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A Novel Approach to the Discovery of Natural Products From Actinobacteria

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A Novel Approach to the Discovery of Natural Products From *Actinobacteria*

by

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Abstract

Actinobacteria, primarily the genus *Streptomyces*, have led to the development of a number of antibiotics, which result from their secondary metabolites or modified derivatives. Secondary metabolite production can result from competition with neighboring microbes in an effort to disrupt growth, aiding in the competition for vital nutrients in impoverished conditions. Such secondary metabolites have the potential to affect a plethora of cellular functions in target cells, including, cell wall development, protein synthesis, protein function and fatty acid synthesis/metabolism. Due to the pandemic spread of antibiotic resistant bacteria, it is imperative to continue the search for new therapeutic agents targeting these deadly organisms. As such, our group explored soil and marine samples from Tampa Bay's surrounding farmlands and waterways for secondary metabolite producing microbes using culture methods specific to *Actinobacteria*. Through these efforts we isolated over 750 bacterial species, of which almost half are confirmed *Actinobacteria*. In an attempt to derive new and novel chemistry from these organisms, we used our novel collection, and developed techniques for epigenetic modification to un-silence dormant and cryptic metabolic pathways. Our work reveals that a number of these *Actinobacteria* produce secondary metabolites that are effective against the ESKAPE pathogens, some at very low concentrations. Although the bioactivity from secondary metabolites is a well-known source for antibiotic drug discovery, our epigenetic methods suggest a potential to isolate previously overlooked compounds that have a very real possibility for use as antibacterial therapeutics.

Introduction

Antibiotics: Costs and Resistance.

Some of the greatest advances in healthcare have been the advent of antimicrobial agents that are safe and effective. This therapeutic option has been associated with winning wars or prolonging life and is an essential part of modern medicine. Through the development of antibiotics human kind has had the benefit of increased food production in the form of preventing large-scale infections that wipe out entire populations of livestock. Anti-infective agents have also allowed for the advancement of surgeries including invasive procedures that may not have been available for fear of disease. There is however, a rather large caveat, which is antibiotic resistance. This issue is not limited to third world countries, it occurs on a global scale, in fact there were over two million antibiotic resistance infections and roughly 23,000 antibiotic resistance related deaths in the United States in 2013 (1). Antibiotics can be described as a double-edged sword being that using them is what drives resistance. While we constantly struggle to identify novel therapeutics, bacteria have inadvertently found numerous mechanisms for escaping the threat of antimicrobials. Bacteria have not only developed resistance to one antibiotic, but have acquired resistance to numerous antibiotics simultaneously, thus bringing about multi-drug resistance and even pan-resistance as seen in *Acinetobacter baumannii* isolates (2). Antimicrobial resistance does not only affect fitness of humans and microbes, but it also creates an extreme economic burden for humanity. Estimates suggest direct healthcare costs as high as $20 billion and lost productivity costs as high as $35 billion annually (3).

There are numerous arguments for how humanity may have misused antibiotics including overuse, inappropriate prescriptions, and use in agriculture. One of the biggest issues with regards to antibiotic resistance is the extensive overuse of these drugs (1). As mentioned earlier, resistance is acquired through the use of antibiotics, however it seems as though this occurrence could have been delayed if antibiotics were not issued unnecessarily. Antibiotic resistance could also be delayed if there were less of a need for antibiotics, this could easily be achieved if humanity were proactive with regards to infection prevention. Inappropriate prescriptions play a gargantuan role in antibiotic resistance as research has estimated that antibiotics are either misused or unwarranted 50% of the time (1). Failure to provide accurate prescriptions by doctors may not even provide relief for the patient, and is causing an extreme level of detriment for the future of humanity. In a 2015 Food and Drug Administration report, 71% of antibiotics sold in the United States were for use in food animals as an aid for production or production/therapeutics while the remaining 29% was used for as a therapeutic only (4). This staggering statistic is known to play a role in antibiotic resistance in many forms including emerging antibiotic resistant organisms and organisms that have acquired much higher tolerances to current antibiotics (5). It is impossible to know what could have been if humanity had used antibiotics appropriately, however in the generation leading up to a potential post-antibiotic era, we may yet rectify some of these deleterious effects.

ESKAPE Pathogens

Since the discovery of penicillin by Alexander Fleming, humanity has entered a non-stop struggle against the constant flow of evolution and genetic transfer that microbes rapidly undergo. This genomic flexibility seen in bacteria has led to an increase of antibiotic-resistance, which has resulted in the need for new bioactive therapeutic compounds (6). The increase in resistance is associated with the overuse of antibiotics, negligent patients and a

misunderstanding of how microbes play an important role governing our homeostasis (7). A group of bacteria, commonly associated with human infection that have developed the ability to escape the antimicrobial effects of modern antibiotics, have been termed the ESKAPE (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter species*) pathogens by the Infectious Disease Society of America (IDSA) (7). These ESKAPE pathogens comprise the majority of nosocomial-acquired infections in the United States (8).

Enterococcus faecium is a Gram-positive, facultative anaerobe and opportunistic pathogen, which reside within the digestive tract of animals. It is a non-spore forming member of the *Firmicutes*, which are characterized by their low G+C content (9). Prior to 1984, this bacterium was known as *Streptococcus faecium* (10). *E. faecium* infections can result in pelvic and urinary tract infections as well as endocarditis (11). Enterococci are well equipped with intrinsic resistance to a number of antibiotics such as aminoglycosides, and are able to mutate or acquire genes in order to deal with other antibiotics, such as β-lactams (12, 13). Prior to the mid 1980s vancomycin resistant *E. faecium* was unheard of, but as of 2007 more than 80% of *E. faecium* isolates possessed vancomycin resistance (14). *E. faecium* doesn't possess the level of pathogenic potential as other organisms such as *Staphylococcus aureus*, however, it is proving to be quite problematic as it has demonstrated resistance to almost every antibiotic used for enterococcal infections (12, 14).

Staphylococcus aureus is a Gram-positive, facultative anaerobe and opportunistic pathogen, which can reside on the skin, or within the anterior nares and throat of humans and also a wide range of animals (15). It is a non-spore forming member of the *Firmicutes* (9). *S. aureus* has been a major public health concern for a long while and is the leading cause of foodborne illness worldwide (16, 17). Staphylococci are also well known for their ability to acquire

resistance to harsh antibiotics such as methicillin or vancomycin as well as the speed with which they acquire such resistance. There are numerous mechanisms with which *S. aureus* is capable acquiring resistance, such as hydrolytically cleaving a compound as seen in β-lactam antibiotic resistance, or alteration of their architecture in order to prevent binding of compounds as seen with glycoside antibiotic resistance (18). Some of the components that make *S. aureus* such a threat are the variety of virulence factors, toxins as well as the ability to survive host immune responses (19).

Klebsiella pneumoniae is a Gram-negative, facultative anaerobe and opportunistic pathogen, which can reside in the mouth, intestines and other mucosal surfaces of healthy humans (20). *K. pneumoniae* can also naturally reside within the soil (21). It is a non-spore forming member of the *Proteobacteria*, specifically the *Gammaproteobacteria*, which includes numerous medically relevant groups of bacteria (22). Majority of *K. pneumoniae* isolates possess a capsule which helps the bacterium evade the immune system (23). Colistin has been a last resort antibiotic for *K. pneumoniae* infections, but with the rise in carbapenum resistance, colistin became a necessary treatment option for carbapenum-resistant *K. pneumoniae* (24). As a result of the continued use of colistin, we are now seeing colistin resistance amongst this bacterium, which will eventually leave humanity vulnerable again (24).

Acinetobacter baumannii is a Gram-negative, facultative anaerobe and opportunistic pathogen, which resides almost exclusively on humans, unlike other *Acinetobacter* species, which reside within soil, water and animals (25). It is a non-spore forming member of the *Proteobacteria*, specifically the *Gammaproteobacteria* (22). *A. baumannii* can cause numerous infections including skin and soft tissue infections, wound infections, urinary tract infections and also has been implicated in pneumonia as well as septicemia (26, 27). Resistance to a broad spectrum of therapeutics has been seen in *A. baumannii* including beta-lactam, quinolone and

aminoglycoside antibiotics (28). These antibiotics are dealt with in a number of ways, including enzyme degradation, as seen in beta-lactam antibiotics, mutations and highly active efflux pumps, as seen with quinolone resistance, or enzyme modification, as seen in aminoglycoside resistance (28).

Pseudomonas aeruginosa is a Gram-negative, facultative anaerobe and opportunistic pathogen, which can reside in almost any environment including soil, water, animals, humans, and plants (29). It is a non-spore forming member of the *Proteobacteria*, specifically the *Gammaproteobacteria* (22). One of the biggest concerns for this bacterium is the low nutrient requirements as well as its uncanny ability to escape antibiotic effects (30). Of the mechanisms for antibiotic resistance, over active efflux pumps play a significant role in preventing drugs from reaching their targets (31). Other mechanisms for antibiotic resistance include modification of drugs or drug targets (31).

Enterobacter cloacae is a Gram-negative, facultative anaerobe and opportunistic pathogen, which can reside in the gastrointestinal tract of humans as well as throughout the environment (32). It is a non-spore forming member of the *Proteobacteria*, specifically *Gammaproteobacteria* (22). *E. cloacae* was originally thought to occur as a nosocomial agent from the environment or hospital staff, however it was later concluded to be a result of the patients own microflora (33). It has been demonstrated that *E. cloacae* possesses resistance to ampicillin and a number of cephalosporin antibiotics, and has the potential to mutate in order to acquire alternative antibiotic resistance (32).

There are many ongoing debates as to which acquisition of infection type is worse, community acquired, or hospital acquired. Some suggest that hospital acquired infectious agents possess less virulence, but are more robust and difficult to treat and therefore impossible to completely

eradicate from the host (34). Others pose that community acquired infectious agents have had limited exposure to antibiotics and are more virulent suggesting that disease onset is much more rapid (34). Original thoughts were that hospitals provided the optimal environment for infection as residents were often ill or had some form of immune deficiency (35). This is no longer the case as number of community acquired infectious agents is on the rise and one mathematical model suggests community acquired methicillin-resistant *S. aureus* will outcompete hospital acquired strains (36). Many reports from hospitals suggest that community acquired strains include the ESKAPE pathogens among others (37, 38). A study of methicillin-resistant *S. aureus* diagnoses in 2013 from California hospitals reported that 1 in 100 inpatient stays possessed a primary or secondary diagnosis (39).

Antibiotic Resistance Mechanisms

There are many methods by which bacteria confer antibiotic resistance. Methods that are utilized by many different bacteria include reduced uptake of antibiotic, antibiotic target modification by mutation or chemical modification, antibiotic modification, overproduction of antibiotic target, enzymatic degradation or efflux of antibiotic (**Figure 1**). Even with this torrent of antibiotic resistance mechanisms, we still have numerous classes of therapeutics that maintain some level of potency today. The antibiotic class that includes sulphonamides, which are still synthetically derived today, need perpetual alterations, as microorganisms continuously acquire resistance, furthermore, a large portion of the human population have allergies to sulpha drugs (40). Sulphonamides are typically used today for treatment of urinary tract infections, as they are broad spectrum and target folate synthesis. Other antibiotic classes used in medicine today include β-lactams (penicillins, cephalosporins, carbapenums etc.), which have undergone numerous structural evolutions through the years, target the bacterial cell wall (41, 42).

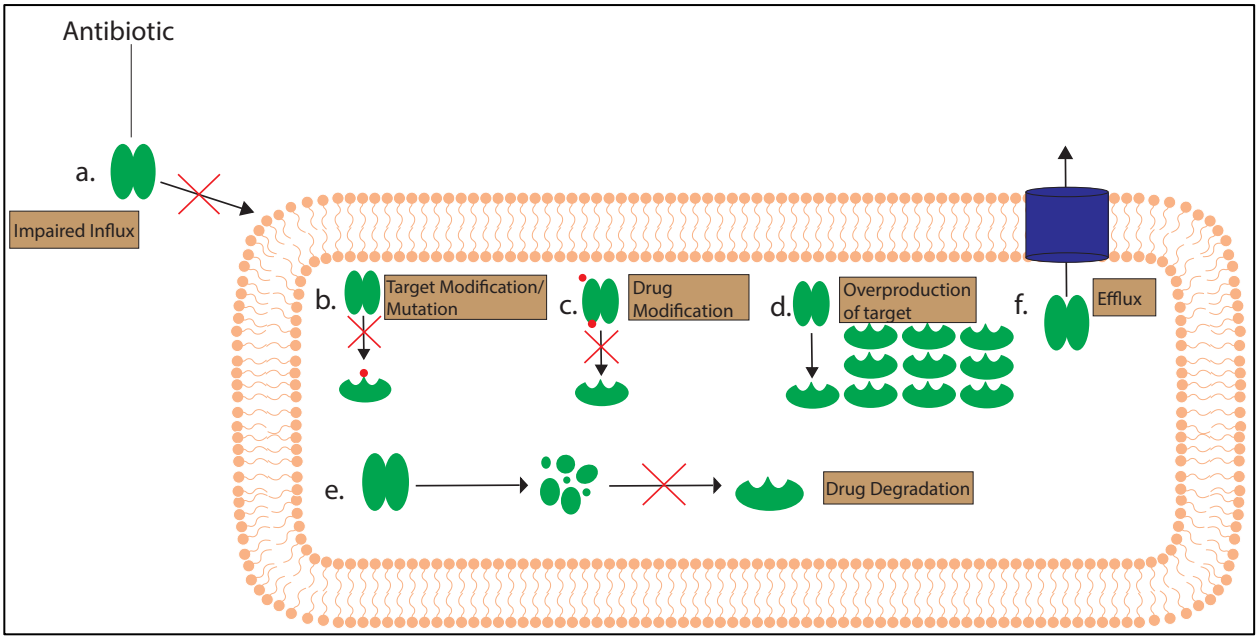

Figure 1. Antibacterial Resistance Mechanisms
Resistance to antibacterial compounds can e achieved in a number of ways. Drugs are not able to permeate through the membrane **(a)**; drug target is modified or mutated **(b)**; drug is modified **(c)**; drug target is overproduced **(d)**; drug is enzymatically degraded **(e);** effluxed immediately after entry **(f)**.

Amendments to β-lactam antibiotics primarily involve prevention of enzymatic degradation via β-lactamase, by addition of substituents that “shield” the β-lactam ring structure (41).

Reducing the uptake of a harmful compound ensures it will never reach its designated target. All antibiotics, regardless of their mechanism of action require some form of uptake into the cell; therefore permeability plays an important role in antibiotic efficiency. Gram-negatives possess a secondary membrane, which provides an extra layer of protection from permeable and semi-permeable compounds. Reducing permeability in Gram-negatives outer membrane proteins also limits antibiotic entry (43). In *Enterobacteriaceae*, it was seen that there was a reduction in carbapenemase expression, the enzyme responsible for enzymatic degradation of carbapenem, yet the bacteria were still exhibiting resistance to the antibiotic (44). It was determined that there was selective pressure by carbapenum that caused a mutation in porin expression genes (45, 46) (**Figure 1a**).

Antibiotics are target specific in that they bind with extreme prejudice to specific sites thereby inhibiting or reducing normal function. Subtle changes to the target site and still allowing for normal function can provide partial or complete resistance to the antibiotic. In an infection, there are many different subpopulations within the overall population. If a single point mutation were to occur in the gene of an antibiotic target, this could be the difference between surviving or succumbing to the onslaught. This has been demonstrated with linezolid resistance in *S. aureus*. Single nucleotide mutations in *S. aureus*’ genes encoding the 23S rRNA ribosomal subunit prevents binding of linezolid, but does not inhibit function and thereby conferring antibiotic resistance (47, 48). Modifications that result in resistance do not always occur by mutations, they can also occur via transformation, or uptake of foreign DNA from the environment that results in “mosaic” genes. An example of such a phenomenon occurred in

Streptococcus pneumoniae with regard to penicillin resistance. *S. pneumoniae* acquired genes which encode penicillin binding proteins that possessed a reduced affinity towards penicillin (49). Another example occurred in methicillin resistant *Staphylococcus aureus* in which the bacterium acquired a staphylococcal cassette chromosome *mec* element, which allowed *S. aureus* to produce β-lactam insensitive penicillin binding protein (50). Acquisition of this insensitive penicillin binding protein allowed for cell wall synthesis to continue as indigenous penicillin binding proteins were inactivated by present antibiotics (50) (**Figure 1b**).

Modification of antibiotic target using the addition of a small chemical group can be just as beneficial to bacteria as mutations or acquisition of modified target genetic material. One method that bacteria have established in the war against antibiotics is methylation of the target site. An example was observed with regard to erythromycin resistance whereby the erythromycin ribosome methylase (*erm*) family of genes would methylate the 16S rRNA ribosomal subunit (51, 52). Another instance where methylation conferred resistance has been observed in chloramphenicol resistance which involves the chloramphenicol-florfenicol resistance (*cfr*) methyltransferase enzyme transferring a methyl group to the 23S rRNA subunit's active site to prevent drug binding not only for chloramphenicol, but to a number of drugs that rely on this site for inhibition (53-55). Modification of drug targets is not limited to methylation, but can also occur with proteins that bind with higher affinity than the drug or bind to facilitate release of the drug. With regard to quinolone resistance, *qnr* genes encode pentapeptide repeat proteins that can bind to the topoisomerase-quinolone complex and result in the release of the antibiotic (56) (**Figure 1c**).

Bacteria have not only unlocked methods modify self to prevent antibiotic binding, but they have also ascertained approaches to modify the antibiotic compound itself. Addition of chemical groups to an antibiotic can result in resistance to the drug. Bacteria modify antibiotics with

proteins called “resistance enzymes,” which transfer chemical groups to the drug. Group transfer can include acetylation, phosphorylation thiol transfer among others. Acetylation as a drug modification system has been seen with aminoglycosides, which prevents the drug from binding to the ribosome (56). Due to their nature, aminoglycosides possess many substituents that can be modified in different ways including phosphorylation, which also prevents the drug from binding to the ribosome (57). Phosphorylation modifications can occur on other antibiotics as well; macrolides such as erythromycin are also susceptible to this modification, which prevents interaction with the 23S rRNA ribosomal subunit (58-60). Thiol transfer inactivation of a drug has been seen in fosfomycin resistance, through which the bacteria can resume peptidoglycan biosynthesis (61). It has been found that a number of these resistance enzymes are plasmid encoded and perhaps originated in the bacterium that produced the antibiotic (61).

A technique used by bacteria to overcome the barrage from antibiotics includes an overproduction of the drug target. Additional production, higher than standard levels, can result in antibiotic resistance as seen with trimethoprim resistance. Typically overproduction of the drug target seems to be pure circumstance. Random mutations in the promoter region of the gene can lead to more efficient binding of the RNA polymerase, which results in higher levels of transcription of the gene. Alternatively, random mutations to the ribosomal binding site can yield the same outcome as more of the mRNA is then translated. In the case of trimethoprim resistance in *Escherichia coli*, both of these events occurred which resulted in an overproduction of dihydrofolate reductase, which is used for tetrahydrofolate production, an important cofactor in amino acid and nucleotide biosynthesis (62). This overproduction allowed for trimethoprim sequestration as well as cellular functions to continue normally (**Figure 1d**).

Since resistance to antibiotics cannot always be obtained through modifications or mutations, bacteria have also demonstrated numerous methods for the destruction of the drugs. Enzymatic

destruction has occurred in bacteria since the introduction of penicillin in 1943 by Alexander Fleming (5). There are a wide variety of β-lactamases, which are able to hydrolyze most of the β-lactam antibiotics such as penicillins, cephalosporins, and carbapenums (41, 42). Attempts have been made to modify β-lactam antibiotics to provide more rigid structures that are less prone to degradation, however bacteria have still demonstrated resistance. An example of such a modification is seen with oxyimino-aminothiazolyl cephalosporins where the β-lactam ring is typically sheltered from hydrolysis, however β-lactamases found in *K. pneumoniae* have demonstrated hydrolytic capabilities (41, 63) (**Figure 1e**).

Another proficient method of escaping the effects of antibiotics is to remove the drug from the cell before it has a chance to reach its target. Efflux pumps are transporters that provide such an act. Many times antibiotics that have bacteria specific targets only affect Gram-positives due to the effectiveness of efflux pumps found in Gram-negatives. Overexpression of efflux pump related genes can provide a significant advantage and ensure high levels of resistance (64) (**Figure 1f**).

Actinobacteria

The name *Actinomycetes* was first proposed in 1916 as a way to categorize bacteria (65). Originally known as the “thread” bacteria, which possess a filamentous form and share very similar characteristics to filamentous fungi, *Actinobacteria* have had many changes with regards to taxonomy overtime (65). This phylum exhibits great diversity with regard to varying morphologies, physiologies, and metabolism. As one of the largest phyla it contains six classes, which are composed of 22 orders. The largest class, *Actinobacteria*, possesses 15 of the orders and is made up by 43 families (66). *Actinobacteria* are Gram-positive filamentous bacteria that typically possess high G+C genomic content (66). *Actinobacteria* can be spore and non-spore

forming and occupy almost all ecological niches including terrestrial, aquatic, and plant or animal associated as either pathogens or commensals (67, 68) Many organisms within this phylum have very diverse secondary metabolism, which has been and continues to be exploited by humans for use in biotechnology, medicine and agriculture. By appearance, many closely resemble filamentous fungi as majority of the *Actinobacteria* develop mycelia and reproduce via sporulation. The varying morphologies of *Actinobacteria* can include coccoid, short-rod, or branched filaments and can possess substrate mycelia, aerial hyphae, both or neither. Spore formers can produce in spore chains containing as many as two through 100's of spores, or as singular spores (69).

Actinobacteria life cycle varies greatly between the classes, however many that include sporulation in their lifecycle such as *Streptomyces*, begin as a free spore. Spore germination occurs and leads to vegetative growth and development of substrate mycelium. As nutrient levels begin to dwindle, aerial hyphae begin to form and finally spore formation occurs, however spore formation can occur without the presence of aerial hyphae (69). Streptomycetes and other *Actinobacteria* do not divide via elongation of the lateral wall as seen in other rod shaped bacteria such as *Escherichia coli* or *Bacillus subtilis*, instead they replicate via tip extension (70-72). Cell wall synthesis occurs at the tip of the growing bacteria due to DivIVA accumulation (70, 71). These tip extensions are segregated into compartments which results in multi-chromosomal filaments which penetrate the substrate known as substrate mycelium (72, 73).

Actinobacteria are characterized via the 16S rRNA gene, which can organize isolates into their respective genera, it is difficult to discern between closely related genera or species. Genome sizes can vary from as small as 0.93 mega base pairs seen in *Tropheryma whipplei* to 12.7 mega base pairs seen in *Streptomyces rapamycinicus* (74, 75). Small genome size is thought to be a result of genome compaction as the bacteria adapt to a host, which provides a relatively

unchanging environment (74). Conversely it is theorized that strains that remain the open environment such as soil maintain their large genome size in order to acclimate to constant change and potential threats (74).

The secondary metabolism of *Actinobacteria* is remarkably diverse and has provided roughly two-thirds of all antibiotics as either native natural products or as natural product derivatives (76). Secondary metabolites are not only classified as antibiotics, they play an immense role in the survival of *Actinobacteria.* Aside from killing or inhibition of other microorganisms, they can aid in metal acquisition, and even have toxic affects against plants and animals (77). It is thought that the number of secondary metabolites being produced by specific bacterium is a mere fraction of the biosynthetic potential of these bugs (78). Gene clusters within the genome that are not expressed under normal growth conditions and cannot be isolated are typically referred to as cryptic or silent. *Actinobacteria* in the environment come into contact with an enormous variety of organisms, including insects, plants, bacteriophages, fungi, and of course other bacteria. Interactions with any of these organisms, or their respective metabolites, could initiate a cascade of transcriptional machinery for cryptic or silenced biosynthetic pathways. It was found that within *Streptomyces coelicolor* alone actinorhodin, streptorubin B, geosmin, 2-methylisoborneol, albaflavenone, calcium-dependent antibiotic 2a, desferrioxamine E, coelimycin P1, methylenomycin A, germicidin A, coelichelin, *Streptomyces coelicolor* butyrolactone 1, methylenomycin furan 1, TW95a, flaviolin dimer, aminotrihydroxybacteriohopane, and isorenieratine could be produced, each of these belonging different classes of secondary metabolite (78-80). In a study of the whole genome of one of the best-characterized *Actinobacteria*, *Streptomyces coelicolor,* Bentley *et al*. described almost 20 theorized secondary metabolite biosynthetic pathways (78). The biggest complication lies within identifying appropriate conditions necessary for transcription of said biosynthetic pathways (78). Large strides have been made in a global effort for the search of novel chemistry from

Actinobacteria including methods such as co-cultivation, genome mining and chemical or small molecule induction (81). Co-cultivation is thought to better mimic environmental interactions of the *Actinobacteria*. One study demonstrated N-acetylglucosamine, a cell wall component of bacteria, could induce more production of actinorhodin in *S. coelicolor* (82). Genome mining involves scouring the entire genome of one bacterium in search for homologous genes to those that have already been annotated for use in secondary metabolite biosynthesis. Bentley et al. utilized this method in unearthing the 20 theorized secondary metabolite biosynthetic pathways (78). Chemical or small molecule induction practices suggest that introduction of a chemical agent can alter the secondary metabolite production of a bacteria. Varying concentrations of dimethyl sulfoxide were used on three different *Streptomyces* strains and demonstrated increased compound production (83).

Natural Products

The field of natural products chemistry encompasses any chemical substances produced by a living organism, however it is notoriously associated with chemical substances that have a pharmacological effect. Before modern medicine, natural products were applied in the form of crude pastes or teas, such as willow bark, which contains salicylic acid, better known as aspirin (84, 85). With the discovery of penicillin in 1928 and its introduction in the early 1940s, natural products chemistry morphed into what we now know as the field of antibiotics (86). Shortly after the introduction of penicillin, *Actinobacteria* entered the scene with the discovery of actinomycin in 1940 by Selman Waksman from a soil organism, *Actinomyces sp.* (87, 88). Waksman spent many years looking at soil content and resident organisms as far back as 1916 in search of whether soil organisms grew the same way in their natural environment as in the laboratory (89). The early years of natural product drug discovery focused on industrial scale culturing of microorganisms in the search for potential therapeutics (90). The time between the 1940s and

1960s are regarded as the “golden age” of antibiotics, where many of the classes of antibiotics we know today were discovered (91). This fleeting moment of history was over all too soon; as humanity entered the 1970s changes were being made in science, medicine and the economy. Antibiotics were becoming increasingly expensive to pursue and too often were they re-discovered (76). We have since left the golden age of antibiotics and entered into a state with which a post-antibiotic era is a potential and terrifying scenario (7). While natural products are still providing as a prolific source of antibiotics, the rigor required for FDA approval combined with the rate of antimicrobial resistance is proving a herculean task (5). Antimicrobial resistance in a few cases has even been identified prior to the introduction of an antibiotic, such as penicillin resistance, which was observed in 1940 and the drug released three years later (5). Current drug pipelines involve the process of screening for products, finding an extract that possesses some form of biological activity, purifying the compound and elucidating the structure, followed by rigorous testing to ensure it maintains activity in its purified state, all of this before the compound is even considered for medicinal chemistry (**Figure 2**). After all of the preliminary data is acquired, compounds can eventually make their way to clinical trials, which could take roughly 15 years, if the drug passes all three phases (92). Aside from the timeline, the average cost of introducing a drug in modern medicine averages almost $2.6 billion (92). As a result of the financial burden and the life span, a number of pharmaceutical companies downsized their natural product divisions and changed focus to synthetic chemistry and the introduction of combinatorial chemistry (90). However there are still many programs that exist today that focus on natural products, which is due to nature’s imagination acting better than any synthetic chemist. The field has evolved through the years to include modern techniques such as high-pressure liquid chromatography (HPLC) and mass spectrometry (93).

An enormous challenge with natural products drug discovery involves the timeline; in order to find a potential therapeutic, it must first be produced by nature. Alternative approaches

mentioned earlier describe current techniques used to modify organisms in an attempt to produce alternative chemistry including chemical, biological or genomic elicitation (78, 82, 83). Another issue lies within the screening of natural product extracts, which usually contain an amalgam of compounds, some which may present issues later in the purification process (90).

Regardless of the daunting reputation of natural products chemistry, it has still provided antibiotics such as penicillin, streptomycin, polymyxin and many more, which are used in the treatment of infectious disease. The alarming rate of resistance developing continually challenges humanity to develop new therapeutics. Of the ~500,000 natural product derived compounds discovered up until 2012, 20% are animal derived, 70% are plant derived, and 10% are microbial derived (76). Although natural products can be isolated from animals and plants, bioactivity is seen from only three and seven-percent, respectively, while 47% of microbial derived natural products maintain bioactivity (76). Roughly 37% of all microbial derived bioactive metabolites are identified from bacteria of the order *Actinomycetales* (76).

DNA Methyltransferase

Of the many regulatory mechanisms possessed by bacteria, methylation is extremely fast as well as efficient. Methylation of DNA provides numerous benefits including control of DNA replication and regulation of cell cycle; indication and administration of mismatch repair, as well as distinguishing self from foreign DNA (such as bacteriophage) (94-96). DNA methylation occurs via enzymes known as DNA methyltransferase. DNA methyltransferases are ubiquitous in nature and occur in all domains of life. There are three primary targets of DNA methyltransferases, which leave behind methylated base pairs. C^5 methyl-cytosine possesses a methyl group on the on the fifth carbon position; this is seen most commonly in Eukaryotes, but can occur in bacteria, however not very often (97). N^6 methyl-adenine possesses a methyl

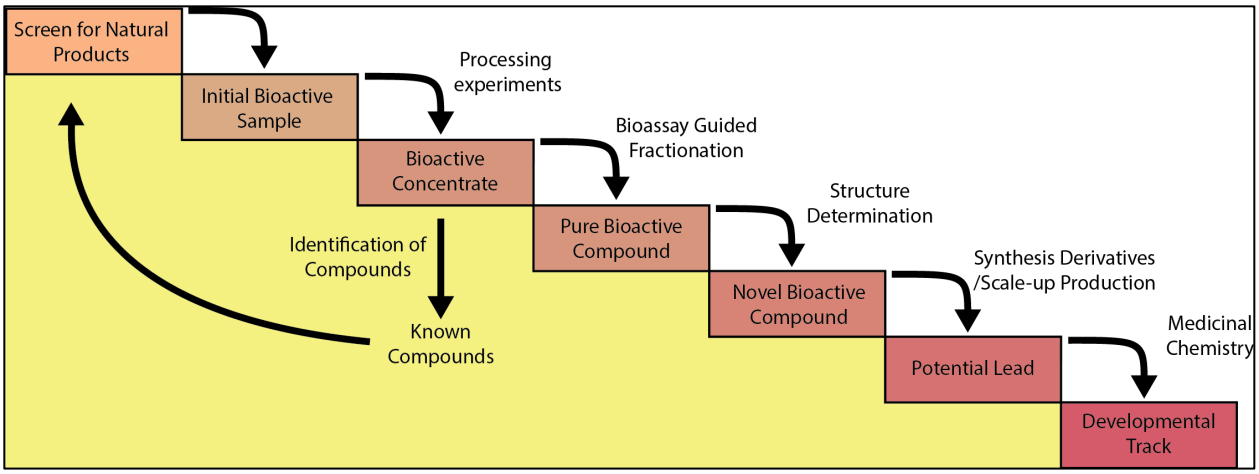

Figure 2. Traditional Pipeline for Drug Discovery
The natural product is extracted from a biological source and tested for bioactivity then purified. The structure is determined and a mechanism of action is established. Novel compounds are scaled or tested to determine if synthetic derivatives can be produced. Once a feasible method for scaled production is determined, the compound proceeds to additional optimization and eventually medicinal chemistry.

group on the sixth nitrogen position; this is seen most commonly in Prokaryotes and lower eukaryotes, but not in vertebrates (97). N^4 methyl-cytosine possesses a methyl group on the fourth nitrogen position; this method of methylation has only been seen in bacteria thus far (97). All DNA methyltransferases utilize S-adenosine-L-methionine (SAM) as the methyl donor. In the case of C^5 methyl-cytosine the transfer of a methyl group begins with a nucleophilic attack by a thiol group (found within the protein complex) on the sixth carbon on a cytosine base, which will result in the transfer of a methyl group from SAM (found within the protein complex), after this transfer a β-elimination reaction will release the DNA methyltransferase from the DNA leaving behind a methylated cytosine base (**Figure 3A**) (98).

Epigenetic Modification

Since *Actinobacteria* have historically been such a prevalent source of bioactive compounds, scientists have exploited many novel techniques for exploring their genomes with the hopes of finding novel secondary metabolites (91). Techniques, such as whole genome sequencing, have changed the way we look at organisms, such that we can now analyze an organism's chromosome and determine homologous genes. This phenomenon was observed with *S. coelicolor*, when Bentley *et al*. scoured the chromosome for any biosynthesis genes that may be to known bioactive secondary metabolite biosynthesis associated genes and found over 20 gene clusters that were known or hypothesized to code for secondary metabolites (78). One of the difficulties with such findings; it raises the question "how can the organism be modified to ensure these cryptic biosynthetic pathway genes are expressed?" The task of ensuring genetic expression sounds daunting enough, but then the transcribed genes also need to exercise their intended function. There are two very generalized approaches to such a challenge 1) pick a specific organism and a target within that organism then begin modifications or 2) prepare a universal technique that can be performed in a high throughput fashion. Both approaches are

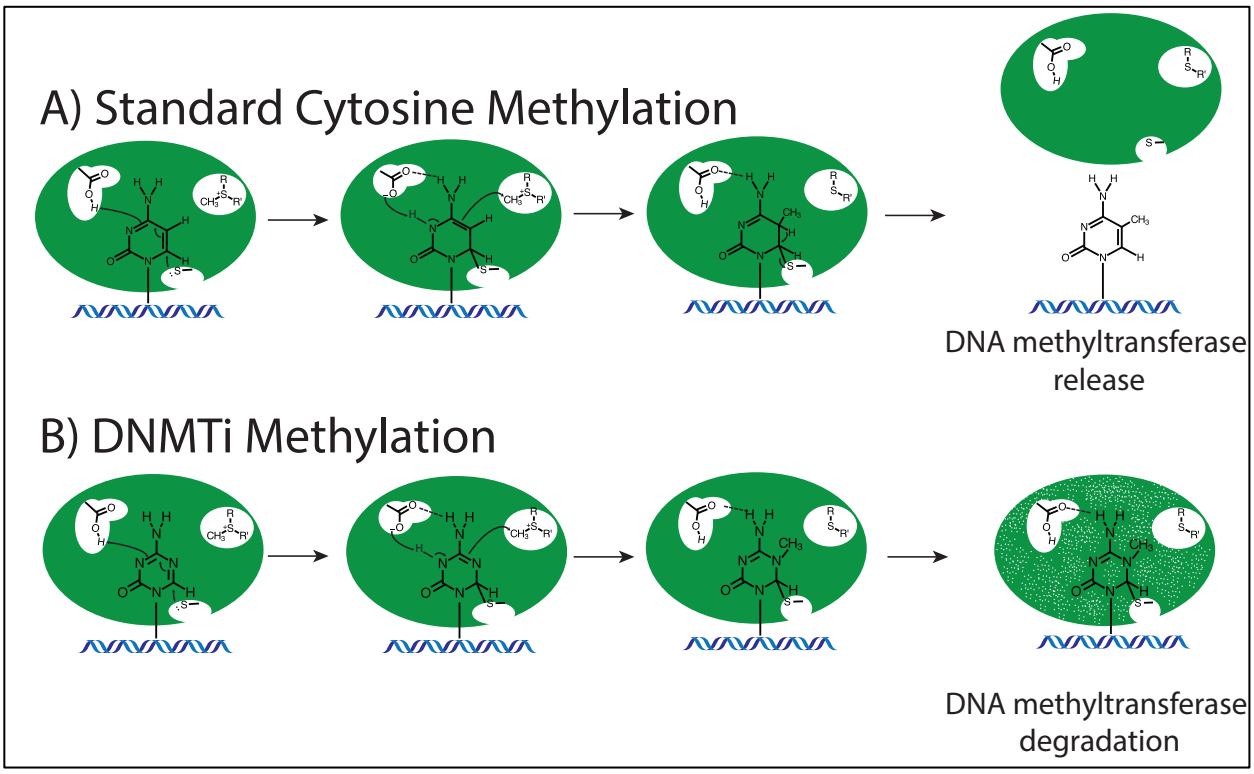

Figure 3. Mechanisms of DNA Methyltransferase

DNA methyltransferase enzymes possess a thiol group, which allows for a nucleophilic attack on the carbon in the sixth position of a cytosine base and through multiple steps of electron transfer a methyl group is transferred from SAM to a carbon in the fifth position of the cytosine base, finally a β-elimination releases the enzyme **(A)**. Using a DNA methyltransferase inhibitor which resembles a cytosine base, the same nucleophilic attack from the thiol group to the carbon in the sixth position and through multiple steps of electron transfer a methyl group is transferred from SAM to a nitrogen in the fifth position, finally there are no further reactions that can take place and the enzyme is unable to be released resulting in its degradation **(B)**.

utilized throughout the world today; however there have been a number of successes identifying novel compounds from well-characterized organisms using epigenetic modification (99, 100).

Epigenetic modification, looking at differences in an organism due to changes in gene expression, involves the addition of a chemical agent designed to inhibit genetic modifiers such as methyltransferase or histone deacetylase, in order to prevent proper gene regulation (99, 101). Proper gene regulation is disrupted when an epigenetic modifier, such as a DNA methyltransferase inhibitor, prevents methylation of specific regions near or within gene promoter regions. This lack of methylation has the potential to increase the affinity with which transcription machinery binds to the promoter regions (102). Substances such as 5-azacytidine can be used as a DNA methyltransferase inhibitor (DNMTi). 5-azacytidine will be converted upon entrance to 5-azacytosine, a molecule analogous to the DNA base pair, cytosine. The difference between the two is in the fifth carbon position of cytosine, which has been replaced with nitrogen. This subtle change in the molecule still allows for methyl transfer, however prevents the DNA methyltransferase enzyme from dissociating, which results in degradation as well as eliminating the enzyme from the cellular environment (97) (**Figure 3B**). Literature searches suggest that epigenetically modifying organisms for elicitation of secondary metabolites has been limited primarily to fungi (100, 103-106). Under a variety of laboratory growth conditions *Aspergillus niger* will suppress transcription of more than 70% of the gene clusters dedicated to secondary metabolite biosynthesis (106). Using a histone deacetylase inhibitor, Henrikson *et al*. were able to demonstrate variation of *A. niger*'s secondary metabolome with the identification of a novel secondary metabolite, nygerone A (106).

These methods for fungal epigenetic modification have utilized primarily DNA methyltransferase inhibitors and histone deacetylase inhibitors. Bacteria do not possess histones; instead they

possess DNA-binding histone-like proteins such as HU or H-NS found in *E. coli.* There have been examinations of the effects of histone deacetylase inhibitors on prokaryotes, which have exhibited variations in gene expression levels, although the mechanism by which this occurs is not fully understood, and therefore we focused our efforts on the DNA methylation (107). Although the literature primarily describes fungal epigenetic modification, bacterial epigenetic modification literature is also available. Kumar *et al*. found that epigenetically modified *S. coelicolor* possessed an additional major protein band when compared to the unmodified control (108). There were also differences within the HPLC chromatogram as the crude extract from the modified bacterium demonstrated 12 compounds and unmodified only displayed five (108). Another group looked at antibiotic production in bacteria using kinase inhibitors and cell transport regulators as a form of epigenetic modification and found that one of the kinase inhibitors actually increased production (109).

Using a DNA methyltransferase inhibitor, we believe a collection of *Actinobacteria* can be modified and extracted in a high throughput screen. We anticipate that by altering the epigenetic regulatory enzymes, the well-characterized strains will be unable to properly regulate previously unidentified and highly sought after genetic pathways, yielding new bioactive secondary metabolites (**Figure 4**).

Anthracyclines

The first anthracycline was isolated from *Streptomyces peucetius* and titled daunomycin or rubidomycin, however quickly changed to daunorubicin (110-112). Farmitalia Research Laboratories of Milan, Italy and the French firm of Rhóne Poulenc of Paris, France founded daunorubicin. The compound received its name for Daunii, a pre-Roman tribe where to soil

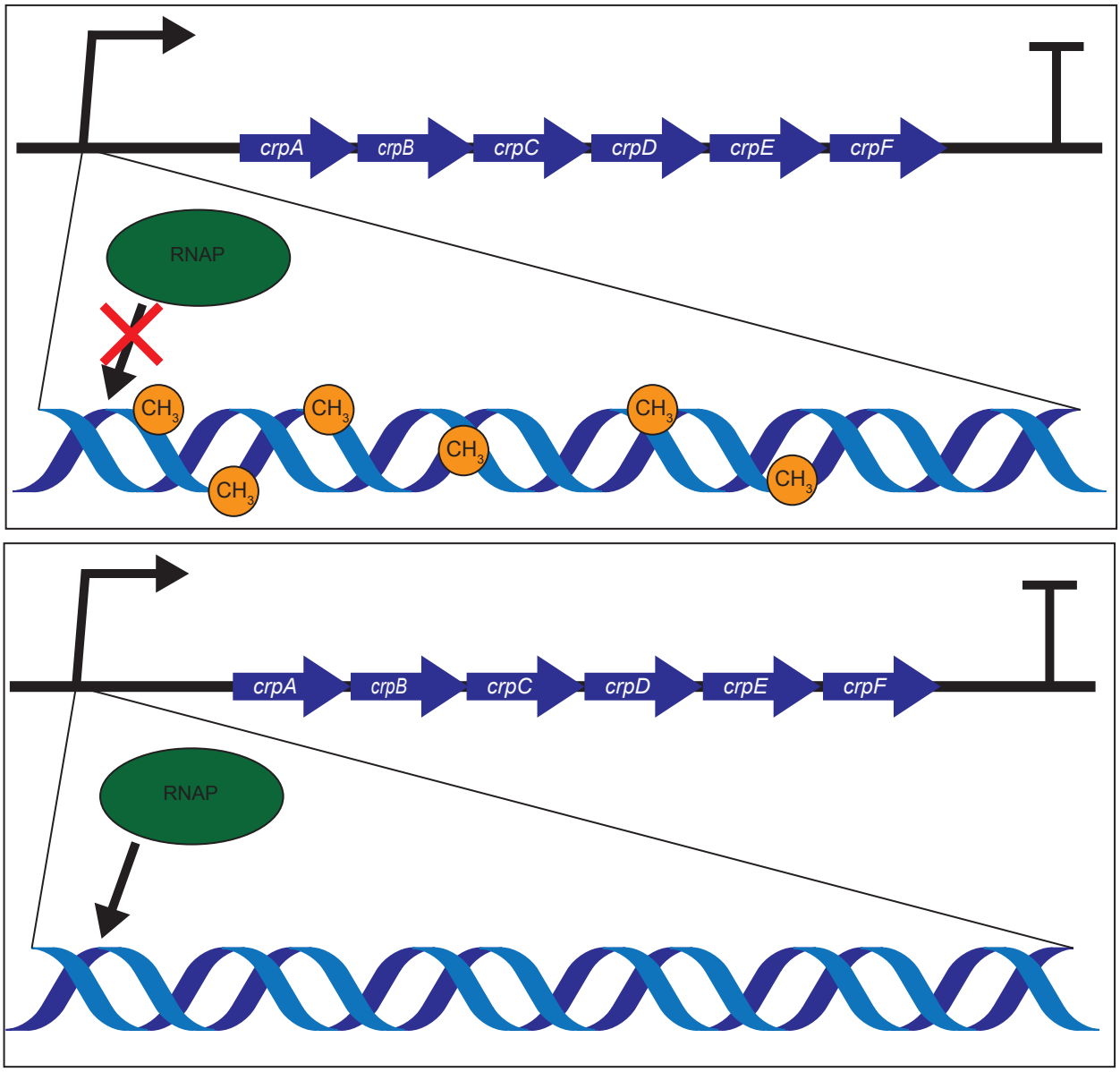

Figure 4. Potential Outcome of Demethylation
Control possesses methyl groups along DNA, which prevents transcription of potential secondary metabolite biosynthesis pathways or their products. Using a DNMTi effectively prevents methylation of DNA, which allows transcription of potential secondary metabolite biosynthesis pathways or their products. Presence or absence of methyl groups on DNA can either induce or repress transcription.

sample was collected, and rubis, the French word for ruby (113). Since the introduction of anthracyclines in the 1960's, many more have been discovered and synthesized including analogs such as doxorubicin (Adriamycin), epirubicin, or idarubicin. Many anthracyclines have been approved for use throughout the world, however doxorubicin was the first to be approved by the Food and Drug Administration in the United States. The only difference between daunorubicin and doxorubicin is a single hydroxyl group (**Figure 5**). This subtle change seems insignificant, however both compounds display different anti-tumor activity (111). Since this subtle change in structure resulted in changes in anti-tumor activity, chemists have synthesized anthracycline analogs with numerous variations on the original structure.

Anthracyclines are characterized as intercalating agents that possess anti-tumor activity as well as mild Gram-positive antibacterial activity (110). This class of antibiotic has been ranked as one of the most effective anti-cancer drugs ever developed and the most utilized worldwide being doxorubicin (113). A major drawback of anthracycline compounds is the inevitable cardiotoxicity. The search and creation of new anthracyclines is held to the standard of doxorubicin, whereby the anti-tumor activity is assessed, followed by the cardiotoxicity. Compounds that exhibit any combination of anti-tumor activity or cardiotoxicity that would be more severe for a patient are dismissed.

A number of methods by which anthracyclines are effective have been proposed, including prevention of topoisomerase II from binding to DNA due to anthracycline intercalating ability, inhibition of religation of DNA after a double strand or single strand break from topoisomerase (114), induction of apoptosis, or free radical generation (115). It is thought that anthracyclines inhibit growth of bacteria using similar mechanisms to those seen in humans (116). Inhibition of

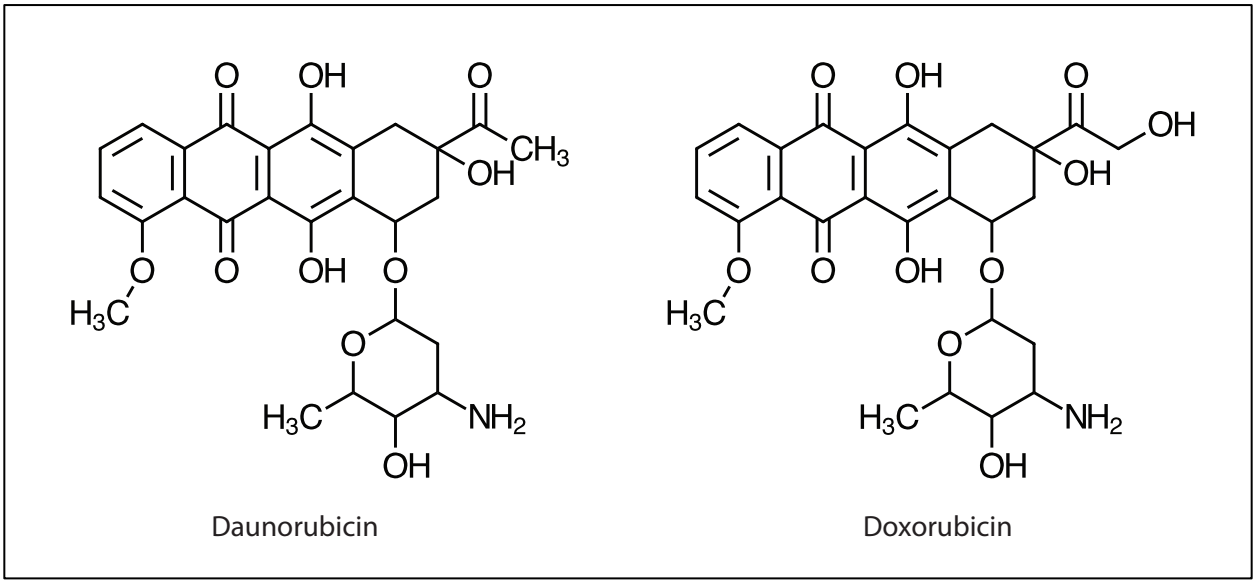

Figure 5. Comparing Two Anthracyclines
Daunorubicin possesses a basic acetyl substituent on the D ring while Doxorubicin is identical except for the hydroxyl substituent attached to the acetyl residue. This subtle change provides significantly higher anti-tumor bioactivity.

growth in bacteria is limited to the Gram-positive organisms, and studies have shown sensitivity to anthracyclines in Gram-negative bacteria lacking efflux systems (117).

Project Aim

One of the major issues humanity faces is the potential of a post-antibiotic era, where currently effective drugs, will no longer be useful and diseases that have been maintained for over 75 years will once again wreak havoc. Natural products have provided many potential therapeutic options throughout the years as well as scaffolds for natural product derivatives. We know that *Actinobacteria* have historically provided many of these natural product compounds, but the difficulty lies within coaxing the bacteria to produce. Genomic studies have found that numerous well-characterized *Actinobacteria* still have cryptic secondary metabolite biosynthesis pathways that are not expressed. Using our DNA methyltransferase inhibitor, we plan to disrupt gene regulation of our *Actinobacteria* strain collection one by one and compare the secondary metabolite crude extract of the modified and unmodified organisms. This comparison will allow us to determine if cryptic secondary metabolite biosynthesis pathways have been unlocked, or remain dormant.

We will first establish isolation protocols for growth of *Actinobacteria* from environmental samples as well as identification techniques. After establishing an *Actinobacteria* strain collection we will optimize methods of secondary metabolite extraction. Epigenetic modification techniques will be optimized as well, which will determine the best parameters for inhibition of DNA methylation including concentration of inhibitors, length of time for fermentation and what media should be used. In order to determine if any of these secondary metabolites are effective inhibitory agents, they will be screened against the multidrug resistant ESKAPE pathogens. When a crude extract displays bioactivity above a specific threshold, it becomes viable for large-

scale fermentation and purification. Using techniques such as high-pressure liquid chromatography (HPLC), liquid chromatography coupled mass spectrometry (LC/MS), H^1, C^{13}, COSY, and HSQC NMR we can elucidate the structure of a bioactive compound. We hope to identify novel secondary metabolites that possess inhibitory activity against one or more of the ESKAPE pathogens. It is possible a secondary metabolite of this nature could lead to identification of a novel therapeutic that would aid in the war against multidrug resistant pathogens.

Materials and Methods

Initial Soil Collection and Processing Optimization

Two soil samples were collected from a farm in Ft. Meyers in May of 2015. The samples were collected in sterile 50mL conical tubes from a depth of at least 15 cm. All future soil samples were collected in the same fashion.

Media

All media were prepared by using deionized water supplemented with 36 g L^{-1} Instant Ocean® and autoclaved for sterility. Media used for isolation and cultivation of *Actinobacteria* were supplemented with antibiotics designated for inhibition of fungal cultures. The antibiotics were added at the following concentrations: 50 µg mL^{-1} nystatin and 50 µg mL^{-1} cycloheximide.

Arginine-Glycerol-Salt Medium (AGS) (118)

0.1% Arginine monochloride	1 g L^{-1}
1.25% Glycerol	12.5 g L^{-1}
0.1% Dibasic potassium phosphate	1 g L^{-1}
1.5% Agar	15 g L^{-1}

Starch Casein Agar (SCA) (119)

0.1% Casein	1 g L^{-1}
1.0% Soluble starch	10 g L^{-1}
0.2% Potassium nitrate (KNO_3)	2 g L^{-1}

1.5% Agar	15 g L^{-1}

Salt-water Yeast Extract Agar (SYE)(120)

0.025% Yeast extract	0.25 g L^{-1}
0.05% Dibasic potassium phosphate (K_2HPO_4)	0.5 g L^{-1}
1.8% Agar	18 g L^{-1}

International *Streptomyces* Project Medium – 2 (ISP-2)(120)

1.0% Malt extract	10 g L^{-1}
0.4% Glucose	4 g L^{-1}
0.4% Yeast extract	4 g L^{-1}
1.5% Agar	15 g L^{-1}

Tryptic Soy Broth (TSB)

3.0% Tryptic soy	30 g L^{-1}

Inoculation Techniques

Method 1 (dilute/heat). One gram of soil samples was added to 5 mL of sterile seawater and shaken vigorously until homogenized. Dilutions of the suspension were made to 10^{-3}. The dilution was heated to 55 °C for six minutes, then 100 µl were inoculated onto a sterile agar plate and spread via autoclave sterilized glass beads (121).

Method 2 (dry/stamp). One gram of soil samples was added to a petri dish and allowed to dry overnight in a laminar flow hood. Using a 2cm foam stamp, sample was stamped six to eight times around the inside perimeter of the plate to simulate serial dilutions (121).

Method 3 (dilute/spread). One gram of soil samples was added to 5 mL of sterile seawater and shaken vigorously until homogenized. The homogenized mixture was then centrifuged 10 minutes at 2900 × g. Dilutions of the supernatant were made to 10^{-3}. 100 µl were inoculated onto a sterile agar plate and spread via autoclave sterilized glass beads (121).

Method 4 (dry/spread). One gram of soil samples was added to a petri dish and allowed to dry overnight in a laminar flow hood. Dry sample was aseptically transferred directly to the agar plate. The plate was flooded with 100 microliters of sterile seawater and spread via autoclave sterilized glass beads (121).

Isolation and Purification of species

Plates were allowed to grow for 2-8 weeks and monitored weekly for growth. After plates demonstrated growth, individual colonies were transferred to petri dishes containing ISP-2 for isolation. Bacteria on plates of ISP-2 were streaked for isolation no fewer than three times to ensure growth was identical to previous growth and isolation had been achieved.

DNA Extraction – Isolated colonies were grown in six mL of tryptic soy broth (TSB) for 3-7 days at 28 °C in a shaking incubator until adequate growth was present to perform a DNA extraction following manufacturer instructions (DNeasy Blood & tissue kit, Qiagen).

PCR Amplification – The 16S rRNA gene was then PCR amplified using the universal 16S rRNA primer pair OL2629 (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG TCT AAC GGA CGA TAG AGT TTG ATC CTG GCT CAG-3') (forward) and OL399 (5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3') (reverse) (122) and the products were purified using QIAquick PCR

purification Kit (Qiagen) following the manufacturer instructions. Sanger sequencing based upon OL2629 was performed and sequences generated were aligned and related to sequences obtained from a NCBI Nucleotide BLAST (BLASTN) search in order to determine identity to at least a genus level or if possible, a species level.

Current *Actinobacteria* Culture Techniques

Modified ISP-2 (120, 123, 124)

1.0% Malt extract	10 g L^{-1}
0.4% Glucose	4 g L^{-1}
0.4% Yeast extract	4 g L^{-1}
1.5% Agar	15 g L^{-1}
0.038% Tyrosine	0.38 g L^{-1}

Isolate Identification

Inoculated agar plates were allowed to incubate for 2-8 weeks at room temperature to allow slow-growing bacteria to form adequate sized colonies. Plates were monitored on a weekly basis and adequate growth was monitored visually. *Actinobacteria* were removed from the low nutrient media after no longer than 8 weeks of incubation based upon colony morphology, visual confirmation of aerial mycelia or substrate hyphae, visual confirmation of spore formation, pigment formation, or the presence of diffusible pigments. Colonies were separated and plated on ISP-2 followed by serial streaking until isolation was achieved. ISP-2 supplemented with tyrosine aided in further characterizing *Actinobacteria*, as there are numerous genera that are able to produce the soluble pigment melanin. Isolates were then Gram-stained, in an effort to eliminate the possibility of further characterization of Gram-negatives. Isolates were then DNA extracted, the 16S rRNA gene was amplified via PCR for Sanger sequencing, followed by

comparison of the generated sequences to known 16S rRNA genes using BLASTN. Isolates that identified as *Actinobacteria* were added to the Shaw lab strain collection and frozen glycerol stocks were prepared.

Isolates Used for Optimization Techniques

Cultures selected for epigenetic modification optimization were chosen for their phenotypic traits as well as their unique metabolism and metabolites. Isolate 11 (*Gordonia namibiensis*) colonies were slimy, smooth, orange in the center and white on the rim, and pinpoint. Isolate 11 was chosen because *Gordonia* species have been found to possess unique metabolism, which allows them to play a role in biodegradation of rubber materials, utilization of hydrocarbons, as well as break down other natural products that are not easily degraded in the environment (125-127). Isolate 39 (*Streptomyces griseorubens*) colonies were leathery, smooth, yellow, and pinpoint that produced the soluble pigment melanin when plated on tyrosine containing media. Isolate 39 was chosen because Streptomycetes are well known producers of bioactive secondary metabolites and *Streptomyces griseorubens* has been noted for its ability to utilize lignocellulose as its sole carbon source (128). Isolate 40 (*Streptomyces fradiae*) colonies were leathery, smooth, white to yellow, pinpoint to large, and irregular. Isolate 40 was chosen also because of Streptomycetes well known nature to produce bioactive secondary metabolites as well as the bacterium's ability to produce a number of well characterized compounds such as neomycin, tylosin and urdamycins A-F (129-131). All three isolates were grown for 21 days in ISP-2 broth (liquid media recipes omitted agar from preparation) prior to commencement of the extraction methods below and performed in triplicate.

Secondary Metabolite Extraction Optimization

Method A (partition/extract). Whole bacterial cultures were transferred from 50 mL bio-reactor conical tubes to 120 mL glass French square bottles and partitioned using 25 mL of EtOAc. Culture and organic layer were homogenized followed by transfer of the organic layer to pre-weighed 20 mL scintillation vials. Organic layer was dried under constant airflow. The addition of EtOAc and transfer of the organic layer was repeated three times in order to obtain as much product as possible.

Method B (freeze dry/extract). Whole bacterial cultures were frozen at -80°C and then lyophilized. Dried material was then extracted using 25 mL of EtOAc. Whole extract was filtered such that only EtOAc-soluble material could be transferred to pre-weighed 20 mL scintillation vials. EtOAc was dried under constant airflow. The addition of EtOAc and whole extract filtration was performed three times in order to obtain as much product as possible.

Method C (centrifuge/extract supernatant). Whole bacterial cultures were centrifuged and the supernatant transferred to 120 mL glass French square bottles and partitioned using 25 mL of EtOAc. Supernatant and organic layer were homogenized followed by transfer of the organic layer to pre-weighed 20 mL scintillation vials. Organic layer was dried under constant airflow. The addition of EtOAc and transfer of the organic layer was repeated three times in order to obtain as much product as possible.

Method D (centrifuge/extract pellet). Whole bacteria cultures were centrifuged and the removed. The pellet was frozen at -80°C and then lyophilized. Dried material was then extracted using 25 mL of EtOAc. Whole extract was then filtered such that only EtOAc-soluble material could be transferred to pre-weighed 20 mL scintillation vials. EtOAc was dried under constant airflow.

The addition of EtOAc and whole extract filtration was repeated three times in order to obtain as much product as possible.

Epigenetic Modification Optimization

DNMTi Concentration Optimization - Cultures of isolates 11, 39 and 40 were grown on ISP-2 media for three days in order to obtain a working stock. Each isolate was inoculated into 50 mL bio-reactor conical tubes containing 35 mL of liquid media. The liquid media used for this optimization was ISP-2 broth. 5-azacytidine was added to each of the flasks at the following concentrations: 0 µM, 1 µM, 10 µM, 50 µM, 100 µM, and 500 µM. Tubes were allowed to incubate for 21 days and were monitored for growth and color changes daily. A second round of DNMTi concentration optimization was performed in conjunction with time optimization.

Time Optimization - Cultures of isolates 11, 39 and 40 were grown on ISP-2 media for three days in order to obtain a working stock. Each isolate was inoculated into 50 mL bio-reactor conical tubes containing 35 mL of ISP-2 broth. 5-azacytidine was added to each of the 50 mL bio-reactor conical tubes at the following concentrations: 0 µM, 1 µM, 5 µM, and 10 µM. Tubes were allowed to incubate for 7, 14, and 21 days.

Media Optimization - Cultures of isolates 11, 39 and 40 were grown on ISP-2 media for three days in order to obtain a working stock. Each isolate was inoculated into 50 mL bio-reactor conical tubes containing 35 mL of SCA broth, SYE broth or ISP-2 broth. 5-azacytidine was added to each of the 50 mL bio-reactor conical tubes at the following concentrations: 0 µM, 1 µM, and 5 µM. Tubes were allowed to incubate for 21 days.

Screening Extracts Against the ESKAPE Pathogens

20 mL scintillation vials containing crude extract from the various extraction methods were re-suspended in DMSO to a concentration of 5 mg mL^{-1}. The extracts were assessed for the ability to inhibit growth against the ESKAPE pathogens. ESKAPE pathogen strains were grown overnight in TSB at 37 °C in a shaking incubator then diluted (10^{-3}) into fresh media. The MIC was determined through a tiered approach beginning with 200 µg mL^{-1} and continually halving the inhibitory concentration until inhibition was no longer seen. Sterile 96-well microtiter plates were loaded with culture and solvated compounds were added such that the total volume was 200 µl.

Small-Scale Growth

Slow growing *Actinobacteria* were grown at 28 °C in 35 mL of SCA broth in 50 mL bio-reaction conical tubes (Cell-Treat) in a shaking incubator under constant agitation for 21 days, in the presence and absence of one µM of our DNMTi (5-azacytidine). Rapidly growing *Actinobacteria* were also grown at 28 °C in 35 mL of ISP-2 broth in 50 mL bio-reaction conical tubes (Cell-Treat) in a shaking incubator under constant agitation for 21 days, in the presence and absence of one µM of 5-azacytidine. The bio-reaction conical tubes were monitored for color changes and secreted pigments throughout the incubation period.

Secondary Metabolite Extraction – after 21 days of growth, actinobacterial cultures were transferred to 120 mL glass French square bottles and 25 mL of ethyl acetate (EtOAc) were added to the glass bottles, the culture/EtOAc mixture was homogenized and filtered. The resulting mixture was allowed to settle for 24 hours and layers were formed, after which the top organic layer was decanted into a pre-weighed 20 mL scintillation vial. Care was taken to ensure only the EtOAc layer was removed and not the aqueous layer containing the media and

bacterial cultures. This process was repeated three times in order to remove as much material potentially containing secondary metabolites as possible. Between removals of the organic layers, the previously removed EtOAc was dried; secondary and tertiary removals of EtOAc were added to the same corresponding scintillation vials. Scintillation vials were weighed after drying to determine total yield of crude extract. Crude extracts weighing >= 1 mg were solvated to 5 mg mL^{-1} in 100% dimethyl sulfoxide (100% DMSO), 0.5 – 0.99 mg were solvated to 2.5 mg mL^{-1} in 100% DMSO, and <0.5 mg were solvated to 1 mg mL^{-1} in 100% DMSO. Aliquots of extracts were transferred into 96-well plates, with stock solutions archived.

ESKAPE Bioactivity Screening – in order to assess the antimicrobial activity of the crude extracts we used the ESKAPE pathogens, which are an excellent representation for multidrug resistant pathogens. All of the ESKAPE strains were grown overnight in TSB at 37 °C in a shaking incubator, and then diluted (10^{-3}) into fresh media. The minimum inhibitory concentration (MIC) was determined through a tiered approach beginning with 200 µg mL^{-1} and continually halving the inhibitory concentration until inhibition was no longer seen. Sterile 96-well microtiter plates were loaded with culture, and solvated compounds were added to equal a total volume of 200 µl. Care was taken not to add more than 5% of the total volume of the compound to any of the wells and a control well was included with 5% total volume being 100% DMSO. Plates were then incubated at 37°C for 24 hours and MICs were determined via visual inspection, inhibition was apparent by a lack of turbidity in the wells.

Large-Scale Growth

Actinobacteria were grown in 1000 mL of SCA broth at 28 °C in a two liter Erlenmeyer flask in a shaking incubator under constant agitation for 28 days. The flasks were monitored for color changes and secreted pigments throughout the incubation period. After 28 days of growth 500

mL of EtOAc were added to the flask and the culture/EtOAc mixture was homogenized by shaking incubator for 24 hours. The resulting mixture was filtered and allowed to settle for 24 hours before decanting the organic layer. This process was repeated twice more using only 250 mL of EtOAc in order to remove as much material potentially containing secondary metabolites as possible. Between removals of the organic layers, the previously removed EtOAc was dried; secondary and tertiary removals of EtOAc were added to the same corresponding glass bottle. Five milligrams of crude extract was removed and placed in a 20 mL scintillation vial and kept for storage.

Partition – The whole crude extract was suspended in EtOAc and transferred to a separatory funnel and partitioned using double distilled H_2O. Three partitions were collected in separate 20mL scintillation vials, the liquid layer (H_2O), the middle layer (insoluble material) and the organic layer (EtOAc). These partitions were dried under air and weighed, then five milligrams of each were removed and placed in separate 20 mL scintillation vials and kept for storage. Following the partition, whole crude extract, as well as each of the partitions were subjected to bioassay to ensure activity of the large-scale extract and partitions were similar to that of the small-scale crude extract as well as determine the active partition for further characterization and testing.

HPLC Purification and Bioassay Guided Fractionation

The bioactive crude extract was subjected to normal-phase high-pressure liquid chromatography (HPLC) purification with two silica columns in tandem as the stationary phase. The mobile phase used a five minute 100% hexanes run, followed by a 35-minute continuous gradient until 100% EtOAc, then a 15-minute 100% EtOAc run, and finally a 10-minute 100% 50:50 EtOAc:IPA wash. An evaporative light scattering (ELS) as well as an ultraviolet (UV)

detector monitored HPLC analysis in order to identify a variety of potential fractions. Fractions were submitted to bioassay to determine which possessed the activity seen in the crude extract.

Modified HPLC Purification and Bioassay Guided Fractionation

Partitioned crude extracts were used as an initial modification of this purification process. The bioactive partition was subjected to normal-phase HPLC purification with a silica stationary phase. The mobile phase used a five minute 100% hexanes run, followed by a 35-minute continuous gradient until 100% 50:50 EtOAc:IPA, then a 15 minute 100% 50:50 EtOAc:IPA run. An evaporative light scattering (ELS) as well as an ultraviolet (UV) detector monitored HPLC analysis in order to identify a variety of potential fractions. Fractions were submitted to bioassay to determine which possessed the activity seen in the active partition.

Bioactive fractions were subjected to reverse-phase HPLC purification with a C8 column, using a five minute 90% H_2O: 10% 1:4 THF:ACN run, followed by a 35 minute continuous gradient until 99% 1:4 THF:ACN, then a 15 minute 1% H_2O 99% 1:4 THF:ACN run. and this process will be repeated until a single bioactive compound has been identified. Purified bioactive fractions will be analyzed by liquid chromatography coupled mass spectrometry (LCMS) in order to identify a molecular weight and potential chemical formula.

Results

Investigating Cultivation Strategies Necessary for *Actinobacteria* Growth

Historically, *Actinobacteria* have been an ample source of antibiotics and bioactive secondary metabolites, the difficulty lying within the recovery rate of isolates from environmental samples. Initially, using the two soil samples from the farm in Ft. Meyers, we attempted to isolate and identify *Actinobacteria*. Using SCA, SYE, and AGS coupled with methods 1-4 and performing each combination in triplicate, we generated 36 plates per sample. After eight weeks of growth, we began serially streaking isolates onto ISP-2 agar plates to ensure isolation. A total of 432 isolates were obtained from the investigation of cultivation strategies **(Table 1).** We selected 36 isolates that possessed at least one of the *Actinobacteria*-like characteristics including rough or leathery colonies, aerial hyphae, substrate mycelia, presence of pigments, presence of spores, or diffusible pigments to perform Sanger sequencing. Media or methods that were considered optimal and use continued resulted in *Actinobacteria* identification for at least 80% of the sequenced isolates from their respective media or method (**Table 2**). The various identified isolates were from both samples, Soil 1 and Soil 2, and both samples possessed identified *Actinobacteria*. Isolates identified as *Actinobacteria* included species from the genera *Rhodococcus*, *Streptomyces*, *Gordonia*, *Nocardia*, *Arthrobacter*, *Aeromicrobium*, and *Mycobacterium* (**Table 3**). These preliminary results demonstrate the omnipresence of *Actinobacteria.* Using this preliminary data, we were able to determine that SCA and SYE in conjunction with methods one and three were optimal for growth and isolation of *Actinobacteria* and were used moving forward with future soil samples.

Table 1. Number of Isolates Obtained Using Various Media and Methods

Method	Media	Number of Isolates from Soil 1	Number of Isolates from Soil 2
1	AGS	23	41
	SYE	29	63
	SCA	25	37
2	AGS	5	13
	SYE	13	10
	SCA	4	15
3	AGS	14	28
	SYE	18	16
	SCA	19	11
4	AGS	4	16
	SYE	8	6
	SCA	7	7
	Total isolates	**169**	**263**

** Listed are the total yields from each media and method combination from environmental samples Soil 1 and Soil 2. Media used includes: AGS – arginine-glycerol-salt; SYE – salt-water yeast-extract; SCA – starch casein agar.*

Table 2. Prevalence of *Actinobacteria*

Media	Percent *Actinobacteria*	Method	Percent Actinobacteria
AGS	22%	1	84%
SCA	95%	2	43%
SYE	80%	3	80%
		4	0%

** Listed are the percentages associated with the number of **Actinobacteria** identified that were cultivated under each media type or method. Media used includes: AGS – arginine-glycerol-salt; SYE – salt-water yeast-extract; SCA – starch casein agar*

Table 3. Identity and Acquisition Method

Strain Number	Identity	Media	Method	Sample
1	*Rhodococcus rhodochrous*	SCA	3	Soil 2
2	*Staphylococcus gallinarum*	SYE	3	Soil 2
3	*Staphylococcus cohnii*	AGS	3	Soil 2
4	*Bacillus aryabhattai*	AGS	2	Soil 2
5	*Rhodococcus rhodochrous*	SCA	1	Soil 2
6	*Streptomyces violarus*	SCA	3	Soil 1
7	*Streptomyces sp.*	SCA	1	Soil 2
8	*Streptomyces beijiangensis*	AGS	1	Soil 2
9	*Halomonas venusta*	AGS	1	Soil 2
10	*Streptomyces coelicoflavus*	SCA	1	Soil 2
11	*Gordonia namibiensis*	SCA	1	Soil 2
12	*Rhodococcus rhodochrous*	SCA	1	Soil 2
13	*Nocardia vermiculata*	SCA	1	Soil 2
14	*Streptomyces platensis*	SCA	1	Soil 1
15	*Streptomyces violarus*	SCA	1	Soil 2
16	*Streptomyces albiflavescens*	SCA	1	Soil 2
17	*Streptomyces corchorusii*	SCA	1	Soil 2
18	*Arthrobacter protophormiae*	SYE	1	Soil 2
19	*Gordonia namibiensis*	SCA	3	Soil 1
20	*Aeromicrobium tamlense*	SCA	2	Soil 2
21	*Rhodococcus phenolicus*	SCA	2	Soil 2
22	*Gordonia terrae*	SCA	3	Soil 1
23	*Bacillus sp.*	AGS	2	Soil 2
24	*Bacillus sp.*	AGS	2	Soil 2
25	*Rhodococcus rhodochrous*	SCA	2	Soil 2
26	*Streptomyces chromofuscus*	SCA	1	Soil 2
27	*Streptomyces acrimycini*	SCA	1	Soil 2
28	*Bacillus aryabhattai*	AGS	2	Soil 2
29	*Mycobacterium parafortuitum*	SCA	3	Soil 2
30	*Streptomyces macrosporeus*	SCA	3	Soil 2
31	*Streptomyces antibioticus*	SYE	3	Soil 1
32	*Streptomyces gardneri*	SCA	3	Soil 1
33	*Streptomyces tumescens*	AGS	1	Soil 1
34	*Nocardia asteroides*	AGS	1	Soil 1
35	*Streptomyces minoensis*	SYE	1	Soil 1
36	*Streptomyces aurantiogriseus*	SYE	1	Soil 2

** Listed are the strain numbers used as an identifier for each addition to the **Actinobacteria** strain collection as well as the respective isolate identity, media, method, and environmental sample used for cultivation. Media used includes: AGS – arginine-glycerol-salt; SYE – salt-water yeast-extract; SCA – starch casein agar.*

Using SCA and SYE with methods one and three, and plating in triplicate, a total of 12 plates were generated per sample in order to accurately cultivate potential microbes harbored within the environmental samples. Environmental samples were acquired from a variety of sources including farmland, deserts, aquatic environments, as well as high altitudes (**Table 4**).

Model for Rapid Identification of Actinobacterial Cultures and Strain Collection Development

Typically, the identification of an unknown bacterium can be determined using 16S rRNA gene sequencing, which can be compared to previously sequenced genes, to provide an identity to a genus and sometimes species level. A common issue within the phylum is the similarity of the 16S rRNA gene sequence, which raises difficulties when organizing isolates at a species level. This common issue has limited our identification to at least a genus level, and in some cases a species level identification was achieved when comparable genetic sequences were available. A number of modifications were made when transitioning between investigating cultivation strategies and current cultivation strategies, which include added steps to rapidly detect *Actinobacteria,* thereby reducing costs associated with identification via Sanger sequencing. Recognizing that a number of bacteria possess tyrosinases, which can be used in the formation of pigments such as melanin, was a crucial step in the rapid identification of *Actinobacteria* (124). As one of the *Actinobacteria*-like indicators was pigment production, the addition of tyrosine to our ISP-2 media aided immensely. The production of melanin was added to the list of *Actinobacteria*-like indicators. Many of the colonies cultivated from environmental samples possessed at least one of the *Actinobacteria*-like indicators, such that upon visual inspection, a colony could be identified as *Actinobacteria*-like or not. This visual identification made it possible to remove the need for Sanger sequencing of each isolate. 16S rRNA gene sequencing was still of use for isolates that did not possess at least one of the *Actinobacteria*-like indicators.

Table 4. List of Environmental Samples

Sample Number	Location	Description	Type of Sample	Number of Isolates
-3	Soil 1	Farm (field) Ft. Myers	Soil	32
-2	Soil 2	Farm (horse) Ft. Myers	Soil	119
-1	Sed 8	Florida Keys	Sediment	N/A
0	Sed 10	Florida Keys	Sediment	N/A
1	TSF	Oak hickory heavy shade	Soil	36
2	TSF	Cow loading area, empty for years	Soil	30
3	TSF	Cow pasture, under hay (feeding area)	Soil	48
4	TSF	Pond 1 edge	Soil	32
5	TSF	Pond 1 edge further out	Soil	16
6	TSF	Pond 2 edge	Soil	13
7	TSF	Arid cow pasture	Soil	29
8	TSF	Organic soil, heavy trees	Soil	1
9	TSF	low lands (wet cow pasture)	Soil	31
10	TSF	Organic soil, cedar trees	Soil	69
11	TSF	Gopher hole	Soil	8
12	TSF	Forest Floor	Soil	14
13	TSF	Deep in the forest	Soil	5
14	TSF	Deeper in the forest	Soil	10
15	TSF	Cave floor	Soil	17
16	TSF	Clay near house	Soil	1
17	SHH	Deep under sprinkler	Soil	10
18	SHH	Front Garden	Soil	60
19	SIS	Ding Darling Island	Soil	66
20	SIS	Beach Dunes	Soil	84
21	SIS	Low tide beach	Soil	N/A
22	SHH	Back garden	Soil	N/A
23	CWR	14	Sponge	1
24	CWR	15-16	Sponge	N/A
25	CWR	15-18	Sponge	N/A
26	CWR	15-19	Sponge	N/A
27	CWR	15-20	Sponge	1
28	CWR	15-21	Sponge	N/A
29	CWR	15-22	Sponge	N/A
30	CWR	15-23	Sponge	N/A
31	CWR	15-SED1	Sediment	1
32	CWR	15-SED2	Sediment	N/A
33	CWR	15-SED3	Sediment	N/A

Table 4. (Continued)

34	BB	15-6	Soil	N/A
35	BB	15-3	Soil	8
36	MFG15	Sed 1	Sediment	N/A
37	MFG15	Sed 2	Sediment	N/A
38	MFG15	Sed 3	Sediment	N/A
39	MFG15	Sed 4	Sediment	N/A
40	MFG15	Sed 5	Sediment	N/A
41	MFG15	Sed 6	Sediment	N/A
42	MFG15	Sed 7	Sediment	N/A
43	MFG15	Sed 8	Sediment	N/A
44	MFG15	Sed 9	Sediment	N/A
45	MFG15	Sed 10	Sediment	N/A
46	MFG15	Sed 11	Sediment	N/A
47	MFG15	Sed 12	Sediment	N/A
48	MFG15	Sed 13	Sediment	N/A
49	MFG15	Sed 14	Sediment	N/A
50	MFG15	Sed 15	Sediment	N/A
51	MFG15	Sed 16	Sediment	N/A
52	MFG15	Sed 17	Sediment	N/A
53	MFG15	Sed 18	Sediment	N/A
54	MFG15	6a	Sponge	N/A
55	MFG15	6b	Sponge	N/A
56	MFG15	8	Sponge	N/A
57	MFG15	10	Sponge	N/A
58	MFG15	12	Sponge	N/A
59	MFG15	14	Sponge	N/A
60	MFG15	16a	Sponge	N/A
61	MFG15	16b	Sponge	N/A
62	MFG15	18	Sponge	N/A
63	MFG15	20	Sponge	N/A
64	MFG15	22	Sponge	N/A
65	MFG15	24	Sponge	N/A
66	MFG15	26	Sponge	N/A
67	MFG15	30	Sponge	N/A
68	MFG15	32	Sponge	N/A
69	MFG15	38	Sponge	N/A
70	MFG15	40	Sponge	N/A
71	MFG15	42	Sponge	N/A

Table 4. (Continued)

72	MFG15	52	Sponge	N/A
73	MFG15	54	Sponge	N/A
74	MFG15	56	Sponge	N/A
75	MFG15	60	Sponge	N/A
76	EG 15	Sand 1	Sand	10
77	EG 15	Sand 2	Sand	9
78	EG 15	Sahel shemally	Sediment	N/A
79	HRT	Memphis, TN	Soil	3
80	HRT	Newalla, OK	Soil	N/A
81	HRT	Muldrow, OK	Soil	N/A
82	HRT	South Rim Grand Canyon, AZ	Sand	N/A
83	HRT	Hatch, UT	Soil	N/A
84	HRT	Death Valley Lakebed	Sand	N/A
85	HRT	Amarillo, TX	Soil	N/A
86	HRT	Fulton, MS	Soil	N/A
87	HRT	Brinkley, AR	Soil	N/A
88	HRT	Moriarty, NM	Soil	N/A
89	HRT	Chambers, AZ	Soil	N/A
90	HRT	Lone Pine, CA	Soil	N/A
91	HRT	Valley of Fire, NV	Soil	N/A
92	TN16	Tri-cities, TN beside boulder	Soil	N/A
93	TN16	Tri-cities, TN rattlesnake trail	Soil	N/A
94	TN16	Tri-cities, TN tree roots	Soil	N/A
95	TN16	Tri-cities, TN under tree	Soil	N/A
96	TN16	Tri-cities, TN on Embankment	Soil	N/A
97	TN16	Church Hill, TN (1509 ft.)	Soil	N/A
98	TN16	Church Hill, TN (1445 ft.)	Soil	N/A
99	TN16	Johnson City, TN (1481 ft.)(soil in root)	Soil	N/A
100	TN16	Church Hill, TN (1466 ft.)	Soil	N/A
101	TN16	Church Hill, TN (1394 ft.) (bottom layer)	Soil	N/A
102	TN16	Johnson City, TN (1481 ft.)(under wagon)	Soil	N/A
103	TN16	Church Hill, TN (1396 ft.) (top layer)	Soil	N/A
104	TN16	Johnson City, TN (1481 ft.) (soil below root)	Soil	N/A
105	RC16	Shamrock Soil	Soil	N/A
106	AS16	Forrest in Newton, PA (#1)	Soil	N/A
107	AS16	Forrest in Newton, PA (#2)	Soil	N/A
108	AS16	Forrest in Newton, PA (#3)	Soil	N/A
109	AS16	Forrest in Newton, PA (#4)	Soil	N/A

Table 4. (Continued)

110	WR16	11,753 ft., Estes Park, CO	Soil	N/A
111	WR16	12,193 ft., Estes Park, CO	Soil	N/A
112	WR16	9,247 ft., Estes Park, CO	Soil	N/A
113	WR16	9,444 ft., Estes Park, CO	Soil	N/A
114	WR16	10,057 ft., Estes Park, CO	Soil	N/A
115	NV16	Nashville, TN 795 ft.	Soil	N/A
116	NV16	Nashville, TN Valve House Trail	Soil	N/A
117	PH16	USF campus, FL	Soil	N/A
118	PH16	Tampa, FL	Soil	N/A
119	PH16	Tampa, FL	Soil	N/A
120	PH16	Tampa, FL	Soil	N/A
121	PH16	Tampa, FL	Soil	N/A
122	PH16	Tampa, FL	Soil	N/A

** Listed are the environmental samples acquired throughout this study as well as the associated number of **Actinobacteria** identified. Location identifiers are: TSF – Te Strake farm; SHH – Shaw House; SIS – Sanibel Island soil; CWR – Clearwater reef; BB – Bill Baker; MFG15 – Florida middle grounds 2015; EG 15 – Egypt 2015; HRT – Hailey road trip; TN16 – Tennessee 2016; RC16 – Ronan Carroll 2016; AS16 – Arielle Sharp 2016; WR16 – Wind river 2016; NV16 – Nashville, Tennessee 2016; PH16 – Phage biology class 2016.*

** N/A indicates samples have not yet been processed.*

Over 750 isolates displaying *Actinobacteria*-like indicators have been added to the Shaw lab *Actinobacteria* strain collection, of which over 200 have a confirmed genus based upon BLASTN confirmation of the 16S rRNA gene (**Appendix I**). The over 200 *Actinobacteria* are represented a variety of genera, including, *Aeromicrobium, Agromyces, Arthrobacter, Brevibacterium, Gordonia, Isoptericola, Leifsonia, Microbacterium, Micrococcus, Mycobacterium, Micromonospora, Nocardia, Nocardioides, Promicromonospora, Pseudonocardia, Rhodococcus, Streptomyces,* and *Williamsia* (**Table 5**). The most abundant of the genera, *Streptomyces*, which comprises more than half of the identified isolates, originated from all sample sites screened for *Actinobacteria*. This finding is concurrent with the literature that suggests that majority of the *Actinobacteria* found within the soil are of the genus *Streptomyces* (68). A circular cladogram was constructed with the over 200-actinobacterial-16S rRNA genes that displays the vast diversity of our collection as well as separates the genera into their respective families and orders (**Figure 6**). All Sanger sequenced isolates belong to the same class, *Actinobacteria*.

Table 5. Order, Family and Genus of Identified Isolates

No. of isolates	Genus	Family	Order
1	*Aeromicrobium*	*Nocardioidaceae*	*Proprionibacteriales*
2	*Agromyces*	*Microbacteriaceae*	*Micrococcales*
2	*Arthrobacter*	*Micrococcaceae*	*Micrococcales*
1	*Brevibacterium*	*Brevibacteriaceae*	*Micrococcales*
6	*Gordonia*	*Nocardiaceae*	*Corynebacteriales*
1	*Isoptericola*	*Promicromonosporaceae*	*Micrococcales*
3	*Leifsonia*	*Microbacteriaceae*	*Micrococcales*
3	*Microbacterium*	*Microbacteriaceae*	*Micrococcales*
2	*Micrococcus*	*Micrococcaceae*	*Micrococcales*
20	*Mycobacterium*	*Mycobacteriaceae*	*Corynebacteriales*
1	*Micromonospora*	*Micromonosporaceae*	*Micromonosporales*
9	*Nocardia*	*Nocardiaceae*	*Corynebacteriales*
2	*Nocardioides*	*Nocardioidaceae*	*Proprionibacteriales*
1	*Promicromonospora*	*Promicromonosporaceae*	*Micrococcales*
1	*Pseudonocardia*	*Pseudonocardiaceae*	*Pseudonocardiales*
20	*Rhodococcus*	*Nocardiaceae*	*Corynebacteriales*
163	*Streptomyces*	*Streptomycetaceae*	*Streptomycetales*
1	*Williamsia*	*Nocardiaceae*	*Corynebacteriales*

** Listed are the different genera identified throughout this study as well as their respective family, order, and the number of isolates obtained that belongs to each genus.*

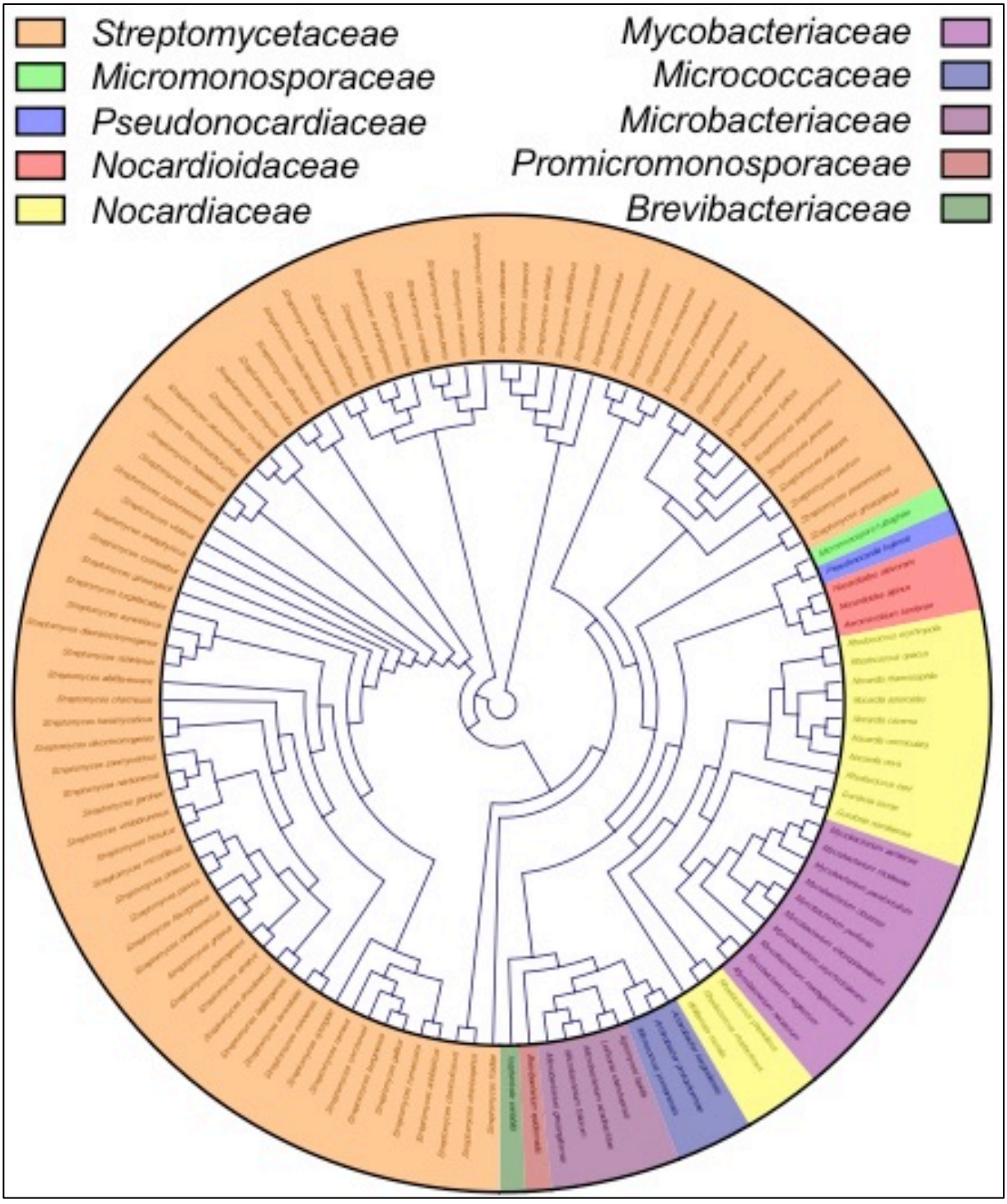

Figure 6. Circular Cladogram of Identified *Actinobacteria*
Cladogram was assembled using the 16S rRNA gene from identified *Actinobacteria* within our strain collection. Colors surrounding the cladogram identify bacteria as displaying specific traits or production of known compounds.

Secondary Metabolite Extraction Optimization

Secondary metabolite extraction results in a crude extract that may contain thousands of compounds, some of which may polar, non-polar, insoluble, temperature sensitive, pH sensitive, or even sensitive to the type of solvent used. As a consequence of the number of variables we strove to identify a method for secondary metabolite extraction that would afford suitable weights with which to work in a high throughput fashion. All three isolates (11, 39 and 40) were grown in triplicate for a period of 21 days and each were extracted following each of the methods above (A-D). The crude extract weights were recorded and used to determine which of the extraction methods would work best in a high throughput manner (**Table 6**). It was determined based upon average weights of all three isolates for each of the extraction methods that extraction method B would work optimally.

Epigenetic Modification Optimization

DNMTi Concentration Optimization – It was not yet known how 5-azacytidine would interact with our three isolates or the necessary concentration to use that would confer a change. Based upon existing literature for epigenetically modified fungi, we decided it would be best to optimize this method to ensure appropriate concentrations were being tested. Initial concentrations of 5-azacytidine included: 0 µM, 1 µM, 10 µM, 50 µM, 100 µM, and 500 µM. Eighteen 50 mL bioreactor conical tubes were used to determine the maximum threshold for unaltered growth of each of the *Actinobacteria*. All three isolates had no visually noticeable growth defects at 0 µM and 1 µM concentrations. All three isolates showed some visually noticeable growth defects at the 10 µM concentration. None of the isolates matured passed inoculation in any of the concentrations greater than 10 µM (**Table 7**).

Table 6. Secondary Metabolite Extraction Results

Extraction Method	Isolate 11 (milligrams)			Average	Isolate 39 (milligrams)			Average	Isolate 40 (milligrams)			Average
A	0.70	0.80	0.30	**0.60**	0.80	0.70	0.60	**0.70**	0.70	0.70	0.30	**0.56**
B	1.20	2.60	4.80	**2.86**	3.10	2.4	1.20	**2.23**	1.90	15.90	3.60	**7.13**
C	1.50	0.50	0.60	**0.86**	2.30	1.90	2.00	**2.06**	0.80	0.90	1.40	**1.03**
D	1.70	2.60	2.20	**2.16**	2.60	1.90	4.90	**3.13**	1.60	3.50	0.80	**1.96**

** Listed are the extraction methods used for extraction optimization; these were performed identically for each of the isolates. As there was no uniformity throughout each of the extraction methods, averages were taken across the isolates as well as within the isolates to determine which of the methods would be optimal for this high throughput screening campaign.*

Table 7. Growth Based Upon Concentration of DNMTi

[DNMTi]	Isolate 11	Isolate 39	Isolate 40
0 µM	Growth	Growth	Growth
1 µM	Growth	Growth	Growth
10 µM	Growth Defect	Growth Defect	Growth Defect
50 µM	No Growth	No Growth	No Growth
100 µM	No Growth	No Growth	No Growth
500 µM	No Growth	No Growth	No Growth

** Listed are the DNA methyltransferase inhibitor (DNMTi) concentrations used. Cultures were assessed based upon growth or no growth. A culture was labeled with growth defect if the culture did not achieve growth comparable to that of the control culture.*

Basing this initial optimization solely on visual inspection, it was determined that another round of DNMTi concentration optimization would be performed following a more narrow scope of concentrations.

Time Optimization – Majority of *Actinobacteria* possess doubling times significantly longer than other bacteria, such as *Staphylococcus aureus*, which can have doubling times as low as 24 minutes under laboratory conditions (132). As such, we needed to optimize a length of time that would suit majority of the *Actinobacteria* collection in a high throughput fashion. Cultures of isolates 11, 39, and 40 represent varying growth rates, which can provide a length of time that suits all isolates. In conjunction with time optimization, we also further specified the concentration of our DNMTi. Each of the isolates were challenged with the following concentrations of 5-azacytidine: 0 μM, 1 μM, 5 μM, and 10 μM. These tubes were allowed to incubate for 7, 14, or 21 days in triplicate. At each of the time points, a tube with each of the combinations of isolates and concentrations of 5-azacytidine were extracted with method B mentioned above. The crude extract weights were measured at each of time points and averaged (**Table 8**). All samples yielded, regardless of concentration of 5-azacytidine, the highest mass after 21 days of incubation. While samples that were inoculated with 10 μM of 5-azacytidine for 21 days averaged the highest mass overall, it was clear that majority of the extracted mass was unusable and contained significant levels of cell material. These results provided an optimal length of time for incubation of 21 days, but still did not provide an optimal concentration of 5-azacytidine that would allow for continuous growth of the isolates. Therefore yet another round of DNMTi concentration optimization would be performed following an even more narrow scope of concentrations.

Media Optimization – Numerous studies have been performed in the hopes of determining optimal media for the production of antibiotics. A study by Cortes *et al.* found that in *Streptomyces* too much glucose could inhibit antibiotic production as it signals to the bacteria that there is an abundance of nutrients (133). Therefore it was essential to determine which of the media (SYE broth, SCA broth or ISP-2 broth) would be optimal for growth and production of secondary metabolites. In conjunction with media optimization, we further explored the optimal concentration of 5-azacytidine to include the following concentrations: 0 μM, 1 μM, and 5 μM in triplicate. With all three media options, a tube with each of the combinations of isolates and concentrations of 5-azacytidine were extracted with method B after 21 days of growth. Averages of the crude extract weights were taken for each of combinations and averaged (**Table 9**). All of the sample's averages yielded the highest crude extract mass in either the SCA and ISP-2 broths. The samples in all instances except one resulted in the highest crude extract mass in either the unmodified or 1 μM concentration of 5-azacytidine. These results provided both the optimal media for the production of secondary metabolites as well as the optimal concentration of 5-azacytidine for modification of *Actinobacteria* isolates.

Before commencement of analysis of secondary metabolite production from our *Actinobacteria* strain collection, we needed to optimize a number of factors. These results aided in identifying the optimal parameters for the growth of these bacteria including two different media (SCA and ISP-2), a time frame with which to grow each batch (21 days) and the appropriate concentration of 5-azacytidine to use in conjunction with an unmodified control (1 μM).

Table 8. Length of Time for Cultivation and DNMTi Concentration Results

Concentration	7 Days (milligrams)			7 Days Average	14 Days (milligrams)			14 Days Average	21 Days (milligrams)			21 Days Average
Isolate 11 (0 µM)	1.50	0.90	1.20	**1.20**	1.10	1.90	3.20	**2.07**	7.30	11.90	5.20	**8.13**
Isolate 11 (1 µM)	3.00	0.20	1.40	**1.53**	1.20	1.80	3.50	**2.17**	5.00	34.70	12.10	**17.27**
Isolate 11 (5 µM)	4.10	2.20	0.60	**2.30**	1.30	0.60	1.00	**0.97**	7.10	5.10	24.00	**12.07**
Isolate 11 (10 µM)	2.20	1.40	0.20	**1.27**	0.00	0.70	0.50	**0.40**	6.30	12.00	32.90	**17.07**
Isolate 39 (0 µM)	0.60	1.40	1.30	**1.10**	0.30	1.20	2.80	**1.43**	9.80	8.80	2.30	**6.97**
Isolate 39 (1 µM)	9.90	8.00	2.40	**6.77**	0.80	0.60	0.00	**0.47**	24.60	30.40	0.40	**18.47**
Isolate 39 (5 µM)	5.70	21.10	2.90	**9.90**	0.30	0.30	0.40	**0.33**	55.20	14.20	29.40	**32.93**
Isolate 39 (10 µM)	0.20	0.00	0.00	**0.07**	2.40	3.20	1.20	**2.27**	4.50	14.70	1.10	**6.77**
Isolate 40 (0 µM)	7.20	0.50	0.20	**2.63**	0.70	0.20	0.70	**0.53**	0.70	40.00	14.70	**18.47**
Isolate 40 (1 µM)	2.00	4.80	1.20	**2.67**	0.50	2.80	2.40	**1.90**	21.30	1.00	3.30	**8.53**
Isolate 40 (5 µM)	1.80	1.20	0.70	**1.23**	0.60	0.50	0.90	**0.67**	45.60	0.20	4.10	**16.63**
Isolate 40 (10 µM)	3.60	3.00	0.40	**2.33**	0.30	4.70	17.30	**7.43**	65.50	33.70	16.40	**38.53**

** Listed are the concentrations of DNA methyltransferase inhibitor (DNMTi) used as well as the different lengths of time for optimization of cultivation period. In all cases, after 21 days of growth yielded the highest mass average.*

Table 9. Media and DNMTi Concentration Results

Concentration	SYE Broth (milligrams)			SYE Broth Average	SCA Broth (milligrams)			SCA Broth Average	ISP-2 Broth (milligrams)			ISP-2 Broth Average
Isolate 11 (0 µM)	0.70	0.10	1.20	**0.67**	0.70	1.00	10.00	**3.90**	0.30	1.20	0.30	**0.60**
Isolate 11 (1 µM)	0.10	1.40	1.40	**0.97**	4.90	1.50	1.60	**2.67**	0.60	0.30	0.90	**0.60**
Isolate 11 (5 µM)	1.40	3.60	2.30	**2.43**	1.10	0.10	0.10	**0.43**	0.20	0.40	1.00	**0.53**
Isolate 39 (0 µM)	0.70	0.10	0.50	**0.43**	0.10	0.20	0.10	**0.13**	1.00	1.90	1.00	**1.30**
Isolate 39 (1 µM)	0.10	0.10	0.10	**0.10**	0.10	0.10	0.50	**0.23**	1.00	0.80	0.70	**0.83**
Isolate 39 (5 µM)	0.10	0.10	0.40	**0.20**	0.30	0.10	0.20	**0.20**	0.10	0.70	0.50	**0.43**
Isolate 40 (0 µM)	0.60	0.50	1.10	**0.73**	0.50	6.20	0.20	**2.30**	0.90	0.10	0.50	**0.50**
Isolate 40 (1 µM)	0.40	0.70	0.20	**0.43**	0.10	1.90	1.60	**1.20**	2.70	0.70	1.00	**1.47**
Isolate 40 (5 µM)	0.10	0.10	0.10	**0.10**	0.10	0.10	0.80	**0.33**	1.20	1.20	1.30	**1.23**

** Listed are the concentrations of DNA methyltransferase inhibitor (DNMTi) used as well as the different media types for optimization. Media used includes: SYE – salt-water yeast-extract; SCA – starch casein agar.*

Quantitative Analysis of Secondary Metabolite Production of *Actinobacteria* Strain Collection

Bacteria utilize DNA methylation as a method to rapidly activate or inactivate gene transcription, which allows them to acclimate to their environment more efficiently (95). As such, using 5-azacytidine to prevent DNA methylation has the potential to disrupt bacterial gene expression including, but not limited to secondary metabolite biosynthesis pathways.

Secondary metabolites have the potential to affect a plethora of cellular functions including cell wall development, protein synthesis, protein function and fatty acid synthesis/metabolism (77). Therefore, using purified bacterial isolates from soil and sediment samples, a total of 148 bacterial isolates have been screened for the presence of bioactive secondary metabolites. After 21 days of incubation in the presence and absence of 1 μM of 5-azacytidine, it was seen that all of the *Actinobacteria* were able to produce some level of crude extract (**Table 10**). The masses of crude extract from each of the bacterial isolates varied greatly, however, there were 40 of the 148 (27%) isolates that had >= +/- 1.0 mg difference between the challenged and unchallenged samples. The remaining 108 (73%) varied between challenged and unchallenged less than +/- 1.0 mg or there were no changes. The changes in crude extract mass observed in the 40 isolates with >= +/- 1.0 mg indicates there may be a difference in regulation between the two. The differences seen in crude extract weight may provide insight into how much material is being produced, however there is no indication as to what is being produced and whether or not bioactive secondary metabolite regulation is being disrupted.

Evaluating the Effects of Crude Secondary Metabolites Against the ESKAPE Pathogens

A major problem with the discovery of bioactive secondary metabolites lies within determination of how to assess such activity and where to focus our efforts. A majority of compounds

Table 10. Comparison of Crude Extract Mass

Strain Number	Identity	Unchallenged (milligrams)	Challenged (milligrams)	Change (milligrams)
1	*Rhodococcus rhodochrous*	2.6	3.5	0.9
5	*Rhodococcus rhodochrous*	3.0	3.1	0.1
6	*Streptomyces violarus*	1.7	1.4	-0.3
7	*Streptomyces sp.*	1.6	1.9	0.3
8	*Streptomyces beijiangjensis*	1.2	0.9	-0.3
10	*Streptomyces coelicoflavus*	2.2	1.6	-0.6
11	*Gordonia namibiensis*	6.4	6.2	-0.2
12	*Rhodococcus rhodochrous*	9.3	6.4	-2.9
13	*Nocardia vermiculata*	4.6	3.5	-1.1
14	*Streptomyces platensis*	9.3	4.7	-4.6
15	*Streptomyces violarus*	3.4	3.0	-0.4
16	*Streptomyces antibioticus*	14.7	13.9	-0.8
17	*Streptomyces corchorusii*	1.8	2.2	0.4
18	*Arthrobacter protophormiae*	4.2	2.2	-2.0
19	*Gordonia namibiensis*	3.1	4.4	1.3
20	*Aeromicrobium tamlense*	16.3	13.3	-3.0
21	*Rhodococcus phenolicus*	13.8	14.8	1.0
22	*Gordonia terrae*	6.5	6.4	-0.1
25	*Rhodococcus rhodochrous*	3.0	2.3	-0.7
26	*Streptomyces chromofuscus*	3.2	8.5	5.3
27	*Streptomyces acrimycini*	6.7	4.3	-2.4
29	*Mycobacterium parafortuitum*	2.0	2.4	0.4
30	*Streptomyces macrosporeus*	6.2	22.5	16.3
31	*Streptomyces antibioticus*	12.0	16.9	4.9
32	*Streptomyces gardneri*	1.6	2.3	0.7
33	*Streptomyces tumescens*	4.0	3.1	-0.9
34	*Nocardia asteroides*	4.1	3.3	-0.8
35	*Streptomyces minoensis*	2.1	2.2	0.1
36	*Streptomyces aurantiogriseus*	3.0	3.1	0.1
37	*Streptomyces matensis*	2.6	3.1	0.5
39	*Streptomyces griseorubens*	4.7	6.8	2.1
40	*Streptomyces fradiae*	2.7	3.0	0.3
41	*Streptomyces violascens*	3.6	4.2	0.6
42	*Streptomyces zaomyceticus*	2.0	2.2	0.2
43	*Streptomyces viridochromogenes*	7.6	6.8	-0.8
44	*Streptomyces beijiangensis*	3.4	4.2	0.8
45	*Streptomyces intermedius*	4.8	4.6	-0.2

Table 10. (Continued)

46	*Streptomyces intermedius*	5.1	4.2	-0.9
47	*Streptomyces albidoflavus*	3.2	3.1	-0.1
48	*Streptomyces matensis*	2.4	1.5	-0.9
49	*Streptomyces griseoplanus*	11.5	10.8	-0.7
50	*Streptomyces drozdowiczii*	1.2	2.4	1.2
51	*Streptomyces drozdowiczii*	1.8	2.6	0.8
52	*Streptomyces coelicoflavus*	2.1	1.4	-0.7
54	*Streptomyces intermedius*	1.4	1.6	0.2
56	*Streptomyces intermedius*	3.3	2.5	-0.8
57	*Streptomyces sampsonii*	2.3	2.5	0.2
58	*Nocardia asteroides*	3.0	5.6	2.6
59	*Nocardia asteroides*	3.4	3.2	-0.2
60	*Streptomyces glebosus*	4.8	1.3	-3.5
61	*Streptomyces hawaiiensis*	2.4	4.9	2.5
62	*Streptomyces variabilis*	2.1	2.0	-0.1
64	*Streptomyces drozdowiczii*	4.5	3.7	-0.8
65	*Streptomyces drozdowiczii*	3.5	3.1	-0.4
66	*Streptomyces griseoaurantiacus*	2.6	3.3	0.7
67	*Streptomyces intermedius*	2.5	2.6	0.1
68	*Streptomyces thermocarboxydus*	2.5	3.1	0.6
69	*Streptomyces viridobrunneus*	1.8	3.2	1.4
70	*Streptomyces intermedius*	2.4	1.9	-0.5
71	*Brevibacterium epidermidis*	2.3	2.5	0.2
72	*Streptomyces intermedius*	4.5	3.8	-0.7
73	*Microbacterium ginsengterrae*	4.0	5.2	1.2
74	*Streptomyces coelicoflavus*	1.6	1.2	-0.4
75	*Streptomyces gardneri*	1.7	1.1	-0.6
76	*Streptomyces intermedius*	2.5	2.4	-0.1
77	*Streptomyces malachitospinus*	1.6	1.0	-0.6
78	*Streptomyces intermedius*	2.9	2.4	-0.5
79	*Streptomyces aurantiacus*	3.2	2.5	-0.7
80	*Streptomyces malachitospinus*	1.1	1.2	0.1
81	*Streptomyces tendae*	0.4	0.5	0.1
82	*Streptomyces drozdowiczii*	1.2	0.7	-0.5
84	*Streptomyces tendae*	0.8	1.1	0.3
85	*Streptomyces tendae*	0.4	0.9	0.5
86	*Streptomyces gardneri*	0.3	0.5	0.2
87	*Streptomyces praecox*	4.6	3.1	-1.5
88	*Streptomyces turgidiscabies*	0.3	0.5	0.2

Table 10. (Continued)

89	*Streptomyces drozdowiczii*	12.6	11.1	-1.5
90	*Streptomyces drozdowiczii*	5.3	9.4	4.1
91	*Streptomyces zaomceticus*	18.1	11.1	-7.0
92	*Gordonia namibiensis*	16.7	14.6	-2.1
93	*Rhodococcus opacus*	5.5	4.0	-1.5
95	*Micrococcus yunnanensis*	15.5	10.4	-5.1
96	*Streptomyces griseorubens*	7.6	11.2	3.6
97	*Streptomyces lividans*	8.7	6.3	-2.4
98	*Nocardia caverna*	0.7	0.6	-0.1
99	*Streptomyces violascens*	2.4	1.8	-0.6
100	*Streptomyces drozdowiczii*	1.8	1.0	-0.8
101	*Streptomyces griseus*	1.1	1.3	0.2
104	*Streptomyces coelicoflavus*	1.6	0.7	-0.9
105	*Streptomyces malachitospinus*	1.7	1.6	-0.1
106	*Streptomyces libani*	1.7	1.8	0.1
107	*Nocardia asteroides*	1.2	1.2	0.0
108	*Streptomyces spongiae*	1.6	0.8	-0.8
109	*Streptomyces parvulus*	1.1	1.9	0.8
110	*Streptomyces olivoverticillatus*	1.5	1.1	-0.4
111	*Streptomyces chartreusis*	1.4	2.1	0.7
112	*N/A*	0.9	0.8	-0.1
113	*Streptomyces hawaiiensis*	0.9	0.6	-0.3
114	*Streptomyces cinereospinus*	1.1	1.5	0.4
115	*Streptomyces intermedius*	3.6	3.5	-0.1
116	*Streptomyces chartreusis*	3.2	1.4	-1.8
117	*Streptomyces chartreusis*	1.2	1.6	0.4
118	*Streptomyces griseocarneus*	0.1	0.1	0.0
121	*Streptomyces cyanoalbus*	0.5	0.3	-0.2
122	*Rhodococcus erythropolis*	2.7	4.3	1.6
123	*Streptomyces atratus*	2.3	1.6	-0.7
124	*Streptomyces chartreusis*	0.9	0.9	0.0
125	*Streptomyces cinereorectus*	1.0	1.5	0.5
126	*Streptomyces diastatochromogenes*	0.8	1.2	0.4
127	*Streptomyces chartreusis*	4.0	2.4	-1.6
128	*Streptomyces drozdowiczii*	2.3	1.3	-1.0
129	*Streptomyces platensis*	0.8	0.9	0.1
130	*Rhodococcus opacus*	1.0	3.8	2.8
131	*Rhodococcus opacus*	5.7	5.6	-0.1
132	*Streptomyces rishiriensis*	0.6	0.9	0.3

Table 10. (Continued)

133	*Streptomyces drozdowiczii*	2.3	9.3	7.0
134	*Rhodococcus opacus*	4.2	5.1	0.9
135	*Streptomyces angustmyceticus*	1.1	0.8	-0.3
136	*Streptomyces drozdowiczii*	1.7	1.7	0.0
137	*Streptomyces purpurescens*	1.4	1.3	-0.1
138	*Streptomyces exfoliatus*	5.5	1.6	-3.9
139	*Streptomyces septatus*	1.3	19.9	18.6
140	*Streptomyces rochei*	1.1	1.1	0.0
141	*Streptomyces angustmyceticus*	0.9	0.8	-0.1
142	*Streptomyces olivoverticillatus*	1.5	1.3	-0.2
143	*Streptomyces hirsutus*	1.3	4.9	3.6
144	*Streptomyces angustmyceticus*	1.1	1.3	0.2
145	*Streptomyces olivochromogenes*	1.0	1.2	0.2
146	*Agromyces lapidis*	2.0	1.7	-0.3
147	*Rhodococcus opacus*	2.3	3.2	0.9
149	*Arthrobacter kerguelensis*	1.3	1.1	-0.2
150	*Streptomyces exfoliatus*	1.0	1.2	0.2
152	*Streptomyces tendae*	1.4	1.1	-0.3
153	*Streptomyces lavendulae*	2.2	1.7	-0.5
154	*Mycobacterium parafortuitum*	3.1	2.6	-0.5
155	*Streptomyces malachitospinus*	1.4	1.7	0.3
156	*Streptomyces pactum*	2.4	1.3	-1.1
157	*Streptomyces olivaceus*	2.2	1.1	-1.1
158	*Streptomyces malachitospinus*	2.7	1.3	-1.4
159	*Streptomyces olivaceus*	2.7	2.0	-0.7
160	*Streptomyces praecox*	1.8	0.5	-1.3
161	*Streptomyces malachitospinus*	0.7	0.7	0.0
162	*Streptomyces griseoaurantiacus*	0.2	0.5	0.3
163	*Streptomyces griseoplanus*	0.1	0.1	0.0
164	*Streptomyces malachitospinus*	0.4	1.0	0.6
165	*Streptomyces malachitospinus*	1.1	1.1	0.0
166	*Streptomyces malachitospinus*	0.1	0.6	0.5
713	*Micrococcus sp.*	1.0	1.1	0.1

** Listed are the strain numbers associated with each of the isolate identities along with the crude extract mass yield from both modified and unmodified cultures as well as the comparison between the two. Positive change indicates the modified culture's crude extract possessed more mass and negative change indicates the unmodified culture's crude extract possessed more mass.*

discovered from *Actinobacteria* confer some form of bioactivity against other bacteria, and the antibiotic resistance seen in pathogenic bacteria is proving to be a worldwide issue. Therefore we decided that in order to properly assess the presence of antibacterial bioactive secondary metabolites, we needed to a panel of bacteria that could be used to do so. We wanted to ensure if any bioactivity were identified, it was novel; therefore we utilized clinical isolates in the screening process. The clinical isolates were comprised of the ESKAPE pathogens, which are responsible for majority of hospital-acquired infections in the United States and well characterized as possessing resistance to antibiotics (8). The 296 crude extracts, when solvated in DMSO to a concentration of 5 mg mL^{-1}, were screened for antimicrobial activity against all of the ESKAPE pathogens using a MIC assay. A heat map was constructed to represent the bioactivity of only the active extracts against the ESKAPE pathogens individually, the combined activity for the Gram-positives, the combined activity for the Gram-negatives and collective activity for all six (**Figure 7**). Of the 296 crude extracts, 78 (~26%) possessed bioactivity against one or more of the ESKAPE pathogens (**Appendix 2**). The genera responsible for the activity seen in these 78 crude extracts are *Streptomyces*, *Nocardia*, and *Mycobacterium*. As suggested earlier, we looked at differences in crude extract mass of each of the *Actinobacteria*, which provided little insight, but may have alluded to differences in gene regulation with a number of the isolates. However, as we established bioactivity, we could further this insight and illustrate actual biochemical differences between modified and unmodified isolates. As such, we looked at changes in ESKAPE bioactivity between only the active extracts (**Figure 8**). Differences seen between modified and unmodified isolate crude extract bioactivities occurred with almost every sample. A few noteworthy crude extracts demonstrated higher levels of bioactivity against the same pathogen when compared to their counterpart and a number showed bioactivity against different pathogens compared to their counterpart. These differences indicate there are changes

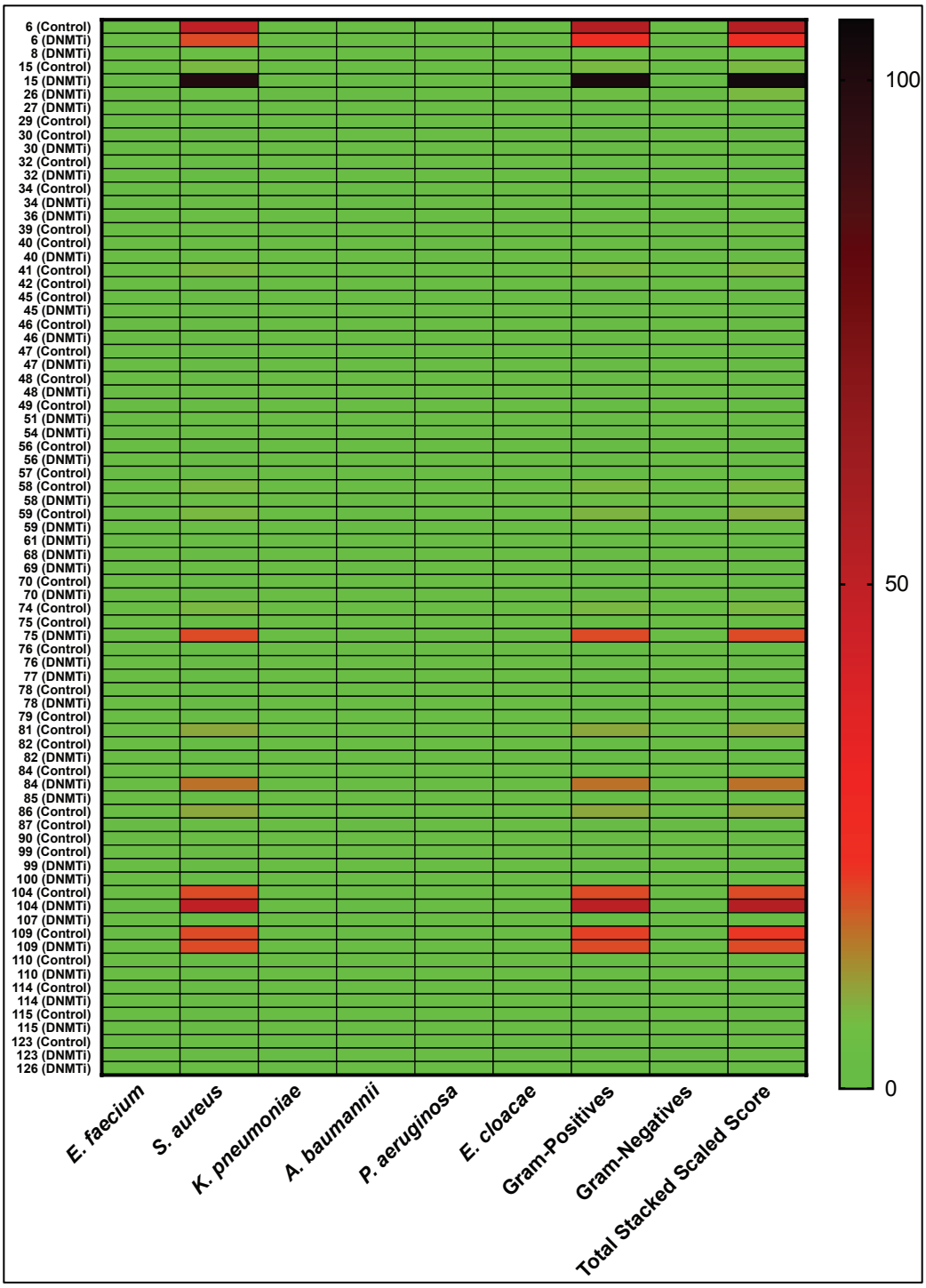

Figure 7. Heat Map of Crude Extract Bioactivity

All crude extracts were screened against the ESKAPE pathogens and the crude extracts with any level of bioactivity were assembled in this heat map. Heat map demonstrates level of activity to each of the individual ESKAPE pathogens, the Gram-positives, the Gram negatives, followed by the compiled activity against all ESKAPE pathogens as a whole.

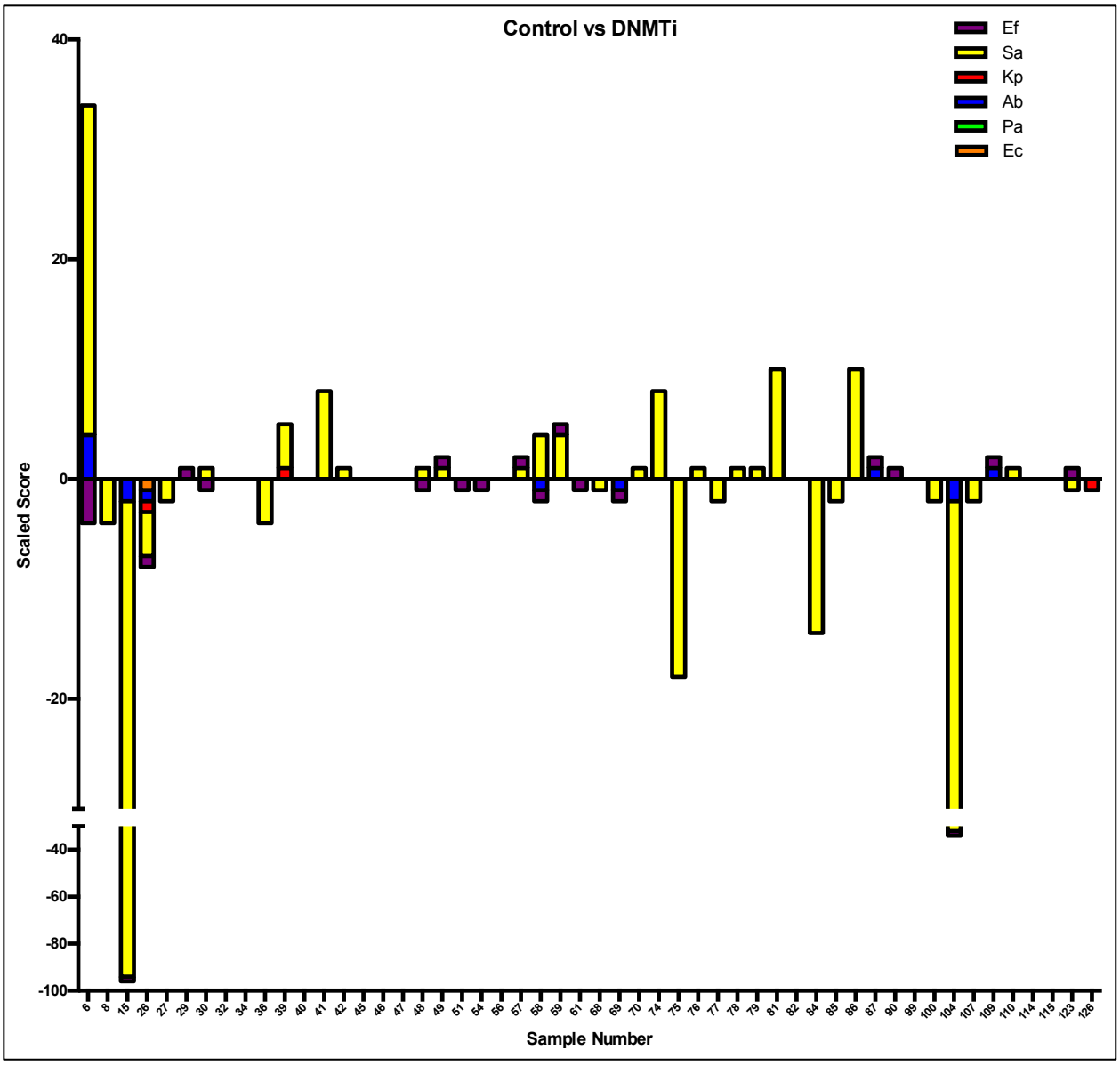

Figure 8. Comparison of Activity Between Modified and Unmodified Crude Extracts
All crude extracts that possessed bioactivity were assembled and compared to their modified or unmodified counterpart. Any portion of the bar graph that falls within the positive spectrum of this graph indicates the unmodified crude extract possessed higher bioactivity than the modified. Any portion of the bar graph that falls within the negative spectrum of this graph indicates the modified crude extract possessed higher bioactivity than the unmodified

being made to the regulation of these isolates, whether it is transcriptional, translational, or post-translational, further characterization is required.

These efforts to disrupt gene regulation via inhibition of DNA methylation have resulted in numerous changes thus far. We have observed differences in crude extract mass as well as changes in bioactivity against the ESKAPE pathogens, however in order to bottleneck the number of isolates we are working with, we needed to identify an approach to do so. We needed to establish a minimum scaled score to ensure the crude extracts we were excavating would provide adequate bioactivity upon purification. Therefore we determined any crude extract possessing a combined scaled score of >= 8 would be a priority for purification and characterization, while the remainder were archived. Of the 78 bioactive crude extracts, 17 (~22%) possessed a combined scaled score >= 8 (**Figure 9**). The genera responsible for these higher scaled scores belonged to *Streptomyces* and *Nocardia,* 15 of which were crude extracts from *Streptomyces* (**Figure 10**). These 17 offered a location with which to begin scaling up growth of our collection for higher yields of crude extract in order to further purify and characterize each of the extracts.

From the initial ranking data, we decided to move forward with sample 6 (*Streptomyces violarus*) as this crude extract possessed a vibrant ruby color and diffusible pigments can be an indicator of bioactive secondary metabolites (134). Aside from the coloration of the crude extract, sample 6 control was able to effectively inhibit *A. baumannii* at 50 µg/mL and *S. aureus* at 4 µg/mL and sample 6 DNMTi effectively inhibited *E. faecium* at 50 µg/mL and *S. aureus* at 10 µg/mL. Therefore we scaled up production and grew sample six in the presence and absence of 1 µM 5-azacytidine in two liter Erlenmeyer flasks containing 1000 mL of SCA broth. These flasks were kept at 28 °C in a shaking incubator under constant agitation for 28 days.

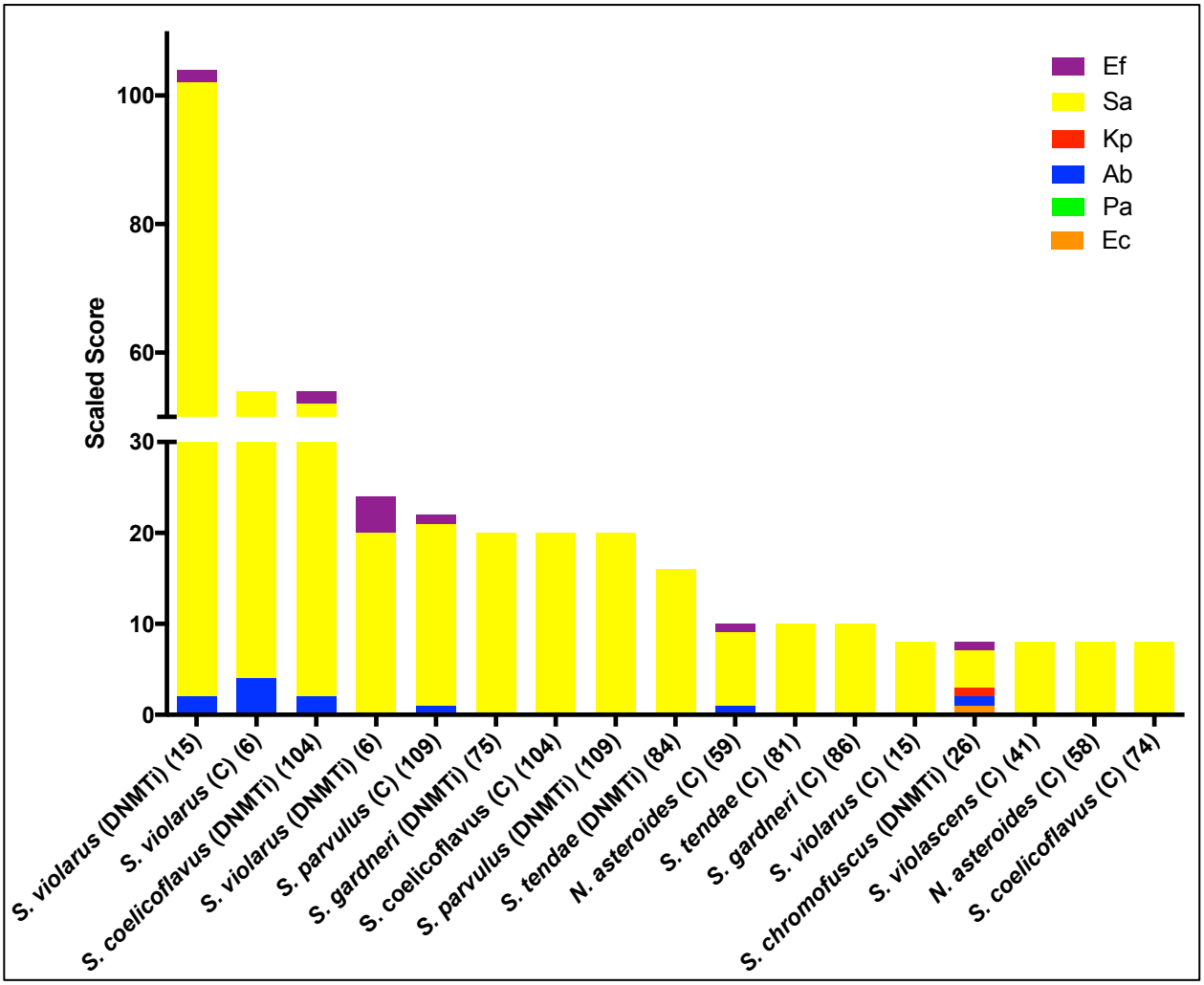

Figure 9. Crude Extracts with High Levels of Bioactivity
Crude extracts were organized based upon level of bioactivity in order to determine a prioritized system for future characterization.

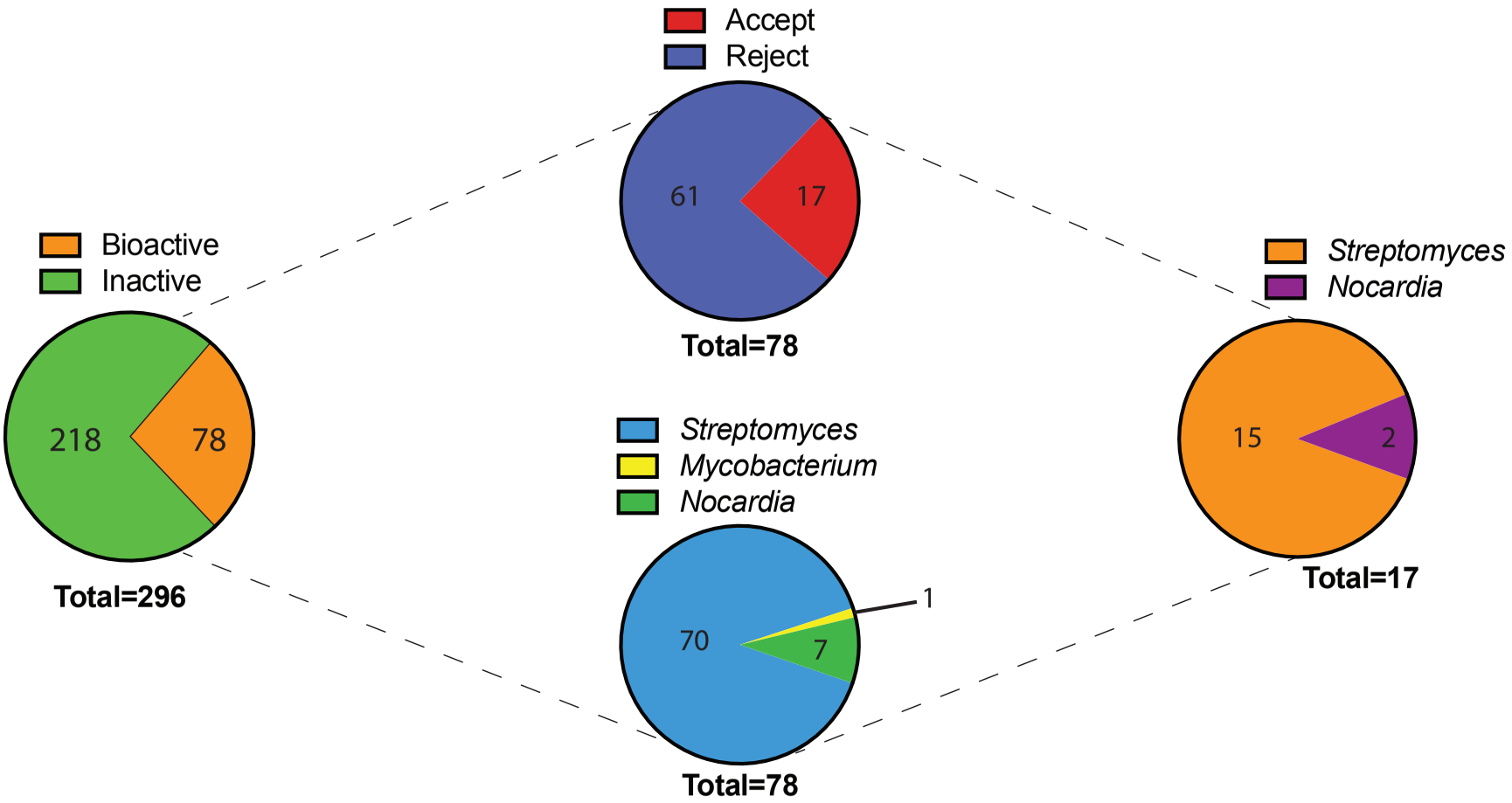

Figure 10. Overall Analysis of Crude Extract Bioactivity.
Total crude extracts used against the ESKAPE pathogens was 296, of which 78 possessed any level of bioactivity. Bioactive genera included *Streptomyces, Mycobacterium* and *Nocardia*. Only 17 of these 78 possessed high levels of bioactivity, which included species of the genera *Streptomyces* and *Nocardia*

The initial elements to analyze were to determine if there were a correlation between the crude extract mass and bioactivities of the small scale and large scale. The difference in volume of media was almost a 30-fold increase, while the fold change in crude mass extracted for the unchallenged and challenged were 32-fold and 42-fold, respectively (**Figure 11A**). The bioactivities of both of the large-scale crude extracts were almost 2-fold, with regards to *S. aureus* bioactivity, of the small-scale (**Figure 11B**). *E. faecium* bioactivity remained the same between small-scale and large-scale when challenged with 5-azacytidine, but bioactivity seen with *A. baumannii* in the unmodified small-scale crude extract was now replaced with bioactivity against *E. faecium* in the unmodified large-scale crude extract. We weren't as concerned with the level of activity as both the modified and unmodified demonstrated the same trend with the primary difference being a 2-fold increase in bioactivity. Therefore we proceeded to purification of the crude extract.

Initial High Performance Liquid Chromatography Coupled with Bioassay Guided Fractionation

High performance (pressure) liquid chromatography is a very beneficial form of column chromatography used by a number of fields for the purpose of separating complex mixtures. The system involves pumping your mixture through a column (stationary phase) in a solvent (mobile phase), mixture of solvents, or solvent-solvent gradient. The chromatogram displays the retention times of the separated compounds. Each of the compounds within the mixture interacts with the column and solvents differently; this interplay is what causes separation. There are numerous detectors used with HPLC analysis, however we focused on ultraviolet (UV) and electronic light scattering (ELS) detectors. We used two semi-preparatory silica columns in tandem as our stationary phase. Our mobile phase used 100% hexanes for 5 minutes, followed by a 35 minute gradient from 0-100% EtOAc, after which 15 minutes of 100%

A

Sample	Small-Scale (milligrams)	Large-Scale (milligrams)	Fold change (Media)	Fold Change (Crude mass)
Sample 6 (Control)	1.7	55	~29	~32
Sample 6 (DNMTi)	1.4	59.3	~29	~42

B

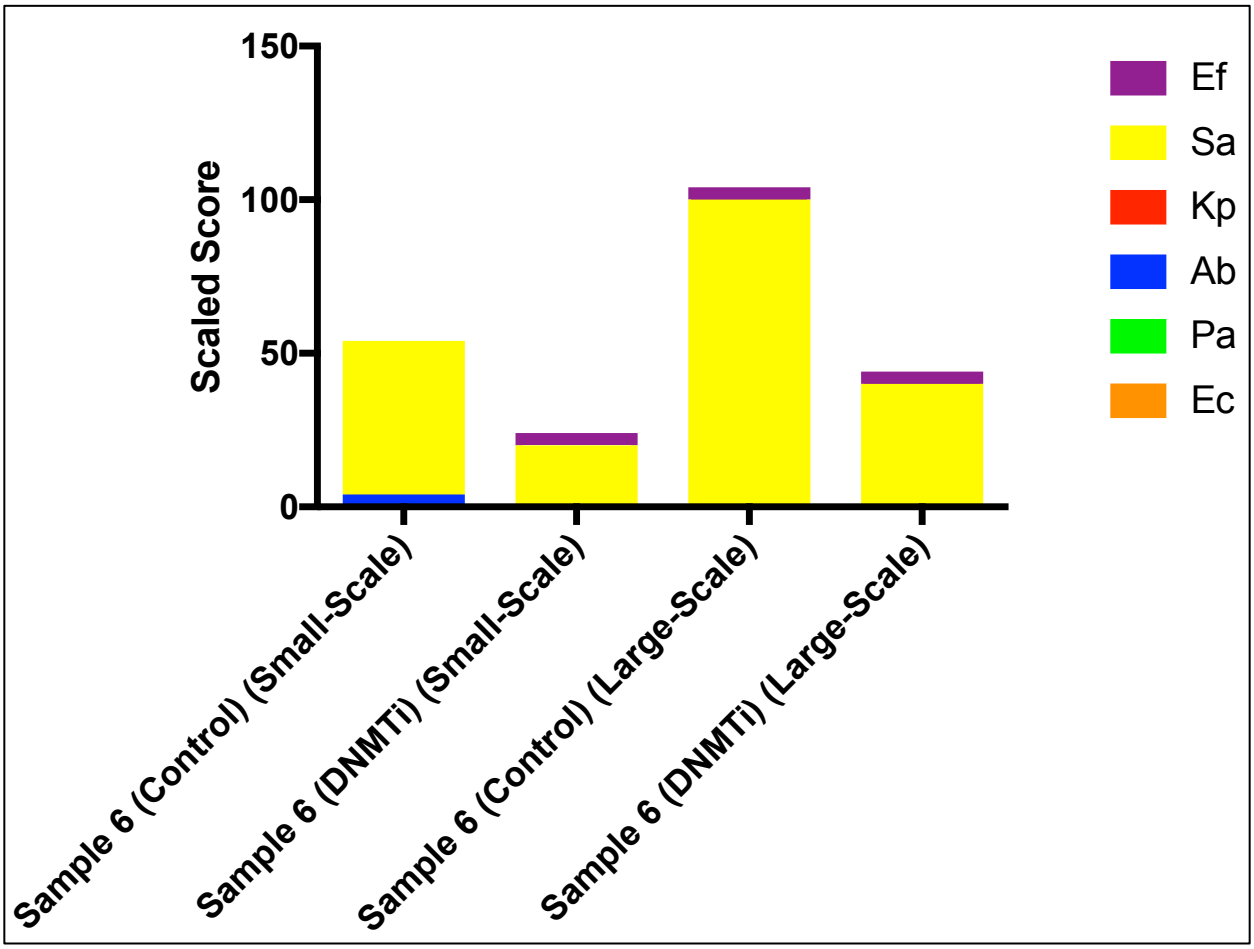

Figure 11. Small-Scale and Large-Scale Variations
Large-scale unmodified and modified crude extract masses were 32 and 42 fold higher than small-scale masses, respectively **(A)**. Large-scale unmodified and modified crude extract level of bioactivity against the ESKAPE pathogens were both ~2-fold higher than small-scale bioactivity **(B)**.

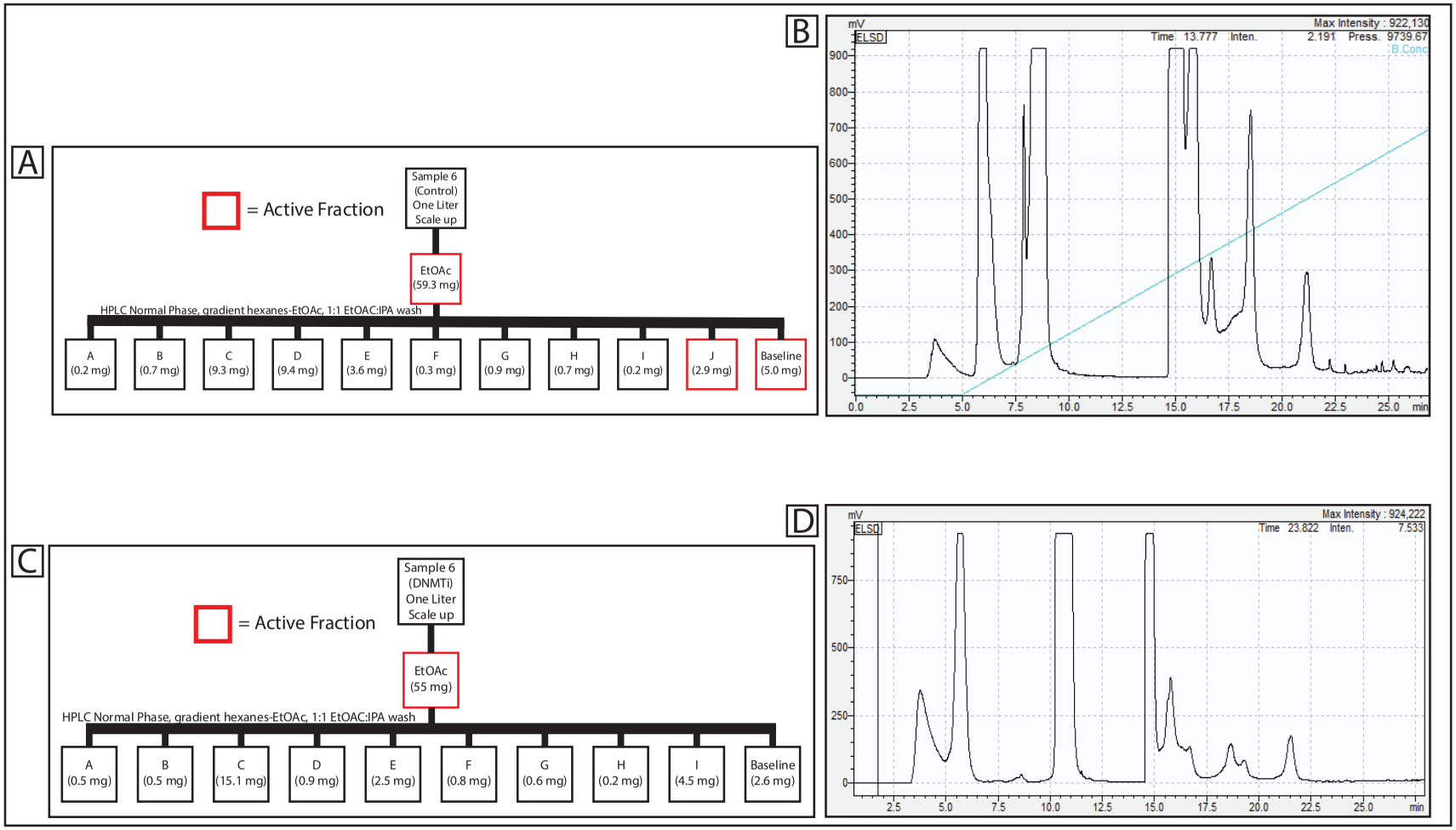

Figure 12. HPLC Outline and Chromatograms
Workflow and results of each of the HPLC purifications for sample 6 modified and unmodified crude extracts. Workflow and resultant fractions of sample 6 unmodified crude extract **(A)**; HPLC chromatogram for sample 6 unmodified crude extract, resulted in 10 fractions as well as baseline collections **(B)**; Workflow and resultant fractions of sample 6 modified crude extract **(C)**; HPLC chromatogram for sample 6 modified crude extract, resulted in 9 fractions as well as baseline collections **(D)**.

EtOAc, and concluded with 15 minutes of 1:1 EtOAc:IPA. This method yielded 10 fractions for the modified crude extract and 11 fractions for the unmodified crude extract (**Figure 12A-D**). After separating the two crude extracts into 21 separate fractions, we needed to determine which portion possessed the bioactivity seen in previous bioassays. The screen was performed against all of the ESKAPE pathogens in the same fashion as above and resulted with bioactivity in two fractions from sample 6 control, F and baseline (**Figure 13**). Bioactivity was only observed against *S. aureus* and was significantly reduced from bioactivity seen in prior screenings, and the bioactivity that was originally observed against *E. faecium* was lost completely. After performing bioassay with all of the fractions, we only possessed ~2.0 mg of the bioactive fraction J, which we attempted to purify further with reverse phase HPLC, however the mass acquired simply was not enough for further purification.

We learned a few important key factors with regard to our initial HPLC purification screening, one of which was that we required much more crude extract mass in order to obtain higher masses of purified fractions. Another important lesson learned was that a method needed to be optimized for purification of the crude extract, as there were a number of issues with regards to mobile phase optimization, stationary phase optimization and collecting of the fractions.

High Performance Liquid Chromatography Coupled with Bioassay Guided Fractionation

With initial large-scale purification we learned a number of valuable lessons, which we intended to improve upon with further attempts at HPLC purification. Our first amendment involved drastically increasing the crude extract mass, whereby we grew two liters of culture every 28 days, and three liters on the final 28 days, to a total of 13 liters for extraction. Each two-liter interval was considered the same and crude extracts were combined, as such we concluded with six individual crude extracts (**Table 11**). Our second amendment involved an extra purification step for each of the samples in an attempt to ensure uniform solubility for our

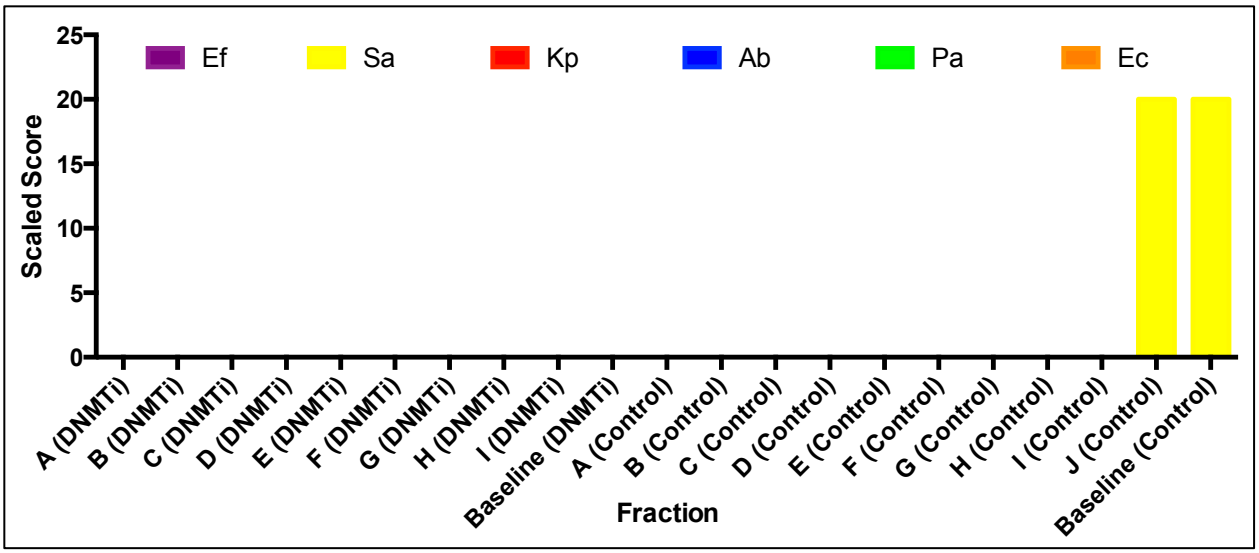

Figure 13. Bioactivity of HPLC Fractions
All fractions from HPLC purification of modified and unmodified sample 6 were screened against the ESKAPE pathogens to determine which of these possessed bioactivity. None of the fractions except for fraction J and the baseline fraction from unmodified sample 6 possessed bioactivity and only against *S. aureus*.

Table 11. Two-Liter Fermentation cycles and Partitioned Masses

Partition	First (mg)	Second (mg)	Third (mg)	Fourth (mg)	Fifth (mg)	Sixth (mg)
Crude	212.0	180.2	135.6	240.3	210.6	330.7
EtOAc	65.2	111.6	56	151.6	110.3	160
Insoluble	25.6	9.6	7.8	20.6	12.3	75.6
H_2O	100.5	40.9	58.1	55.6	66.9	59.6

** Listed above are the mass yields for each of the two-liter fermentation cycles including crude mass as well as the mass for each of the partitions. Slight overall losses can be attributed to residual crude extract within the original collection vials.*

complex mixture of compounds. This involved re-suspending each of our crude extracts in EtOAc followed by partitioning with an equal volume of double distilled H_2O in a separatory funnel, and collecting each of the layers (EtOAc soluble, insoluble, and H_2O soluble) into separate pre-weighed 20 mL scintillation vials, this was repeated three times to ensure complete separation. The collections were dried under constant flow of air and weighed. After an initial weigh in, a small mass of each partition was collected for bioassay (**Table 12**). Each of the partitions were screened against the ESKAPE pathogens to ensure bioactivity towards *E. faecium* and *S. aureus* were still present amongst each extraction as well as to identify which partition possessed bioactivity. For the purpose of identifying whether or not activity was present, MICs were not performed; instead all partitions were screened at a test concentration of 25 μg mL^{-1}. At this concentration, all of the crude extracts, as well as the EtOAc and insoluble partitions possessed bioactivity towards the two pathogens, while the H_2O layer only possessed activity in a few of the extractions. This circumstantial activity alluded to a number of possibilities, the first being we were unable to completely remove all bioactive compounds from the H_2O partition in all of the extractions, a second being multiple compounds possessing bioactivity and varying polarities are present in our complex mixture and therefore our partitions. Our most likely scenario is the former, as a result of bioactivity being seen in only a few of the H_2O partitions and not all.

The increased number of fermentations alongside partitioning provided ~650 mg of the EtOAc partitioned crude extract to be purified via HPLC. This mass was suspended in 4:6 hexanes: EtOAc to a final concentration of 75 mg mL^{-1} for normal-phase HPLC using one semi-preparatory silica column as the stationary phase. Our mobile phase used 100% hexanes for 5 minutes, followed by a 35 minute gradient from 0-100% 1:1 EtOAc:IPA, and concluded with 15 minutes of 100% 1:1 EtOAc:IPA. This method is slightly modified from our former method, which

Table 12. Total Mass for Each Two-Liter Fermentation Cycle

Partition	First Extraction (mg)	Second Extraction (mg)	Third Extraction (mg)	Fourth Extraction (mg)	Fifth Extraction (mg)	Sixth Extraction (mg)
Crude mass removed for bioassay	5.0	5.0	3.3	7.8	4.9	6.0
Crude mass remaining	8.7	5.2	7.1	2.3	12.7	5.2
EtOAc mass removed for bioassay	5.0	4.3	5.0	6.6	7.4	5.0
EtOAc mass remaining	60.2	107.3	51.0	145.0	102.9	155.0
Insoluble mass removed for bioassay	5.0	2.7	1.3	4.5	5.0	6.9
Insoluble mass remaining	20.6	6.9	5.5	16.1	7.3	68.7
H_2O mass removed for bioassay	2.5	4.3	5.3	7.8	8.3	8.1
H_2O mass remaining	98.0	36.6	52.8	47.8	58.6	51.1
Sum	205.0	172.3	131.3	237.9	207.1	306.4
Overall Percent lost	3.3%	4.4%	2.4%	1.0%	1.7%	7.3%

** Listed above are the total masses from the partitions from each of the two-liter fermentation cycles. This includes all of the masses removed for bioassay as well as the remaining mass and finally the total mass to determine the amount lost from each partition.*

used a mobile phase of EtOAc followed by a 1:1 EtOAc:IPA wash and a single silica column as opposed to two in tandem for our stationary phase. These minor modifications provided significantly better separation of the compounds present in the mixture and yielded 18 fractions (**Figure 14A-B, B not present yet**). In order to determine which of these fractions possessed the bioactivity seen in previous screenings, they were challenged against the ESKAPE pathogens. Fractions N and O retained activity against both *E. faecium* and *S. aureus*, which therefore required even further HPLC purification. We were unconcerned with the MIC of these fractions due to the limited masses we acquired, and therefore only analyzed each of the fractions as bioactive or inactive. The retention times for both fractions N and O indicated that the compounds present were more polar as they did not elute until majority of the mobile phase was 1:1 EtOAc:IPA. Further purification via HPLC utilized the system in reverse phase, which involves a hydrophobic stationary phase.

We began by suspending fractions N and O in a 20% solution of tetrahydrofuran (THF) in acetonitrile (ACN) and used an analytical C8 column as our stationary phase. Our mobile phase used 9:1 H_2O:20%THF:ACN for five minutes, followed by a 35 minute gradient from 10-99% of 20%THF:ACN, and concluded with 15 minutes of 99% of 20%THF:ACN. This method was used for both fractions for purification and yielded seven sub-fractions each (**Figure 14C, Chromatogram needs to be obtained**). Once again we needed to determine which of these sub-fractions possessed the bioactivity seen in previous screenings, as such, they were all challenged against the ESKAPE pathogens. Fractions 2ND and 2OD retained bioactivity against both *E. faecium* and *S. aureus*, while fraction 2OC displayed a hint of bioactivity as there was a severe growth defect with both *E. faecium* and *S. aureus*. As we now had even more limited masses than prior ESKAPE screenings, we once again limited our analysis to determine if the fractions were bioactive or inactive. This clear indication of which fractions possess bioactivity

has allowed for further characterization of our compound using mass spectrometry, H^1 NMR and C^{13} NMR.

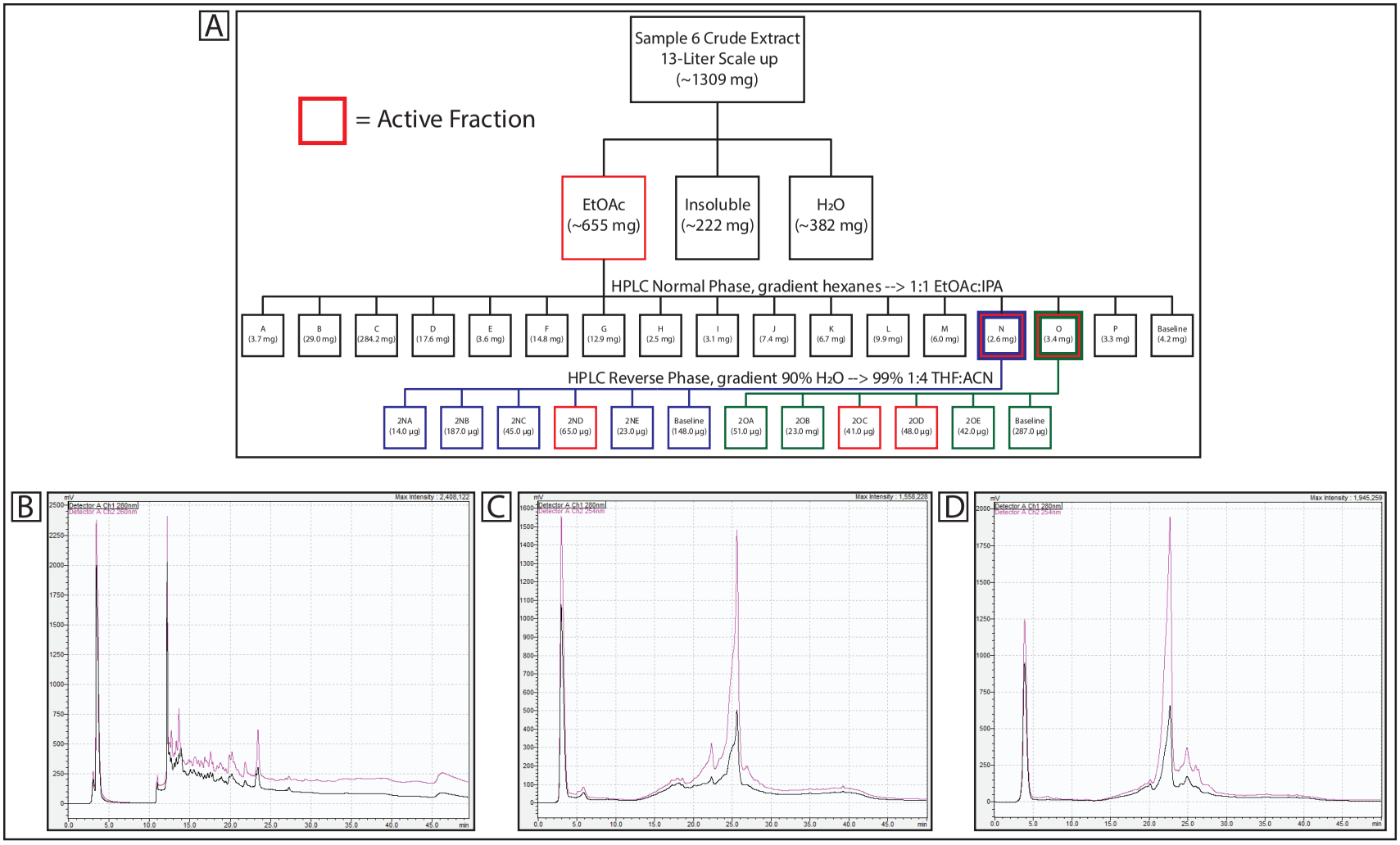

Figure 14. Modified HPLC Workflow and Chromatograms
Workflow and resultant fractions from EtOAc partition of sample six 13-L scale-up fermentations for normal phase as well as reverse phase HPLC purification **(A)**; HPLC chromatogram for sample 6 unmodified EtOAc partition **(B)**; HPLC chromatogram for sample 6 unmodified fraction N **(C)**; HPLC chromatogram for sample 6 unmodified fraction O **(D)**.

Discussion

It has long been known that *Actinobacteria* in the soil can provide a plethora of bioactive secondary metabolites, but have we just scratched the surface? We know that many species within the phylum *Actinobacteria* possess very diverse secondary metabolism, which can aid in their survival (77). Although some of the best described and most helpful for humanity have been antibiotics, which inhibit enzymes and cellular processes, *Actinobacteria* can also manufacture siderophores, for iron acquisition (135), spore pigments, which have been shown to provide some level of UV protection (136), as well as enzymes specialized for degradation, such as chitin binding proteins, which can aid in chitin degradation as a carbon and nitrogen source (137). The multitude of secondary metabolic potentials arise when *Actinobacteria* are stressed in the form of threats by other micro- or macroorganisms, nutrient depletion, and environmental stressors such as temperature and pH. These secondary metabolite products provide the structures and scaffolds we need to identify novel therapeutics and encouraging these organisms to produce them is only the first step.

Actinobacteria Diversity

Actinobacteria as a phylum have undergone numerous taxonomical classification events, only to be adjusted as technology advances. Originally classified as thread bacteria that closely resemble filamentous fungi, their organization has been adapted to include cell wall composition, morphological features, biochemical analysis, 16S rRNA gene sequencing, and nowadays we fall to whole genome sequencing (65, 138, 139). The constant evolution of *Actinobacteria* taxonomy is a result due to the remarkable diversity within this phylum. As each

new method of classification arises, we find outliers that do not match the necessary descriptions that fall within certain classes, genera, or species. An issue seen with cell wall composition as a mode of classification is that bacteria are typically classified as Gram-positive or –negative. This is based upon a Gram's stain, which will either indicate a thick peptidoglycan layer and single membrane (Gram-positive) or a thin peptidoglycan layer and two membranes (Gram-negative) (140). However the issue that arises is when bacteria don't match either of these descriptions and are considered Gram-variable, this can include *Actinobacteria* such as *Actinomyces, Corynebacterium,* and *Arthrobacter* (141). Biochemical analysis for classification saw similar issues as seen with cell wall composition taxonomy. Between the species there were a number of isolates that demonstrated similar cell-wall characteristics, and while the biochemical analyses could differentiate between a few, overlap was still observed, as seen with *Mycobacterium spp.* (142). Along with the advent of gene sequencing, the 16S rRNA gene became the method by which prokaryotes would be organized (143). As there are conserved regions of the 16S rRNA gene within a genus, there are also regions of variability that can be species specific, which is what gives this technique such remarkable cataloguing potential. As with all methods thus far, problems have arisen and while it is well known that bacteria possess 16S rRNA genes in numerous locations scattered throughout their chromosome, it was found that bacteria can also possess 16S rRNA genes that vary from one another as seen in *Thermomonospora chromogena*, which also happens to be a member of *Actinobacteria* (144). With whole genome sequencing becoming increasingly affordable, 16S rRNA gene classification may eventually lose its place, however it can still organize prokaryotes based upon their phylum, class, order, family and in a majority of instances, genus (66).

Within our unique collection of *Actinobacteria* we utilized 16S rRNA gene sequencing in order to identify the strains. With the objective of identifying a high throughput technique for isolating *Actinobacteria* we began by using 16S rRNA gene sequencing as an initial method for

identifying the bacteria we were working with. Using this technique, we were able to become familiar with *Actinobacteria* characteristics, which aided in rapid detection thereby eliminating the need for 16s rRNA gene sequencing. As we were isolating these bacteria with the intention of modifying already characterized isolates to unlock dormant or overlooked metabolism, we only resorted to 16S rRNA gene sequencing only when isolates possessed characteristics unlike those of *Actinobacteria*. In such cases we first ensured they were Gram-positives or repeatedly demonstrated a Gram-variable phenotype. Concurrent with literature, we have found that majority of our isolates for which we have identified belong to the genus *Streptomyces*. The majority of the isolates within the strain collection possess some level of semblance to the identified *Streptomycetes* therefore these bacteria which share a likeness were added to the collection (68). Regardless of the dominance of *Streptomycetes* in our environmental samples, we have still managed to isolate and identify a variety *Actinobacteria*, which provides alternative secondary metabolite potential in our search. We believe the diversity in our *Actinobacteria* collection is due to the variety of locations our environmental samples were obtained. Thus far we have primarily focused on terrestrial samples, however we have scavenged a few *Actinobacteria* in our collection from other areas including aquatic and arid environments. As we continue to isolate and identify *Actinobacteria*, we anticipate that the diversity will continue and grow as we move to more unique environments such as deep-sea sediments, sponges, and high-altitude soils.

Many researchers in the *Actinobacteria* world focus their efforts on locating novel genera and species from exotic locations in an effort to discover new chemistry that allows the bacteria to survive in these unique environments. We however are combining this common approach while simultaneously focusing on cultivation of as many isolates as possible from environmental samples. We have looked at a variety of locations for *Actinobacteria* including in our back yard or a farm, which has the potential to provide immense diversity. For numerous soils it is

estimated that the population density is ~ 1 billion bacteria per gram, while the variety of species can be between 4,000 and 50,000 (145, 146). *Actinobacteria* are only thought to account for between 2 – 9% of the overall population, this would suggest a large range of potential actinobacterial isolates (80-4,500 *Actinobacteria* g^{-1}) (145). Most of our samples yielded a number of isolates that falls within this range perfectly, as shown with our Soil 1 environmental sample from a Ft. Meyers farm. Soil 1 was able to provide 32 *Actinobacteria* that we were able to identify into our strain collection. Taking into consideration the dilution series undergone and volume plated, we see that the estimated number present in the original sample was ~1,600 *Actinobacteria* g^{-1}. This number only represents the *Actinobacteria* we were able to culture within the laboratory, which suggests we may be missing potential additions to our collection. One study proposed that the use of many different media types would increase the chance of identifying novel members of the phylum, their results suggest that the number of isolates acquired was higher when more media types were used (147). We attempted to use a greater variety of media and methods initially, however it did not seem a feasible option with the number of environmental samples we possess. Although the exact media composition for optimal growth of *Actinobacteria* is unknown, our two low-nutrient media provide an excellent basis for high throughput isolation.

Drug Discovery

With the introduction of penicillin by Alexander Fleming in 1928 and its subsequent release to the public in the 1940s, so began the era of natural products drug discovery (86). The field delved further with the introduction of actinomycin and streptomycin by Selman Waksman shortly after (87, 88). Antimicrobial resistance spurred drug discovery into a never-ending endeavor to prevent infection and soon it seemed all pharmaceutical companies and drug discovery researchers were growing microorganisms in the search for antibiotics. This search

for anti-infective agents did not come without a price tag or regulations, which were becoming more and more stringent. In the 1970s, many pharmaceutical companies were realizing that natural product drug discovery branches were becoming a financial burden as the proverbial "low hanging fruit" was now out of reach (148). As the 1990s came about, we were looking at a revolution within the drug discovery world and that was combinatorial chemistry, a method by which tens of thousands of chemical structures can be tested at once as potential therapeutics (90). One of the issues with combinatorial chemistry is that the synthetic capabilities of chemists cannot compete with that of nature, and therefore natural products chemistry has produced scaffolds and compounds that cannot be mimicked in the laboratory. As seen with *Actinobacteria* classification, new technologies provided alternative approaches to searching for novel bioactive compounds. Technological advancements have led to resurgence in natural product drug discovery. Liquid chromatography advancements now allow for accurate and replicative separating of soluble compounds and mass spectrometry has provided analysis of crude extracts, which can detect trace amounts of individual compounds. Novel cultivation techniques cater the isolation of previously unattainable microbes to be grown in lab conditions and whole genome sequencing has allowed for genome mining in the search for genes homologous to those found in known biosynthetic pathways. All of these coupled to one another have thrust natural product drug discovery in new directions previously unexplored.

Today, drug discovery looks very different than before as novel techniques are used in numerous applications. Bioactive secondary metabolites have been found in unorthodox locations such as a Colorado iron-rich freshwater spring by a group from Oklahoma (149). Six new compounds were identified from fungi found within a microbial mat and the group was able to characterize clearanols A-E and disulochrin (149). Of these six natural products disulochrin demonstrated bioactivity against methicillin-resistant *Staphylococcus aureus* and clearanol C showed weak bioactivity against *Candida albicans* biofilm formation (149). Identifying microbes

from unique locations can lead to remarkable chemistry as these organisms have developed methods for thriving in such environments. An alternative habitat that has been explored to yield new secondary metabolites is sea life. Former explorations of marine life such as tunicates suggested that compounds found within were synthesized by the Ascidians (150). While they are a prolific source of natural products, it was later found that these compounds were actually produced by the endophytic bacteria. This was elucidated using genome sequencing where it was found that the *Prochloron didemni* did not possess the necessary genes for synthesis of the antitumor polyketides known as patellazoles (151). The combination of new habitats and whole genome sequencing allowed natural products to identify the specific producer of the compounds.

In the constant struggle of imitating the natural environment within the lab there have been numerous attempts at replicating such conditions. In order to mirror nutrient fluctuations seen in the environment, groups in Germany have devised a clever way including continuous culture of *Aspergillus nidulans*, one of the model organisms used to study fungal genetics. This continuous culture could be modified to mimic nitrogen, phosphorous and carbon limiting conditions that could occur in the environment (152). Under nitrogen and phosphorous limitations silent polyketide synthase genes were differentially expressed when compared to standard growth conditions (152). A search that yielded novel secondary metabolites utilized a novel growth technique that allowed bacteria to grow in their natural environment. Using a multi-channel device known as an isolation chip or iChip, the group was able to isolate previously unculturable microbes (153). This iChip consists of hundreds of diffusion chambers made up of through holes with semi-permeable membranes on either side (153). The process involves loading each of the wells with a sample of soil that has been diluted to roughly one cell per 20 microliters of molten agar, assembling the remainder of the iChip, and then burying it where the original soil sample was obtained (153). This method was used by Ling *et al*. to isolate and

identify the novel cell wall inhibitor, teixobactin from the new species *Eleftheria terrae* (154). Prior to identification of this compound, this group of Gram-negatives were not known to produce any antibiotics, which exposes how little is known about bacteria in their natural environment (154).

Although bacteria possess seemingly limitless diversity when it comes to secondary metabolites, a struggle commonly seen is the abundance with which they are synthesized. Advancements in instrumentation used for detection and analysis of metabolite production has given rise to the term “metabolome.” The metabolome is in reference to the overall metabolite production by an organism, much the same, as the genome and the associated genes or the proteome and proteins produced. Improvements to mass spectrometry have allowed for detection of trace loads of a compound in a mixture. Using HPLC and mass spectrometry in tandem grants separation as well as mass detection of crude mixtures resulting in a metabolic profile. After further purification, even the insignificant portions of the amalgam of compounds could be identified. Many metabalomic profiles exist from bacteria today; they can be used to determine effect of nutrient starvation, mutation, temperature or pH stressors on metabolite production. A group at the University of Wisconsin – Madison were able to discover analogs of a known compound using liquid chromatography coupled mass spectrometry (LCMS) (155). This analog, bottromycin D possessed slightly lower levels of bioactivity than the original bottromycin A_2, however identifying the compound cements LCMS as a promising technique for future analysis of metabolites (155).

Our research has utilized a number of these methods listed above, which could be what drives the immense diversity of our collection. We have not only obtained samples from diverse locations, but we also are using HPLC coupled with mass spectrometry as well as NMR techniques to elucidate the structure of a compound. Our environmental samples include soil

from numerous locations including farms (higher nitrogen levels), high altitudes (lower oxygen levels) and forests (higher organic content). Not only does our environmental sample collection have a variety of soils, but we also possess numerous sponges, sand from arid and high temperature regions, as well as shallow and deep-sea sediments. This variety of conditions can play a significant role in the behavior and lifestyle of the native microbes and by using such a wide variety environmental samples we have been able to demonstrate similarities to literature in acquiring a diverse collection of *Actinobacteria*. Interestingly, some of our environmental samples that have provide the most numerous isolates of *Actinobacteria* have been locations that have had ample traffic by grazing animals. Our biggest contributor with regards to the *Actinobacteria* collection was from a soil sample procured from horse stables, however this has been only an observation thus far. A hypothesis that could provide insight to this observation is that grazing animals leave in their wake a myriad of particulate organic matter, which is readily colonized by *Actinobacteria,* as they possess the necessary metabolism to decompose such substances. Of the 13 different *Actinobacteria* that possessed high levels of bioactivity against the ESKAPE pathogens, all were from soil samples that had interaction with grazing animals. This suggests the bacteria within the soil samples may have had interactions with mammalian associated bacteria, therefore requiring them to mount defensive strategies that persisted throughout lab cultivation. An alternative theory suggests the soil may have been supplemented with lime, which is commonly used as a remedy for low soil pH and help plants and fields flourish, and has also been shown to enhanced presence of *Streptomycetes* (156, 157). Furthermore majority of the crude extracts that possessed high levels of bioactivity were from the genus *Streptomyces*, which has historically been a primary source of antibiotics. Majority of our bioactivity is towards our Gram-positive organisms *E. faecium* and *S. aureus*, which seems fairly common with drug discovery campaigns. Within the literature it seems that bioactive compounds trend towards the Gram-positives for a number of reasons including two attributes of the Gram-negatives, an outer cell membrane and efflux pumps (158). It has been

demonstrated that antibiotics with intracellular targets possessed by both Gram-positives and negatives can affect only the Gram-positives and efflux deficient Gram-negatives (117).

Although we have successfully extracted bioactive secondary metabolites from our cultured *Actinobacteria*, there are numerous improvements that could provide better yields. One such improvement could be to increase aeration within our flasks. It is suggested that for cultivation of Streptomycetes, there should be 4-10 fold volumes of air compared to that of the liquid medium. Currently in our methods we are using a 1:1 ratio of liquid media to air, which may not be enough for the bacteria to properly thrive. We have also noticed significant clumping of our bacteria in liquid cultures, primarily the Streptomycetes. This is a common issue that can be dealt with in numerous ways including addition of sucrose, polyethylene glycol, or using baffled flasks (159). As such we will be optimizing a method to prevent clumping and perhaps optimizing even further the necessary volumes for increased production of bioactive secondary metabolites. Additional to our potentially sub-optimal aeration, our extraction methodology focuses solely on secondary metabolites, which are soluble in EtOAc. This could be rectified with modifications to procedure that involve drying the entire growth culture prior to extraction.

Epigenetics

Many efforts have been devoted to discovering new methods of unlocking silent metabolic pathways in *Actinobacteria* and other natural product-producing organisms. Of these methods there have been attempts of small molecule or chemical, co-cultivation, or genome mining elicitation of these cryptic genes. Much of the literature regarding co-cultivation suggests that quorum sensing molecules or presence of a nutrient competitor elicits the production of secondary metabolites (160). Genome mining has also proved a potential for novel metabolites, as shown with *S. coelicolor*, which displayed over 20 theorized secondary metabolite

biosynthetic pathways (78). However, our search for silent metabolic pathways involves epigenetic modification of *Actinobacteria* using small molecules. This has only been performed a handful of times, however for fungal elicitation it has been very successful with identification of novel metabolites. A study performed in 1995 by Fernandez *et al*. and Novella and Sanchez discussed the differences in production of rhodomycin from *S. antibioticus* (161, 162). It was noticed that the cultures with 5-azacytidine began producing the anthracycline after only six hours of growth, while the control didn't display any signs of production even after four days of growth (161). The study by Fernandez *et al*. attributed the changes in anthracycline production to low levels of methyltransferase in the 5-azacytidine challenged culture stating that methyltransferase activity was as low as 50% of that seen in the control (161). Fernandez *et al*. were looking for effects of DNA methyltransferase inhibitors on the overall growth and development of *Streptomyces*, which was not limited to secondary metabolite production, but other characteristics as well. In the subsequent study by Novella and Sanchez, it was demonstrated that neither DNA, RNA or protein synthesis in *S. antibioticus* were inhibited by 5-azacytidine, yet this study confirmed the low level of activity from methyltransferases (162). Another study that used 5-azacytidine as a small molecule elicitor of secondary metabolite biosynthesis was performed at Banaras Hindu University in Varanasi, India. This study by Kumar *et al*. involved exposing *S. coelicolor* to varying concentrations of 5-azacytidine to compare crude extracts bioactivity to untreated cultures. Kumar *et al*. tested crude extracts from each of the *S. coelicolor* cultures at the varying concentrations against five human pathogenic bacteria (108). Using a combination of Kirby-Bauer disk diffusion and MTT assays, they determined that a concentration of 25 μM was sufficient to induce antibacterial activity (108). The group continued to HPLC and MALDI-TOF/mass spectrometry analysis of the crude extracts to derive differences between the 25 μM and untreated growth cultures. Their conclusion alludes to antibacterial activation after epigenetic modification as their control culture produced only five compounds, while their modified produced 12 (108). Although the study

provided compelling evidence to suggest epigenetically modifying *S. coelicolor* induced inhibitory agents after a threshold concentration was achieved, there was no mention of the inhibitory action of 5-azacytidine against the human pathogens at the concentrations mentioned. In our study we overcame this obstacle by ensuring 5-azacytidine concentrations used would not affect growth of our pathogens. We also ensured the concentration of 5-azacytidine would not inhibit growth of our *Actinobacteria* as we optimized the concentration used rigorously. In our studies of epigenetic modification of *Actinobacteria* we have attempted to ensure each of our processes are optimal for a high throughput screening campaign. By identifying optimal the optimal cultivation period, media, concentration of epigenetic modifier, and catering to multiple growth types present in *Actinobacteria*, we believe this high throughput endeavor will successfully unlock cryptic metabolic pathways.

As a result of our rigor, we have successfully observed differences in production and antibacterial activity of our crude extracts from modified and unmodified *Actinobacteria*. As mentioned previously, the masses associated with our crude extracts vary greatly, however the differences in mass between modified and unmodified cultures demonstrates the level of change each of the *Actinobacteria* are undergoing. These changes in mass primarily indicate that the proportions of product are different, which we believe is a direct effect of our epigenetic modifier. We have also established diversity in the level of antibacterial bioactivity between modified and unmodified cultures indicating different compound production between the two. It has been demonstrated that mutations in gene promoters and changes in methylation patterns can alter expression of the subsequent genes (45, 46, 95). Therefore we hypothesize that the variations in crude extract mass and bioactivity against the ESKAPE pathogens could be a result of augmented regulation due to gene promoter de-methylation. This of course suggests three outcomes for secondary metabolite biosynthesis gene promoters; they can be expressed more frequently, less frequently, or can be unaffected. This interpretation would provide

adequate rationale for our modified crude extracts yielding higher, lower or equal mass and bioactivity compared to the unmodified crude extracts.

As we near the potential of a post-antibiotic era, we require new therapeutics that can combat the rise of resistance. *Actinobacteria* have historically contributed to majority of the antibiotics we possess today, and continue to provide natural products and natural product derivatives. Our results demonstrate the abundance of compounds yet to be identified in both new organisms as well as the well established. The identification of our lead compound has and will remain our top priority as it has demonstrated such inhibitory potential of *S. aureus* and *E. faecium*. Thus far we have successfully purified our crude extract from the large-scale fermentation of *S. violarus* via HPLC and have moved to structure elucidation. The structure of our compound thus far, when comparing the NMR and mass spectrometry data, seems to possess characteristics often associated with anthracyclines. The phenotype of our compound also suggests anthracycline as it retains the same ruby pigmentation often associated with this class of drug. Anthracyclines are characterized as possessing anti-tumor capabilities as well as have been observed to inhibit Gram-positives, although we have not yet performed cytotoxic screening of our compound, we have noticed inhibition of only Gram-positives thus far. Although the exact mechanism of action of anthracyclines is perpetually debated, it has been shown resistance in Gram-negatives may be due to efflux pumps (117).

Future Directions

Preliminary results suggest the bioactivity of our extract is due to an anthracycline compound based upon the ruby pigment, the Gram-positive specific bioactivity, and initial NMR data. In order to properly elucidate the structure of our lead compound, it would be beneficial to obtain significantly more crude extract for purification. We have performed numerous purifications via HPLC in order to obtain as much pure compound as possible, yet we seem to be left with minuscule amounts. Regrettably the long cultivation period and low extraction yield results in providing only preliminary data that suggests subtle hints of the structure of our lead compound. Therefore significantly more crude extract is needed in order to purify higher more. Alternatively, we could also optimize our growth period and determine at what point *S. violarus* is producing the highest yield of our bioactive compound. This can be done in a few ways including a bioactivity based approach or a mass spectrometry approach, either would provide insight into the abundance at different growth stages. Alternatively, it would also be worthwhile performing whole genome sequencing on the isolate to determine the gene clusters responsible for our compound. This information would potentially allow us to identify biosynthesis and regulatory pathways, which would allow for optimized growth and production. Genome mining could also help identify otherwise unknown alternative secondary metabolite biosynthesis pathways, which could harbor previously unseen chemistry. After establishing the structure of our lead compound we will focus on further characterizing the bioactivity seen including whether the compound is bacteriostatic or bactericidal in nature, cytotoxicity of the compound and whether this compound has the ability to affect bacteria within a biofilm.

We also plan to continue growing and modifying the *Actinobacteria* strain collection followed by determining antibacterial activity against the ESKAPE pathogens. Thus far we have successfully grown and extracted a large number of isolates, however, as the collection continues to grow, so must the extractions. We possess a number of environmental samples we have been unable to cultivate organisms from, however, this requires further optimization that we will achieve. We have identified a number of high activity crude extracts, which have provided a number of avenues with which to pursue in further large-scale culturing followed by purification and characterization. In an attempt to further identify the impact of our DNMTi, we would also like to establish a metabolome profile for our unmodified and modified organisms. This of course could not be performed on all organisms we cultivate; however it would be interesting to analyze our crude extracts that possess high levels of bioactivity against the ESKAPE pathogens and demonstrate differences between the modified and unmodified samples.

References

1. Centers for Disease Control and Prevention, Office of Infectious Disease. Antibiotic resistance threats in the United States, 2013. April 2013. Available at: http://www.cdc.gov/drugresistance/ threat-report-2013. Accessed March 30, 2016. .

2. Wang SH, Sheng WH, Chang YY, Wang LH, Lin HC, Chen ML, Pan HJ, Ko WJ, Chang SC, Lin FY. Healthcare-associated outbreak due to pan-drug resistant Acinetobacter baumannii in a surgical intensive care unit. J Hosp Infect. 2003;53(2):97-102. PubMed PMID: 12586567.

3. Roberts RR, Hota B, Ahmad I, Scott RD, 2nd, Foster SD, Abbasi F, Schabowski S, Kampe LM, Ciavarella GG, Supino M, Naples J, Cordell R, Levy SB, Weinstein RA. Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. Clin Infect Dis. 2009;49(8):1175-84. doi: 10.1086/605630. PubMed PMID: 19739972.

4. US Food and Drug Administration. 2015 summary report on antimicrobials sold or distributed for use in food producing animals, 2016. Available at: http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM534243.pdf. Accessed 7 February 2017.

5. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-83. PubMed PMID: 25859123; PMCID: PMC4378521.

6. Duncan KR, Haltli B, Gill KA, Correa H, Berrue F, Kerr RG. Exploring the diversity and metabolic potential of actinomycetes from temperate marine sediments from Newfoundland, Canada. J Ind Microbiol Biotechnol. 2015;42(1):57-72. doi: 10.1007/s10295-014-1529-x. PubMed PMID: 25371290.

7. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis. 2008;197(8):1079-81. doi: 10.1086/533452. PubMed PMID: 18419525.

8. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(1):1-12. doi: 10.1086/595011. PubMed PMID: 19035777.

9. Wolf M, Muller T, Dandekar T, Pollack JD. Phylogeny of Firmicutes with special reference to Mycoplasma (Mollicutes) as inferred from phosphoglycerate kinase amino acid sequence data. Int J Syst Evol Microbiol. 2004;54(Pt 3):871-5. doi: 10.1099/ijs.0.02868-0. PubMed PMID: 15143038.

10. Schleifer KH, Kilpper-Bälz R. Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. International Journal of Systematic and Evolutionary Microbiology. 1984;34(1):31-4. doi: doi:10.1099/00207713-34-1-31.

11. Maccallum WG, Hastings TW. A Case of Acute Endocarditis Caused by Micrococcus Zymogenes (Nov. Spec.), with a Description of the Microorganism. J Exp Med. 1899;4(5-6):521-34. PubMed PMID: 19866921; PMCID: PMC2118017.

12. Arias CA, Murray BE. The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10(4):266-78. doi: 10.1038/nrmicro2761. PubMed PMID: 22421879; PMCID: PMC3621121.

13. Chow JW. Aminoglycoside resistance in enterococci. Clin Infect Dis. 2000;31(2):586-9. doi: 10.1086/313949. PubMed PMID: 10987725.

14. Arias CA, Murray BE. Emergence and management of drug-resistant enterococcal infections. Expert Rev Anti Infect Ther. 2008;6(5):637-55. doi: 10.1586/14787210.6.5.637. PubMed PMID: 18847403.

15. Sivaraman K, Venkataraman N, Cole AM. Staphylococcus aureus nasal carriage and its contributing factors. Future Microbiol. 2009;4(8):999-1008. doi: 10.2217/fmb.09.79. PubMed PMID: 19824791; PMCID: PMC2908500.

16. Ortega E, Abriouel H, Lucas R, Galvez A. Multiple roles of Staphylococcus aureus enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins (Basel). 2010;2(8):2117-31. doi: 10.3390/toxins2082117. PubMed PMID: 22069676; PMCID: PMC3153285.

17. Genigeorgis CA. Present state of knowledge on staphylococcal intoxication. Int J Food Microbiol. 1989;9(4):327-60. PubMed PMID: 2701861.

18. McCallum N, Berger-Bachi B, Senn MM. Regulation of antibiotic resistance in Staphylococcus aureus. Int J Med Microbiol. 2010;300(2-3):118-29. doi: 10.1016/j.ijmm.2009.08.015. PubMed PMID: 19800843.

19. Kong C, Neoh HM, Nathan S. Targeting Staphylococcus aureus Toxins: A Potential form of Anti-Virulence Therapy. Toxins (Basel). 2016;8(3). doi: 10.3390/toxins8030072. PubMed PMID: 26999200; PMCID: PMC4810217.

20. Ryan KJ, Ray CG, Sherris JC. Sherris medical microbiology : an introduction to infectious diseases. 4th ed. New York: McGraw-Hill; 2004. xiii, 979 p. p.

21. Postgate JR. Nitrogen fixation. 3rd ed. Cambridge, U.K. ; New York, NY, USA: Cambridge University Press; 1998. vi, 112 p. p.

22. Williams KP, Gillespie JJ, Sobral BW, Nordberg EK, Snyder EE, Shallom JM, Dickerman AW. Phylogeny of gammaproteobacteria. J Bacteriol. 2010;192(9):2305-14. doi: 10.1128/JB.01480-09. PubMed PMID: 20207755; PMCID: PMC2863478.

23. Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev. 1998;11(4):589-603. PubMed PMID: 9767057; PMCID: PMC88898.

24. Ah YM, Kim AJ, Lee JY. Colistin resistance in Klebsiella pneumoniae. Int J Antimicrob Agents. 2014;44(1):8-15. doi: 10.1016/j.ijantimicag.2014.02.016. PubMed PMID: 24794735.

25. Towner KJ. Acinetobacter: an old friend, but a new enemy. J Hosp Infect. 2009;73(4):355-63. doi: 10.1016/j.jhin.2009.03.032. PubMed PMID: 19700220.

26. Antunes LC, Visca P, Towner KJ. Acinetobacter baumannii: evolution of a global pathogen. Pathog Dis. 2014;71(3):292-301. doi: 10.1111/2049-632X.12125. PubMed PMID: 24376225.

27. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nat Rev Microbiol. 2007;5(12):939-51. doi: 10.1038/nrmicro1789. PubMed PMID: 18007677.

28. Poirel L, Bonnin RA, Nordmann P. Genetic basis of antibiotic resistance in pathogenic Acinetobacter species. IUBMB Life. 2011;63(12):1061-7. doi: 10.1002/iub.532. PubMed PMID: 21990280.

29. Lederberg J. Encyclopedia of microbiology. 2nd ed. San Diego: Academic Press; 2000.

30. Chatterjee M, Anju CP, Biswas L, Anil Kumar V, Gopi Mohan C, Biswas R. Antibiotic resistance in Pseudomonas aeruginosa and alternative therapeutic options. Int J Med Microbiol. 2016;306(1):48-58. doi: 10.1016/j.ijmm.2015.11.004. PubMed PMID: 26687205.

31. Livermore DM. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? Clin Infect Dis. 2002;34(5):634-40. doi: 10.1086/338782. PubMed PMID: 11823954.

32. Keller R, Pedroso MZ, Ritchmann R, Silva RM. Occurrence of virulence-associated properties in Enterobacter cloacae. Infect Immun. 1998;66(2):645-9. PubMed PMID: 9453621; PMCID: PMC113501.

33. Flynn DM, Weinstein RA, Nathan C, Gaston MA, Kabins SA. Patients' endogenous flora as the source of "nosocomial" Enterobacter in cardiac surgery. J Infect Dis. 1987;156(2):363-8. PubMed PMID: 3598234.

34. Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, Leitch CD, Daum RS. Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. JAMA. 1998;279(8):593-8. PubMed PMID: 9486753.

35. Kuehnert MJ, Hill HA, Kupronis BA, Tokars JI, Solomon SL, Jernigan DB. Methicillin-resistant-Staphylococcus aureus hospitalizations, United States. Emerg Infect Dis. 2005;11(6):868-72. doi: 10.3201/eid1106.040831. PubMed PMID: 15963281; PMCID: PMC3367609.

36. Webb GF, Horn MA, D'Agata EM, Moellering RC, Jr., Ruan S. Competition of hospital-acquired and community-acquired methicillin-resistant Staphylococcus aureus strains in hospitals. J Biol Dyn. 2010;4(1):115-29. doi: 10.1080/17513750903026411. PubMed PMID: 19587839; PMCID: PMC2706519.

37. Giske CG, Monnet DL, Cars O, Carmeli Y, ReAct-Action on Antibiotic R. Clinical and economic impact of common multidrug-resistant gram-negative bacilli. Antimicrob Agents Chemother. 2008;52(3):813-21. doi: 10.1128/AAC.01169-07. PubMed PMID: 18070961; PMCID: PMC2258516.

38. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards J, Jr., Infectious Diseases Society of A. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clin Infect Dis. 2008;46(2):155-64. doi: 10.1086/524891. PubMed PMID: 18171244.

39. Sutton JP, Steiner CA. Hospital-, Health Care-, and Community-Acquired MRSA: Estimates From California Hospitals, 2013: Statistical Brief #212. Healthcare Cost and Utilization Project (HCUP) Statistical Briefs. Rockville (MD)2006.

40. Aminov R. History of antimicrobial drug discovery - Major classes and health impact. Biochem Pharmacol. 2016. doi: 10.1016/j.bcp.2016.10.001. PubMed PMID: 27720719.

41. Livermore DM. Defining an extended-spectrum beta-lactamase. Clin Microbiol Infect. 2008;14 Suppl 1:3-10. doi: 10.1111/j.1469-0691.2007.01857.x. PubMed PMID: 18154524.

42. Nordmann P, Guibert M. Extended-spectrum beta-lactamases in Pseudomonas aeruginosa. J Antimicrob Chemother. 1998;42(2):128-31. PubMed PMID: 9738828.

43. Tamber S, Hancock RE. On the mechanism of solute uptake in Pseudomonas. Front Biosci. 2003;8:s472-83. PubMed PMID: 12700103.

44. Wozniak A, Villagra NA, Undabarrena A, Gallardo N, Keller N, Moraga M, Roman JC, Mora GC, Garcia P. Porin alterations present in non-carbapenemase-producing Enterobacteriaceae with high and intermediate levels of carbapenem resistance in Chile. J Med Microbiol. 2012;61(Pt 9):1270-9. doi: 10.1099/jmm.0.045799-0. PubMed PMID: 22700549.

45. Lavigne JP, Sotto A, Nicolas-Chanoine MH, Bouziges N, Pages JM, Davin-Regli A. An adaptive response of Enterobacter aerogenes to imipenem: regulation of porin balance in clinical isolates. Int J Antimicrob Agents. 2013;41(2):130-6. doi: 10.1016/j.ijantimicag.2012.10.010. PubMed PMID: 23280442.

46. Tangden T, Adler M, Cars O, Sandegren L, Lowdin E. Frequent emergence of porin-deficient subpopulations with reduced carbapenem susceptibility in ESBL-producing Escherichia coli during exposure to ertapenem in an in vitro pharmacokinetic model. J Antimicrob Chemother. 2013;68(6):1319-26. doi: 10.1093/jac/dkt044. PubMed PMID: 23478794.

47. Meka VG, Gold HS. Antimicrobial resistance to linezolid. Clin Infect Dis. 2004;39(7):1010-5. doi: 10.1086/423841. PubMed PMID: 15472854.

48. Wolter N, Smith AM, Farrell DJ, Schaffner W, Moore M, Whitney CG, Jorgensen JH, Klugman KP. Novel mechanism of resistance to oxazolidinones, macrolides, and chloramphenicol in ribosomal protein L4 of the pneumococcus. Antimicrob Agents Chemother. 2005;49(8):3554-7. doi: 10.1128/AAC.49.8.3554-3557.2005. PubMed PMID: 16048983; PMCID: PMC1196237.

49. Hakenbeck R, Bruckner R, Denapaite D, Maurer P. Molecular mechanisms of beta-lactam resistance in Streptococcus pneumoniae. Future Microbiol. 2012;7(3):395-410. doi: 10.2217/fmb.12.2. PubMed PMID: 22393892.

50. Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 2000;44(6):1549-55. PubMed PMID: 10817707; PMCID: PMC89911.

51. Poirel L, Ros A, Carricajo A, Berthelot P, Pozzetto B, Bernabeu S, Nordmann P. Extremely drug-resistant Citrobacter freundii isolate producing NDM-1 and other carbapenemases identified in a patient returning from India. Antimicrob Agents Chemother. 2011;55(1):447-8. doi: 10.1128/AAC.01305-10. PubMed PMID: 20974865; PMCID: PMC3019622.

52. Dolejska M, Villa L, Poirel L, Nordmann P, Carattoli A. Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. J Antimicrob Chemother. 2013;68(1):34-9. doi: 10.1093/jac/dks357. PubMed PMID: 22969080.

53. Long KS, Porse BT. A conserved chloramphenicol binding site at the entrance to the ribosomal peptide exit tunnel. Nucleic Acids Res. 2003;31(24):7208-15. PubMed PMID: 14654696; PMCID: PMC291879.

54. Shen J, Xia X, Jiang H, Li C, Li J, Li X, Ding S. Determination of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in poultry and porcine muscle and liver by gas chromatography-negative chemical ionization mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877(14-15):1523-9. doi: 10.1016/j.jchromb.2009.03.040. PubMed PMID: 19395324.

55. Zhang WJ, Xu XR, Schwarz S, Wang XM, Dai L, Zheng HJ, Liu S. Characterization of the IncA/C plasmid pSCEC2 from Escherichia coli of swine origin that harbours the multiresistance gene cfr. J Antimicrob Chemother. 2014;69(2):385-9. doi: 10.1093/jac/dkt355. PubMed PMID: 24013193; PMCID: PMC3937595.

56. Vetting MW, Hegde SS, Zhang Y, Blanchard JS. Pentapeptide-repeat proteins that act as topoisomerase poison resistance factors have a common dimer interface. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2011;67(Pt 3):296-302. doi: 10.1107/S1744309110053315. PubMed PMID: 21393830; PMCID: PMC3053150.

57. Llano-Sotelo B, Chow CS. RNA-aminoglycoside antibiotic interactions: fluorescence detection of binding and conformational change. Bioorg Med Chem Lett. 1999;9(2):213-6. PubMed PMID: 10021931.

58. Nakamura A, Miyakozawa I, Nakazawa K, K OH, Sawai T. Detection and characterization of a macrolide 2'-phosphotransferase from a Pseudomonas aeruginosa clinical isolate. Antimicrob Agents Chemother. 2000;44(11):3241-2. PubMed PMID: 11184231; PMCID: PMC101646.

59. Nakamura A, Naito Y, Nakazawa K, Ohara K, Sawai T. [Selective suppression of aminoglycoside 3'-phosphorylating enzyme activities by macrolides--a study by using macrolide-resistant bacteria]. Jpn J Antibiot. 2000;53 Suppl A:28-31. PubMed PMID: 10756446.

60. Nakamura A, Nakazawa K, Miyakozawa I, Mizukoshi S, Tsurubuchi K, Nakagawa M, O'Hara K, Sawai T. Macrolide esterase-producing Escherichia coli clinically isolated in Japan. J Antibiot (Tokyo). 2000;53(5):516-24. PubMed PMID: 10908116.

61. Llaneza J, Villar CJ, Salas JA, Suarez JE, Mendoza MC, Hardisson C. Plasmid-mediated fosfomycin resistance is due to enzymatic modification of the antibiotic. Antimicrob Agents Chemother. 1985;28(1):163-4. PubMed PMID: 3899003; PMCID: PMC176334.

62. Flensburg J, Skold O. Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim. Eur J Biochem. 1987;162(3):473-6. PubMed PMID: 3549289.

63. Yuan M, Hall LM, Hoogkamp-Korstanje J, Livermore DM. SHV-14, a novel beta-lactamase variant in Klebsiella pneumoniae isolates from Nijmegen, The Netherlands. Antimicrob Agents Chemother. 2001;45(1):309-11. doi: 10.1128/AAC.45.1.309-311.2001. PubMed PMID: 11120985; PMCID: PMC90280.

64. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015;13(1):42-51. doi: 10.1038/nrmicro3380. PubMed PMID: 25435309.

65. Buchanan RE. Studies in the Nomenclature and Classification of Bacteria: The Problem of Bacterial Nomenclature. J Bacteriol. 1916;1(6):591-6. PubMed PMID: 16558720; PMCID: PMC378679.

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

67. Adnani N, Vazquez-Rivera E, Adibhatla SN, Ellis GA, Braun DR, Bugni TS. Investigation of Interspecies Interactions within Marine Micromonosporaceae Using an Improved Co-Culture Approach. Mar Drugs. 2015;13(10):6082-98. doi: 10.3390/md13106082. PubMed PMID: 26404321; PMCID: PMC4626680.

68. Goodfellow M, Williams ST. Ecology of actinomycetes. Annu Rev Microbiol. 1983;37:189-216. doi: 10.1146/annurev.mi.37.100183.001201. PubMed PMID: 6357051.

69. Cross T, Goodfellow M. Taxonomy and classification of the actinomycetes. Soc Appl Bacteriol Symp Ser. 1973;2:11-112. PubMed PMID: 4584092.

70. Flardh K. Essential role of DivIVA in polar growth and morphogenesis in Streptomyces coelicolor A3(2). Mol Microbiol. 2003;49(6):1523-36. PubMed PMID: 12950918.

71. Flardh K. Growth polarity and cell division in Streptomyces. Curr Opin Microbiol. 2003;6(6):564-71. PubMed PMID: 14662351.

72. Flardh K, Buttner MJ. Streptomyces morphogenetics: dissecting differentiation in a filamentous bacterium. Nat Rev Microbiol. 2009;7(1):36-49. doi: 10.1038/nrmicro1968. PubMed PMID: 19079351.

73. Sigle S, Ladwig N, Wohlleben W, Muth G. Synthesis of the spore envelope in the developmental life cycle of Streptomyces coelicolor. Int J Med Microbiol. 2015;305(2):183-9. doi: 10.1016/j.ijmm.2014.12.014. PubMed PMID: 25595023.

74. Raoult D, Ogata H, Audic S, Robert C, Suhre K, Drancourt M, Claverie JM. Tropheryma whipplei Twist: a human pathogenic Actinobacteria with a reduced genome. Genome Res. 2003;13(8):1800-9. doi: 10.1101/gr.1474603. PubMed PMID: 12902375; PMCID: PMC403771.

75. Baranasic D, Gacesa R, Starcevic A, Zucko J, Blazic M, Horvat M, Gjuracic K, Fujs S, Hranueli D, Kosec G, Cullum J, Petkovic H. Draft Genome Sequence of Streptomyces rapamycinicus Strain NRRL 5491, the Producer of the Immunosuppressant Rapamycin. Genome Announc. 2013;1(4). doi: 10.1128/genomeA.00581-13. PubMed PMID: 23929477; PMCID: PMC3738893.

76. Berdy J. Thoughts and facts about antibiotics: Where we are now and where we are heading. J Antibiot (Tokyo). 2012;65(8):441. doi: 10.1038/ja.2012.54. PubMed PMID: 22922479.

77. Challis GL, Hopwood DA. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by Streptomyces species. Proc Natl Acad Sci U S A. 2003;100 Suppl 2:14555-61. doi: 10.1073/pnas.1934677100. PubMed PMID: 12970466; PMCID: PMC304118.

78. Bentley SD, Chater KF, Cerdeno-Tarraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). Nature. 2002;417(6885):141-7. doi: 10.1038/417141a. PubMed PMID: 12000953.

79. Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat Prod Rep. 2009;26(11):1362-84. doi: 10.1039/b817069j. PubMed PMID: 19844637; PMCID: PMC3063060.

80. Challis GL. Exploitation of the Streptomyces coelicolor A3(2) genome sequence for discovery of new natural products and biosynthetic pathways. J Ind Microbiol Biotechnol. 2014;41(2):219-32. doi: 10.1007/s10295-013-1383-2. PubMed PMID: 24322202.

81. Abdelmohsen UR, Grkovic T, Balasubramanian S, Kamel MS, Quinn RJ, Hentschel U. Elicitation of secondary metabolism in actinomycetes. Biotechnol Adv. 2015;33(6 Pt 1):798-811. doi: 10.1016/j.biotechadv.2015.06.003. PubMed PMID: 26087412.

82. Rigali S, Titgemeyer F, Barends S, Mulder S, Thomae AW, Hopwood DA, van Wezel GP. Feast or famine: the global regulator DasR links nutrient stress to antibiotic production by Streptomyces. EMBO Rep. 2008;9(7):670-5. doi: 10.1038/embor.2008.83. PubMed PMID: 18511939; PMCID: PMC2475330.

83. Chen G, Wang GY, Li X, Waters B, Davies J. Enhanced production of microbial metabolites in the presence of dimethyl sulfoxide. J Antibiot (Tokyo). 2000;53(10):1145-53. PubMed PMID: 11132960.

84. Norn S, Permin H, Kruse PR, Kruse E. [From willow bark to acetylsalicylic acid]. Dan Medicinhist Arbog. 2009;37:79-98. PubMed PMID: 20509453.

85. Gonzalez J. Medicinal plants in Colombia. J Ethnopharmacol. 1980;2(1):43-7. PubMed PMID: 7464182.

86. Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. 1929. Bull World Health Organ. 2001;79(8):780-90. PubMed PMID: 11545337; PMCID: PMC2566493.

87. Jones D, Metzger HJ, Schatz A, Waksman SA. Control of Gram-Negative Bacteria in Experimental Animals by Streptomycin. Science. 1944;100(2588):103-5. doi: 10.1126/science.100.2588.103. PubMed PMID: 17788929.

88. Waksman SA, Woodruff HB. The Soil as a Source of Microorganisms Antagonistic to Disease-Producing Bacteria. J Bacteriol. 1940;40(4):581-600. PubMed PMID: 16560371; PMCID: PMC374661.

89. Waksman SA. Do Fungi Live and Produce Mycelium in the Soil? Science. 1916;44(1131):320-2. doi: 10.1126/science.44.1131.320. PubMed PMID: 17840079.

90. Butler MS. The role of natural product chemistry in drug discovery. J Nat Prod. 2004;67(12):2141-53. doi: 10.1021/np040106y. PubMed PMID: 15620274.

91. Berdy J. Bioactive microbial metabolites. J Antibiot (Tokyo). 2005;58(1):1-26. doi: 10.1038/ja.2005.1. PubMed PMID: 15813176.

92. DiMasi JA, Grabowski HG, Hansen RW. Innovation in the pharmaceutical industry: New estimates of R&D costs. J Health Econ. 2016;47:20-33. doi: 10.1016/j.jhealeco.2016.01.012. PubMed PMID: 26928437.

93. Bindseil KU, Jakupovic J, Wolf D, Lavayre J, Leboul J, van der Pyl D. Pure compound libraries; a new perspective for natural product based drug discovery. Drug Discov Today. 2001;6(16):840-7. PubMed PMID: 11495757.

94. Wilson DN. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. Nat Rev Microbiol. 2014;12(1):35-48. doi: 10.1038/nrmicro3155. PubMed PMID: 24336183.

95. Marinus MG. DNA methylation in Escherichia coli. Annu Rev Genet. 1987;21:113-31. doi: 10.1146/annurev.ge.21.120187.000553. PubMed PMID: 3327459.

96. Heitman J, Model P. Site-specific methylases induce the SOS DNA repair response in Escherichia coli. J Bacteriol. 1987;169(7):3243-50. PubMed PMID: 3036779; PMCID: PMC212376.

97. Cheng X. DNA modification by methyltransferases. Curr Opin Struct Biol. 1995;5(1):4-10. PubMed PMID: 7773746.

98. Stresemann C, Lyko F. Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. Int J Cancer. 2008;123(1):8-13. doi: 10.1002/ijc.23607. PubMed PMID: 18425818.

99. Wang X, Sena Filho JG, Hoover AR, King JB, Ellis TK, Powell DR, Cichewicz RH. Chemical epigenetics alters the secondary metabolite composition of guttate excreted by an atlantic-forest-soil-derived Penicillium citreonigrum. J Nat Prod. 2010;73(5):942-8. doi: 10.1021/np100142h. PubMed PMID: 20450206; PMCID: PMC2878378.

100. Beau J, Mahid N, Burda WN, Harrington L, Shaw LN, Mutka T, Kyle DE, Barisic B, van Olphen A, Baker BJ. Epigenetic tailoring for the production of anti-infective cytosporones from the marine fungus Leucostoma persoonii. Mar Drugs. 2012;10(4):762-74. doi: 10.3390/md10040762. PubMed PMID: 22690142; PMCID: PMC3366674.

101. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004;429(6990):457-63. doi: 10.1038/nature02625. PubMed PMID: 15164071.

102. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. Nat Rev Genet. 2008;9(6):465-76. doi: 10.1038/nrg2341. PubMed PMID: 18463664.

103. VanderMolen KM, Darveaux BA, Chen WL, Swanson SM, Pearce CJ, Oberlies NH. Epigenetic Manipulation of a Filamentous Fungus by the Proteasome-Inhibitor Bortezomib Induces the Production of an Additional Secondary Metabolite. RSC Adv. 2014;4(35):18329-35. doi: 10.1039/C4RA00274A. PubMed PMID: 24955237; PMCID: PMC4061710.

104. Yang XL, Huang L, Ruan XL. Epigenetic modifiers alter the secondary metabolite composition of a plant endophytic fungus, Pestalotiopsis crassiuscula obtained from the leaves of Fragaria chiloensis. J Asian Nat Prod Res. 2014;16(4):412-7. doi: 10.1080/10286020.2014.881356. PubMed PMID: 24498889.

105. Zutz C, Bandian D, Neumayer B, Speringer F, Gorfer M, Wagner M, Strauss J, Rychli K. Fungi treated with small chemicals exhibit increased antimicrobial activity against facultative bacterial and yeast pathogens. Biomed Res Int. 2014;2014:540292. doi: 10.1155/2014/540292. PubMed PMID: 25121102; PMCID: PMC4119895.

106. Henrikson JC, Hoover AR, Joyner PM, Cichewicz RH. A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from Aspergillus niger. Org Biomol Chem. 2009;7(3):435-8. doi: 10.1039/b819208a. PubMed PMID: 19156306.

107. Moore JM, Bradshaw E, Seipke RF, Hutchings MI, McArthur M. Use and discovery of chemical elicitors that stimulate biosynthetic gene clusters in Streptomyces bacteria. Methods Enzymol. 2012;517:367-85. doi: 10.1016/B978-0-12-404634-4.00018-8. PubMed PMID: 23084948.

108. Kumar J, Sharma VK, Singh DK, Mishra A, Gond SK, Verma SK, Kumar A, Kharwar RN. Epigenetic Activation of Antibacterial Property of an Endophytic Streptomyces coelicolor Strain AZRA 37 and Identification of the Induced Protein Using MALDI TOF MS/MS. PLoS One. 2016;11(2):e0147876. doi: 10.1371/journal.pone.0147876. PubMed PMID: 26844762; PMCID: PMC4742224.

109. Wang B, Waters AL, Sims JW, Fullmer A, Ellison S, Hamann MT. Complex marine natural products as potential epigenetic and production regulators of antibiotics from a marine Pseudomonas aeruginosa. Microb Ecol. 2013;65(4):1068-75. doi: 10.1007/s00248-013-0213-4. PubMed PMID: 23563743; PMCID: PMC3650628.

110. Tan C, Tasaka H, Yu KP, Murphy ML, Karnofsky DA. Daunomycin, an antitumor antibiotic, in the treatment of neoplastic disease. Clinical evaluation with special reference to childhood leukemia. Cancer. 1967;20(3):333-53. PubMed PMID: 4290058.

111. Weiss RB, Bruno S. Daunorubicin treatment of adult solid tumors. Cancer Treat Rep. 1981;65 Suppl 4:25-8. PubMed PMID: 7049378.

112. Di Marco A, Silvestrini R, Di Marco S, Dasdia T. Inhibiting effect of the new cytotoxic antibiotic daunomycin on nucleic acids and mitotic activity of HeLa cells. J Cell Biol. 1965;27(3):545-50. PubMed PMID: 5885429; PMCID: PMC2106764.

113. Weiss RB, Sarosy G, Clagett-Carr K, Russo M, Leyland-Jones B. Anthracycline analogs: the past, present, and future. Cancer Chemother Pharmacol. 1986;18(3):185-97. PubMed PMID: 2948729.

114. Pommier Y, Leo E, Zhang H, Marchand C. DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem Biol. 2010;17(5):421-33. doi: 10.1016/j.chembiol.2010.04.012. PubMed PMID: 20534341.

115. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev. 2004;56(2):185-229. doi: 10.1124/pr.56.2.6. PubMed PMID: 15169927.

116. Westman EL, Canova MJ, Radhi IJ, Koteva K, Kireeva I, Waglechner N, Wright GD. Bacterial inactivation of the anticancer drug doxorubicin. Chem Biol. 2012;19(10):1255-64. doi: 10.1016/j.chembiol.2012.08.011. PubMed PMID: 23102220.

117. Kaur P. Expression and characterization of DrrA and DrrB proteins of Streptomyces peucetius in Escherichia coli: DrrA is an ATP binding protein. J Bacteriol. 1997;179(3):569-75. PubMed PMID: 9006006; PMCID: PMC178733.

118. El-Nakeeb MA, Lechevalier HA. Selective isolation of aerobic Actinomycetes. Appl Microbiol. 1963;11:75-7. PubMed PMID: 13937509; PMCID: PMC1057945.

119. Kuester E, Williams ST. Selection of Media for Isolation of Streptomycetes. Nature. 1964;202:928-9. PubMed PMID: 14190108.

120. Pridham TG, Anderson P, Foley C, Lindenfelser LA, Hesseltine CW, Benedict RG. A selection of media for maintenance and taxonomic study of Streptomyces. Antibiot Annu. 1956:947-53. PubMed PMID: 13425486.

121. Jensen PR, Gontang E, Mafnas C, Mincer TJ, Fenical W. Culturable marine actinomycete diversity from tropical Pacific Ocean sediments. Environ Microbiol. 2005;7(7):1039-48. doi: 10.1111/j.1462-2920.2005.00785.x. PubMed PMID: 15946301.

122. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. 2002;148(Pt 1):257-66. doi: 10.1099/00221287-148-1-257. PubMed PMID: 11782518.

123. Claus H, Decker H. Bacterial tyrosinases. Syst Appl Microbiol. 2006;29(1):3-14. doi: 10.1016/j.syapm.2005.07.012. PubMed PMID: 16423650.

124. Plonka PM, Grabacka M. Melanin synthesis in microorganisms--biotechnological and medical aspects. Acta Biochim Pol. 2006;53(3):429-43. PubMed PMID: 16951740.

125. Linos A, Berekaa MM, Reichelt R, Keller U, Schmitt J, Flemming HC, Kroppenstedt RM, Steinbuchel A. Biodegradation of cis-1,4-polyisoprene rubbers by distinct actinomycetes: microbial strategies and detailed surface analysis. Appl Environ Microbiol. 2000;66(4):1639-45. PubMed PMID: 10742254; PMCID: PMC92035.

126. Linos A, Steinbuchel A, Sproer C, Kroppenstedt RM. Gordonia polyisoprenivorans sp. nov., a rubber-degrading actinomycete isolated from an automobile tyre. Int J Syst Bacteriol. 1999;49 Pt 4:1785-91. doi: 10.1099/00207713-49-4-1785. PubMed PMID: 10555361.

127. Arenskotter M, Broker D, Steinbuchel A. Biology of the metabolically diverse genus Gordonia. Appl Environ Microbiol. 2004;70(6):3195-204. doi: 10.1128/AEM.70.6.3195-3204.2004. PubMed PMID: 15184112; PMCID: PMC427784.

128. Feng H, Sun Y, Zhi Y, Mao L, Luo Y, Wei X, Zhou P. Lignocellulose degradation by the isolate of Streptomyces griseorubens JSD-1. Funct Integr Genomics. 2015;15(2):163-73. doi: 10.1007/s10142-014-0425-9. PubMed PMID: 25452226.

129. Waksman SA, Lechevalier HA. Neomycin, a New Antibiotic Active against Streptomycin-Resistant Bacteria, including Tuberculosis Organisms. Science. 1949;109(2830):305-7. doi: 10.1126/science.109.2830.305. PubMed PMID: 17782716.

130. Drautz H, Zahner H, Rohr J, Zeeck A. Metabolic products of microorganisms. 234. Urdamycins, new angucycline antibiotics from Streptomyces fradiae. I. Isolation, characterization and biological properties. J Antibiot (Tokyo). 1986;39(12):1657-69. PubMed PMID: 3818439.

131. Stark WM, Daily WA, Mc GJ. A fermentation study of the biosynthesis of tylosin in synthetic media. Sci Rep Ist Super Sanita. 1961;1:340-54. PubMed PMID: 13916323.

132. Domingue G, Costerton JW, Brown MR. Bacterial doubling time modulates the effects of opsonisation and available iron upon interactions between Staphylococcus aureus and human neutrophils. FEMS Immunol Med Microbiol. 1996;16(3-4):223-8. PubMed PMID: 9116639.

133. Cortes J, Liras P, Castro JM, Martin JF. Glucose regulation of cephamycin biosynthesis in Streptomyces lactamdurans is exerted on the formation of alpha-aminoadipyl-cysteinyl-valine and deacetoxycephalosporin C synthase. J Gen Microbiol. 1986;132(7):1805-14. doi: 10.1099/00221287-132-7-1805. PubMed PMID: 3794640.

134. Chi WJ, Lee SY, Lee J. Functional analysis of SGR4635-induced enhancement of pigmented antibiotic production in Streptomyces lividans. J Microbiol. 2011;49(5):828-33. doi: 10.1007/s12275-011-1100-7. PubMed PMID: 22068502.

135. Barona-Gomez F, Lautru S, Francou FX, Leblond P, Pernodet JL, Challis GL. Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in Streptomyces coelicolor A3(2) and Streptomyces ambofaciens ATCC 23877. Microbiology. 2006;152(Pt 11):3355-66. doi: 10.1099/mic.0.29161-0. PubMed PMID: 17074905.

136. Funa N, Funabashi M, Ohnishi Y, Horinouchi S. Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in Streptomyces griseus. J Bacteriol. 2005;187(23):8149-55. doi: 10.1128/JB.187.23.8149-8155.2005. PubMed PMID: 16291687; PMCID: PMC1291289.

137. Schnellmann J, Zeltins A, Blaak H, Schrempf H. The novel lectin-like protein CHB1 is encoded by a chitin-inducible Streptomyces olivaceoviridis gene and binds specifically to crystalline alpha-chitin of fungi and other organisms. Mol Microbiol. 1994;13(5):807-19. PubMed PMID: 7815940.

138. Sen A, Daubin V, Abrouk D, Gifford I, Berry AM, Normand P. Phylogeny of the class Actinobacteria revisited in the light of complete genomes. The orders 'Frankiales' and Micrococcales should be split into coherent entities: proposal of Frankiales ord. nov., Geodermatophilales ord. nov., Acidothermales ord. nov. and Nakamurellales ord. nov. Int J Syst Evol Microbiol. 2014;64(Pt 11):3821-32. doi: 10.1099/ijs.0.063966-0. PubMed PMID: 25168610.

139. Wu D, Hugenholtz P, Mavromatis K, Pukall R, Dalin E, Ivanova NN, Kunin V, Goodwin L, Wu M, Tindall BJ, Hooper SD, Pati A, Lykidis A, Spring S, Anderson IJ, D'Haeseleer P, Zemla A, Singer M, Lapidus A, Nolan M, Copeland A, Han C, Chen F, Cheng JF, Lucas S, Kerfeld C, Lang E, Gronow S, Chain P, Bruce D, Rubin EM, Kyrpides NC, Klenk HP, Eisen JA. A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. Nature. 2009;462(7276):1056-60. doi: 10.1038/nature08656. PubMed PMID: 20033048; PMCID: PMC3073058.

140. Coico R. Gram staining. Curr Protoc Microbiol. 2005;Appendix 3:Appendix 3C. doi: 10.1002/9780471729259.mca03cs00. PubMed PMID: 18770544.

141. Beveridge TJ. Mechanism of gram variability in select bacteria. J Bacteriol. 1990;172(3):1609-20. PubMed PMID: 1689718; PMCID: PMC208639.

142. Tsukamura M, Mizuno S, Tsukamura S. Numerical classification of slowly growing mycobacteria. Am Rev Respir Dis. 1969;99(2):299-303. doi: 10.1164/arrd.1969.99.2.299. PubMed PMID: 4975013.

143. Woese CR, Fox GE. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci U S A. 1977;74(11):5088-90. PubMed PMID: 270744; PMCID: PMC432104.

144. Yap WH, Zhang Z, Wang Y. Distinct types of rRNA operons exist in the genome of the actinomycete Thermomonospora chromogena and evidence for horizontal transfer of an entire rRNA operon. J Bacteriol. 1999;181(17):5201-9. PubMed PMID: 10464188; PMCID: PMC94023.

145. Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AK, Kent AD, Daroub SH, Camargo FA, Farmerie WG, Triplett EW. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J. 2007;1(4):283-90. doi: 10.1038/ismej.2007.53. PubMed PMID: 18043639; PMCID: PMC2970868.

146. Torsvik V, Goksoyr J, Daae FL. High diversity in DNA of soil bacteria. Appl Environ Microbiol. 1990;56(3):782-7. PubMed PMID: 2317046; PMCID: PMC183421.

147. Maldonado LA, Fragoso-Yanez D, Perez-Garcia A, Rosellon-Druker J, Quintana ET. Actinobacterial diversity from marine sediments collected in Mexico. Antonie Van Leeuwenhoek. 2009;95(2):111-20. doi: 10.1007/s10482-008-9294-3. PubMed PMID: 19023674.

148. Ikeda H. Natural products discovery from micro-organisms in the post-genome era. Biosci Biotechnol Biochem. 2017;81(1):13-22. doi: 10.1080/09168451.2016.1248366. PubMed PMID: 27885932.

149. Gerea AL, Branscum KM, King JB, You J, Powell DR, Miller AN, Spear JR, Cichewicz RH. Secondary metabolites produced by fungi derived from a microbial mat encountered in an iron-rich natural spring. Tetrahedron Lett. 2012;53(32):4202-5. doi: 10.1016/j.tetlet.2012.05.156. PubMed PMID: 22844162; PMCID: PMC3404852.

150. Schmidt EW, Donia MS, McIntosh JA, Fricke WF, Ravel J. Origin and variation of tunicate secondary metabolites. J Nat Prod. 2012;75(2):295-304. doi: 10.1021/np200665k. PubMed PMID: 22233390; PMCID: PMC3288725.

151. Piel J. Biosynthesis of polyketides by trans-AT polyketide synthases. Nat Prod Rep. 2010;27(7):996-1047. doi: 10.1039/b816430b. PubMed PMID: 20464003.

152. Sarkar A, Funk AN, Scherlach K, Horn F, Schroeckh V, Chankhamjon P, Westermann M, Roth M, Brakhage AA, Hertweck C, Horn U. Differential expression of silent polyketide biosynthesis gene clusters in chemostat cultures of Aspergillus nidulans. J Biotechnol. 2012;160(1-2):64-71. doi: 10.1016/j.jbiotec.2012.01.015. PubMed PMID: 22306112.

153. Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, Kanigan T, Lewis K, Epstein SS. Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. Appl Environ Microbiol. 2010;76(8):2445-50. doi: 10.1128/AEM.01754-09. PubMed PMID: 20173072; PMCID: PMC2849220.

154. Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schaberle TF, Hughes DE, Epstein S, Jones M, Lazarides L, Steadman VA, Cohen DR, Felix CR, Fetterman KA, Millett WP, Nitti AG, Zullo AM, Chen C, Lewis K. A new antibiotic kills pathogens without detectable resistance. Nature. 2015;517(7535):455-9. doi: 10.1038/nature14098. PubMed PMID: 25561178.

155. Hou Y, Tianero MD, Kwan JC, Wyche TP, Michel CR, Ellis GA, Vazquez-Rivera E, Braun DR, Rose WE, Schmidt EW, Bugni TS. Structure and biosynthesis of the antibiotic bottromycin D. Org Lett. 2012;14(19):5050-3. doi: 10.1021/ol3022758. PubMed PMID: 22984777; PMCID: PMC3466018.

156. Dai Z, Zhang X, Tang C, Muhammad N, Wu J, Brookes PC, Xu J. Potential role of biochars in decreasing soil acidification - A critical review. Sci Total Environ. 2017;581-582:601-11. doi: 10.1016/j.scitotenv.2016.12.169. PubMed PMID: 28063658.

157. Weaver RW, Soil Science Society of America. Methods of soil analysis. Part 2, Microbiological and biochemical properties. Madison, Wis., USA: Soil Science Society of America; 1994. xxvii, 1121 p. p.

158. Silver LL. Challenges of antibacterial discovery. Clin Microbiol Rev. 2011;24(1):71-109. doi: 10.1128/CMR.00030-10. PubMed PMID: 21233508; PMCID: PMC3021209.

159. Kieser T, Hopwood DA. Genetic manipulation of Streptomyces: integrating vectors and gene replacement. Methods Enzymol. 1991;204:430-58. PubMed PMID: 1943784.

160. Konig CC, Scherlach K, Schroeckh V, Horn F, Nietzsche S, Brakhage AA, Hertweck C. Bacterium induces cryptic meroterpenoid pathway in the pathogenic fungus Aspergillus fumigatus. Chembiochem. 2013;14(8):938-42. doi: 10.1002/cbic.201300070. PubMed PMID: 23649940.

161. Fernandez M, Soliveri J, Novella IS, Yebra MJ, Barbes C, Sanchez J. Effect of 5-azacytidine and sinefungin on Streptomyces development. Gene. 1995;157(1-2):221-3. PubMed PMID: 7541762.

162. Novella IS, Sanchez J. Effects of 5-azacytidine on physiological differentiation of Streptomyces antibioticus. Res Microbiol. 1995;146(9):721-8. PubMed PMID: 8584794.

Appendix

Table A - 1. Identified Isolates in *Actinobacteria* Strain Collection

Strain Collection ID	Genus	Species	Media	Method	Sample
1	*Rhodococcus*	*Rhodochrous*	SCA	Method 3	Soil 2
5	*Rhodococcus*	*Rhodochrous*	SCA	Method 1	Soil 2
6	*Streptomyces*	*Violarus*	SCA	Method 3	Soil 1
8	*Streptomyces*	*Beijiangensis*	AGS	Method 1	Soil 2
10	*Streptomyces*	*Coelicoflavus*	SCA	Method 1	Soil 2
11	*Gordonia*	*Namibiensis*	SCA	Method 1	Soil 2
12	*Rhodococcus*	*Rhodochrous*	SCA	Method 1	Soil 2
13	*Nocardia*	*Vermiculata*	SCA	Method 1	Soil 2
14	*Streptomyces*	*Platensis*	SCA	Method 1	Soil 1
15	*Streptomyces*	*Violarus*	SCA	Method 1	Soil 1
16	*Streptomyces*	*Albiflavescens*	SCA	Method 1	Soil 2
17	*Streptomyces*	*Corchorusii*	SCA	Method 1	Soil 2
18	*Arthrobacter*	*Protophormiae*	SYE	Method 1	Soil 2
19	*Gordonia*	*Namibiensis*	SCA	Method 3	Soil 1
20	*Aeromicrobium*	*Tamlense*	SCA	Method 2	Soil 2
21	*Rhodococcus*	*Phenolicus*	SCA	Method 2	Soil 2
22	*Gordonia*	*Terrae*	SCA	Method 3	Soil 1
25	*Rhodococcus*	*Rhodochrous*	SCA	Method 2	Soil 2
26	*Streptomyces*	*Chromofuscus*	SCA	Method 1	Soil 2
27	*Streptomyces*	*Acrimycini*	SCA	Method 1	Soil 2
29	*Mycobacterium*	*Parafortuitum*	SCA	Method 3	Soil 2
30	*Streptomyces*	*Macrosporeus*	SCA	Method 3	Soil 2

Table A - 1. (Continued)

31	*Streptomyces*	*Antibioticus*	SYE	Method 3	Soil 1
32	*Streptomyces*	*Gardneri*	SCA	Method 3	Soil 1
33	*Streptomyces*	*Tumescens*	AGS	Method 1	Soil 1
34	*Nocardia*	*Asteroides*	AGS	Method 1	Soil 1
35	*Streptomyces*	*Minoensis*	SYE	Method 1	Soil 1
36	*Streptomyces*	*aurantiogriseus*	SYE	Method 1	Soil 2
37	*Streptomyces*	*Matensis*	SCA	Method 3	Soil 2
39	*Streptomyces*	*griseorubens*	SCA	Method 1	Soil 2
40	*Streptomyces*	*Fradiae*	SCA	Method 1	Soil 2
41	*Streptomyces*	*Violascens*	SYE	Method 3	Soil 2
42	*Streptomyces*	*zaomyceticus*	AGS	Method 3	Soil 2
43	*Streptomyces*	*viridochromogenes*	SCA	Method 3	Soil 2
44	*Streptomyces*	*beijiangensis*	AGS	Method 1	Soil 2
45	*Streptomyces*	*intermedius*	SYE	Method 1	Soil 2
46	*Streptomyces*	*intermedius*	SYE	Method 1	Soil 2
47	*Streptomyces*	*albidoflavus*	SYE	Method 1	Soil 2
48	*Streptomyces*	*Matensis*	SCA	Method 3	Soil 2
49	*Streptomyces*	*griseoplanus*	SYE	Method 3	Soil 2
50	*Streptomyces*	*drozdowiczii*	SYE	Method 1	Soil 2
51	*Streptomyces*	*drozdowiczii*	AGS	Method 1	Soil 2
52	*Streptomyces*	*coelicoflavus*	AGS	Method 2	Soil 2
54	*Streptomyces*	*intermedius*	SYE	Method 2	Soil 2
56	*Streptomyces*	*intermedius*	SCA	Method 1	Soil 2
57	*Streptomyces*	*Sampsonii*	SCA	Method 1	Soil 2
58	*Nocardia*	*Asteroides*	AGS	Method 1	Soil 1
59	*Nocardia*	*Asteroides*	SYE	Method 1	Soil 1
60	*Streptomyces*	*Glebosus*	AGS	Method 1	Soil 1

Table A - 1. (Continued)

61	*Streptomyces*	*hawaiiensis*	SYE	Method 1	Soil 1
62	*Streptomyces*	*Variabilis*	SCA	Method 1	Soil 1
63	*Streptomyces*	*drozdowiczii*	AGS	Method 1	Soil 2
64	*Streptomyces*	*drozdowiczii*	AGS	Method 1	Soil 2
65	*Streptomyces*	*drozdowiczii*	AGS	Method 1	Soil 2
66	*Streptomyces*	*griseoaurantiacus*	AGS	Method 1	Soil 2
67	*Streptomyces*	*intermedius*	AGS	Method 1	Soil 2
68	*Streptomyces*	*thermocarboxydus*	AGS	Method 1	Soil 2
69	*Streptomyces*	*viridobrunneus*	AGS	Method 1	Soil 2
70	*Streptomyces*	*intermedius*	AGS	Method 1	Soil 2
71	*Brevibacterium*	*epidermidis*	AGS	Method 1	Soil 2
72	*Streptomyces*	*intermedius*	AGS	Method 1	Soil 2
73	*Microbacterium*	*ginsengiterrae*	AGS	Method 1	Soil 2
74	*Streptomyces*	*coelicoflavus*	SCA	Method 1	Soil 2
75	*Streptomyces*	*Gardneri*	AGS	Method 3	Soil 1
76	*Streptomyces*	*intermedius*	SCA	Method 4	Soil 2
77	*Streptomyces*	*malachitospinus*	AGS	Method 4	Soil 1
78	*Streptomyces*	*intermedius*	SCA	Method 4	Soil 2
79	*Streptomyces*	*aurantiacus*	SCA	Method 4	Soil 2
80	*Streptomyces*	*malachitospinus*	AGS	Method 4	Soil 1
81	*Streptomyces*	*Tendae*	SYE	Method 1	Soil 2
82	*Streptomyces*	*drozdowiczii*	SYE	Method 1	Soil 2
84	*Streptomyces*	*Tendae*	SYE	Method 1	Soil 2
85	*Streptomyces*	*Tendae*	SYE	Method 1	Soil 2
86	*Streptomyces*	*Gardneri*	AGS	Method 3	Soil 1
87	*Streptomyces*	*Praecox*	SYE	Method 3	Soil 2
88	*Streptomyces*	*turgidiscabies*	AGS	Method 3	Soil 1

Table A - 1. (Continued)

89	*Streptomyces*	*drozdowiczii*	AGS	Method 3	Soil 2
90	*Streptomyces*	*drozdowiczii*	AGS	Method 3	Soil 2
91	*Streptomyces*	*zaomyceticus*	AGS	Method 3	Soil 2
92	*Gordonia*	*namibiensis*	SCA	Method 3	Soil 1
93	*Rhodococcus*	*Opacus*	SCA	Method 3	Soil 1
94	*Streptomyces*	*glebosus*	SYE	Method 3	Soil 1
95	*Micrococcus*	*yunnanensis*	SCA	Method 1	Soil 2
96	*Streptomyces*	*griseorubens*	SCA	Method 1	Soil 2
97	*Streptomyces*	*Lividans*	SCA	Method 1	Soil 2
98	*Nocardia*	*caverna*	SCA	Method 1	Soil 2
99	*Streptomyces*	*violascens*	SCA	Method 1	Soil 2
100	*Streptomyces*	*drozdowiczii*	SCA	Method 1	Soil 2
101	*Streptomyces*	*Griseus*	SCA	Method 1	Soil 2
104	*Streptomyces*	*coelicoflavus*	SCA	Method 1	Soil 2
105	*Streptomyces*	*malachitospinus*	SCA	Method 1	Soil 2
106	*Streptomyces*	*Libani*	AGS	Method 1	Soil 1
107	*Nocardia*	*asteroides*	AGS	Method 3	Soil 1
108	*Streptomyces*	*spongiae*	AGS	Method 1	Soil 1
109	*Streptomyces*	*parvulus*	SCA	Method 3	TSF 3
110	*Streptomyces*	*olivoverticillatus*	AGS	Method 1	Soil 1
111	*Streptomyces*	*chartreusis*	AGS	Method 1	Soil 1
113	*Streptomyces*	*hawaiiensis*	AGS	Method 1	Soil 1
114	*Streptomyces*	*cinereospinus*	AGS	Method 1	Soil 1
115	*Streptomyces*	*intermedius*	AGS	Method 1	Soil 1
116	*Streptomyces*	*chartreusis*	SYE	Method 1	TSF 1
117	*Streptomyces*	*chartreusis*	SCA	Method 1	TSF 1
118	*Streptomyces*	*griseocarneus*	SYE	Method 3	TSF 1

Table A - 1. (Continued)

121	*Streptomyces*	*cyanoalbus*	SYE	Method 1	TSF 1
122	*Rhodococcus*	*erythropolis*	SCA	Method 1	TSF 1
123	*Streptomyces*	*Atratus*	SYE	Method 3	TSF 1
124	*Streptomyces*	*chartreusis*	SYE	Method 3	TSF 1
125	*Streptomyces*	*cinereorectus*	SCA	Method 1	TSF 1
126	*Streptomyces*	*diastatochromogenes*	SCA	Method 3	TSF 1
127	*Streptomyces*	*chartreusis*	SCA	Method 3	TSF 1
128	*Streptomyces*	*drozdowiczii*	SYE	Method 1	TSF 1
129	*Streptomyces*	*platensis*	SYE	Method 1	TSF 1
130	*Rhodococcus*	*opacus*	SCA	Method 3	TSF 1
131	*Rhodococcus*	*opacus*	SYE	Method 3	TSF 1
132	*Streptomyces*	*rishiriensis*	SCA	Method 1	TSF 1
133	*Streptomyces*	*drozdowiczii*	SCA	Method 1	TSF 1
134	*Rhodococcus*	*opacus*	SCA	Method 1	TSF 1
135	*Streptomyces*	*angustmyceticus*	SCA	Method 1	TSF 1
136	*Streptomyces*	*drozdowiczii*	SCA	Method 3	TSF 1
137	*Streptomyces*	*purpurescens*	SCA	Method 1	TSF 1
138	*Streptomyces*	*exfoliatus*	SCA	Method 1	TSF 1
139	*Streptomyces*	*septatus*	SYE	Method 3	TSF 1
140	*Streptomyces*	*Rochei*	SCA	Method 1	TSF 1
141	*Streptomyces*	*angustmyceticus*	SCA	Method 3	TSF 1
142	*Streptomyces*	*olivoverticillatus*	SCA	Method 3	TSF 1
143	*Streptomyces*	*hirsutus*	SCA	Method 1	TSF 1
144	*Streptomyces*	*angustmyceticus*	SCA	Method 3	TSF 1
145	*Streptomyces*	*olivochromogenes*	SCA	Method 3	TSF 1
146	*Agromyces*	*Lapidis*	SCA	Method 1	TSF 1
147	*Rhodococcus*	*opacus*	SCA	Method 3	TSF 1

Table A - 1. (Continued)

149	*Arthrobacter*	*kerguelensis*	SYE	Method 3	TSF 1
150	*Streptomyces*	*exfoliatus*	SCA	Method 3	TSF 1
152	*Streptomyces*	*Tendae*	SYE	Method 1	TSF 1
153	*Streptomyces*	*lavendulae*	SCA	Method 1	TSF 3
154	*Mycobacterium*	*parafortuitum*	SCA	Method 1	TSF 3
155	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
156	*Streptomyces*	*pactum*	SCA	Method 1	TSF 3
157	*Streptomyces*	*olivaceus*	SCA	Method 1	TSF 3
158	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
159	*Streptomyces*	*olivaceus*	SCA	Method 1	TSF 3
160	*Streptomyces*	*praecox*	SCA	Method 1	TSF 3
161	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
162	*Streptomyces*	*griseoaurantiacus*	SCA	Method 1	TSF 3
163	*Streptomyces*	*griseoplanus*	SCA	Method 1	TSF 3
164	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
165	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
166	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
167	*Streptomyces*	*griseoplanus*	SCA	Method 1	TSF 3
168	*Streptomyces*	*viridochromogenes*	SCA	Method 1	TSF 3
169	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
170	*Streptomyces*	*malachitospinus*	SCA	Method 3	TSF 3
171	*Streptomyces*	*bungoensis*	SCA	Method 1	TSF 3
172	*Streptomyces*	*psammoticus*	SCA	Method 1	TSF 2
173	*Streptomyces*	*malachitospinus*	SCA	Method 1	TSF 3
174	*Streptomyces*	*rameus*	SYE	Method 1	TSF 2
175	*Mycobacterium*	*obuense*	SYE	Method 1	TSF 2
177	*Gordonia*	*Terrae*	SCA	Method 1	TSF 2

Table A - 1. (Continued)

178	*Streptomyces*	*rameus*	SCA	Method 1	TSF 2
180	*Streptomyces*	*champavatii*	SYE	Method 1	TSF 3
181	*Streptomyces*	*griseoplanus*	SYE	Method 1	TSF 3
182	*Streptomyces*	*flavogriseus*	SYE	Method 1	TSF 3
183	*Streptomyces*	*parvus*	SYE	Method 1	TSF 3
185	*Streptomyces*	*viridochromogenes*	SCA	Method 1	TSF 3
186	*Streptomyces*	*microflavus*	SCA	Method 1	TSF 3
187	*Streptomyces*	*olivaceus*	SCA	Method 1	TSF 3
188	*Nocardioides*	*alpinus*	SYE	Method 3	TSF 3
189	*Streptomyces*	*galbus*	SCA	Method 1	TSF 2
317	*Streptomyces*	*praecox*	SYE	Method 3	SIS 19
318	*Streptomyces*	*endophyticus*	SCA	Method 3	SIS 19
319	*Streptomyces*	*endophyticus*	SCA	Method 3	SIS 19
320	*Streptomyces*	*narbonensis*	SYE	Method 3	SIS 19
321	*Isoptericola*	*variabilis*	SYE	Method 1	SIS 19
322	*Streptomyces*	*philanthi*	SCA	Method 3	SIS 19
323	*Streptomyces*	*philanthi*	SYE	Method 3	SIS 19
334	*Streptomyces*	*psammoticus*	SCA	Method 3	TSF 7
335	*Streptomyces*	*lydicus*	SCA	Method 3	TSF 7
336	*Streptomyces*	*costaricanus*	SYE	Method 3	TSF 7
337	*Streptomyces*	*shenzhensis*	SYE	Method 3	TSF 7
338	*Streptomyces*	*gardneri*	SYE	Method 3	SIS 19
339	*Streptomyces*	*kanamyceticus*	SYE	Method 1	SIS 19
340	*Streptomyces*	*drozdowiczii*	SCA	Method 3	SIS 19
341	*Streptomyces*	*drozdowiczii*	SCA	Method 3	SIS 19
342	*Streptomyces*	*endophyticus*	SYE	Method 1	SIS 19
343	*Streptomyces*	*shenzhensis*	SYE	Method 1	TSF 7

Table A - 1. (Continued)

344	*Streptomyces*	*lydicus*	SYE	Method 1	TSF 7
345	*Streptomyces*	*lydicus*	SYE	Method 1	TSF 7
346	*Streptomyces*	*ginsengisoli*	SYE	Method 1	TSF 7
347	*Streptomyces*	*prasinopilosus*	SYE	Method 1	TSF 7
348	*Streptomyces*	*indiaensis*	SYE	Method 1	TSF 7
356	*Streptomyces*	*malachitospinus*	SCA	Method 1	CWR 23
357	*Streptomyces*	*violascens*	SCA	Method 1	CWR 31
358	*Streptomyces*	*javensis*	SCA	Method 1	CWR 27
408	*Mycobacterium*	*neoaurum*	SCA	Method 3	TSF 2
409	*Nocardioides*	*sp.*	SCA	Method 3	TSF 7
410	*Mycobacterium*	*obuense*	SCA	Method 3	TSF 7
411	*Rhodococcus*	*equi*	SCA	Method 1	TSF 7
412	*Streptomyces*	*philanthi*	SCA	Method 3	SIS 19
435	*Streptomyces*	*zaomyceticus*	SYE	Method 3	SIS 19
436	*Mycobacterium*	*obuense*	SYE	Method 3	TSF 2
437	*Mycobacterium*	*obuense*	SYE	Method 3	TSF 2
445	*Microbacterium*	*foliorum*	SYE	Method 3	SHH 18
446	*Streptomyces*	*sp.*	SYE	Method 3	SHH 18
447	*Agromyces*	*sp.*	SCA	Method 1	SHH 18
448	*Leifsonia*	*sp.*	SYE	Method 1	TSF 7
449	*Streptomyces*	*piomogenus*	SYE	Method 1	TSF 7
450	*Rhodococcus*	*sp.*	SCA	Method 1	SHH 18
451	*Rhodococcus*	*equi*	SYE	Method 1	TSF 4
452	*Streptomyces*	*sp.*	SYE	Method 3	SHH 18
453	*Mycobacterium*	*rhodesiae*	SCA	Method 3	TSF 4
454	*Rhodococcus*	*equi*	SYE	Method 1	TSF 4
455	*Williamsia*	*sp.*	SYE	Method 3	SHH 18

Table A - 1. (Continued)

475	*Leifsonia*	*sp.*	SCA	Method 1	TSF 5
476	*Microbacterium*	*azadirachtae*	SCA	Method 3	TSF 5
478	*Mycobacterium*	*neglectum*	SCA	Method 3	TSF 5
571	*Mycobacterium*	*poriferae*	SYE	Method 1	TSF 9
572	*Rhodococcus*	*equi*	SCA	Method 1	TSF 9
573	*Mycobacterium*	*poriferae*	SYE	Method 3	TSF 9
574	*Mycobacterium*	*psychrotolerans*	SYE	Method 1	TSF 9
575	*Mycobacterium*	*aichiense*	SCA	Method 1	TSF 9
586	*Micromonospora*	*tulbaghiae*	SCA	Method 1	TSF 4
587	*Mycobacterium*	*chlorophenolicum*	SCA	Method 1	TSF 5
588	*Mycobacterium*	*madagascariense*	SCA	Method 3	TSF 7
589	*Leifsonia*	*shinshuensis*	SYE	Method 3	TSF 4
590	*Nocardia*	*nova*	SCA	Method 1	TSF 2
591	*Mycobacterium*	*sp.*	SCA	Method 1	TSF 4
657	*Mycobacterium*	*rhodesiae*	SCA	Method 3	TSF 4
658	*Mycobacterium*	*sp.*	SYE	Method 1	TSF 9
682	*Gordonia*	*terrae*	SCA	Method 3	HRT 79
683	*Rhodococcus*	*rhodochrous*	SYE	Method 1	HRT 79
684	*Pseudonocardia*	*kujensis*	SYE	Method 1	HRT 79
707	*Mycobacterium*	*sp.*	SCA	Method 1	SIS 20
708	*Promicromonospora*	*sp.*	SYE	Method 3	TSF 12
709	*Mycobacterium*	*sp.*	SCA	Method 1	TSF 12
710	*Rhodococcus*	*sp.*	SYE	Method 3	TSF 13
711	*Nocardia*	*oleivorans*	SYE	Method 1	TSF 10
712	*Nocardia*	*rhamnosiphila*	SYE	Method 1	TSF 10
713	*Micrococcus*	*sp.*	Dishaw	N/A	Seasquirt
714	*Rhodococcus*	*sp.*	SCA	Method 3	TSF 13

Table A - 2. Bioactivity of All Crude Extracts

Sample	*E. faecium*	*S. aureus*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*E. cloacae*
1 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
1 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
5 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
5 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
6 (Control)	0 μg mL^{-1}	4 μg mL^{-1}	0 μg mL^{-1}	50 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
6 (DNMTi)	50 μg mL^{-1}	10 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
7 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
7 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
8 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
8 (DNMTi)	0 μg mL^{-1}	50 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
10 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
10 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
11 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
11 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
12 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
12 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
13 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
13 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
14 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
14 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
15 (Control)	0 μg mL^{-1}	25 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
15 (DNMTi)	100 μg mL^{-1}	2 μg mL^{-1}	0 μg mL^{-1}	100 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
16 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
16 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
17 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
17 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}

Table A - 2. (Continued)

18 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
18 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
19 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
19 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
20 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
20 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
21 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
21 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
22 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
22 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
25 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
25 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
26 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
26 (DNMTi)	200 µg mL^{-1}	50 µg mL^{-1}	200 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}
27 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
27 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
29 (Control)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
29 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
30 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
30 (DNMTi)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
31 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
31 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
32 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
32 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
33 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
33 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
34 (Control)	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

34 (DNMTi)	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
35 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
35 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
36 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
36 (DNMTi)	0 µg mL^{-1}	50 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
37 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
37 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
39 (Control)	0 µg mL^{-1}	50 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
39 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
40 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}
40 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}
41 (Control)	0 µg mL^{-1}	25 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
41 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
42 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
42 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
43 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
43 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
44 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
44 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
45 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
45 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
46 (Control)	200 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
46 (DNMTi)	200 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
47 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
47 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
48 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
48 (DNMTi)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

49 (Control)	200 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
49 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
50 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
50 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
51 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
51 (DNMTi)	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
52 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
52 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
54 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
54 (DNMTi)	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
56 (Control)	0 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
56 (DNMTi)	0 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
57 (Control)	200 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
57 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
58 (Control)	0 µg mL $^{-1}$	25 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
58 (DNMTi)	200 µg mL $^{-1}$	50 µg mL $^{-1}$	0 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
59 (Control)	200 µg mL $^{-1}$	25 µg mL $^{-1}$	0 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
59 (DNMTi)	0 µg mL $^{-1}$	50 µg mL $^{-1}$	0 µg mL $^{-1}$	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
60 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
60 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
61 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
61 (DNMTi)	200 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
62 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
62 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
64 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
64 (DNMTi)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$
65 (Control)	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$	0 µg mL $^{-1}$

Table A - 2. (Continued)

65 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
66 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
66 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
67 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
67 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
68 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
68 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
69 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
69 (DNMTi)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
70 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
70 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
71 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
71 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
72 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
72 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
73 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
73 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
74 (Control)	0 µg mL^{-1}	25 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
74 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
75 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
75 (DNMTi)	0 µg mL^{-1}	10 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
76 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
76 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
77 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
77 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
78 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
78 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

79 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
79 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
80 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
80 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
81 (Control)	0 µg mL^{-1}	20 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
81 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
82 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
82 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
84 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
84 (DNMTi)	0 µg mL^{-1}	12.5 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
85 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
85 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
86 (Control)	0 µg mL^{-1}	20 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
86 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
87 (Control)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
87 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
88 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
88 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
89 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
89 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
90 (Control)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
90 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
91 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
91 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
92 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
92 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
93 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

93 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
95 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
95 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
96 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
96 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
97 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
97 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
98 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
98 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
99 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
99 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
100 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
100 (DNMTi)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
101 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
101 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
104 (Control)	0 µg mL^{-1}	10 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
104 (DNMTi)	100 µg mL^{-1}	4 µg mL^{-1}	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
105 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
105 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
106 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
106 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
107 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
107 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
108 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
108 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
109 (Control)	200 µg mL^{-1}	10 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
109 (DNMTi)	0 µg mL^{-1}	10 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

110 (Control)	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
110 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
111 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
111 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
112 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
112 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
113 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
113 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
114 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
114 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
115 (Control)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
115 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
116 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
116 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
117 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
117 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
118 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
118 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
121 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
121 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
122 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
122 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
123 (Control)	200 µg mL^{-1}	0 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}
123 (DNMTi)	0 µg mL^{-1}	200 µg mL^{-1}	100 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}
124 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
124 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
125 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

125 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
126 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
126 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
127 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
127 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
128 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
128 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
129 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
129 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
130 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
130 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
131 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
131 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
132 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
132 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
133 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
133 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
134 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
134 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
135 (Control)	200 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
135 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
136 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
136 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
137 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
137 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
138 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
138 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

139 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
139 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
140 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
140 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
141 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
141 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
142 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
142 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
143 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
143 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
144 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
144 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
145 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
145 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
146 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
146 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
147 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
147 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
149 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
149 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
150 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
150 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
152 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
152 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
153 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
153 (DNMTi)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}
154 (Control)	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}	0 µg mL^{-1}

Table A - 2. (Continued)

154 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
155 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
155 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
156 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
156 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
157 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
157 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
158 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
158 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
159 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
159 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
160 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
160 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
161 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
161 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
162 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
162 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
163 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
163 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
164 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
164 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
165 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
165 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
166 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
166 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
713 (Control)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}
713 (DNMTi)	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}	0 μg mL^{-1}