Production of Kibdelomycin by *Kibdelosporangium sp.* and Discovery of Potential Structural Analogues

A Thesis in Biology

By

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Abstract

Kibdelomycin is a natural product produced by the actinomycete *Kibdelosporangium sp.*, and was discovered by Merck & Co. in the late-2000s (Phillips et al. 2011). It is a type II topoisomerase inhibitor with antibiotic activity against Gram-positive bacteria. In this study, fermentation conditions were established for *Kibdelosporangium sp.* to improve kibdelomycin production, with galactose currently the best carbon source in a complex medium. To further improve production of kibdelomycin for future chemical modification studies, we have successfully implemented a strain improvement procedure involving the selection of production strains resistant to aminoglycosides such as streptomycin and gentamicin. The generation of cumulative antibiotic resistance has previously worked for other actinomycete bacteria, and is correlated to ribosomal mutations that cause overproduction of natural products (Hu and Ochi 2001; Wang et al. 2008; Tanaka et al. 2013). Utilizing this method in *Kibdelosporangium sp.* resulted in improved strains which produce up to 50.4% more kibdelomycin. LC-MS analysis of fermentation extracts revealed production of kibdelomycin and five potential structural analogues as well as a kibdelomycin isomer. These analogues share characteristic UV profiles of kibdelomycin but differ in retention time and molecular weight as well as mass spectrometry fragmentation patterns. Overall, media development and strain improvement were important steps taken for the future development of kibdelomycin, and identification of structural analogues may assist in finding a compound with maximum antibiotic activity and minimum serum antagonism.

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Chapter 1: Review of the Global Antibiotic Resistance Crisis

I. Antibiotic Resistance Background

Prior to the discovery and implementation of antibiotics, infectious disease was the leading cause of death the United States in 1900, causing 33% of all deaths or around 400,000 (Tippet 2014). The discovery of penicillin and other antibiotics in the mid-1900s seemed to put an end to this era, enough so, that William Stewart, the Surgeon General of the United States in the 1960s, declared "It's time to close the books on infectious diseases, declare the war against pestilence won, and shift national resources to such chronic problems as cancer and heart disease" (Spellberg et al. 2008). The quote reflected the population's attitudes toward antibiotics at the time. Antibiotics were perceived as the end-all cure to infectious disease; however, no one considered the effects of the rise in prevalence of antibiotic resistance that would develop. When bacteria develop antibiotic resistance, they are no longer susceptible to that antibiotic, meaning treatment with that antibiotic becomes ineffective. Within five years of penicillin's first use, 50% of the *Staphylococcus aureus* (clinical) isolates were resistant (Abboud and Waisbren 1959). In fact, resistance has been observed for every antibiotic drug that has ever been developed (Ventola 2015). Drug-resistant bacteria were reported to have infected over 2 million leading to 23,000 deaths in the U.S in 2013 (CDC 2013). Although drug resistant bacterial infections may occur anywhere, they are most commonly acquired in healthcare settings, making this an even more pressing issue.

The acquisition of antibiotic resistance by microbes is a natural process (D'Costa et al. 2011) that is being exacerbated by antibiotic overuse (Ventola 2015). Resistance to penicillin was seen before the drug was even employed widely in humans (Wenzel 2004; Clatworthy et al. 2007; Ventola 2015). Since the beginning of antibiotic discovery, resistance has developed for all antibiotics currently in use (Figure 1-1). Microbes acquire resistance by one or more of the following methods: enzymatically inactivating the antibiotic, increasing the efflux of the antibiotic (transport out of cell), reducing the transport of antibiotic into cell, mutating the antibiotic's cellular target, metabolically bypassing the target (making it not necessary), or overproducing the target so that the effect of the antibiotic is minimal (Singh and Barrett 2005).

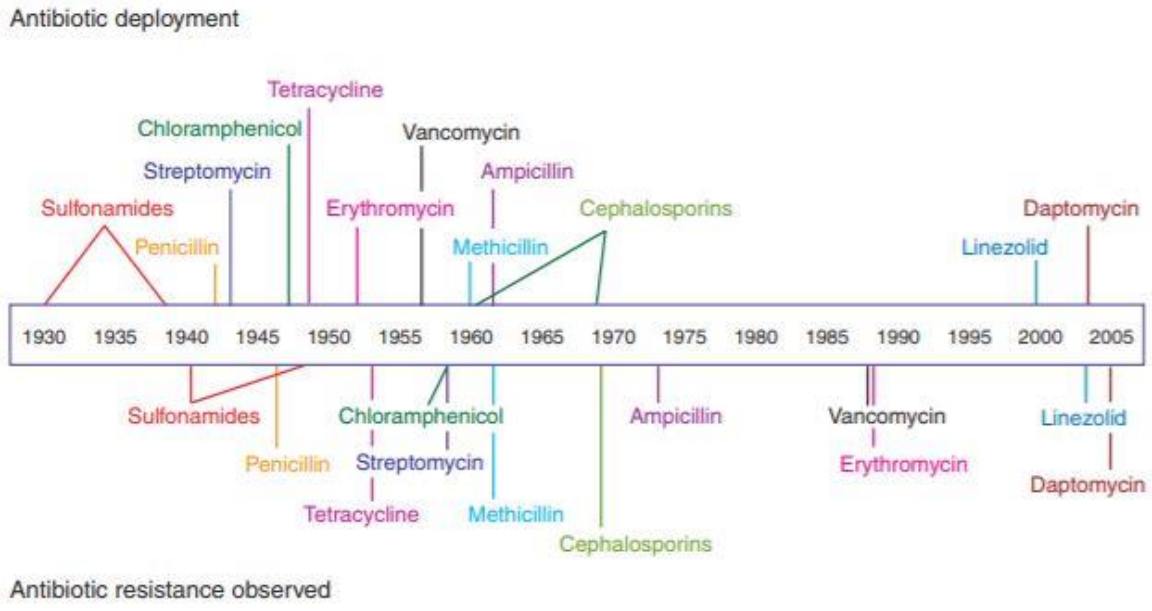

Figure 1-1: A comparison of antibiotic discovery dates and dates at which resistance to that antibiotic was found. (Figure from Clatworthy et al. 2007)

When antibiotics are used, most bacteria are killed or made inactive, however a very small percentage of resistant bacteria remain alive and active. The removal of non-

resistant bacteria allows for the resistant-bacteria to grow and replicate more readily. This process of selection allows the bacteria to practice ‘vertical’ manner of passing resistance on to offspring as well as the ‘horizontal’ manner of antibiotic resistance acquisition when microbes share their genes that encode resistance with other microbes; this can occur between microbes of the same or different species (Davies and Davies 2010; Ventola 2015). Both methods of antibiotic resistance transfer are shown in Figure 1-2.

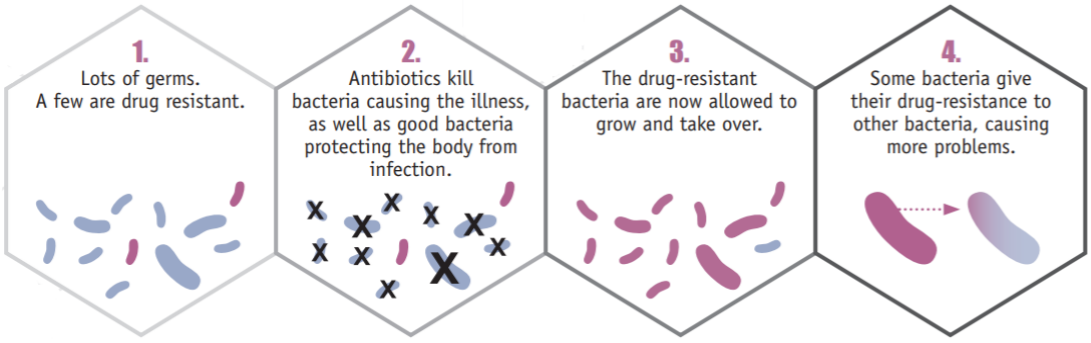

Figure 1-2: The effect of antibiotic overuse on microbial populations
The use of antibiotics causes natural selection for antibiotic-resistant microbes, creating an enriched population of antibiotic-resistant microbes (adapted from CDC 2013).

II. The Antibiotic Resistance Crisis

The world is currently experiencing an antibiotic resistance crisis, exacerbated by lack of discovery and development of novel, effective antibiotics. The committee which developed the Review on Antimicrobial Resistance (RAR), led by Jim O’Neill, produced a comprehensive report, “Tackling Drug-Resistant Infections Globally: Final Report and Recommendations,” detailing the effects of antibiotic resistance as well as recommending several steps to diminish the effects of the antibiotic crisis (RAR 2016). They found that antibiotic-resistant infections currently cause 700,000 deaths globally each year and is forecasted to be 10 million deaths per year worldwide by 2050, passing the current

number of deaths caused annually by cancer- of 8.2 million (Figure 1-3), if no action is taken to resolve the antibiotic resistance problem (RAR 2016). Within the report, O'Neill looked not only at the medical burden, but at the economic burden as well, taking into account the money that would be spent by hospitals on extended visits and countless treatments, lost wages by family members, and the decreased income from the inability to perform common procedures that require antibiotics such as surgery. Overall, the economic burden is estimated to be 100 trillion U.S. dollars (USD) by 2050. Already, methicillin-resistant *Staphylococcus aureus* (MRSA) is killing more Americans each year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Ventola 2015). The global antibiotic resistance problem is a pertinent issue to human lives and immediate action needs to be taken against it.

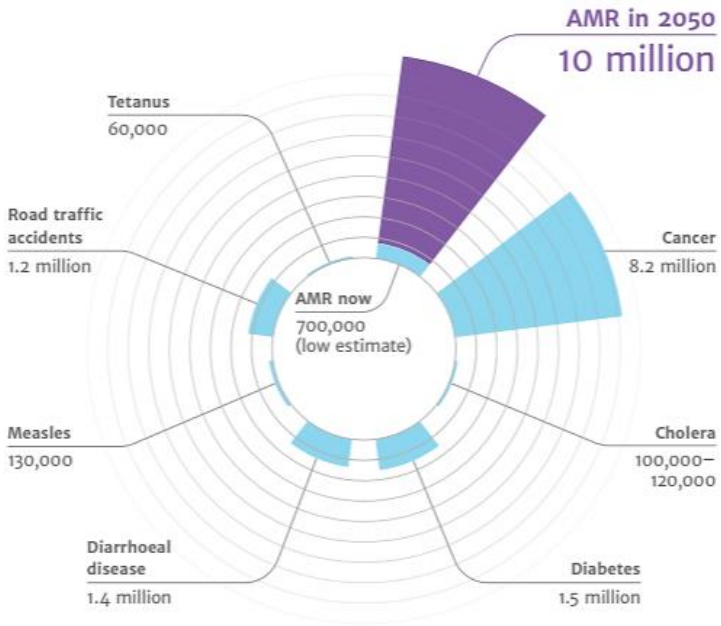

Figure 1-3: Projected number of antimicrobial resistance related deaths in 2050 compared to current most common causes of death
Figure from RAR 2016

The demand for antibiotics is higher than ever while the current antibiotics are becoming less effective against resistant bacteria (Clatworthy et al. 2007; Ventola 2015). Without a steady pipeline of new antibiotics, pathogenic and opportunistic bacteria will pose a major threat to the well-being of humans (Lewis 2013). The lack of new antibiotics will leave humans defenseless against infectious disease, and return advances in medicine to the pre-antibiotic era of 1900, where simple ailments such as pneumonia or an infected wound could be a death sentence. Steps must be taken to reduce antibiotic resistance and discover new antibiotics. But, the causes of the antibiotic resistance crisis must be better understood before effective solutions can be put in place.

A. Causes

At the rate that antibiotic resistance is now growing, the current arsenal of effective antibiotics will soon run out (Lewis 2013; Ventola 2015; RAR 2016). Finding new antibiotics is a primary concern, however they too will may become ineffective if the rate at which microbes are acquiring antibiotic resistance is not controlled. To prevent known antibiotics from becoming ineffective, the causes of resistance acquisition must be slowed down. Major contributors to the antibiotic resistance crisis include both the medical and agricultural fields. The overuse of antibiotics (whether it is in the medical field or in agriculture) greatly increases the rate at which microbes acquire resistance (RAR 2016).

Additionally, the writing of antibiotic prescriptions in healthcare settings is left solely to the discretion of doctors. Rapid diagnostic testing for confirming the presence of

a bacterial infection has not yet been developed enough to provide quantifiable data for doctors to diagnose bacterial versus non-bacterial infections in real time (RAR 2016). This results in antibiotics being prescribed and used when they are not necessary; it is estimated that 30% of antibiotics prescribed in outpatient centers in 2010-2011 were unnecessary (Fleming-Dutra et al. 2016). Often, physicians feel pressure to prescribe antibiotics preemptively so that patients do not have to suffer symptoms while waiting for proper diagnosis of a bacterial infection. This is problematic because excess exposure of a microbe to an antibiotic can increase the number of resistant organisms in the population through the mechanisms of horizontal and vertical gene transfer, as described previously.

Physicians are not the only cause for the increased spread of antibiotic resistance. Patients often misuse antibiotics, contributing to the overall crisis. Almost 40% of patients are non-compliant with their antibiotic prescriptions (Kardas et al. 2005). Once symptoms of the infection dissipate, the antibiotic no longer seems necessary so patient don't finish their prescription. This, again, aids in the selective pressure that allows for an increase in the number of resistant microbes in the population. Patients must finish the entire prescribed treatment to kill off or inactivate the whole population of microbes and limit any selection for resistant microbes.

Another prominent cause of increased resistance is the use of antibiotics in agriculture (Davies and Davies 2010, Ventola 2015). According to the Review on Antimicrobial Resistance (RAR), 70% (by weight) of antibiotics medically important to humans are used to prevent and treat infections as well as promote growth in livestock

(2016), meaning that the majority of antibiotics (those used to treat humans) produced are actually going towards agriculture. This excessive use of antibiotics can be problematic, as it increases microbe exposure to antibiotics and creates selective pressure for resistant microbes.

B. Potential Solutions

The causes of the antibiotic resistance crisis must be addressed in developing potential solutions. Steps need to be taken to reduce the overuse of antibiotics in both healthcare and agricultural settings, increase efficacy of current antibiotics, and lower the public's reliance on antibiotics through development of nontraditional treatment methods.

Antibiotic over use in the medical field can be addressed by developing rapid diagnostic technologies, antibiotic prescriptions could only be written when a bacterial infection has been confirmed, thus lowering the amount of unnecessary antibiotics being prescribed. In turn, less microbes would be exposed to antibiotics, decreasing the number of resistant microbes in a population.

Additionally, antibiotic use needs to be diminished in agriculture to remove selective pressure for resistant microbes, and thus to preserve antibiotic efficacy long enough to find novel antibiotics. As of 2017, the use of several medically important antibiotics for growth promotion purposes in livestock has been prohibited in the United States (Hoelzer et al. 2017). This is a big step in reducing the rate of resistance acquisition, however more drastic policies have been put in place in other countries to ensure the use of antibiotics in livestock only when medically necessary for the well-

being of the animal (Hoelzer et al. 2017). If more countries adopted similar policies, perhaps a significant effect on the antibiotic resistance crisis would be made.

Antibiotic adjuvants (or antibiotic enhancers) could be administered with antibiotics to increase efficacy of the antibiotic against resistant microbes. Adjuvants assist antibiotics by making the target more susceptible to the antibiotic or by reducing the resistance built up to the target (Cox et al. 2017, Melander and Melander 2017). Adjuvants do this through inhibition of antibiotic modification, inhibition of antibiotic target modification, enhancement of antibiotic uptake, inhibition of efflux, and other mechanisms (Melander and Melander 2017). One successful group of antibiotic adjuvants are β-lactamase inhibitors. Microbes developed resistance to β-lactam antibiotics, such as penicillin. The resistant microbes produced B-lactamase enzymes which break the β-lactam structure, inhibiting the antibiotic function (Drawz and Bonomo 2010). The use of β-lactamase inhibitors as adjuvants has assisted in prolonging the effectiveness of β-lactam antibiotics. Implementation of adjuvants requires a significant understanding of resistance mechanisms acquired by the microbe but may be helpful in lowering the rate of resistance acquisition, even after new antibiotics are discovered (Cox et al. 2017).

Additionally, the development of new treatments (non-antibiotic) to combat infections would assist in alleviating our dependence on antibiotics. The Pew Charitable Trust, an organization which compiles information about current antibacterial treatments (both traditional and non-traditional) in initial and clinical development, asserts that these “out-of-the-box” or nontraditional methods would be beneficial in preventing and treating

bacterial infections without antibiotics (PEW 2019). Among the nontraditional treatments in clinical trials are lysins, antibodies, and probiotics. Perhaps novel treatments will limit the acquisition of resistance by bacteria, further decreasing the demand for antibiotics. If successful, these treatments could buy researchers time in the race to develop novel antibiotics. It is also possible that non-traditional treatments could be used in combination with future antibiotics to limit the acquisition of resistance.

Although slowing down the rate of resistance acquisition will significantly decrease the demand for antibiotics, the problem of finding new antibiotics must be discussed. Antibiotic discovery has decreased significantly since the mid-1900s (Clatworthy et al. 2007; Lewis 2013; RAR 2016; Ventola 2015). The members of the Review on Antimicrobial Resistance recommend governments provide financial incentives for antibiotic research to persuade pharmaceutical companies and small research groups to produce the next line of antibiotics. Although it would require large sums of money to reward each group, the cost would likely be less than the economic burden of the antibiotic crisis by 2050: 10 trillion USD (RAR 2016).

III. Summary

The antibiotic crisis is a truly pressing issue in the world today. If nothing is done, the world risks the chance of falling back into the pre-antibiotic era, with a significant decrease in life expectancy (RAR 2016). So many surgical procedures are taken for granted today. They are thought of as simple, but they could be lifesaving. Some

procedures, such as cesarean sections, hip replacements, or gut surgeries, would be impossible without antibiotics because post-operative infections would be uncontrollable.

To maintain modern lifestyles and quality of life, action against antibiotic resistance must be taken. The demand for antibiotics must be lowered by decreasing the rate at which microbes acquire resistance. This can be done by developing quick diagnostic testing for use by doctors, informing patients of the dangers of antibiotic misuse, and prohibiting the unnecessary use of certain antibiotics in agriculture. Looking at alternative treatments to control or eliminate infections would decrease our dependence on antibiotics and lessen the rate of resistance acquisition. Last, and most importantly, the supply of new antibiotics must be increased. Providing financial incentives to researchers who work in the field of antibiotic discovery would bring much more attention to the issue and aid in the production of novel antibiotics by alleviating the financial burden associated with antibiotic development.

Chapter 2: Paradigms in Antibiotic Discovery- Old and New

I. Introduction

Recently, an article was published in the Los Angeles Times newspaper by Melissa Healy titled, “In soil-dwelling bacteria, scientists find a new weapon to fight drug-resistant superbugs” (Healy 2018). If one were to replace ‘drug-resistant superbugs’ with ‘bacteria,’ the title could easily be used to report the majority of antibiotic discoveries during the mid-1900s. During this time, soil bacteria were the most common sources of antibiotics (Hover et al. 2018).

However, the methods by which soil bacteria were screened for antibiotic production varied drastically between the time periods. During the mid-1900s, random screens of compounds extracted from microbial fermentations determined if any compounds contained in the extract showed antibiotic activity (Lewis 2013). New methods turn to advances in genetic studies to look at encoded secondary metabolite pathways (Hover et al. 2018). The similarities and differences between past and current methods highlight recent changes in methods of antibiotic discovery, fueled by leaders in the field and their different approaches to the problem. The next sections will analyze the old and new paradigms as well as provide explanations for the change.

II. Old Paradigms

A. Phenotypic Antibiotic Discovery

After Alexander Fleming's discovery of penicillin in 1929 and its development into a successful antibiotic by Ernst Boris Chain and Howard Walter Florey (Chain et al. 1940; Abraham et al. 1941), scientists began sampling many microbes to isolate more natural product antibiotics (Lewis 2013). Natural product antibiotics are molecules that a microbe produces that exhibit antibacterial activity. The molecules are produced by enzymes that are encoded by genes as secondary metabolites, i.e., genes that are not essential to growth or reproduction of the organism. During the 'Golden Era' of antibiotic discovery (1940-1970), the lab of Selman Waksman of Rutgers University in New Jersey developed a discovery platform, based on the discovery of penicillin, which was later used by the pharmaceutical industry in the search of new antibiotics (Lewis 2013). This method was responsible for the discovery of streptomycin, the first effective drug against *Mycobacterium tuberculosis* which causes tuberculosis. The Waksman laboratory screened for anti-bacterial activity of soil bacteria against susceptible microbes grown on an agar plate, looking for zones of inhibition (ZOI), i.e., areas surrounding the soil bacteria where the microbe could not grow. Later on, this procedure was standardized by W.M. Kirby and A. W. Bauer (Kirby-Bauer Disk Diffusion Susceptibility Test) so that antibiotic activity could be compared between different labs (Hudzicki 2009).

Pharmaceutical companies adapted the Waksman Platform to find more antibiotics using their advanced technology and large workforces (Lewis 2013). They isolated microbes from natural sources (e.g., soil samples), fermented (grown in liquid culture) the microbes, extracted the fermentation broths with organic solvents, and tested extracts for antibacterial activity using bioassays via high throughput screening (Singh

and Barrett 2005). If antibiotic activity was detected in an extract, they would attempt to purify the compound from the mixture, and retest it to confirm the antibacterial activity seen in the bioassay. This allowed them to use 'brute force' to screen many samples at once for antibacterial activity. The issue with this method for finding *novel* compounds, is that it finds compounds produced by microbes in high quantities, not compounds produced in trace amounts by those same microbes (Lewis 2013; Cox et al. 2017). The "easy-to-find" or "low-hanging-fruit" antibiotics have already been discovered and developed for clinical use. Antibiotic screening assays were so overrun with these easy-to-find antibiotics, that newer, less common ones were unlikely to be identified (Cox et al. 2017). The screening assays were simply discovering the same antibiotic compounds repeatedly. This led to the creation of a post 'Golden Era' antibiotic discovery void, and natural product-based phenotypic discovery began to fall out of favor (Singh and Barret 2005).

B. Synthetic Antibiotic Discovery

To fill the antibiotic discovery void and turn a profit in this therapeutic area, companies abandoned the search for natural product antibiotics and began sifting through synthetic, combinatorial compound libraries (Payne et al. 2007; Lewis 2013). Libraries of successful drug structures had been put together by companies and research groups; many companies grouped compounds into subsets based on "Lipinski's Rule of 5" (Payne et al. 2007; Lewis 2013). This is a set of characteristics- molecular weight less than 500 Daltons (Da), lipophilicity or partition coefficient less than five, less than five hydrogen bond acceptors, and less than five hydrogen bond donors- found to be shared by

successful drugs (Lipinski 2001). However, these characteristics were based around oral-available activity in humans, which means the availability of the drug in the body after oral administration, absorption, and first-line metabolism (Lewis 2013) and were never proven to be shared by antibiotics (Monaghan and Barrett 2005). In fact, many of the five rules were broken by most of the commonly used antibiotics at the time. One example is streptomycin which had a molecular weight of 581 Da, 15 hydrogen bond acceptors, and 12 hydrogen bond donors (PubChem). However, streptomycin was a highly successful antibiotic. Lipinski admits that the Rule of 5 was based on the success of several thousand drugs, leaving room for exceptions that fall outside of the parameters (Lipinski et al. 2001). Certain groups of drugs were consistent exceptions- antibiotics, antifungals, vitamins, and cardiac glycosides, all of which are orally active despite breaking the "rules".

Companies also compared chemical structures of known antibiotics to those found in the synthetic compound libraries in an attempt to find similar structures that would inhibit a common antibiotic target (Coates and Hu 2007). Antibiotic targets are cell functions that are necessary for cell life (Singh 2014). Antibiotics kill or inhibit growth of microbial cells by inhibiting that function. Some common targets are DNA synthesis, cell wall synthesis, protein synthesis, cell membranes, and metabolic pathways (Monaghan and Barrett 2005). Synthetic compounds with structures similar to antibiotics known to inhibit a specific target could be tested for antibacterial activity via sensitivity or fitness assays. These assays look for compounds with specific cellular targets by testing the activity of compounds against microbes that are more susceptible to growth inhibition at

the particular target of interest (Singh et al. 2007). If activity was observed, scientists would then alter the structures of the active compound through chemical reactions to yield a compound with optimal activity.

This older fitness assay method was not highly successful in identifying new antibiotics (Singh and Barrett 2005). Its lack of success can be attributed to the inability of many of these molecules to enter microbial cells where many of the antibiotic targets reside. Not all successfully developed drugs (evaluated to develop Lipinski's Rule of 5) need to enter bacterial cells to be successful; however, this is a requirement of many antibiotics battling bacteria (Lewis 2013). *In vitro* assays showed compounds which followed Lipinski's Rule of 5 to be active against isolated bacterial targets (e.g., inhibition of enzyme activity), but inactive when tested in whole cell assays (Monaghan and Barrett 2005). This showed that antibiotics discovered with these methods were often ineffective because they cannot hit their intracellular targets. Some scientists proposed that the development of a set of rules based on drug penetration into cells would be more effective in screening libraries for antibiotics (Lewis 2013). Re-screening chemical libraries with rules for penetration may bring about compounds that were overlooked previously due to the breaking of Lipinski's Rule of 5.

After being unable to discover antibiotics from synthetic compound libraries, research groups attempted to optimize existing compounds, natural product or synthetic, rather than discover new ones (Singh and Barrett 2005). They looked to overcome the antibiotic resistance crisis by chemically modifying the structures of existing antibiotics. This approach (dubbed semi-synthetic antibiotic discovery) created next generations of

previously-developed antibiotics (some, now up to six generations); however, there is eventually a limit to structural alterations aimed at improving potency and overcoming resistance to the predecessor compound (Singh 2014). It is possible that this approach has reached the point of diminishing returns.

III. Reasons for Changing the Paradigm

Part of the issue with the old paradigm of antibiotic discovery is pharmaceutical companies and other large research groups have shifted their focus to chronic medical problems, such as high cholesterol or rheumatoid arthritis, rather than antibiotic discovery (Lewis 2013). Antibiotics simply do not produce enough revenue to sustain these large companies. When comparing the chronic illness drug market and antibiotic market, the best-selling cholesterol-lowering drug (atorvastatin) brings in $12 billion annually whereas the best-selling antibiotic levofloxacin has annual sales of $2.5 billion (Lewis 2013). The case of levofloxacin is rare, as the majority of antibiotics have sales of a few hundred million. In a publication prior their “Final Report and Recommendations,” the Review on Antimicrobial Resistance discussed the costs of antibiotic research and development as well as the total profit (RAR 2015). After an initial cost of approximately $700 million, researchers will only start profiting on a successful antibiotic 10 to 12 years after beginning research, and will not break even on their investment until year 23. After this point, net gains stay around $100 million (Figure 2-1).

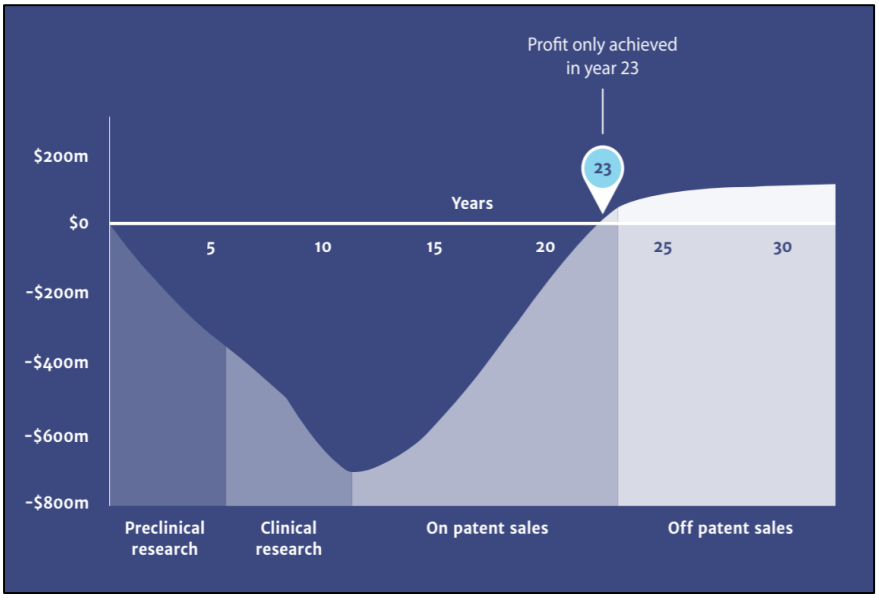

Figure 2-1: Costs of antibiotic research and development compared to overall profit. (Figure from RAR 2015)

The delay in profit can be attributed to the amount of time it takes for an antibiotic to be discovered and developed. This process can take up to 15 years for discovery, development, and clinical trials (Payne et al. 2007). It also requires a lot of money to pursue multiple potential antibiotics because many drugs fail over the course of the process.

Even if a successful drug is developed, there are other reasons profits could be low. For example, antibiotics are only used for a treatment course of 5 to 7 days, while other drugs for chronic medical problems could be used for 5 to 7 years so antibiotics are bought less frequently. Also, when a new antibiotic is approved, physicians try not to use it right away (Ventola 2015). It is saved on the "top shelf" and reserved only for use against infections which cannot be controlled by any other antibiotic. This is a common

and necessary practice for keeping resistance to a new antibiotic low. Nonetheless, it limits the profitability of new antibiotics.

Many pharmaceutical companies were unable to sustain research in antibiotics due to the lack of return on investment (Projan 2003). Their techniques and technology were unsuccessful in discovering novel antibacterials, leading to a loss in capital investments. Even if they had produced antibiotics, it is likely that the revenue for a single antibiotic would have not financially supported such large companies, as the cost of clinical trials as well as regulations on pricing limit the profitability of new antibiotics (Projan 2003).

Overall, the low success rate, lengthy development process, and limited use of antibiotics makes for a poor investment in the eyes of pharmaceutical company executives. Because of this, many pharmaceutical companies have moved away from antibiotic discovery as of the early 2000s.

Although pharmaceutical companies are not capable of being financially supported by the revenue of antibiotics, there are groups that can be (Projan 2003). Academia and small companies do not rely on such large revenues and could potentially benefit from a net profit of $100 million if the research and development costs are kept minimal. These groups are limited in capital and must handle their costs smartly, forcing them to take on the issue of antibiotic discovery from a new perspective rather than continue the failed paradigms of the large pharmaceutical companies. The old methods were no longer churning out results, resulting in a standstill in antibiotic discovery.

Instead of keeping to old methods of discovery, these academic groups and small companies focused on small niches of antibiotic discovery to keep investments minimal. The unique approaches of these groups have the possibility of successfully bringing about new compounds, that could have a lasting impact on humanity. Yet, these unique approaches could not be possible without advancement in technologies.

Advancements in the field of spectrometry, particularly liquid chromatography-mass spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy, have increased the rates at which compounds can be detected and analyzed, which rapidly determines if a biological activity is due to a natural product with a novel structure or one that has been discovered previously. LC-MS works by separating compounds from an extract based on polarity – in the case of reverse phase chromatography - and then characterizes each compound by molecular weight and fragmentation pattern. Advancements allowing finer separations have greatly improved purification procedures, and advancements in automation have allowed researchers to test extracts much quicker (Singh and Barrett 2005). Once a compound is purified, NMR can be used in multiple ways (Proton NMR/Carbon NMR) to determine the compounds structure, because molecular weight alone does not reveal it. Advancements in NMR have allowed structural determinations with minimal amounts of purified compound. Although as little as 3 to 5 micrograms (μg) is considered detectable, it is more efficient to test 3 to 5 milligrams (mg) (Singh and Barrett 2005). Still, this is an improvement to previous instrumentation that was not as sensitive and required substantially more material. Natural products are generally produced in milligram or sub-milligram per liter

concentrations by the wild-type culture so large quantities of material cannot always be purified, especially in the initial stages of research when there may be a large number of cultures and potential activities being evaluated. Therefore, advancements that have allowed natural products to be detected and purified during the initial stages of drug discovery have helped tremendously in antibiotic discovery

One byproduct of advanced instrumental analysis is the ability to dereplicate antibiotics rapidly; dereplication links a compound's structure with its antibiotic activity, and determines if the compound has been previously identified, a problem encountered by many pharmaceutical companies. By dereplicating common, previously discovered antibiotic compounds, researchers can focus on the unknowns with potentially novel structures (Cox et al. 2017). It solves the problem faced by many scientists early on when looking at natural products: the difficult task of weeding out the large quantity of known antibiotics. Previously, dereplication required the lengthy process of extraction from the fermentation, purification, and structure elucidation requiring substantial quantities of material. However, with improved LC-MS and NMR spectroscopy, this process becomes much easier, quicker, and resource sparing (Cox et al. 2017). Knowing structures at an early stage allows known antibiotics to be avoided and novel compounds to be further investigated. The advent of genetic sequencing has had an even greater impact, giving researchers the ability to distinguish between known and novel antibiotics in the microbe's genome before the microbe is even cultured (Singh and Barrett 2005).

Advances in whole genome sequencing have had, likely, the largest impact on antibiotic discovery. Genome sequencing has become quicker, cheaper, and more reliable

in the last two decades (Land et al. 2015). Today, human genome sequencing only costs a fraction of what it did in earlier years and can be accomplished in mere hours compared to years. The cost and time for bacterial genome sequencing is even less, as the genomes are much smaller – e.g., eight million base pairs (mbp) for a typical *Streptomycete* species. Although these advances are incredible, the data they reveal would be useless without advancements in bioinformatic software that has emerged in parallel (Land et al. 2015). Bioinformatics gives researchers the tools to understand the information encoded by genome sequences, turning large amounts of data into comprehendible information. Combined, these breakthroughs have allowed research to focus on finding new antibiotic targets in microbes, locating genetically encoded antibacterial secondary metabolites, and expressing these secondary metabolite gene sequences in transformed host microbes that do not naturally contain that gene (heterologous expression). In general, advances in genomics has made the search for new antibiotics much more efficient and cost effective. Perhaps this will entice more research groups to join the field of antibiotic discovery.

IV. New Paradigm- Genomic Discovery via Microbial Natural Products

Microbial natural products have contributed to the majority of successful drugs. From 1981-2014, there were 1,328 new drug approvals, of which 686 were natural products, natural product-derivatives (i.e., semi-synthetics), or natural product mimics produced synthetically (Newman and Cragg 2016). Only 359 were entirely synthetic, with the remainder being vaccines or other therapeutic proteins. This is a ratio of 2:1 in favor of natural products in general. Natural products dominate drug discovery for all therapeutic classes, but even more so in the discovery of antibiotics.

When looking specifically at natural product antibacterial drug approvals for 1981-2014, 83 out of 140 were natural products, natural product-derived, or mimicked natural products while only 29 were synthetic (Newman and Cragg 2016). This gives a ratio of almost 3:1 in favor of natural antibacterial products.

This trend continues with antibiotics in clinical trials, with 67% of antibiotics in clinical trials in 2010 being natural products or natural product-derived (Donadio et al. 2010). Clearly, natural product antibiotics have an advantage over synthetic antibiotics as drug candidates.

The success of natural products can be attributed to many factors. Researchers theorize that microbes developed antibacterial compounds through evolution as a means of competing with one another (Rouhi 2003). Microbes have existed for over 3.4 billion more years than humans (Rogers and Kadner 2019), allowing them to have an advantage. Through evolution, microbes have developed the ability to naturally produce compounds that aid in their survival when in microbial warfare, or competition with other microbes. These compounds could also assist humans when combatting microbes, creating a shortcut in antibiotic discovery. Another advantage of natural products is that rather than extensive, sequential chemical reactions to produce the antibiotic, natural products simply require the fermentation of the microbe which produces them; the microbe assembles the natural product by a series of enzymes coded for by the genes in a particular natural product pathway. Natural products have an inherent uniqueness and complexity about them, that has not been matched by synthetic chemistry (Payne et al. 2007). As mentioned earlier, antibiotic compounds share characteristics different from other

successful drug groups. They usually have higher molecular weights, contain fewer nitrogen, halogen and sulfur atoms, contain more oxygen atoms and chiral centers, and have complex steric interactions (Rouhi 2003). Whether it is due to their long history of microbial warfare, the ease of their synthesis by microbes, or their unique and complex structures, natural products have proven to be successful drug leads in the past and present. Current and future researchers in the field of antibiotic discovery should look to natural products.

Small companies and academia have brought about a new paradigm of antibiotic discovery, searching for natural product antibiotics with new methods and technologies. Limited investment funds combined with advanced technologies pushes small companies and academic research groups to search for microbial natural products differently than the way pharmaceutical companies did so in the past. Implementation of genomic technologies has allowed them to look at the field from a fresh perspective. These groups utilize advancements in genome sequencing, molecular genetics, and bioinformatics to search microbial natural products in more detail than ever before.

A recent study at Rockefeller University highlights how academic research groups utilize new technology in the new paradigm on antibiotic discovery. Scientists sequenced DNA from soil samples (for the microbes they contain) and used computers to screen the genetic material for DNA sequences indicative of calcium dependence, a characteristic of the known antibiotic daptomycin (Hover et al. 2018). When a cluster of genes was found to contain the calcium dependence trait, the relevant genes were cloned and transformed into a heterologous host microbe. The recombinant host was then fermented and

produced malacidin, a genetically-encoded, calcium-dependent, antibiotic. The method used in this project is characteristic of the new paradigm of antibiotic discovery, which has been brought about by small companies and academic research groups.

The genes for the biosynthetic pathway enzymes for many antibacterial compounds are typically clustered in a single locus in a microbe's genome, similar to an operon (Donadio et al. 2010). Scientists can sequence many microbial genome samples at the same time and search this DNA sequence data using bioinformatic algorithms for encoded secondary metabolite pathways (Chandra Mohana et al. 2018). For example, anti-SMASH is an online program which compares DNA sequences to a database of known secondary metabolite, biosynthetic gene clusters, and aligns them with close relatives with the hopes of identifying new gene clusters (Medema et al. 2011). This method of antibacterial discovery allows researchers to determine the genetic capability of the bacteria to produce antibacterial compounds without culturing the bacteria beforehand, if the bacteria is culturable at all. This is beneficial because many microbes only produce a fraction of the natural products that they are genetically capable of producing (Monaghan and Barrett 2005).

When large pharmaceutical companies made the switch from microbial natural products to synthetics and semi synthetics, they assumed searching natural products was no longer worth the trouble because the same antibiotics continued to be rediscovered (Singh 2014). Past scientists mistook this to mean that most, if not all, notable natural product antibiotics had been found. However, the overwhelming majority of microbes have not yet been cultured (Lewis 2013). In fact, only 1% of all microbes in existence are

readily culturable, or able to be grown in a laboratory setting, meaning that 99% of microbes remain uncultured and understudied. Metagenomics, the study of genetic material recovered from the environment, has determined that a clear majority of microbes have not yet been explored for their capability to produce natural product antibiotics (Chandra Mohana et al. 2018). Potential metabolites can be "detected" through DNA sequencing of unculturable microbes. When the genes of the potential antibiotic are identified, scientists can further investigate the genome to determine culture conditions that might allow for that specific metabolite to be produced, or the gene sequence of the biosynthetic pathway for the metabolite can be transferred from the microbe into a culturable bacterial host and produced heterologously (Figure 2-2). Through this method, the genetic sequences for the biosynthetic pathway of the secondary metabolites that are encoded in unculturable microbes can be expressed in culturable microbes. The culturable microbes would then, in theory, produce the secondary metabolite. The discovery of the antibiotic teixobactin attests to this. Scientists used iChip technology to grow previously unculturable soil microbes in a multi-channeled device (Ling et al. 2015). The unculturable bacteria were introduced into the device from their native environment and then their natural products were extracted. In this study, the novel antibiotic teixobactin was discovered which inhibits bacterial growth via a new mechanism of blocking cell wall synthesis.

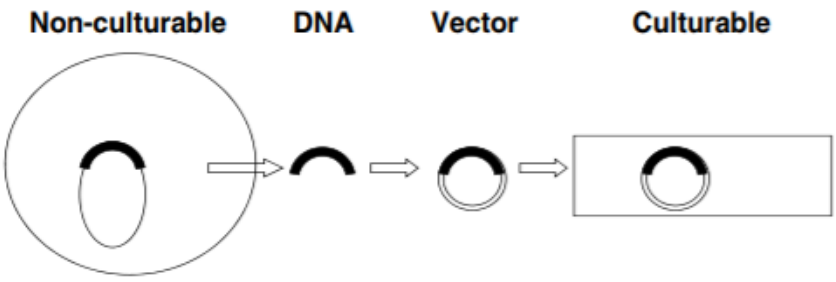

Figure 2-2: Transformation of genes from unculturable bacteria into culturable bacteria. DNA believed to code for enzymes to synthesize a natural product can be copied from the non-culturable bacterium (first arrow) and inserted into a genetic vector such as a plasmid (second arrow). A culturable bacterium can then be transformed with the plasmid carrying the DNA required for synthesis of the novel natural product (final arrow). (Figure adapted from Coates & Hu 2007)

Through the same method, the genes for silent or cryptic pathways (genetic sequences for secondary metabolite pathways that are not expressed by the microbe) can be isolated from the microbe and inserted into genetically facile microbial hosts (Chandra Mohana et al. 2018). Sometimes, genetic expression of the secondary metabolite pathway might be connected to a promoter sequence, or to particular growth conditions. Scientists can use these promoters or growth conditions to their advantage, expressing a secondary metabolite pathway that was transformed into a heterologous host microbe.

Genome sequencing can also assist in identification of antibiotic targets. An antibiotic target can be defined as the essential cell mechanism that an antibiotic inhibits which leads to death of a microbe (bacteriocidal) or at inhibits bacterial growth (bacteriostatic). Given the whole genome sequence of *Staphylococcus aureus,* there are 265 to 350 genetically validated antibiotic targets (Singh 2014); 60% of these targets are broadly conserved in related bacteria (Singh and Barrett 2005). Current antibiotics exploit less than 20 of them, leaving hundreds of targets to develop antibiotics against. Not all

these new targets are likely to be inhibited by an antibiotic; further research is necessary to find which targets are druggable (Singh and Barrett 2005). Some antibiotics, referred to as multi-target ligands, affect multiple targets at the same time and are very successful, indicating that the single antibiotics with multiple targets would be ideal in drug discovery (East and Silver 2013). Examples of multi-target ligands are penicillin, which have multiple penicillin binding proteins and ciprofloxacin, which targets DNA gyrase and topoisomerase IV (Singh et al. 2017). Additionally, multi-target antibiotics are hypothesized to slow down the evolution of resistance because the probability of acquiring resistance to two targets is the product of their separate probabilities because microbes would have to acquire resistance to both target inhibitors. Previously, targets were determined after the antibiotic was purified (Singh and Barrett 2005). Now, scientists can anticipate certain targets based on an antibiotic's structure, which can be roughly determined from the sequence of the genes for the enzymes responsible for its biosynthesis (Chandra Mohana et al. 2018).

Technological advances and the interest of small companies and academia give hope to finding more antibiotics from microbes. Earth contains an incredible amount of biodiversity, and up until now, scientists have only scratched the surface in natural product antibiotic discovery. The scientific community has recognized a need for not only new antibiotics, but new structural classes of antibiotics, as resistance to current classes exist and could rapidly develop for new antibiotics of the same class. Perhaps the unique, complex structures of microbial natural products could allow researchers to

discover these new antibiotic structural classes. Now, more than ever, scientists must look to microbial natural products for new antibiotics.

V. In Between Paradigms- The Discovery of Kibdelomycin

One product of the surge into antibiotic discovery is the novel antibiotic, kibdelomycin. It is a natural product produced by the actinomycete *Kibdelosporangium sp.* and was discovered in the Central African Republic by Merck & Co. in the late-2000s using a *Staphylococcus aureus* fitness test as a screening tool. The fitness test used antisense RNA to down regulate certain targets essential for *S. aureus* growth thus making the strains more sensitive to antibiotics affecting these targets (Singh et al. 2007). Antisense RNA is complementary to the RNA transcribed from a cell's genes and binds to the RNA reducing its translation into a protein (Figure 2-3). The cell now has less of that protein and is therefore more susceptible to antibiotics that target that protein. Essentially, this allows researchers to hand-pick compounds that inhibit the function of specific molecular targets. Experimental samples were screened against *S. aureus* with

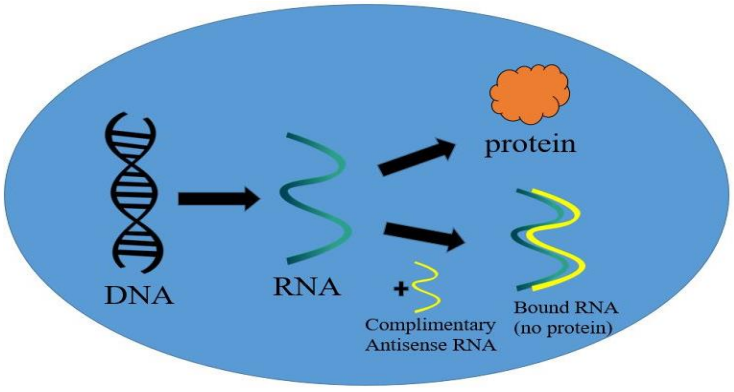

Figure 2-3: Down regulation of bacterial cell targets
Complimentary antisense RNA can be used to down regulate target proteins in a cell by blocking some translation, which makes the cell more susceptible to compounds that attack these proteins

these down-regulated targets, allowing researchers to find antibacterial compounds specific to each target.

While this discovery model is still reminiscent of older antibiotic discovery platforms, it is target specific and was implemented in a manner which favored discovery of novel compounds, lowering chances of re-discovery of known natural products (Phillips et al. 2011). In the study responsible for kibdelomycin's discovery, soil samples were taken from diverse geographic locations, increasing the chances of discovering novel antibiotics. Sampling from areas of high biodiversity, allows for the discovery of more diverse chemical structures. *Kibdelosporangium*, in fact, is native to the rainforests of the Central African Republic, an area known for its biodiversity.

Kibdelosporangium is a member of the actinobacteria phylum (Phillips et al. 2011), a taxonomic class containing the bacteria responsible for producing 90% of natural product antibiotics used in medicine (Martens and Demain 2017). *Kibdelosporangium* is a filamentous bacterium which grows in the soil and produces spores in under certain conditions. Kibdelomycin, a natural product of *Kibdelosporangium*, shows antibacterial activity against several species of bacteria (Phillips et al. 2011). Specifically, studies show activity against Gram-positive microbes: *S. aureus* (Minimum Inhibitory Concentration -MIC- of 2 μg/mL), methicillin-resistant *S. aureus* (MIC = 0.5 μg/mL), *Streptococcus pneumoniae* (MIC = 1 μg/mL), and *Enterococcus faecalis* (MIC = 2 μg/mL). A gram-negative microbe, *Haemophilus influenzae*, was also susceptible to kibdelomycin (MIC = 2 μg/mL).

The structure of kibdelomycin (Figure 2-4) contains a decalin tetramic acid core which is attached to a modified epiallose residue (6-methyl-2-methoxy3-acetoxy-4-carbamoyl substitutions) by an N-glycosidic linkage (Phillips et al. 2011). On the other side is a modified hexopyranose (3-a-aminoethyl-3,6-dideoxy substitutions) connected by an O-glycosidic linkage. The amino group of the amino ethyl substitution forms an amide bond with modified pyrazole (2-carboxylic acid-3,4-dichloro-5-methyl). Tetramic acid is a common structure found in other bacterial natural products and can be responsible for serum antagonism, making it problematic as a drug for use in humans (Royles 1995).

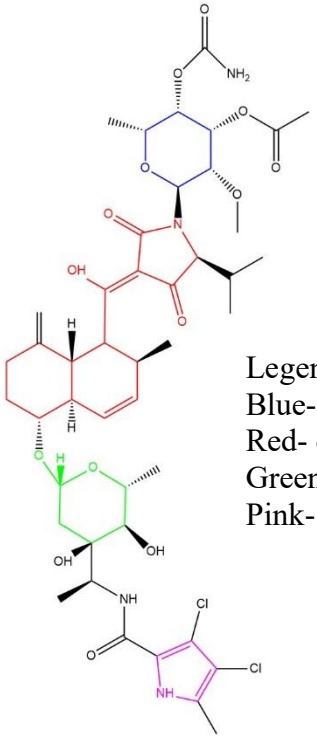

Figure 2-4: Structure of kibdelomycin with highlighted key structures

Kibdelomycin inhibits DNA synthesis, targeting DNA gyrase, a type II topoisomerase (Phillips 2011). Kibdelomycin is the first type II topoisomerase inhibitor discovered from a natural product source in over 60 years and works through a

mechanism similar to the coumarin antibiotic, novobiocin, inhibiting DNA gyrase and topoisomerase IV. However, kibdelomycin is effective against novobiocin-resistant bacteria. In other words, bacteria that have evolved to be resistant to novobiocin are not resistant to kibdelomycin. The fact that it is effective against novobiocin-resistant bacteria makes kibdelomycin a molecule of interest for future antibiotic development. However, the antibacterial activity of kibdelomycin was significantly reduced when tested in the presence of 50% human serum (Phillips et al. 2011). This means that proteins and other factors in human blood inhibit the activity of kibdelomycin. In order for kibdelomycin to be developed into an antibiotic drug, this serum antagonism would need to be reduced. Serum antagonism can potentially be reduced by making structural modifications to the compound (Phillips et al. 2011). However, experimenting with structural modifications of kibdelomycin will require sufficient quantities of purified antibiotic to work with. This requires developing a fermentation process specific to the organism.

Typically, the fermentation process begins with inoculation of a preserved culture into a seed medium (Connors 2003). The seed medium allows for rapid growth of the organism as a means to expand the culture. Once a seed is established (usually a couple of days), an aliquot of seed can be inoculated into production media, where it will grow and produce its natural products. Production media do not necessarily promote rapid growth, but rather production of the compound of interest (Connors 2003). It is important to select and/or develop a production medium that maximizes the amount of product being produced and not the amount of biomass (cell growth). The length of fermentation

in production medium can vary greatly, from hours to weeks (Connors 2003). This typical fermentation process is depicted in Figure 2-5.

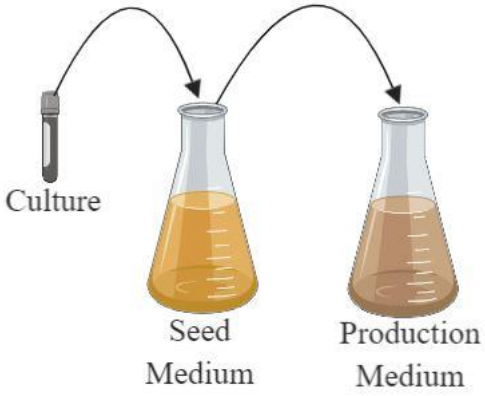

Figure 2-5: Fermentation process for natural product production. Preserved culture is inoculated into seed medium (first arrow) and fermented to expand culture. The seed culture is transferred to production medium (second arrow) which promotes production of the product of interest.

Production media may also be optimized to increase production. One way to do so is through statistical experimental design. All nutrients within the medium are varied at high and low levels in a way that determines the effect of individual nutrients as well as combined effects of several nutrients. From these results, the media can be optimized to contain more or less of each nutrient, promoting production of the natural product of interest. Before larger experiments can take place, an effective fermentation process must be established for kibdelomycin production. Additionally, media development can further increase production of kibdelomycin by *Kibdelosporangium sp.*

The focus of this project was to develop a fermentation process for kibdelomycin production as well as optimize fermentation conditions via media development strategies. Once the fermentation process was established, strain improvement would be the next step in increasing kibdelomycin production.

Chapter 3: Media Development for *Kibdelosporangium sp.*

I. Introduction

Bacteria depend on different nutrients to grow and survive; these various nutrients differ for each organism. These nutrients also affect what is produced by the organism. In order to determine the best conditions for growth of *Kibdelosporangium* and production of kibdelomycin, several liquid seed and production media formulations were tested. Media types published with the discovery of kibdelomycin (Phillips et al. 2011) as well as several new formulations were considered during experimentation. Cultures begin in a seed medium and were moved to production medium after sufficient growth has begun. Superior production media formulations were determined based on overall growth and by comparing the zone of inhibition in disk diffusion assays against *S. aureus* for whole broth extractions of each liquid fermentation. Disk diffusion assays reveal relative amounts of antibiotic activity within an extract sample. Agar-filled Petri dishes were seeded with a layer of bacteria, in this case *S. aureus*, and small filter paper disks containing extract samples – up to 20 µL – were placed on top of the bacterial layer. During incubation, the extract diffuses into the agar, creating a concentration gradient of the compounds within the extract. The concentration of antibiotic is higher closer to the disk, leaving a circular zone of clearance where *S. aureus* cannot grow if *S. aureus* is susceptible to that compound. The size of the zone of inhibition is proportional to the total amount of antibiotic in the sample and can be measured to compare relative amounts of antibiotic in different samples.

II. Seed and Production Media Trials

Kibdelosporangium sp., strains F109 and SAM3A, the original kibdelomycin-producing strains isolated by Merck & Co, were obtained from Fundación MEDINA (Granada, Spain) via Sulagen LLC (Edison, NJ). The strains were not known to differ from each other in any regard other than lab origin. The frozen stocks for each strain provided by MEDINA were inoculated into 20 mL of ATCC-2 medium [per liter: 10 g glucose, 20 g soluble starch, 5 g NZ Amine Type A, 5 g beef extract, 5 g Bacto Peptone, 5 g yeast extract, 1 g calcium carbonate, pH 7.0] (adapted from Phillips et al. 2011) in a sterile 125-mL Erlenmeyer flask, and incubated at 28 °C with 250 rpm rotary shaking for 3 days. Time periods and temperatures for incubations were previously established by Phillips and colleagues (2011). After 3 days, the seed culture was mixed with 60% (v/v) glycerol at a 5:1 ratio (seed to glycerol) to give a 10% final glycerol concentration. The mixture was subdivided into 1 mL aliquots in cryogenic vials and stored at -80°C.

Seed and production fermentations were performed according to the following procedure. One milliliter of each strains frozen stock was thawed and inoculated into sterile 125-mL Erlenmeyer flasks containing 20 mL of seed medium: ATCC-2 or ISM-3 [per liter: 20 g glucose added post-sterile, 15 g yeast extract, 10 g malt extract, 0.5 g magnesium sulfate heptahydrate, 0.03 g iron (III) chloride, pH 7.0 (Sun et al. 1998)]. The flasks were incubated at 28 °C and 250 rpm rotary shaking. After 3 days, 1 mL of each seed culture was inoculated into 20 mL of 4 different production media in sterile 125-mL Erlenmeyer flasks: SPIN-1 [per liter: 16 g glucose, 12 g soluble starch, 16 g methyl oleate, 4 g Pharmamedia, 8 g peptonized milk, 2 g calcium carbonate, pH 7.0 (Sheo

Singh, personal communication)], FR23 [per liter: 5 g glucose, 30 g soluble potato starch, 20 g cane molasses, 20 g Pharmamedia (Phillips et al. 2011)], ½ X FR23 [1/2 concentration of FR23 (adapted from Phillips et al. 2011)], or ½ X AMM [per liter: 10 g galactose, 10 g dextrin, 5 g Bacto Soytone, 2.5 g corn steep solids, 1 g ammonium sulfate, 1 g calcium carbonate, pH 7.4 (Sawa et al. 2012)]. The flasks were incubated at 28 °C and 250 rpm rotary shaking for 14 days. One milliliter of sterile water was added to each flask every 2 days to account for evaporation.

In order to perform extractions of the fermentations on day 14, 2 mL of whole broth were added to an equal volume of acetone and allowed to mix for 30 minutes before centrifugation for 15 minutes at 2400 x g. One-milliliter of extract supernatant was evaporated to dryness overnight in a chemical fume hood and the residue was resuspended in 1 mL of 100% methanol before use in the disk diffusion assays. Luria Broth (LB) agar plates [10 g peptone, 5 g yeast extract, 5 g sodium chloride, 12 g agar (Thermo Fisher Scientific)] were inoculated with *S. aureus* in PBS at a calculated seeding density of OD_{600} 0.01 (*S. aureus* stock was prepared at a density of OD_{600} = 10 and diluted to 0.01 with PBS); three-milliliters of the PBS/*S. aureus* mixture were washed over the surface of the LB plates. Excess liquid was removed with a serological pipette and the plates were left to dry for about 10 minutes at 23°C. Ten microliters of re-dissolved extract were added to a 6-mm filter paper disk and placed on the surface of the *S. aureus* seeded LB agar plates. Control disks were run with Ampicillin (Amp) and Ciprofloxacin (Cip). The plates were incubated at 37°C 16-18 hours overnight and zones

of inhibition (ZOI) diameters (Figure 3-1) were measured the following morning (Table 3-1).

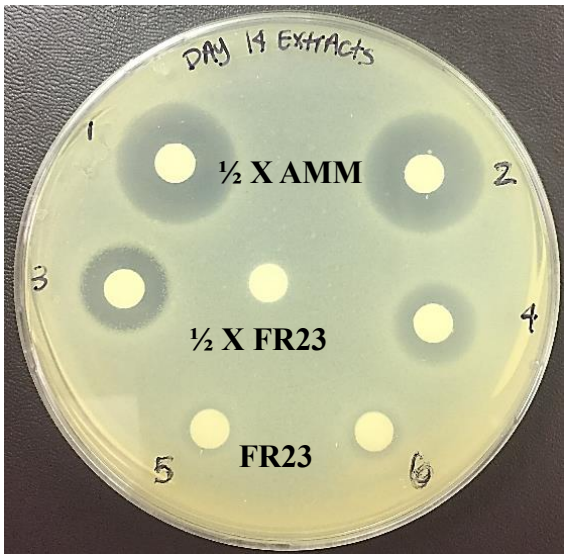

Figure 3-1: Disk diffusion assay on *S. aureus* of kibdelomycin extracts from *Kibdelosporangium* fermented in various seed and production media. (ATCC-2 and SPIN-1 media samples not shown); evens- strain SAM3A, odds- strain F109; center disk- methanol control

Table 3-1: Zone of Inhibition Diameters for Kibdelomycin Extracts

Strain:	Seed Media:	Production Media			
		SPIN-1	FR23	½ X FR23	½ X AMM
F109	ISM-3	16	13	14	17
	ATCC-2	0	7	7	15
SAM3A	ISM-3	10	13	13	19
	ATCC-2	0	12	10	16

Note: *Kibdelosporangium* fermented in various combinations of seed and production media. Extracts were tested against *S. aureus* and ZOI's were measured in millimeters. ZOI's include the diameter of the disk, except in cases where no zone was visible (indicated by zero). ZOI's of 28 and 35 millimeters were observed for Cip and Amp controls, respectively (not displayed in Table 3-1).

While other seed and production media combinations produced zones of inhibition, the best combination was determined to be ISM-3 seed medium and ½ X AMM production medium for both strains tested (Table 3-1). The extracts which show larger ZOI's are presumed to have more kibdelomycin, meaning that *Kibdelosporangium* produces more kibdelomycin when cultured in ISM-3 seed medium and ½ X AMM production medium. Cip and Amp controls were within the published limits (Cip 22-30 mm; Amp 27-35 mm) (BBL Sensi-Disc Antimicrobial Susceptibility Test Discs Zone Diameter Interpretive Chart), demonstrating success in bacterial seeding of the plate.

III. Production Medium Optimization

Steps were taken to optimize the ½ X AMM production medium formulation to further increase the amounts of kibdelomycin being produced by *Kibdelosporangium sp.* Design-Expert® software was used to create a fractional factorial experimental design which varied the concentrations of each component of the medium in order to determine that medium components that had the biggest effect on production.

The five components of the ½ X AMM production medium formulation (galactose, dextrin, Bacto-Soytone, corn steep solids, and ammonium sulfate) were varied at two levels, low or high, compared to the initial formulation above, in a Resolution III, 2^{5-1} fractional factorial design, generated using Design-Expert® software. This design, consisted of 16 media formulations to test to assess which media components have the greatest effect on kibdelomycin production. Two fermentations of the original ½ X AMM formulation (midpoints of each component's high/low variations) were set up as a

control. The experimental media formulations are shown in Table 3-2. One milliliter stock of Kibdelosporangium sp. F109 was inoculated into 20 mL ISM-3 seed medium

Table 3- 2: Formulations for Media Optimization with their Extract's ZOI

Galactose (g/L)	Dextrin (g/L)	Bacto Soytone (g/L)	Corn Steep Solids (g/L)	Ammonium Sulfate (g/L)	**Zone of Inhibition (mm)**
5	5	7.5	3.75	1.5	**0**
5	15	7.5	3.75	0.5	**0**
5	5	2.5	1.25	1.5	**0**
5	15	2.5	3.75	1.5	**0**
5	5	7.5	1.25	0.5	**0**
5	15	7.5	1.25	1.5	**0**
5	5	2.5	3.75	0.5	**0**
5	15	2.5	1.25	0.5	**11**
10	10	5	2.5	1	**11**
10	10	5	2.5	1	**10**
15	5	2.5	3.75	1.5	**16**
15	5	2.5	1.25	0.5	**17**
15	5	7.5	1.25	1.5	**11**
15	15	2.5	3.75	0.5	**16**
15	15	7.5	3.75	1.5	**15**
15	15	2.5	1.25	1.5	**15**
15	5	7.5	3.75	0.5	**13**
15	15	7.5	1.25	0.5	**16**

and incubated at 28 °C and 250 rpm rotary shaking for 3 days. The choice to go forward with strain F109 only was based on better seed growth of this strain and limited incubator capacity. One milliliter of seed was inoculated into 16 125-mL Erlenmeyer flasks containing 20 mL of different production media formulations as well as the two control

formulations with the original formulations of ½ X AMM (Table 3-2). The flasks were incubated at 28 °C and 250 rpm rotary shaking for 14 days. Whole broth extractions and disk diffusion assays were performed as described previously, with the exception that Mueller Hinton agar plates – typically used for disk diffusion assays - were used in place of the LB agar plates. Mueller Hinton agar plates are more standardized for the evaluation of antibacterials via the Kirby-Bauer Susceptibility Test (Hudzicki 2009).

Media formulations with the low concentration of galactose (5 g/L) no zones of inhibition, while formulations with the high (15 g/L) concentration of galactose resulted in larger zones of inhibition (Table 3-2). The mid-point formulations (10 g/L galactose) resulted in zones of inhibition between the highest and lowest (Table 3-2). Zones of inhibition were used in the Design-Expert® software to generate a half-normal plot (Figure 3-2) to reveal which variables had an impact on the production of kibdelomycin.

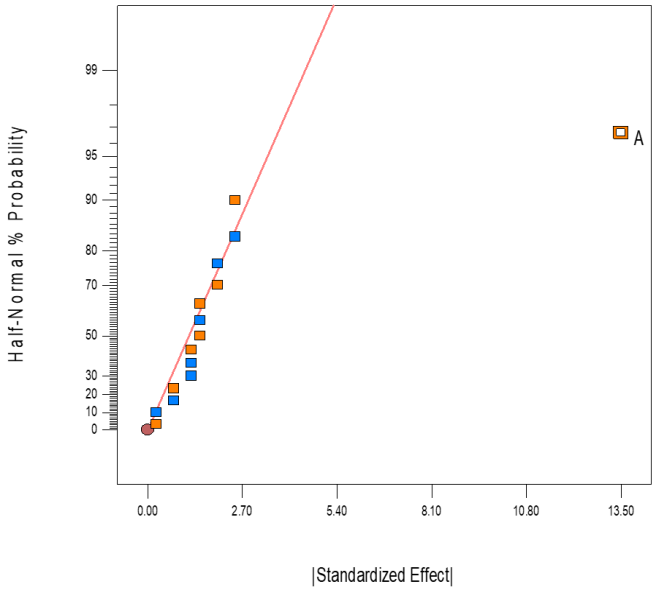

Figure 3-2: Half-Normal plot to determine effect of each component of ½ X AMM production medium. Orange- positive effects, blue- negative effects; A represents galactose (graph created using Design Expert® Software)

Half-normal plot is a linear representation of a normal distribution (i.e. bell-shape curve). Points that fall along the line of best fit represent variables that do not affect the production of kibdelomycin (variables that do not change the normal distribution kibdelomycin production of zone sizes, when fluctuated). Point A, which represents the positive effect of galactose, was the only component within the study that had a statistically significant impact on kibdelomycin production (Figure 3-2). The model equation generated by Design Expert, ZOI = 1.35 (Galactose, g/L) – 5.375, was created with a p value less than 0.0001. This equation indicated that kibdelomycin production increases with higher amounts of galactose in the production medium, and that galactose was the only medium component to impact antibiotic production.

IV. Galactose Optimization

Further studies were performed to determine the ideal concentration of galactose. One milliliter stock of *Kibdelosporangium sp*. F109 was inoculated into 20 mL ISM-3 seed medium and incubated at 28 °C and 250 rpm rotary shaking for 3 days. After which, 1 mL of seed was inoculated into 10 flasks containing 20 mL of ½ X AMM production medium with 10, 15, 25, or 35 g/L concentrations of galactose. Two flasks were made for each concentration, except four flasks were made at the 15 g/L concentration (see below). After inoculation, the flasks were incubated at 28 °C with 250 rpm rotary shaking for 14 days. Sterile water (1 mL) was added every 2 days of fermentation to off-set evaporation. On days 6 and 10, 1 mL of 200 g/L galactose stock (10 g/L final concentration) was added to two of the 15 g/L galactose flasks to test the effect of 35 g/L galactose added incrementally versus adding the same concentration at the beginning of the fermentation.

Extractions were performed at day 10 and day 14 for each sample according to the previous procedure. Disk diffusion assays were performed as described previously, with the exception that the extracts were diluted 1:10 with water before being added to the disks. Cip and Amp disks were again run as controls. Zones of inhibition diameters were measured and are shown in Table 3-3.

Table 3- 3: Zones of inhibition for extracts of *Kibdelosporangium sp.* grown in varying concentrations of galactose

Galactose Concentration	Day 10 Extract ZOI (mm)	Day 14 Extract ZOI (mm)
10 g/L	trace	trace
15 g/L	trace	trace
15 g/L + additions*	11	13
25 g/L	13	14
35 g/L	14	14

Note: Trace signifies a small, unmeasurable zone surrounding the disk. Cip and Amp ZOI's were measured at 26 mm and 35 mm, respectively.

*A 1mL addition of 200 g/L galactose was added at days 6 and 10 bringing the final added concentration of galactose to 35 g/L

With each increase in galactose concentration, more kibdelomycin was produced, with the exception of the day 14 extracts at 25 and 35 g/L (Table 3-3); there appeared to be no difference in ZOI's for 14-day extracts from samples grown in 25 and 35 g/L galactose. Adding 35 g/L galactose at the beginning of the fermentation did not seem to reduce kibdelomycin production (compared to incremental additions of galactose) based on ZOI data (Table 3-3). Cip and Amp controls were, once again, within the published limits (BBL Sensi-Disc Antimicrobial Susceptibility Test Discs Zone Diameter Interpretive Chart), demonstrating success in bacterial seeding of the plate. The effect of

galactose concentration on kibdelomycin production did not appear to have a maximum; however, for future experiments, the optimized ½ X AMM medium was adjusted to contain 35 g/L galactose as a means of remaining cost and time efficient. Within the range of our study, 35 g/L of galactose produced the highest concentration of kibdelomycin in ten days.

This experiment developed an optimized media formulation for the increased production of kibdelomycin. Overall, it helped to find effective seed and production media as well as an establish fermentation and extraction procedures, necessary for the future development of kibdelomycin.

Chapter 4: Strain Improvement

I. Introduction

Although kibdelomycin is naturally produced by *Kibdelosporangium sp*., the observed titer (from preliminary LC-MS screenings of extracts from the previous experiment) in optimized media did not appear to be sufficient to produce enough material for subsequent structural modification experiments (data not shown). The future development of kibdelomycin as an antibiotic requires large amounts of kibdelomycin to be purified in order to carry out chemical synthesis experiments. One method of increasing natural product production is to improve the strain's capability of producing more antibiotic (Hu and Ochi 2001).

Strain improvement involves increasing production of natural products through the introduction of genetic variability - i.e., mutation(s)- followed by selection and/or screening for improved production. Past methods involve treating bacterial strains with chemical agents or UV light that induce random mutations (Parekh et al. 2000). Surviving strains were then screened in small-scale fermenters to determine if the mutations resulted in an increase in natural product production. Those with increased production become the starting point for a subsequent round of strain improvement. However, the frequency of improvement using these methods is usually ≤0.1% per round. With this frequency of strain improvement, the process can be very long and inefficient and requires the screening of a large number of strains.

A different method strain improvement that has proven to effectively increase the rate of occurrence of improved actinomycete strains is the selection of cumulative aminoglycoside antibiotic resistance mutations, which has resulted in a 5-10% frequency of improved strains (Hu and Ochi 2001). This method involves the introduction of multiple, sequential antibiotic resistance mutations into the genome of an actinomycete strain. Hu and Ochi first used this technique in 2001to increase actinorhodin production in *Streptomyces coelicolor* strain A3(2). *S. coelicolor* was grown on agar containing streptomycin and spontaneous resistant colonies formed. The best actinorhodin producers from streptomycin selection were then taken forward to select for gentamicin resistance followed by screening for increased actinorhodin production. The best actinorhodin producer from gentamicin selection was then selected for rifampin resistance. With each round of antibiotic resistance selection, the strain gained mutations that resulted in sequential increases in actinorhodin production. In the end, the triple antibiotic-resistant mutants were producing up to 48 times more actinorhodin than the wildtype strain. This strain improvement method was extended to select for combined resistance to paramomycin, geneticin, fusidic acid, thiostrepton, and lincomycin starting with the streptomycin-gentamicin-rifampin triple resistant mutant of *S. coelicolor* as the starting point (Wang et al. 2008). This study showed up to a further 10-fold increase in actinorhodin production. The genetic mutations in these strains have been studied and researchers discovered that mutations in the *rpsL* and *rpoB* ribosomal genes allowed for drug resistance and correlated with the increase in antibiotic production (Hu and Ochi 2001). Many of the antibiotics used in this method target the ribosome, so it makes sense

that ribosomal mutations would allow for resistance to the drugs. However, it is still unclear as to why these ribosomal mutations would lead to increased natural product (antibiotic) production. Most importantly, this was a more successful method of increasing natural product production of a bacterial strain than random mutagenesis and screening. The generation of cumulative antibiotic resistance has been shown to be a great technique to increase the amount of natural product being produced.

Kibdelosporangium sp, like *Streptomyces*, is an actinomycete and implementing the technique of ribosome engineering could have similar benefits for increasing the production of kibdelomycin. We chose to select for streptomycin and gentamicin resistance, in accordance with the original study by Hu and Ochi in 2001. Streptomycin and gentamicin are both aminoglycoside antibiotics which target the ribosome and inhibit protein translation (Hu and Ochi 2001). A successful result would yield a multi-resistant mutant strain that is resistant to both antibiotics and has increased production of kibdelomycin with each successive round of selection.

II. Streptomycin Resistance Selection

One milliliter of F109 and SAM3A stocks were thawed and inoculated into 20 mL of ISM-3 seed medium in sterile 125-mL flasks. The flasks were incubated at 250 rpm rotary shaking and 28 °C for 3 days before being diluted 1:5 in sterile deionized water. Aliquots (0.1 mL) were spread onto ISP-2 agar plates with different concentrations of streptomycin antibiotic (0, 25, 50, 75, and 100 mg/L). These concentrations were decided upon after starting with the concentrations used by Hu and Ochi (2001) and

increasing the concentration until isolated colonies were produced (data not shown). The plates were incubated at 28 °C for around 7 days.

The amount of confluent biomass or isolated colonies growing on the plates decreased as a function of increasing streptomycin concentration: isolated colonies grew on the plates with higher concentrations of antibiotic while lawn growth was observed on the plates with lower concentrations (Figure 4-1).

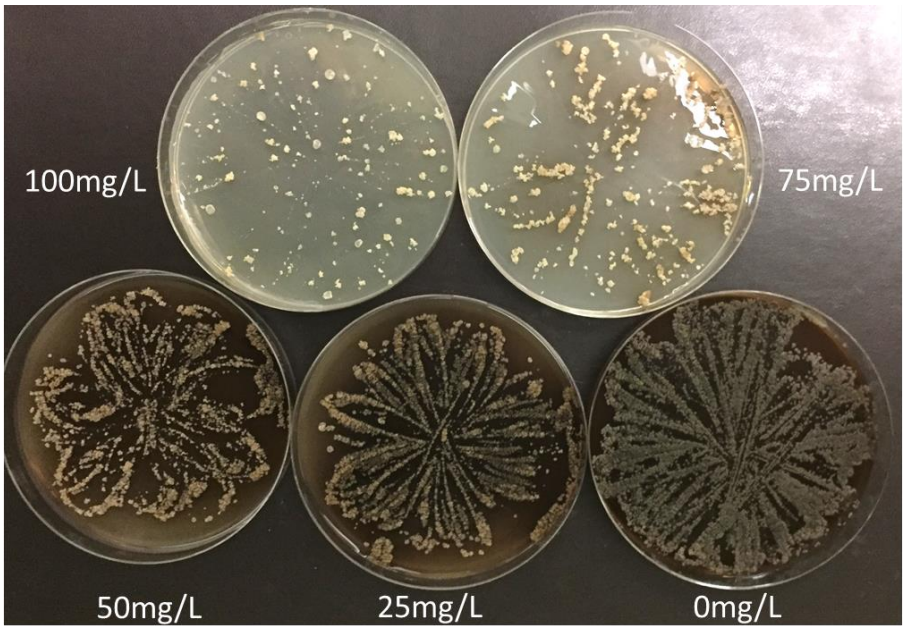

Figure 4-1: *Kibdelosporangium* sp. grown on LB agar plates containing different concentrations of streptomycin

Isolated colonies, or resistant strains, were to be screened for increased production of kibdelomycin. To do this, isolated colonies were transferred from the 75 and 100 mg/L plates with sterile loops and spread onto separate ISP-2 plates containing no antibiotic. These plates were incubated at 28°C for about one week until lawn growth appeared. Two 6-mm diameter agar plugs (removed with transfer tubes) of each lawn were

inoculated into 125-mL flasks containing 20-mL of ISM-3 seed medium and grown for 4 days (250 rpm, 28 °C) before 1 mL of seed was transferred to 125-mL flasks containing 20 mL optimized ½ X AMM production medium – i.e., 35 g/L galactose. These flasks were incubated for 10 days (250 rpm and 28 °C).

The kibdelomycin was then extracted from the fermentations. The extraction methods were altered based on experiments that showed that methyl ethyl ketone (MEK) provided a cleaner extract than acetone because MEK is a more nonpolar organic solvent, so less compounds are soluble in it (unpublished data, Connors and Ortega). A biomass extraction was performed instead of a whole broth extraction because kibdelomycin was found to be produced intracellularly, and not secreted into the production media (data not shown). Aliquots (10 mL) of broth from each flask were centrifuged at 2400 x g for 15 minutes to separate the biomass from the supernatant medium. The supernatant was removed, and the biomass was extracted using 2.5 mL of MEK. The mixture was centrifuged at 2400 x g for 15 minutes to separate the solid biomass and MEK layer. A 1-mL aliquot of the MEK layer was removed and evaporated to dryness. The residue was reconstituted in 1 mL methanol, centrifuged at 9400 x g to remove any insoluble material and then 750 µL of supernatant was transferred to an auto-sampler vial and subsequently analyzed by LC-MS. The extracts were run on a Waters 2695 Separations Module LC-MS with an Echelon C18 column [100 x 4.6mm, 4µm particle size] at a 1.5 mL/min flow rate using a gradient of 0.1% formic acid/H_2O (A) and acetonitrile (B): 0-1 min 50% B, 1-11 minutes increase to 90% B, 11-11.5 min 90% B, 11.5-12 min decrease to 50%B, 12-

15 min 50% B. Mass spectrometry was run in positive ion mode (M+H) with electrospray ionization.

LC-MS analysis was used in place of disk diffusion assays to quantify relative amounts of kibdelomycin being produced as well as confirm that the bio-activity of the assays was the result of kibdelomycin. Extracts can be analyzed via LC-MS to also give ultraviolet (UV) profiles, which display unique peaks for each compound within the extract, as well as mass spectrometry, which displays molecular weight of the parent ion and the molecular weights of any ions it may fragment into.

The UV profile and mass spectrometry of the extracts aligned with that of kibdelomycin which had been previously published (Phillips et al. 2011). Kibdelomycin's UV profile shows a maximum at 280 nm with a shoulder around 240 nm (Figure 4-2A). Mass-spectrometry reveals a parent ion of 939 Da (M+H) and fragment ions at 591 Da (fragment A) and 331 Da (fragment B) (Figure 4-2B). Because the UV profile and mass-spectrometry pattern match those that have previously been published for kibdelomycin, the compound within our extract can be identified as kibdelomycin.

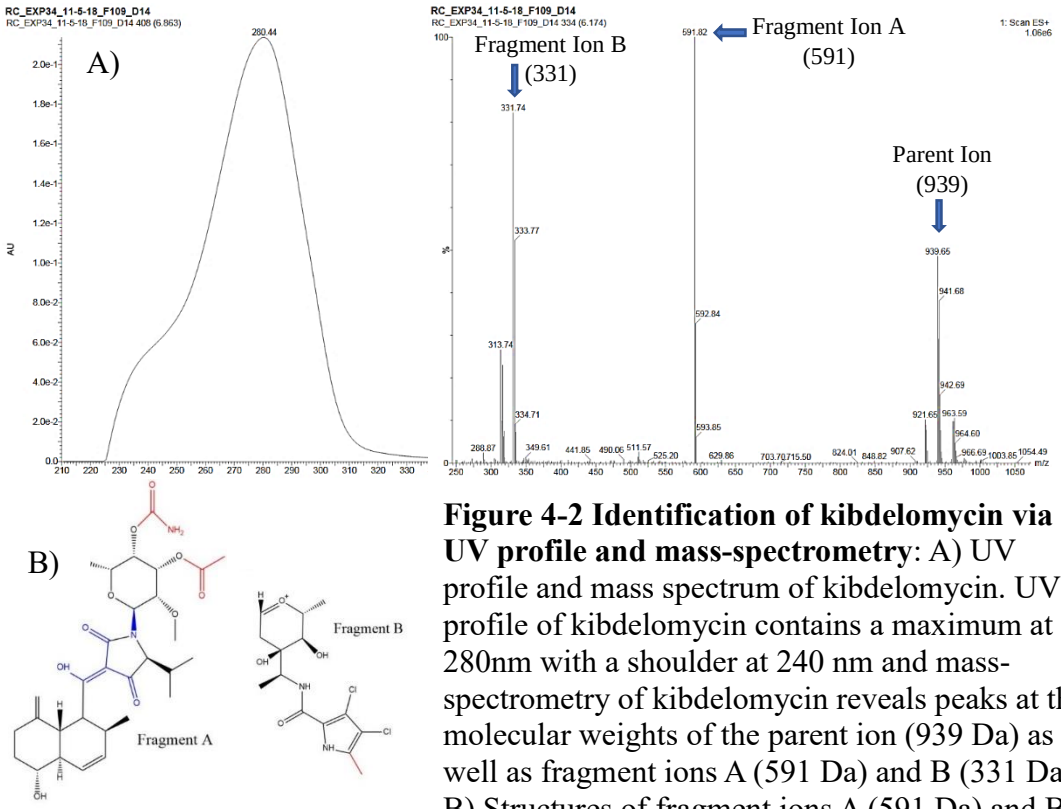

Figure 4-2 Identification of kibdelomycin via UV profile and mass-spectrometry: A) UV profile and mass spectrum of kibdelomycin. UV profile of kibdelomycin contains a maximum at 280nm with a shoulder at 240 nm and mass-spectrometry of kibdelomycin reveals peaks at the molecular weights of the parent ion (939 Da) as well as fragment ions A (591 Da) and B (331 Da); B) Structures of fragment ions A (591 Da) and B (331 Da), formed during mass spectrometry analysis

LC-MS analysis of fermentation extracts revealed production of kibdelomycin and several potential analogues based on molecular weight and fragmentation patterns. These analogues share characteristic UV profiles of kibdelomycin but differ in retention time and molecular weight as well as mass spectrometry fragmentation patterns (to be discussed further in Ch. 5). Without a known concentration standard for kibdelomycin, exact titers in mg/L could not be calculated for each extract. Instead, total UV area counts were used to determine and compare relative amounts of kibdelomycin in the extracts by using the combined area counts of kibdelomycin as well as potential analogues. UV area counts were graphed during LC-MS analysis as curves for each unique compound found in the extract. The area underneath each curve can be used to quantitate relative amounts

of each compound and compare them. Kibdelomycin production was assessed via total UV area counts. Sixty streptomycin-resistant strains (30 SAM3A, 30 F109) were extracted; five appeared to be high-producing compared to the control (had a total area count 5,500,000 greater than the control- an arbitrarily chosen value) (Figure 4-3). One of

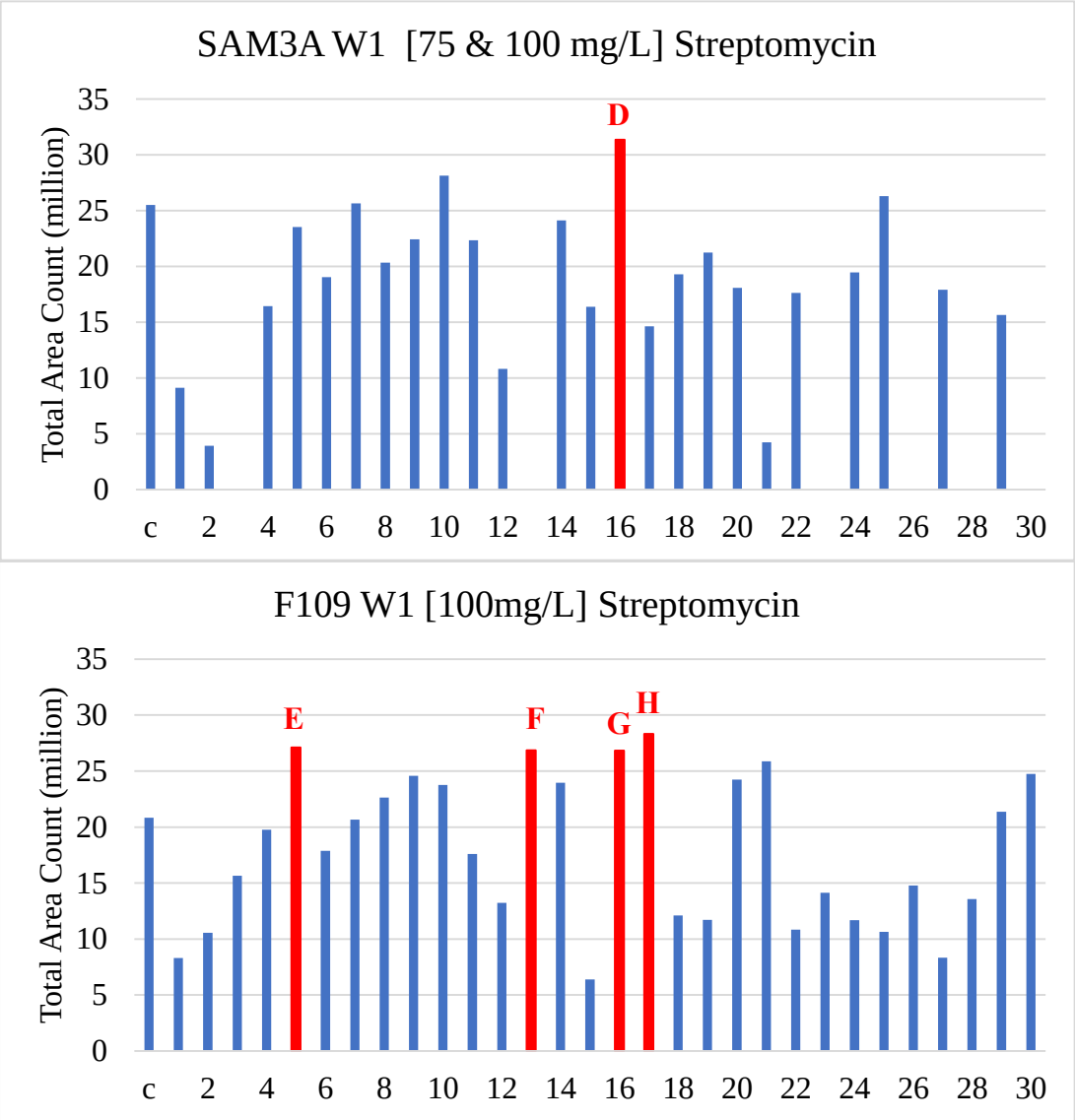

Figure 4-3: Total UV area counts of kibdelomycin production for streptomycin resistant strains. Samples in red showed increased production of kibdelomycin compared to their relative controls.

these putative high producers came from the SAM3A strain while the other four came from F109. They were arbitrarily named D, E, F, G, and H, as depicted in Figure 4-3.

III. Streptomycin Resistance Verification

Stock vials of the high-producing strains were made according to the previously described procedure starting with two 6-mm agar plugs from the ISP-2 plates instead of frozen stocks to inoculate the ISM-3. The seeds were incubated for 4 days instead of 3, because inoculation with agar plugs required a longer seed fermentation to produce a seed density similar to when frozen stocks were used for inoculation.

More experimentation was necessary to validate the improvement of the five strains. Further analysis of the primary screening of strain D (from SAM3A parent) showed that higher ratios of kibdelomycin analogs were being produced compared to kibdelomycin (data not shown), so the strain was not pursued further, as the main focus of the project was increasing production of kibdelomycin. Strains E, F, G, and H, isolated from strain F109 grown on ISP-2 plates containing 100 mg/L streptomycin were grown and compared to the control culture F109 to verify improved production. One milliliter of each frozen stock was thawed and inoculated into 20 mL of ISM-3 seed medium in sterile 125-mL flasks in replicate (n = 5). The flasks were incubated at 250 rpm rotary shaking and 28 °C for 3 days before 1mL of seed was transferred to 125-mL flasks containing 20 mL optimized ½ X AMM production medium with 75 mmol MOPS buffer. A separate line of experimentation revealed that more kibdelomycin was produced when the fermentation was kept above a pH of 5.5-6 which could be maintained by 75 mmol

MOPS buffer (unpublished data from Connors and Ortega). After 10 days incubation, 2.5 mL of fermentation broth was mixed with 2.5 mL of MEK for 30 minutes. Whole broth extractions were performed because additional experimentation on the extraction procedure showed that whole broth extraction with MEK led to a more consistent extraction of kibdelomycin between samples (i.e., higher precision) than biomass extraction (data not shown). All subsequent experiments utilized this extraction method. The mixture was centrifuged at 2400 x g for 15 minutes to separate the MEK layer. In a 1-mL aliquot, the MEK layer was removed and was evaporated to dryness, while the remainder was reconstituted in 1 mL methanol, and centrifuged at 9400 x g. The supernatant (750 µL) was subsequently analyzed by LC-MS according to the previously described method. Of the strains, only strain F was found to produce 13% more kibdelomycin than the F109 control strain (Figure 4-4).

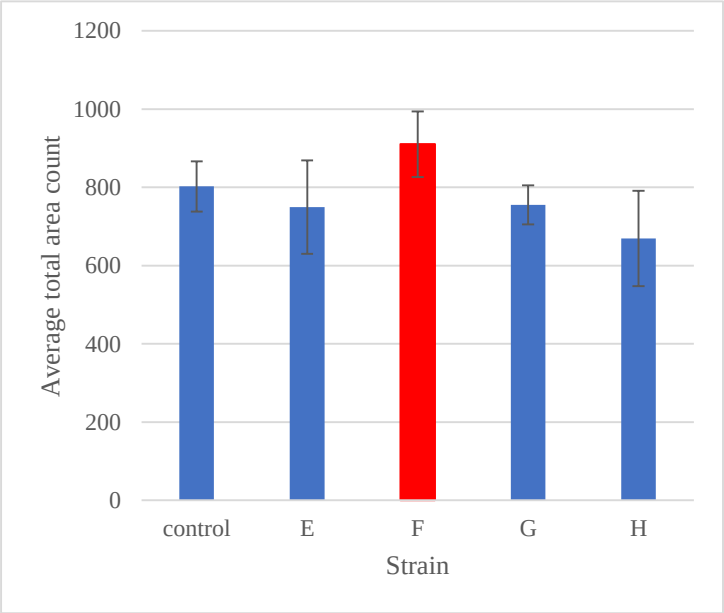

Figure 4-4: Average total area counts of kibdelomycin production for streptomycin-resistant strains compared to control (strain F109)
n=5, error bars represent +/- one standard deviation

Surprisingly, strain F was the only strain that appeared to produce more than the control, despite preliminary screens showing all four to be higher producers. The error bars associated with each area count represent standard deviations. While there is some overlap between F and the control strain (F109), F appears to be producing more kibdelomycin than the control. Strains E, G, and H on the other hand appear to produce as much if not less kibdelomycin than the control F109. Although the differences in kibdelomycin were not found to be significant, Strain F was taken forward to select for gentamicin resistance.

IV. Gentamicin Resistance Selection

Strain F was used as a starting point to develop a strain that was both streptomycin-resistant and gentamicin-resistant. Strain F from the streptomycin selection was grown in 20 mL of ISM-3 seed medium, in 125-mL flasks. The flasks were incubated at 250 rpm and 28 °C for 3 days before being diluted 1:5 with sterile deionized water. Aliquots (0.1 mL) of the diluted seed were spread on ISP-2 agar plates containing various concentrations of gentamicin (0, 50, 100, 200, and 300 mg/L). These concentrations were again decided upon after starting with the concentrations used by Hu and Ochi (2001) and increasing the concentration until isolated colonies were produced (data not shown). The plates were grown at 28 °C for about 7 days, after which, isolated colonies from the 300 mg/L gentamicin concentration plates were transferred with sterile loops and spread onto ISP-2 plates without antibiotic. These plates were grown for 7-10 days before lawn growth appeared. Two 6-mm circular agar plugs from lawns produced on these plates were inoculated into 20 mL ISM-3 seed medium in 125-mL flasks and

incubated at 28 °C 250 rpm. One milliliter aliquots of seed were inoculated into 20 mL optimized ½ X AMM production medium with 75 mmol MOPS buffer in 125-mL flasks and after 10 days, whole broth extractions were performed, and the extracts were analyzed via LC-MS using the previously described method.

Total area counts for each extract are displayed in Figure 4-5. Because no control was run with the experiment, the three highest producing samples, F12, F16, and F18, were selected to move forward (Figure 4-5). Without the control strain, relative improvement could not be evaluated.

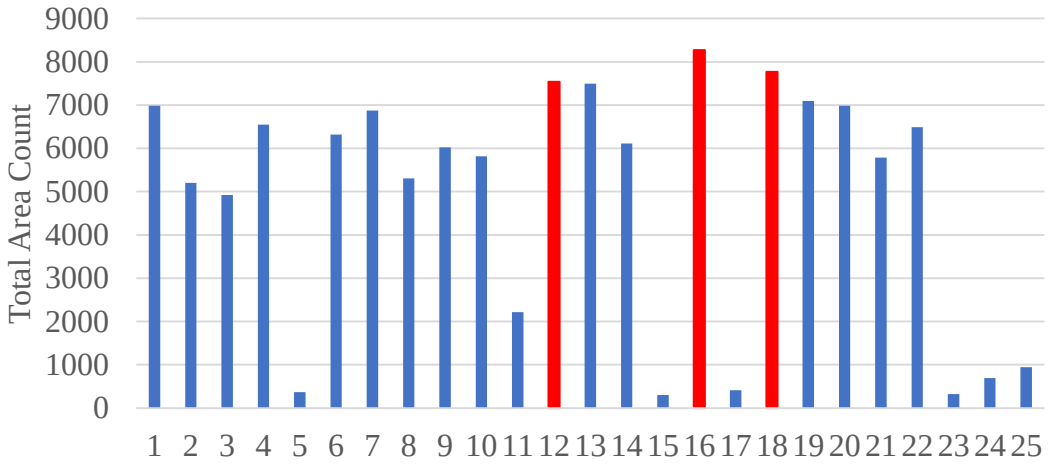

Figure 4-5: Total UV area counts of kibdelomycin production for gentamicin resistant strains. Red indicates high-producing mutant strains; n=1; data from Stine.

V. Gentamicin Resistance Verification

Stock vials were created for later use to verify improved kibdelomycin production. Two 6-mm agar plugs from the original plates for each strain with lawn growth were inoculated into 20 mL ISM-3 seed medium in 125-mL flasks and incubated at 28 °C and 250 rpm rotary shaking. After 4 days, the seed was mixed with 60% (v/v)

glycerol at a 5:1 ratio (seed: 60% glycerol) to give a 10% final glycerol concentration. The mixture was subdivided into 1 mL aliquots in cryogenic vials and stored at -80°C.

Increased production of strains F12, F16, F18 were verified by re-growing them in replicate (n = 5) in comparison to the F109 parent culture. One milliliter of each frozen stock was thawed and inoculated into 20 mL of ISM-3 seed medium, in sterile 125-mL flasks. The flasks were incubated with 250 rpm rotary shaking and 28 °C for 3 days before 1 mL of seed was transferred to 125-mL flasks containing 20 mL optimized ½ X AMM production medium with 75 mmol MOPS buffer. After 10 days of incubation, whole broth extractions were performed and subsequently analyzed by LC-MS according to the previously described method.

Total area counts showed strain F16 produced 50% more kibdelomycin than the F109 control (Figure 4-6). Strain F12, upon verification, did not have improved production of kibdelomycin. The error bars associated with strain F18 overlap with those of the control, indicating that any improvement may not be significantly greater than the control F109 strain.

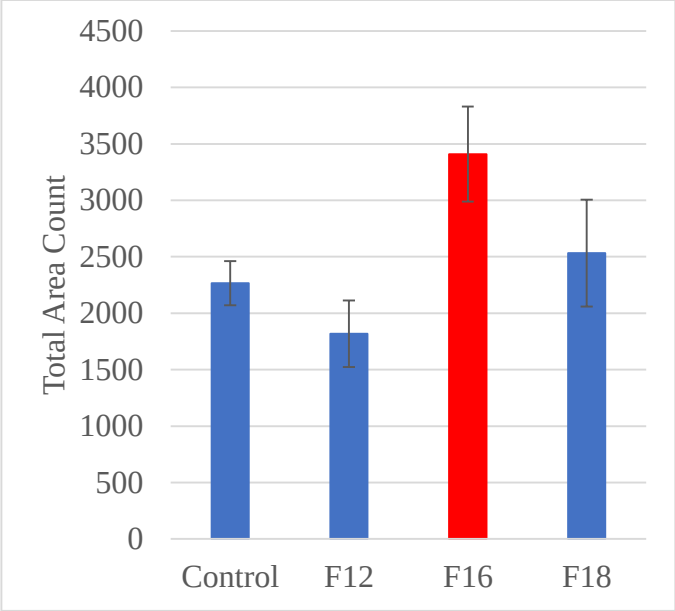

Figure 4-6: Average total UV area counts of kibdelomycin production for streptomycin, gentamicin-resistant strains
n=5, error bars represent +/- one standard deviation

VI. Yield Comparison

A final experiment was performed to compare the kibdelomycin production of the wild type strain, streptomycin resistant (single) mutant, and the streptomycin-gentamicin resistant (double) mutant. One milliliter aliquots of each stock were inoculated into 125-mL flasks containing 20 mL of ISM-3 seed medium. They were incubated at 28°C and 250 rpm rotary shaking. After 3 days, 1 mL of seed for each was inoculated into 20 mL of optimized ½ X AMM with 75 mM MOPS buffer in separate 125-mL flasks. They were incubated at 28°C with 250 rpm rotary shaking for 10 days. Extractions were performed and analyzed via LC-MS as previously described. The comparative total area counts were recorded.

A small amount of purified kibdelomycin was available at this time (Stine) and used to make a standard curve to convert total area counts into titers. The purification was performed at a semi-preparative scale, using reverse phase chromatography on a C18 column and a method similar to the analytical method described previously (unpublished data from Connors and Stine). Pure kibdelomycin (3.2 mg) was weighed out and dissolved in 10 mL of 100% methanol. Dilutions were prepared in 100% methanol to create 256, 160, 96, and 32 mg/L standard concentrations. All dilutions were analyzed via LC-MS on the kibdelomycin 15-minute gradient method. Results can be seen in Figure 4-7A. Kibdelomycin titer in samples was calculated from total area counts using the equation: $Total\ Area\ Count\ = 5.7 \times (kibdelomycin\ yield)$ (Figure 4-7A). The equation was used to convert total area counts for each strain into titer in mg/L (Figure 4-7B).

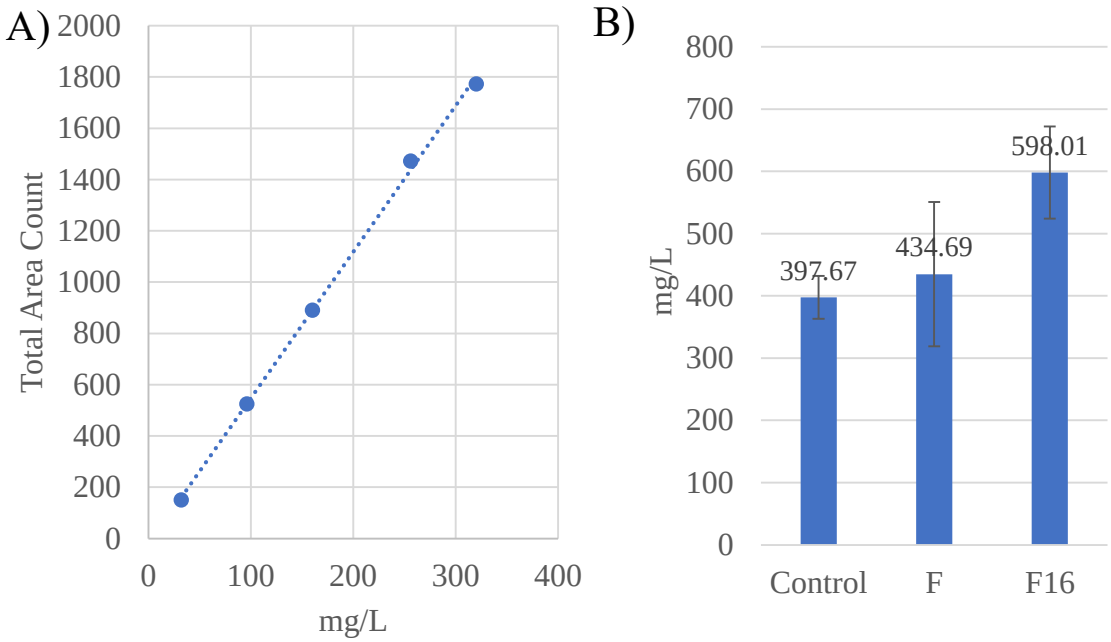

Figure 4-7: Kibdelomycin standard curve and kibdelomycin yield of various strains A) Standard curve relating total area count to mg/L of kibdelomycin, total area count = 5.7 x (kibdelomycin yield), and B) Comparison of kibdelomycin yield between strains in milligrams per liter; n=5, error bars represent percent coefficient of variation, control is strain F109

The kibdelomycin standard curve was key in understanding the amount of kibdelomycin being produced by each strain. Strain F16 clearly exhibits improved production of kibdelomycin compared to the control F109 strain (Figure 4-7B). A 50.4 % increase in kibdelomycin production was seen between strain F16 (streptomycin and gentamicin resistant) and the control (Figure 4-7B). It is unclear whether strain F is significantly improved compared to the control; however, it is likely that the result of strain F16 is contingent on the mutation arisen in strain F.

Further studies could attempt to add rifampin resistance, using F16 as a starting point, thus creating a triple mutant. Ochi and colleagues layered resistance to several antibiotics within their strains to increase production of natural products (Hu and Ochi 2001; Wang et al. 2008; Tanaka et al. 2013). *Kibdelosporangium* could possibly be pushed further to create even more kibdelomycin by continuing to use this method of strain improvement.

DNA sequencing of the control and mutant *Kibdelosporangium* strains –either whole genome or limited to rRNA genes- would provide insight into the specific mutations that took place within *Kibdelosporangium* that resulted in streptomycin and gentamicin resistance. However, it would leave the question of how these mutations increase production of kibdelomycin. Although knowing which specific mutations that occurred would be interesting, the differences in strains most likely depends on the expression of the genes.

Another direction for future work would be determining global gene expression levels using RNA-Seq. RNA-Seq is a method of analyzing an organisms' transcriptome,

or complete set of transcripts (Wang et al. 2009). Gentamicin. We hypothesize that the ribosomal mutations, revealed by Ochi (Hu and Ochi 2001) to cause resistance to streptomycin and gentamicin, may play a role in regulating global transcription of the biosynthetic gene cluster responsible for kibdelomycin. For this reason, RNA-seq would provide more information on the reason that the improved strains produce more kibdelomycin.

Chapter 5: Kibdelomycin Analogues and Fermentation Time Course

I. Kibdelomycin Analogue Discovery

Throughout the media development and strain improvement experiments, a number of new kibdelomycin structural analogues were proposed based on the characteristic UV profile of kibdelomycin but differences in retention time, parent ion molecular weight, and mass spectrometry of fragment ions published by Phillips and colleagues (2011) and Singh and colleagues (2012). Based on LC-MS analysis of fermentation extracts, the kibdelomycin molecule is hypothesized to vary at four different sites (Figure 5-1): the carbamoyl group on the epiallose residue, the acetyl group on the epiallose residue, the methyl on the pyrazole, and tautamerization of the tetramic acid

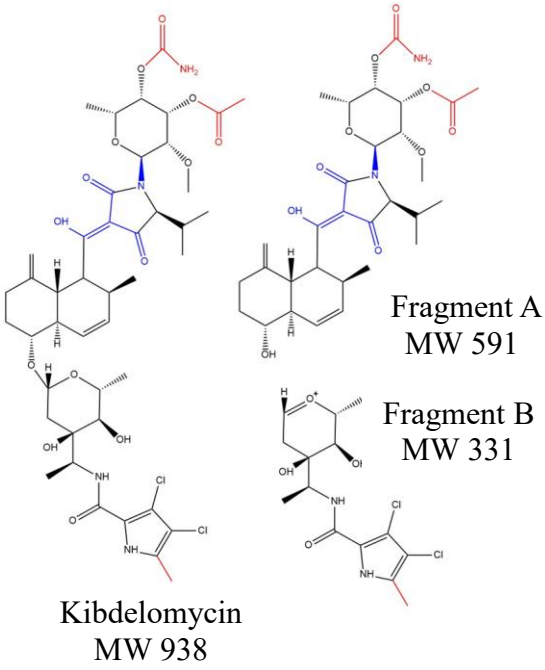

Figure 5-1: Kibdelomycin structure, along with fragment ion. Blue shows areas of suspected tautomerization (about the carbonyl and hydroxyl groups or the N-glycosidic bond); red indicates structures that are removed in analogues.

core. These same modifications are seen in mass spectrometry analysis of the respective fragment ions.

The existence of a kibdelomycin tautomer (Iso-kibdelomycin) is indicated by LC-MS. A molecule with the same molecular weight of kibdelomycin is eluted over half a minute later (6.8 min) than the elution of kibdelomycin (6.2 min), and appears to have a lower ratio of fragment ion A to fragment ion B in mass spectrometry (Figure 5-2). The reason for this ratio difference is undetermined; it is used mainly as an identifier of Iso-kibdelomycin. We believe it is a kibdelomycin tautomer, meaning a molecule with the same molecular formula as kibdelomycin but with different connectivity. The site at which the molecule may produce this tautomer is unknown. The tautomer may be simple switch in the stereochemistry of the nitrogen within the glycosidic bond attaching the

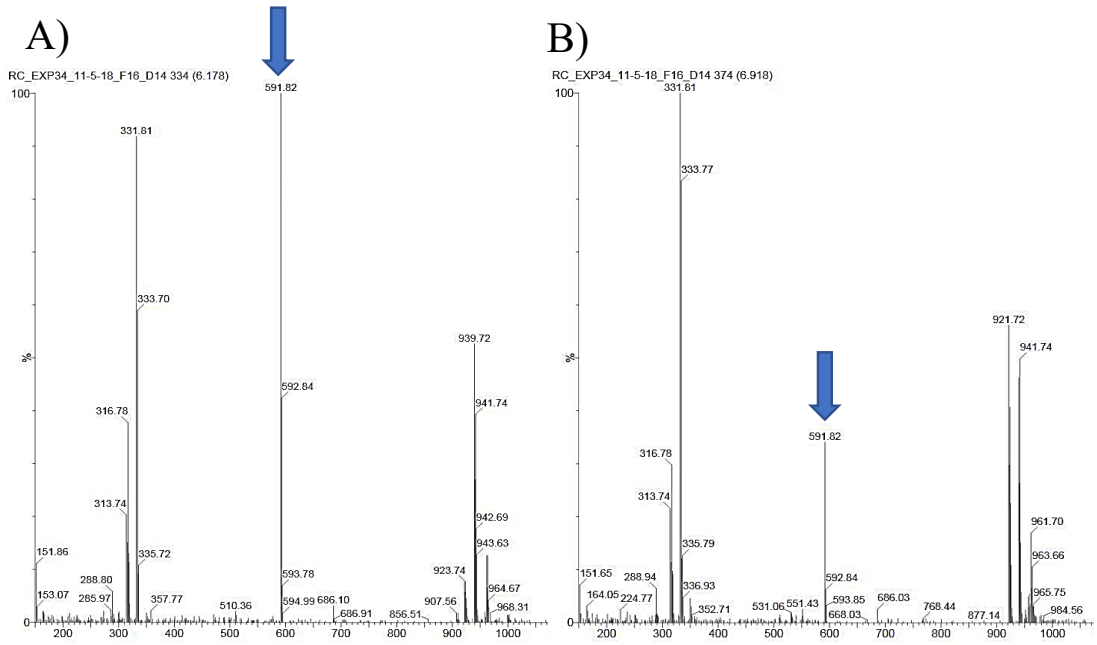

Figure 5-2: Comparing mass spectrometry for kibdelomycin and iso-kibdelomycin
A) Mass spectrometry of kibdelomycin, Retention Time (RT) = 6.2 min; B) Mass spectrometry of iso-kibdelomycin. Arrows in both indicate points of comparison for relative abundancy of fragment ion A (MW=591 Da), RT = 6.8 min.

epiallose residue to the tetramic acid core or enolization of the ketones of the tetramic acid moiety. Several tautomer structures are hypothesized in Figure 5-3. More experiments need to be done to determine the true structure of this iso-kibdelomycin analogue.

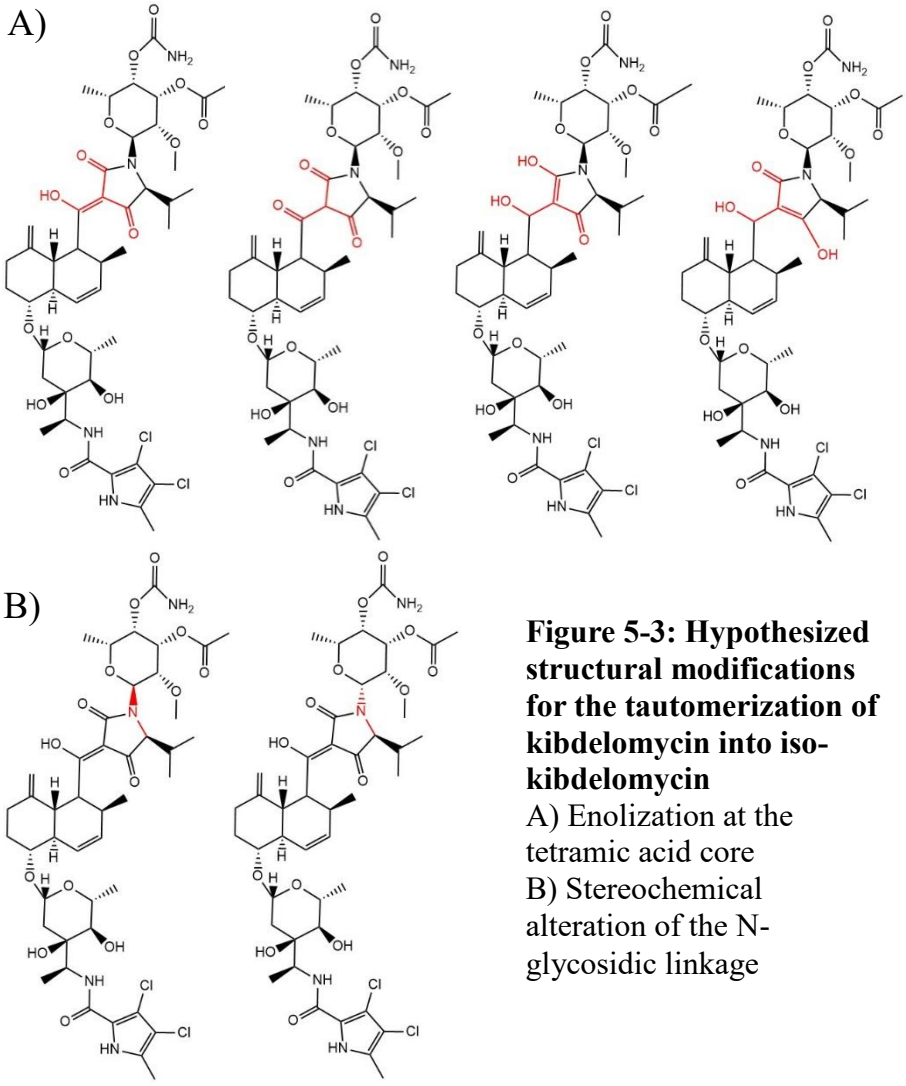

Figure 5-3: Hypothesized structural modifications for the tautomerization of kibdelomycin into iso-kibdelomycin
A) Enolization at the tetramic acid core
B) Stereochemical alteration of the N-glycosidic linkage

Only one analogue has been described previously: 'Kibdelomycin A' which is a desmethyl analogue described by Singh and colleagues (2012); this compound should not

to be confused with fragment ion A which is a fragment created in LC-MS analysis conditions and not a compound created in fermentation. In our extracts, a kibdelomycin analogue elutes at 5.5 minutes with a molecular weight of 925 Da, which is consistent with the previously published desmethyl analogue (Figure 5-4). Fragment ion A remained at the molecular weight 591 Da while the molecular weight of fragment ion B (331 Da M+H) was reduced by 14 Da to 317 Da, indicating the loss of the methyl group from the fragment B portion of the kibdelomycin molecule (Figure 5-4). The change in the molecular weight of the parent ion and fragment ions were consistent with replacing a methyl group with a hydrogen (-14 Da from 939 M+H kibdelomycin molecule). A methyl group consists of a carbon and three hydrogens, which combined total 15 Da. If

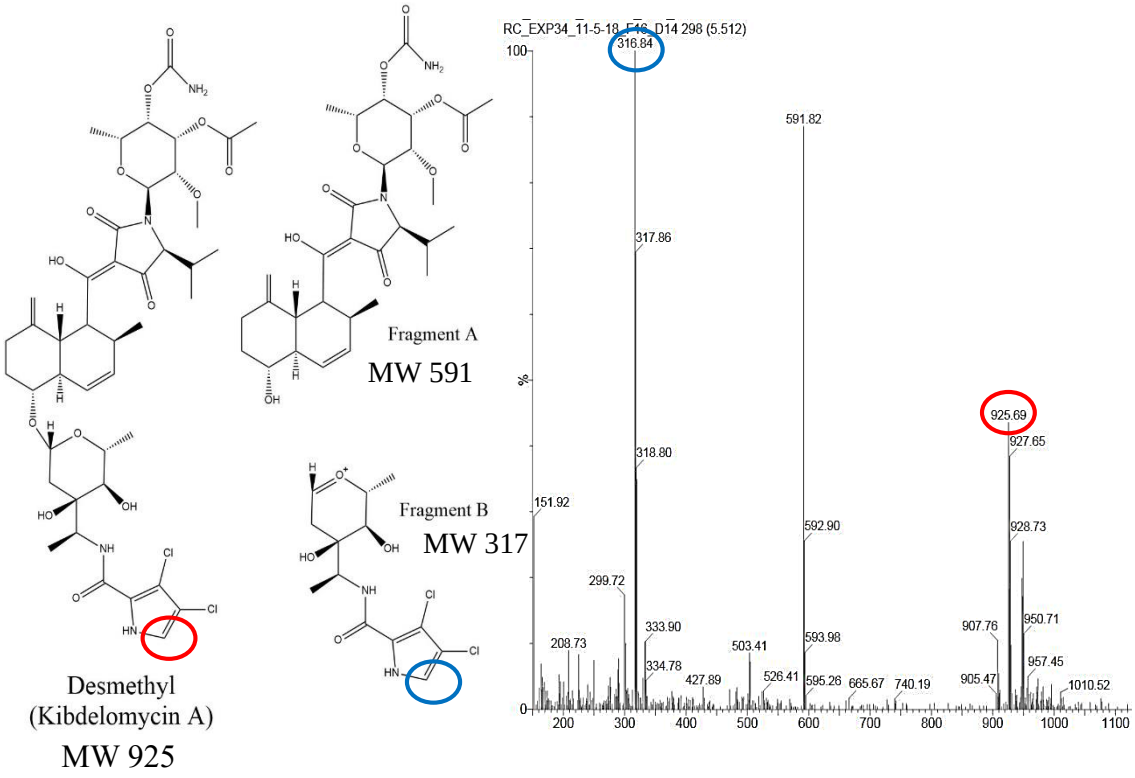

Figure 5-4: Structure and mass spectrometry of desmethyl kibdelomycin. RT = 5.5 min. Red circles highlight the loss of a methyl group in the parent ion while red circles highlight the loss of a methyl group in Fragment B.

this group is removed from kibdelomycin and replaced with a hydrogen, the net loss in molecular weight would be 14 Da, as seen in both the parent ion and fragment ion B during LC-MS analysis (Figure 5-4). The proposed structure of desmethyl kibdelomycin is shown in Figure 5-4.

Next, LC-MS revealed additional analogues which are believed to be a putative desacetyl and a putative desacetyl-descarbamoyl kibdelomycin. The desacetyl analogue produced a parent ion with a molecular weight of 897 Da and eluted at 4.6 minutes (Figure 5-5). Fragment ion A was reduced to 549 Da. These are consistent with the loss of an acetyl group (-42) from the epiallose moiety from the fragment A portion of the molecule. Surprisingly, two molecular weights for fragment B were detected: 317 Da and

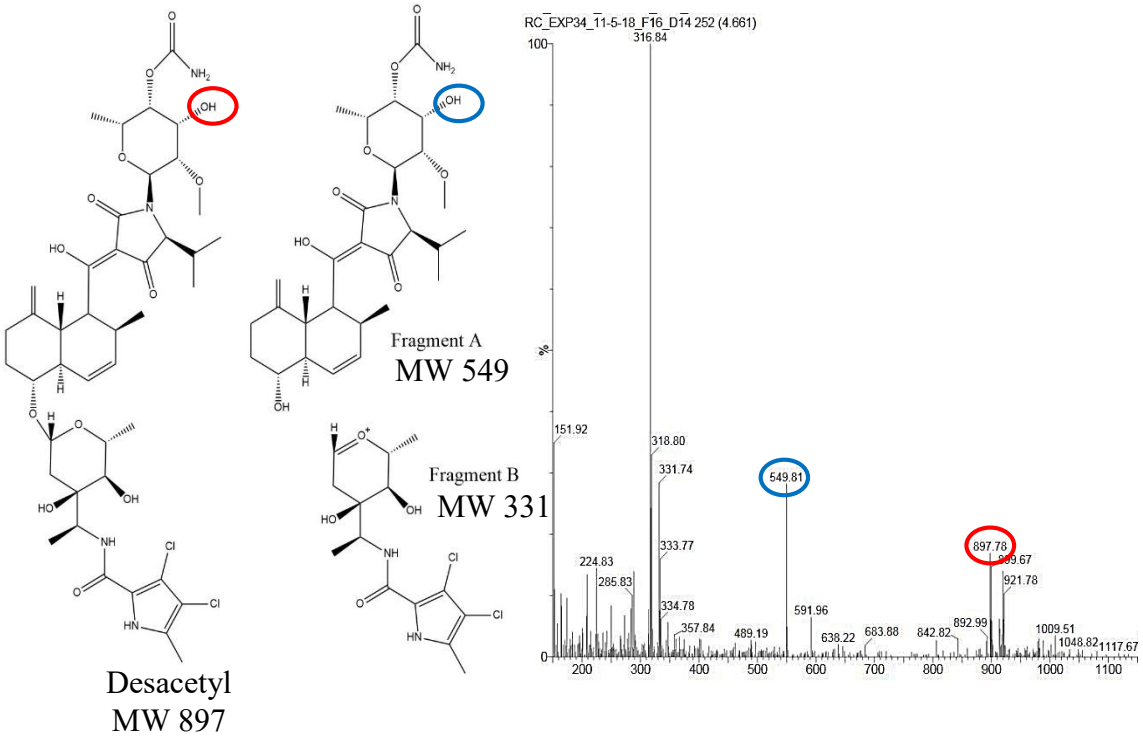

Figure 5-5: Mass spectrometry and proposed structure of desacetyl kibdelomycin
RT = 4.6min; Red circles highlight the loss of an acetyl group in the parent ion while blue circles highlight the loss of an acetyl group in fragment ion A. Fragment ion B can be found at both 317 Da and 331 Da.

331 Da. This would be consistent with a mixture of desacetyl and desmethyl desacetyl analogues eluting together. Trace amounts of an 883 Da parent ion (-56 Da) supported this hypothesis (883 Da parent ion not visible in Figure 5-5). The putative desacetyl descarbamoyl kibdelomycin analogue with an 854 Da parent ion (-85 Da) eluted at 5.7 minutes (Figure 5-6). Fragment A also had an 85 Da molecular weight loss consistent with the loss of both the acetyl and carbamoyl groups from the epiallose residue. However, fragment B appeared to be another mixture of 331 Da and 317 Da. We hypothesized that a desmethyl desacetyl descarbamoyl kibdelomycin analogue elutes at the same time, giving rise to the 317 Da fragment ion B, but mass spectrometry did not reveal a parent ion with a molecular weight of 840 Da that would be consistent with this proposed analogue. More extracts will need to be analyzed to confirm or deny the co-elution of a desmethyl desacetyl descarbamoyl analogue along with purifying small amounts of material and purifying by NMR. The structure of desacetyl kibdelomycin and desacetyl, descarbamoyl kibdelomycin are proposed in Figures 5-5 and 5-6, respectively.

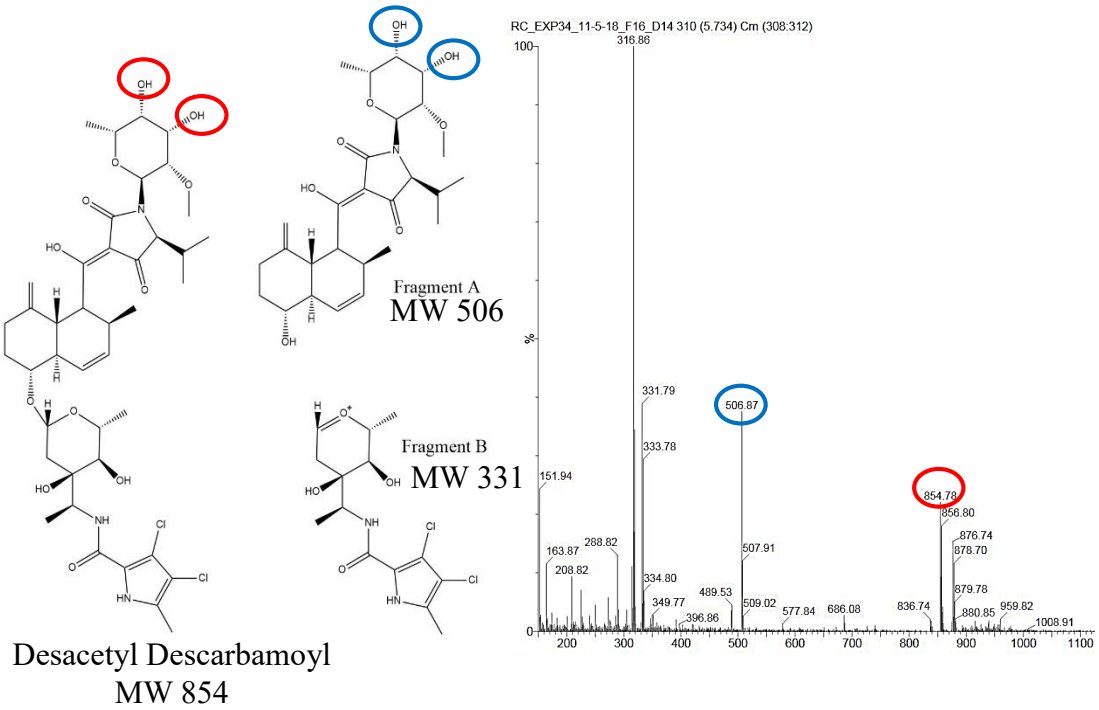

Figure 5-6: Mass spectrometry and proposed structure of desacetyl descarbamoyl kibdelomycin. RT = 5.7 min. Red circles highlight the loss of an acetyl and carbamoyl group in the parent ion while blue circles highlight the loss of an acetyl and carbamoyl group in fragment ion A. Fragment ion B can be found at both 317 Da and 331 Da.

Lastly, desacetyl kibdelomycin and desmethyl desacetyl kibdelomycin also can tautomerize into iso-desacetyl kibdelomycin and iso-desmethyl desacetyl kibdelomycin (data not shown). Iso-desacetyl kibdelomycin and iso-desmethyl desacetyl kibdelomycin analogues both elute at 5.1 minutes which is half a minute after their non-isoforms (Figures 5-4 and 5-5). A different LC-MS gradient may help to separate these analogues as well as other previously described analogues that co-eluted. This could prove useful in the future when looking to purify these analogues. Retention times and molecular weights of iso-desacetyl and iso-desmethyl desacetyl kibdelomycin, as well as the previously described analogues are summarized in Table 5-1.

Table 5-1: Kibdelomycin Analogue Characterizations

Name	RT (min)	Parent Ion (M+H)	Fragment Ion A	Fragment Ion B	Description
Kibdelomycin	6.2	939	591	331	original, unmodified kibdelomycin
Iso-	6.8	939	591	331	isomerization at either OH or N sites; identified by low presence of fragment A and later elution time
Desmethyl	5.5	925	591	317	loss of methyl group (-14) "Kibdelomycin A" (Singh 2012)
Desacetyl	4.6	897	549	331	loss of acetyl group (-42)
Desmethyl, Desacetyl	4.6	883	549	317	loss of acetyl group (-42) and methyl group (-14)
Iso-Desacetyl	5.1	897	549	331	isomerization of desacetyl; identified by the low presence of fragment A and later elution time
Iso-Desmethyl, Desacetyl	5.1	883	549	317	loss of acetyl group (-42) and methyl group (-14)
Desacetyl, Descarbamoyl	5.7	854	506	331	loss of acetyl (-42) and carbamoyl (-43)
Desmethyl, Desacetyl, Descarbamoyl	5.7	840	506	317	loss of methyl (-14), acetyl (-42), and carbomyl (-43)

Note: RT (Retention time)

The presence of structural analogues is fairly common with natural products (Parekh et al. 2000). The analogues are often produced as families and are usually condition and strain specific. They can be helpful or harmful, depending on one's perspective. From an antibiotic discovery standpoint, analogues offer variation which can aid in finding the best possible compound as not all analogues will have the same biological activity. For example, one analogue may be a more potent antibiotic, or

another may have less side effects in human trials. Additionally, variation in the compound can aid in keeping the antibiotic resistance at bay. Resistance to one analogue may be overcome by switching to another analogue. However, from a chemist's perspective, analogues may pose some issues. The development of a new antibiotic requires purification of a single compound and clinical trials on that single compound. Purifying a single natural product antibiotic from its structural analogues can be difficult, resulting in reduced purification yields that can limit experiments aiming to modify the compounds structure through chemical synthesis. Overall, the presence of kibdelomycin analogues is beneficial to the overall goal of developing a successful antibiotic, but will present a challenge when looking to purify kibdelomycin for experiments with structural modifications.

II. Fermentation Time Course

A time course experiment was set up to compare strain growth as well as determine if different analogues were produced at different stages of fermentation. Frozen stocks of strain F109, F, and F16 (1 mL) were inoculated into 20 mL of ISM-3 seed medium in 125-mL flasks. After 3 days of incubation at 28 °C and 250 rpm, 1 mL aliquots of each seed were inoculated into 8 flasks of 20 mL optimized ½ X AMM with 75 mmol MOPS buffer, for a total of 24 flasks. These flasks were incubated at 28 °C and 250 rpm for various time points between 3 and 14 days. At their respective time points, percent wet cell weight was measured as a means of estimating biomass levels because the production medium contains insoluble components. Briefly, whole broth (10 mL) was weighed in a tared conical tube (gross weight) before centrifugation at 2400 x g for 15

minutes. The supernatant was poured off and the biomass was weighed (net weight). Percent wet cell weight was calculated using the following formula:

$\%\ wet\ cell\ weight = \left(\frac{net\ weight}{gross\ weight} \times 100\right)$. Percent wet cell weight at each time point is shown in Figure 5-7.

Strain F16 grew best overall (Figure 5-7). Strain F initially had a slow rate of growth, while the F109 parent strain and strain F16 had equally high growth rates. F109 and strain F's growth rate plateaued between 5 and 10 days and strain F16 continued to grow all the way through day 14.

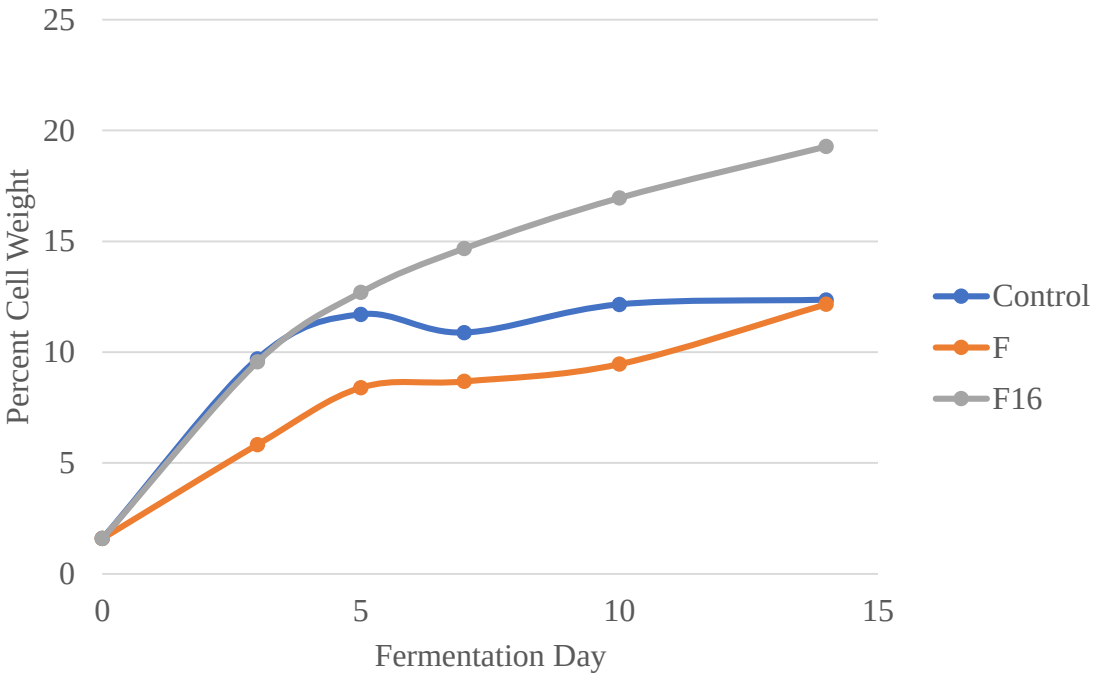

Figure 5-7: Comparison of percent wet cell weight (growth) of control (strain F109) and mutants (F and F16) at various stages of fermentation. n=1

Kibdelomycin was also extracted from the whole broth according to the extraction method described in Chapter 5, and extracts were analyzed via LC-MS. Total area counts of each analogue were recorded and converted into titers via a kibdelomycin standard. The cumulative sum of kibdelomycin and kibdelomycin analogue titers of each strain are shown for each time point in Figure 5-8. These data do not follow the pattern observed for *Kibdelosporangium* growth (Figure 5-7) in the three strains, reaffirming that growth does not necessarily correlate to kibdelomycin production. The F109 control and F (single mutant) strains have roughly equal amounts of biomass (growth) after 14 days

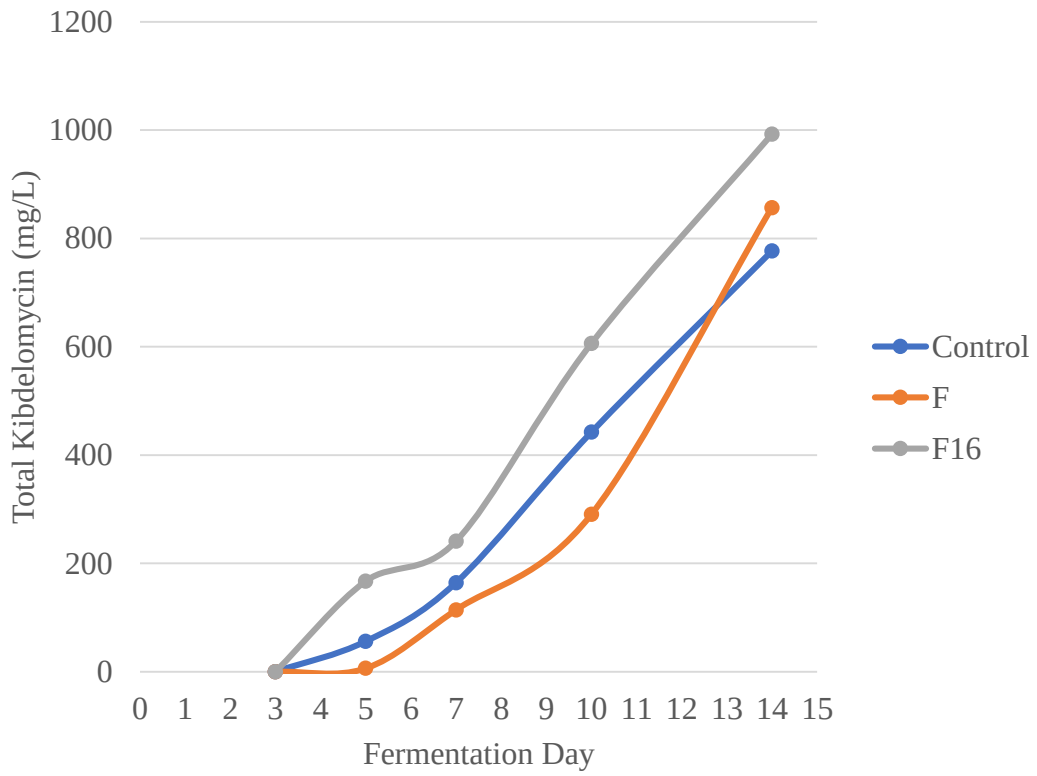

Figure 5-8: Comparison of kibdelomycin production in *Kibdelosporangium* strains F109 (control) and mutants (F and F16) at various stages of fermentation (n=1)

(Figure 5-7), despite strain F appearing to produce more kibdelomycin than F109 (Figure

5-8). In this context, *Kibdelosporangium* growth should not be mistaken to mean kibdelomycin production. A strain does not have to grow abundantly in fermentation to produce substantial amounts of kibdelomycin and in fact, a reduced growth rate is typically a queue for natural product production. However, strain F16 does happen to grow abundantly and produce the highest titer of kibdelomycin.

Kibdelomycin production was also analyzed in terms of comparing analogue ratios at different points in the fermentation. Titers of each individual kibdelomycin analogue are displayed in Figure 5-9. Iso-kibdelomycin dominated the fermentation extracts at each time point for all three strains, while titers for all other analogues and for kibdelomycin remained consistently low and hard to distinguish.

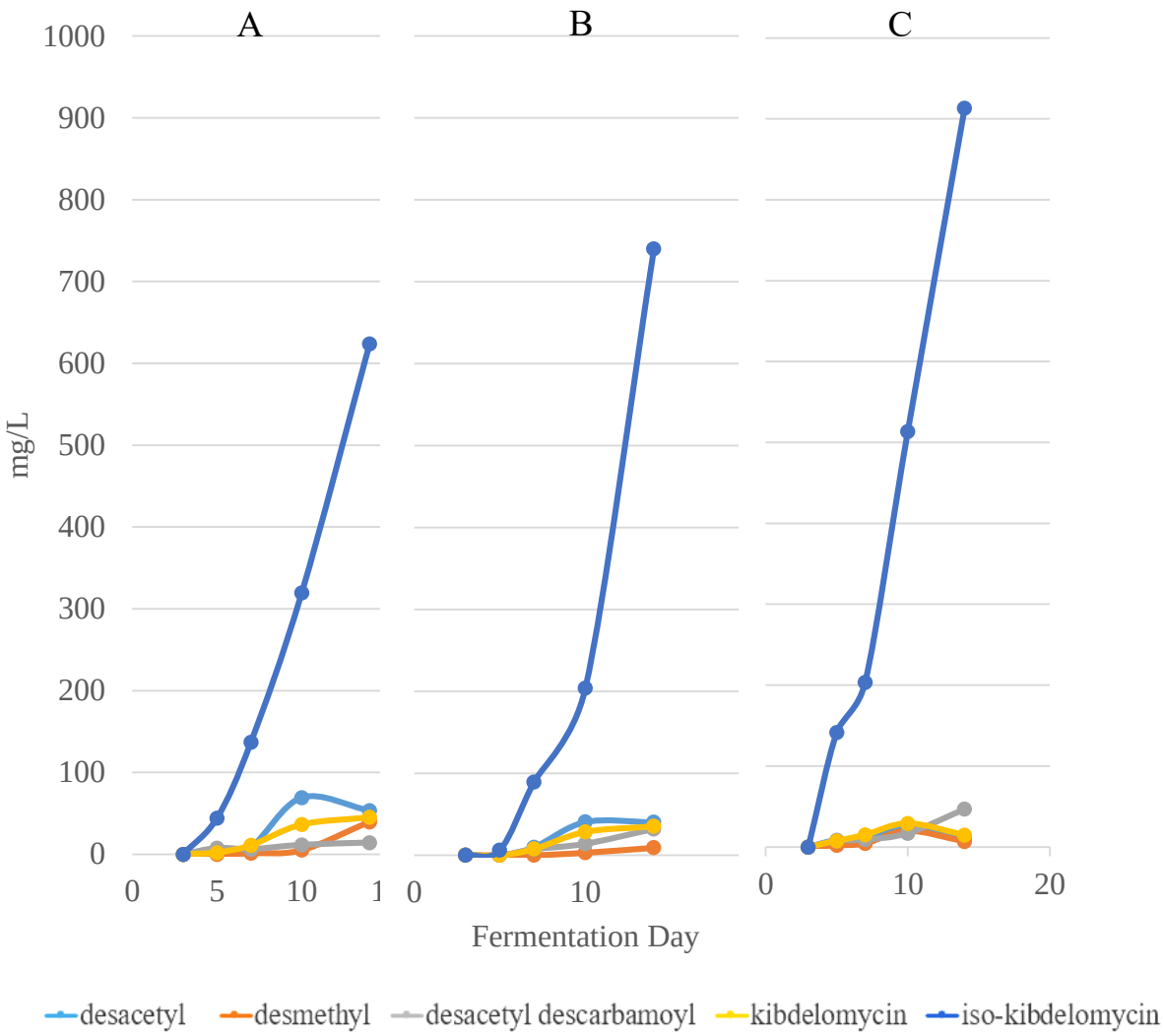

Figure 5-9: Comparison of kibdelomycin and kibdelomycin analogue production in *Kibdelosporangium* strains. A) control- strain F109, B) strain F, and C) strain F16 at various stages of fermentation.

The high levels of iso-kibdelomycin and low levels of kibdelomycin were unexpected, as the goal of the strain improvement project was to increase kibdelomycin production. However, iso-kibdelomycin was not only abundant in the mutant strains, but also in the control F109 strain. Curiosity led to the extracts being reanalyzed via LC-MS after sitting in sealed autosampler vials for two weeks in 100% methanol at room

temperature. After being re-analyzed, the titers of kibdelomycin and iso-kibdelomycin were compared again (Figure 5-10). After two weeks, and with no interference, the ratio of kibdelomycin to iso-kibdelomycin changed. An increase in kibdelomycin corresponded a decrease in iso-kibdelomycin suggesting that iso-kibdelomycin spontaneously converts into kibdelomycin in methanol with time. Further work will need to be done to determine the exact mechanism by which this occurs.

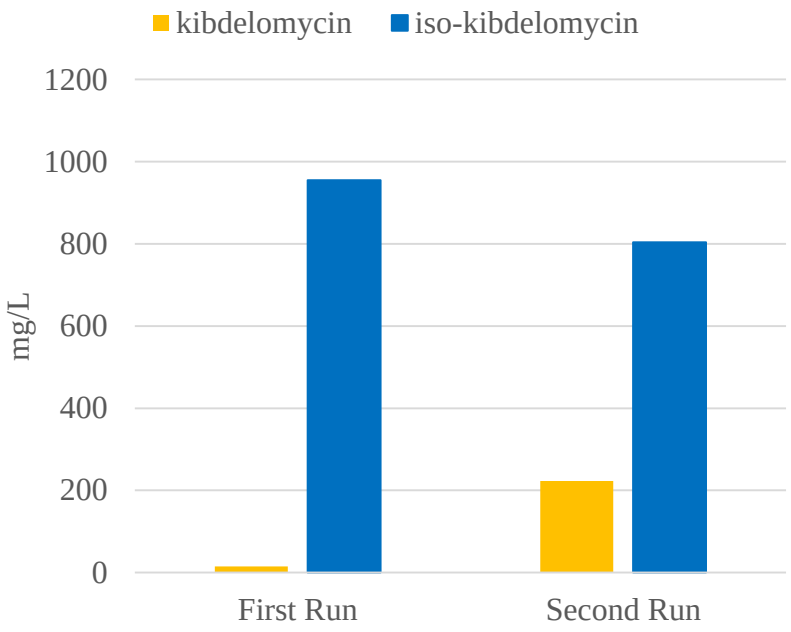

Figure 5-10: Ratio of kibdelomycin to iso-kibdelomycin in the same strain F16 extract, analyzed 2 weeks apart. Yellow represents kibdelomycin quantity and blue represents iso-kibdelomycin quantity

Chapter 6: Conclusions and Future Directions

The antibiotic resistance crisis is a growing threat caused by the misuse of antibiotics both in medicine and in agriculture (Ventola 2015). If nothing is done about it, the world may face a post-antibiotic era, where all known antibiotics have been overcome by resistance microbes. Small infections could be life threatening. Simple medical procedures would be extremely dangerous as there would be limited options for preventing post procedure infections. Total deaths by antibiotic resistant microbes could reach up to 10 million annually across the globe and the economic burden brought on by this could amount to 100 trillion USD (RAR 2016). However, this does not have to become reality. Many actions can and need to be taken to combat resistance, however, discovery of novel antibiotics will be most important in combatting the antibiotic resistance crisis.

Antibiotic discovery is a very important field of research which started in the 20th century. Beginning with the discovery of penicillin by Alexander Fleming, and its development into a therapeutic medicine by Florey and Chain (Chain et al. 1940; Abraham et al. 1941), transitioning into the work of the Waksman lab and their development of streptomycin, natural product antibiotics have dominated the field (Lewis 2013). During the 1940s-1970s, pharmaceutical companies flourished, finding and developing countless natural product antibiotics. Soon, however, the number of discoveries began to fall. Novel compounds could not be found within screens of microbial natural products because they were dominated by previously discovered compounds (the low-hanging-fruit). Companies turned to synthetic chemistry to produce

more antibiotics (Singh 2014). Compound libraries were screened repeatedly in in an attempt to find novel compounds with antibacterial activity. One issue associated with this method was the implementation of Lipinski's rule of 5 (Lipinski 2001; Lewis 2013), which did not consider that successful antibiotics differ from other successful drugs (Payne et al. 2007). This attributed to the lack of antibiotics discovered via the synthetic approach to antibiotic discovery. The semi-synthetic approach was successful in bringing about a next generation of some antibiotics, but the amount of chemical modifications one can make to a compound is limited (Singh 2014). Larger pharmaceutical companies eventually stopped pursuing antibiotic research as it was not profitable enough. The antibiotic market size could not provide enough profit to sustain a large pharmaceutical company; however, it could sustain small companies and academic research groups (RAR 2015).

Small companies and academic research groups have brought about a new era of antibiotic discovery. They acknowledge the success of natural product antibiotics of the past and use recent advances in technology to discover novel compounds (Singh and Barrett 2005). One key technological development is genome sequencing. Researchers can now run analyses on a large number of microbial genome sequences and sift through their data to find biosynthetic gene clusters that encode natural products. The producing organism can be isolated and fermented, or the gene cluster can be introduced into another microbe for fermentation and heterologous production, allowing us to explore the natural products of unculturable microbes (Coates and Hu 2007). These advancements have created a surge into the field of antibiotic discovery.

One product of this surge, although discovered via old methods combined with new techniques, is kibdelomycin. Soil samples from diverse areas were extracted for natural products and tested in a *S. aureus* fitness assay and modern approaches helped to isolate kibdelomycin from a crude extract as well as determine its novel mechanism of action. Kibdelomycin is a natural product of *Kibdelosporangium sp.* that shows antibacterial activity against several Gram-positive microbes, such as *S. aureus* (Phillips et al. 2011). Though kibdelomycin has been shown to be a potentially a useful antibiotic, it has a long way to go before potentially being useful to humans. In particular, it has been shown to be a serum antagonist (Phillips et al. 2011), meaning it would be almost ineffective if used as discovered. Some of the functional groups within kibdelomycin could be altered or removed to reduce serum antagonism. Experiments need to be performed to determine which structures are problematic. But without a consistent source of kibdelomycin, these studies are not possible. For this reason, we need to increase the amount of kibdelomycin. Media studies were performed to determine an optimized fermentation procedure. Galactose levels were found to be the main factor affecting kibdelomycin production (Figure 3-2). With this information, a production medium was created for *Kibdelosporangium sp.*to optimize kibdelomycin production.

Moreover, the original *Kibdelosporangium sp*. strains were improved using a method accumulating antibiotic resistance mutations (Hu and Ochi 2001; Wang et al. 2008). The streptomycin and gentamicin resistant strain (F16) produces 50% more kibdelomycin than the original F109 strain (Figure 4-7) Successful strain improvement is a large step necessary for moving kibdelomycin through development, as increased

production in a strain will allow for more kibdelomycin to be produced per fermentation. Strain improvement by cumulative antibiotic resistance mutations is an achievable goal that could significantly advance the development of kibdelomycin (and future natural products) into a commercially available antibiotic in the future.

It could also be beneficial to continue improving the strain by creating a mutant resistant to additional antibiotics. The papers from which *Kibdelosporangium sp*. strain improvement was based on (Hu and Ochi 2001; Wang et al. 2008) saw 10 to 48 times higher production while kibdelomycin production was only improved 1.5-fold by a streptomycin and gentamicin resistant *Kibdelosporangium sp*. mutant (Figure 4-7). A logical next step would be to take the F16 mutant strain and select for rifampin resistance, generating a mutant resistant to streptomycin, gentamicin, and rifampin. As mentioned earlier, an RNA-Seq analysis could also be performed to determine whether the mutations associated with strain improvement increase production of natural products through regulation of global transcription.

Further work still needs to be done to better separate the kibdelomycin analogues by liquid chromatography so that the structures of these putative analogues can be verified by NMR and assayed individually to determine any structure-activity relationships. It is possible that one of the analogues, though produced in low amounts, may prove to be a better starting point for chemists to eliminate serum antagonism through structural modifications. Minimum inhibitory concentrations could be determined for each analogue via disk diffusion assays, allowing for a comparison of their individual efficacy against target microbes.

Kibdelomycin is in its initial stages of development with a long road in front of it. Advancement of production media quality, generation of an improved strain, characterization of analogues, and development of a preliminary purification procedure are necessary steps for pushing kibdelomycin through its initial phases of development. With the fermentation process in place, kibdelomycin production can be scaled up to produce large quantities of kibdelomycin, permitting further development of kibdelomycin as an antibiotic.

References

Abboud FM, Waisbren BA. 1959. Correlation between in vitro studies and response to antibiotic therapy in Staphylococcic Bacteremia. AMA Arch Intern Med 104(2): 226-233.

Abraham EP, Chain E, Fletcher CM, Gardner AD, Heatley NG, Jennings MA, Florey HW. 1941. Further observations on penicillin. Lancet. 238(6155): 177-189.

BBL Sensi-Disc Antimicrobial Susceptibility Test Discs Zone Diameter Interpretive Chart. Becton, Dickinson & Company. MD, USA.

Center for Disease Control (CDC). 2013. Antibiotic resistance threats in the United States. U.S. Department of Health and Human Services 1-114.

Chain E, Florey HW, Gardner AD, Heatly NG, Jennings MA, Orr-Ewing J, Sanders AG. 1940. Penicillin as a chemotherapeutic agent. Lancet 236(6104):226-228.

Chandra Mohana N, Yashavantha Rao HC, Rakshith D, Mithun PR, Nuthan BR, Satish S. 2018. Omics based approach for biodiscovery of microbial natural products in antibiotic resistance era. J Genet Engin Biotechnol 16:1-8.

Clatworthy AE, Pierson E, Hung DT. 2007. Targeting virulence: a new paradigm for antimicrobial therapy. Nat Chem Biol 3(9):541-548.

Coates ARM, Hu Y. 2007. Novel approaches to developing new antibiotics for bacterial infections. Brit J Pharmacol 152: 1147-1154.

Connors NC. 2003. Culture Medium Optimization and Scale-Up for Microbial Fermentations Mammalian, Microbial, and Plant Cells. In: Vinci VA, Parekh SR, editors. Handbook of Industrial Cell Culture. New Jersey: Humana Press. p. 171-193.

Cox G, Sieron A, King AM, De Pascale G, Pawlowski AC, Koteva K, Wright GD. 2017. A common platform for antibiotic dereplication and adjuvant discovery. Cell Chem Biol 24:98-109.

Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev 74: 417-433.

D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. 2011. Antibiotic Resistance is Ancient. Nat 477: 457–461

Donadio S, Maffioli S, Monciardini P, Sosio M, Jabes D. 2010. Antibiotic discovery in the twenty-first century: current trends and future perspectives. J Antibiot 63, 423-430.

Drawz SM, Bonomo RA. 2010. Three decades of β-lactamase inhibitors. Clin Microbiol Rev 23(1):160-201.

East SP, Silver LL. 2013. Multitarget ligands in antibacterial research: progress and opportunities. Expert Opin Drug Dis 8(2): 143-156

Fleming-Dutra KE, Hersh AL, Shapiro DJ, Bartoces M, Enns EA, File Jr. TM, Finkelstein JA, Gerber JS, Hyun DY, Linder JA et al. Prevalence of inappropriate antibiotic prescriptions among US ambulatory care visits, 2010-2011. 2016. J Am Med Assoc 315(17): 1865-1873.

Healy M. 2018. In soil-dwelling bacteria, scientists find a new weapon to fight drug-resistant superbugs. Los Angeles Times. Feb. 13, 2018.

Hoelzer K, Wong N, Thomas J, Talkington K, Jungman E, Coukell A. 2017. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence?. BMC Vet Res 13(211):1-38.

Hover BM, Kim SW, Katz M, Charlop-Powers Z, Owen JG, Ternei MA, Maniko J, Estrela AB, Molina H, Park S, Perlin DS, Brady SF. 2018. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. Nat Microbiol 3: 415-422.

Hu H and Ochi K. 2001. Novel Approach for Improving the Productivity of Antibiotic-Producing Strains by Inducing Combined Resistant Mutations. Appl Environ Microbiol 67 (4):1885–1892.

Hudzicki J. 2009 Kiby-Bauer disk diffusion susceptibility test protocol. American Society for Microbiology. [Internet]. [cited 23Available from http://www.asmscience.org/content/education/protocol/protocol.3189?crawler=redirect&mimetype=application/pdf

Kardas P, Devine S, Golembesky A, Roberts C. 2005. A systematic review and meta-analysis of misuse of antibiotic therapies in the community. Int J Antimicrob Ag 26(2):106-113.

Land M, Hauser L, Jun SR, Nookaew I, Leuze MR, Ahn TH, Karpinets T, Lund O, Kora G, Wassenaar T, Poudel S. 2015. Insights from 20 years of bacterial genome sequencing. Funct Integr Genomics 15(2):141-61.

Lewis K. 2013. Platforms for antibiotic discovery. Nat Rev Drug Discov 12:371-387.

Ling LL, Schneider T, Peoples AJ, Spoering AL, Engles I, Conlon BP, Mueller A, Schaberle TF, Hughes DE, Epstein S, et al. 2015. A new antibiotic kills pathogens without detectable resistance. Nat. 517:455-459.

Lipinski CA, Lombardo F, Dominy BW, Feeny PJ. 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliver Rev 46(1–3):3-26.

Martens E, Demain AL. 2017. The antibiotic resistance crisis, with a focus on the United States. J Antibiot 70:520-526.

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T. Takano E, Breitling R. 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39:339-46.

Melander RJ, Melander C. 2017. The challenge of overcoming antibiotic resistance: an adjuvant approach?. ACS Infect Dis 3(8):559-63.

Monaghan RL, Barrett JF. 2005. Antibacterial drug discovery- Then, now and the genomics future. Biochem Pharmacol 71, 901-909.

Newman DJ, Cragg GM. 2016. Natural products as sources of new drugs from 1981-2014. J Nat Prod 79(3):629-661.

Tanaka Y, Kasahara K, Hirose Y, Murakami K, Kugimiya R, Ochi K. 2013. Activation and Products of the Cryptic Secondary Metabolite Biosyntheic Gene Clusters by Rifampin Resistance (rpoB) Mutations in Actinomycetes. J Bacteriol 195 (13): 2959-2970.

Parekh S, Vinci VA, Strobel RJ. 2000. Improvement of microbial strain and fermentation processes. Appl Microbiol Biotechnol 54: 287-301.

Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. 2007. Drugs for bad bugs. Nat Rev Drug Discov 6:29-40.

PEW. 2019. Nontraditional products for bacterial infections in clinical development. The PEW Charitable Trust [Internet]. [Cited 15 March 2019.] Available from https://www.pewtrusts.org/en/research-and-analysis/data-visualizations/2017/nontraditional-products-for-bacterial-infections-in-clinical-development.

Phillips JW, Goetz MA, Smith SK, Zink DL, Polishook J, Onishi R, Salowe S, Wiltsie J, Allocco J, Sigmund J, et al. 2011. Discovery of Kibdelomycin, A Potent New Class of Bacterial Type II Topoisomerase Inhibitor by Chemical-Genetic Profiling in *Staphylococcus aureus*. Chem Biol 18: 955–965.

Projan SJ. 2003. Why is big Pharma getting out of antibacterial drug discovery? Curr Opin Microbiol 6, 427-430.

PubChem Compound Database. National Center for Biotechnology Information. CID=19649, https://pubchem.ncbi.nlm.nih.gov/compound/19649

RAR (Review on Antimicrobial Resistance), Government of the United Kingdom and Wellcome Trust. 2015. Securing new drugs for future generations: the pipeline of antibiotics. [Internet]. [Cited 21 November 2018.] Available from https://amr-review.org/publications.

RAR (Review on Antimicrobial Resistance), Government of the United Kingdom and Wellcome Trust. 2016. Tackling drug-resistant infections globally: final report and recommendations. [Internet]. [Cited 20 October 2018.] Available from https://amr-review.org/publications.

Rogers K, Kadner RJ. 2019. Bacteria: Evolution of Bacteria. Encyclopaedia Britannica. [Internet]. [Cited 20 March 2019.] Available from https://www.britannica.com/science/bacteria/Evolution-of-bacteria

Rouhi AM. 2003. Rediscovering natural products. Chem Eng News 81(41):77-78, 82-83, 86-91.

Royles BJL. 1995. Naturally occurring tetramic acids: structure, isolation, and synthesis. Chem Rev. 95(6):1981–2001.

Sawa R, Takahashi Y, Hashizume H, Sasaki K, Ishizaki Y, Umekita M, Hatano M, Abe H, Watanabe T, Kinoshita N, et al. 2012. Amycolamicin: A Novel Broad-Spectrum Antibiotic Inhibiting Bacterial Topoisomerase. Chem Eur J 18(49):947-6539.

Singh SB. 2014. Confronting the challenges of discovery of novel antibacterial agents. Bioorg Med Chem Lett 24:3683-3689.

Singh SB, Barrett JF. 2005. Empirical antibacterial drug discovery- Foundation in natural products. Biochem Parmacol 71:1006-1015.

Singh SB, Goetz MA, Smith SK, Zink DL, Polishook J, Onishi R, Salowe S, Wiltsie J, Allocco J, Sigmund J, et al. 2012. Kibdelomycin A, A Congener of Kibdelomycin, Derivatives and Their Antibacterial Activities. Bioorg Med Chem Lett. 22: 7127–7130.

Singh SB, Phillips JW, Wang J. 2007. Highly sensitive target-based whole-cell antibacterial discovery strategy by antisense RNA silencing. Curr Opin Drug Discov Devel 10(2):160-166.

Singh SB, Young K, Silver LL. 2017. What is an “ideal” antibiotic? Discovery challenges and path forward. Biochem Pharmacol 113:63-73.

Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards J. 2008. The epidemic of antibiotic-resistant infections: a call to action

for the medical community from the Infectious Diseases Society of America. Clin Infect Dis 46(2):155–164.

Sun WJ, Salmon P, Wilson J, Connors N. 1998. Crotonic acid-directed biosynthesis of the immunosuppressants produced by *Streptomyces hygroscopicus var. ascomyceticus*. J Ferment Bioeng 86(3): 261-265.

Thermo Fisher Scientific. LB Broth and LB Agar: LB Agar (Lennox). [Internet] https://www.thermofisher.com/us/en/home/life-science/cell-culture/microbiological-culture/bacterial-growth-media/lb-broth-and-lb-agar

Tippet R. 2014. Mortality and cause of death, 1900 v. 2010. Carolina Demography: Carolina Population Center, UNC-Chapel Hill [Internet].

Ventola CL. 2015. The antibiotic resistance crisis part 1: causes and threats. P T 40(4):277-283.

Wang G, Hosaka T, Ochi K. 2008. Dramatic Activation of Antibiotic Production in *Streptomyces coelicolor* by Cumulative Drug Resistance Mutations. App Environ Microbiol 74: 2834-2840.

Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 10(1):57-63.

Wenzel RP. 2004. The antibiotic pipeline- challenges, costs, and values. N Engl J Med 351(6): 523-526.