that of coral reef habitats. The activation of MXR mechanisms such as cytochrome P450 and efflux pumps in other invertebrates can indicate the presence of hydrocarbons and other pollutants hazardous to marine ecosystems.[7]

Human beings may not inhabit the oceans, but these impacts can be felt by human populations none the less. Traditional coastal populations may feel the

greatest effects. Through restricted access to ancestral fishing locations, pollution from hotels and tourist water sports, and other consequences of modern development, many native islanders are finding their traditional lifestyles in jeopardy. These things can have profound effects on both physical and emotional health. The native populations of Micronesia now have developed surprising rates of heart disease that can be attributed to a shift from a fish-based diet to one which depends on alternate and nutritionally inferior protein sources such as Spam® and other tinned meats high in saturated fats and cholesterol. Rates of alcoholism, depression, spousal abuse and sexually transmitted diseases have risen dramatically among these and other native island populations as they suffer the consequences of the alteration of the marine environment brought about by development.[8]

When all of these factors are considered, the picture of human health becomes a continuous, complex and multi-faceted mosaic, rather than the snap shot in time we have traditionally considered it to be. The ecological and biological components which affect us translate far beyond our physical environment. They impact our nutritional, societal, political, mental and spiritual well-being, which in turn impact our physical well-being.

All of the above may seem far removed from the field of marine natural products drug discovery which is the subject of this dissertation, but on the contrary, all of these factors are intimately connected. Just as on land, agriculture presents the biggest threat to marine ecosystems. The same development that threatens to destroy the diversity of Earth's rainforests also threatens that of the

rainforests of the sea. Loss of biodiversity means loss of chemical diversity, and therefore the possible loss of potent new medicines with the potential to save lives and to ease human suffering before they are ever discovered. Ironically, increasing human population is concurrently driving the degradation of the biosphere upon which we depend, fueling the emergence and spread of infectious diseases and threatening the very sources from which the medicines to treat them may be found.

This, however challenging, does not have to translate into a grim forecast for our future. It simply means that as researchers, we must learn new ways with which to approach the problems which confront human health. We can no longer conduct research in the isolation of our own laboratories, or within the limited boundaries of collaboration with like-minded and similarly trained colleagues. We must learn to step outside of traditional scientific bounds and to take a new look at our world and the role that we play in it. Only a multi-disciplinary approach to solving the problems which face us will allow us to conquer and to re-conquer the infectious diseases which threaten us. In order to prevent the return of the pre-antimicrobial era, we must not only discover and develop new drugs to combat both emerging infectious diseases and newly resistant forms of old diseases, but we must consider the social, economic, political and environmental components of human health in order to move successfully move forward into the future.

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APPENDIX A

PRIMER SEQUENCES
(5'-3')

***mefA* forward external**	ATGGAAAAATACAACAATTGGAAA
***mefA* reverse external**	TTTTAAATCTAATTTTCTAATCT
***mefA* forward internal**	AGTATCATTAATCACTAGTGC
***mefA* reverse internal**	TTCTTCTGGTACTAAAAGTGG
***mefA* up forward**	GGAGGAACCGAAACTATGAC
***mefA* down reverse**	AGGCAAGTTCACCCAGATGA
***mefA* up I/O**	TGCTGCGATAATTAAATCAG
***mefA* down I/O**	GATAAGCTTAACCATTTCAG

APPENDIX B

MEDIA COMPONENTS
(per liter)

THB	Beef heart infusion	500 g
	Peptic digest of animal tissue	20 g
	Dextrose	2 g
	Sodium chloride	2 g
	Disodium phosphate	0.4 g
	Sodium carbonate	2.5 g
	H_20	1 L
LB	NaCl	10 g
	Tryptone	10 g
	Yeast extract	5g
	H_20	1 L
A1BFe+C	Starch	10 g
	Yeast extract	4 g
	Peptone	2 g
	$CaCO_3$	1 g
	$Fe_2(SO_4)_3$	5 mL (8 g/L in DI H_2O)
	KBr	5 mL (20 g/L in DI H_2O)
	Seawater	1 L
TCG	Tryptone	3 g
	Casitone	5 g
	Glucose	4 g
	Seawater	1 L