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A Possible luxR Solo Type Regulator of an Antibiotic-Like Compound from the Soil Bacterium Rhodococcus

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A Possible *luxR* Solo Type Regulator of an Antibiotic-Like Compound from the Soil Bacterium *Rhodococcus*

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by

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Keywords: *Rhodococcus*, antibiotic, *luxR* solo, merodiploid

ABSTRACT

A Possible *luxR* solo Type Regulator of an Antibiotic-Like Compound from the Soil Bacterium *Rhodococcus*

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Rhodococcus, a species of bacteria commonly found in the soil, is an under-explored producer of small bioactive compounds including siderophores, pigments and antibiotics. MTM3W5.2 is a strain of *Rhodococcus* that was previously discovered to produce an antibiotic-like compound that has inhibitory effects on other Rhodococcus strains, including the veterinary pathogen, *R. equi*. The biosynthetic gene cluster responsible for production of the antibiotic has been identified, and a small gene, BTZ20_3964 at the start of the operon is believed to be a *luxR* solo regulator of the gene cluster. The goal of this project was to determine this gene's status as a regulator for the gene cluster. Merodiploids were constructed using the deletion construct, pEX18Km3964AD to obtain a double crossover recombination event to replace the functional gene with the deletion construct. However, evidence indicates that an illegitimate recombination event occurred to produce a merodiploid strain.

DEDICATION

To my family.

ACKNOWLEDGMENTS

I would like to thank my committee chair, Dr. Bert Lampson for being a great mentor and allowing me to work on this project. Thank you for the encouragement you have provided through my graduate career and teaching me how to overcome hardships in the lab. I would also like to thank my committee members, Dr. Christopher Pritchett and Dr. Dhirendra Kumar for the support and input on this project. I would like to thank the Health Sciences department for teaching me research and teaching me about microbiology, which has always been a passion of mine. I want to thank Dr. Sean Fox and Robin Grindstaff for all their help in the media lab and answering my research related questions. I also want to thank the undergraduate researchers in this lab, Mateusz Dróżdż and Jaimin Kapadia for helping me with my project and lending an ear when needed. I would also like to thank my family for all their support and encouragement. Thank you for believing in me as I pursued an education in the field I am truly passionate about.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

Antibiotic Resistance

Antibiotic resistance is an impending crisis in the health care field where antibiotics are no longer effective against many dangerous pathogens. There are critical health challenges associated with antibiotic resistant infections, such as longer hospital stays and more expensive treatments. Antibiotic resistance is where antibiotics are no longer effective against their targets due to some gene acquired by bacteria. Bacteria evolve rapidly and can acquire antibiotic resistance genes through conjugation, bacteriophage infection, transformation, or through spontaneous mutation. The mechanism of resistance varies with the gene and type of antibiotic, but can include mechanisms such as changing the antibiotic's target, inactivating the antibiotic, or block access to the target molecule via changes in permeability or efflux pumps. (Giedraitienė et al. 2011). Table 1 shows types of antibiotics and the types of resistance mechanisms against the antibiotics The rise of antibiotic resistance is likely due to the overuse of antibiotics in agricultural and food industries, and the inappropriate use of antibiotics in health care such as the wrong antibiotic being prescribed, patients not taking them as directed, or when antibiotics are not needed and still prescribed. In 2013, the CDC estimated that at least 2 million people acquired an antibiotic resistant infection, and at least 23,000 of the patients died as a result of the infection. It was estimated that antibiotic resistant infections caused over $20 billion in excess direct health care costs (CDC 2013).

Table 1: Classes of Antibiotics and Mechanisms of Resistance (Kapoor et al. 2017).

Antibiotic Class	**Resistance type**
Aminoglycosides	Antibiotic inactivation (Enzyme modification)
	Decreased uptake
Beta-Lactams	Antibiotic inactivation (Enzyme degradation)
	Altered PBP
Chloramphenicol	Antibiotic inactivation
	Efflux pumps
Glycopeptides	Altered targets
Macrolides	Efflux pumps
	Altered targets
Tetracyclines	Efflux pumps
	Altered targets
Sulfa drugs	Altered targets

Once a bacterium acquires an antibiotic resistance gene, the gene is often selected for and preserved even outside of antibiotic treatment because the gene sometimes provides some benefit to the bacterium, such as allowing them to survive other high stress situations (Berry et al. 2006). Many studies have determined that many water sources have low, nonlethal concentrations of antibiotics present which can place a low selective pressure for bacteria to maintain their resistance gene (Xi et al. 2009). In both humans and animals, a high percentage of antibiotics can be excreted in an unaltered state, so they are still active in wastewater and agricultural runoffs. Antibiotic resistant bacteria and antibiotic resistance genes are considered environmental

pollutants that can persist in aquatic and soil environments, contributing to the high levels of antibiotic resistance (Pruden et al. 2006).

As levels of antibiotic resistance continue to rise, rate of discovery of new antibiotics has slowed drastically, almost to a complete halt. The first true antibiotic, penicillin, was discovered by Alexander Fleming in 1928 though it was not available commercially until the 1940s (Quinn 2013). There are many different inhibitory molecules that are active against microbial pathogens, but a true antibiotic must have special properties: an antibiotic is selectively toxic to a microbial cell (usually a bacterium) but does not harm human cells or tissues. The antibacterial properties of penicillin revolutionized treatment of bacterial infections. By the end of World War II, many pharmaceutical companies were mass producing penicillin and working to discover more antibiotics as they quickly discovered antibiotic resistance developed within years of the release of new antibiotics. The first documented case of a penicillin-resistant *E. coli* strain occurred in 1940, and by 1945, Fleming predicted an "era of abuse" would occur due to the high demand for antibiotics (Lobanovska and Pilla 2017). If new antibiotics could not be found, a post-antibiotic era would come about and once treatable infections would become deadly again.

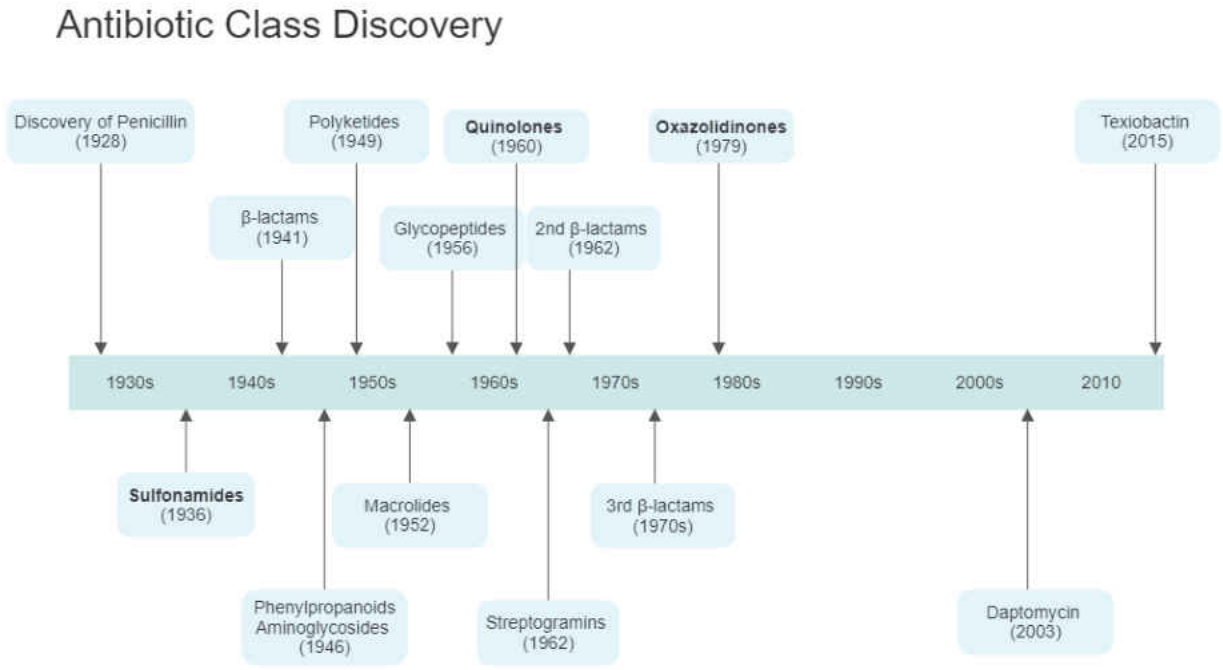


Figure 1: A timeline of discovery for novel antibiotics. Bolded classes are synthetic in origin, and normal font is natural in origin (Singh and Barrett 2006).

Discovery of New Antibiotics

A "golden age" of antibiotic discovery occurred during the 1940s-1960s, with many new classes of antibiotics being discovered during this time. Penicillin was produced by a mold, *Penicillium notaum*, but many research efforts focused on prokaryotes in the production of inhibitory molecules (Singh and Barrett 2006). Traditionally, bacteria were cultured and screened for the presence of inhibitory compounds, but the issue was the high rediscovery rates. Compounds with similar structures and functions were re-isolated over and over, and the financial success dwindled. By the 1990s, half of the pharmaceutical companies abandoned their research in natural antibiotics and the remaining companies cut funding so antibiotic research accounted for less than 2% of overall research and development (Quinn 2013). Instead of

looking for naturally produced molecules, the pharmaceutical industry began to screen large libraries of chemically synthesized compounds. Indeed, the discovery of new molecules that bind to novel bacterial targets were discovered by this method, but most of these new compounds fail to cross the bacterial cell wall and thus are not therapeutically useful (Wright 2018). Daptomycin was the last novel (i.e., binds to a novel bacterial target molecule) antibiotic discovered that has been approved for clinical use in the early 2000s and was the first new class of antibiotic in decades. Texiobactin is a new antibiotic that was discovered in 2015 that is produced by *Eleftheria terrae* and looks to be a promising treatment for MRSA, but is still in early trials and is not yet being used for clinical treatment (Arooj and Koh 2017).

Discovery of new antibiotics is challenging; traditionally bacteria are cultured and screened for inhibitory activity, but the problem with this is that it is predicted that less than 2% of known environmental bacteria can be cultured in typical laboratory growth conditions (Wade 2002). Many bacteria might produce antibiotics, but cannot be cultured in a lab setting, so previously, there was no way to screen for antibiotic production in unculturable bacteria. Today, culture-free genomic sequencing of environmental samples can indicate the presence of biosynthetic gene clusters (BGCs), which could indicate a natural product may be produced by the microbe (Hover et al. 2018). The issue with this method is that many BGCs are uncharacterized and the function of their products are unknown. Secondly, it can be difficult to determine the function of these products if they are produced under a cryptic pathway, meaning they are not produced *in vitro*. Many bacteria may produce clinically useful products, but they may not be produced or detected under typical growth conditions, so awakening cryptic pathways *in vitro* may be beneficial in future searches for new antibiotics, so traditional culturing methods can only detect a small percentage of these small products. It is predicted that up to 90%

of small molecules that can be produced by bacteria remain unknown (Walsh and Fischbach 2010). It is for this reason that many researchers have returned to searching for natural products in the search for new antibiotics. Rather than focusing on discovering new natural products produced in typical lab conditions, focus has shifted to methods to discover this unknown reserve of natural products such as turning on expression of cryptic genes *in vitro,* expression of environmental DNA (Kallifidas et al. 2012), growing species in co-cultures (Molloy and Hertweck 2017), and finding chemical elicitors to activate expression (Okada and Seyedsayamdost 2017).

Expression of Antibiotics

Secondary metabolites are natural products (usually small organic molecules) that are produced by a microbe that are not essential to the growth or reproduction of the microbe, but may help the microbe compete and survive in a natural setting (Tyc et al. 2017). They include a variety of different products that can have a broad range of functions. Secondary metabolites include things such as siderophores, which help microbes acquire enough iron from the environment, pigments, toxins, and inhibitory molecules.

Natural products make up a large percentage of clinically used drugs including aspirin and morphine. Many of our clinically used antibiotics comes from the *Actinobacteria* family, such as from *Streptomyces* species. According to Elsayed et al. (2017), around 23,000 antibiotics have been discovered from microbes, and about 10,000 of those come from the members of the Actinomyces family. Culture-based studied have shown that many bacterial species can produce several secondary metabolites, but often do so under cryptic pathways; cryptic pathways may

control up to 90% of all metabolites (Tyc et al. 2017) (Walsh and Fischbach 2010). The reason for these cryptic pathways may be that secondary metabolites often cost a lot of energy and resources to produce, so having constant expression of the metabolites would be quite wasteful for the cell when they are not needed; for example, a species does not need to make antibiotics in an environment free of competitors.

One way that these genes required to produce antibiotics may be controlled is through a quorum-sensing system (QS), where microbes can "communicate" with each other and alter gene expression based on signals they receive from nearby cells. Bacteria may want to repress or activate transcription of certain metabolites based on the population density of neighboring cells, and quorum sensing is a way they can communicate to make a coordinated response to stimuli. In a typical QS system, such as the *luxR* system, transcription is controlled by a *luxR* type regulator protein. A *luxI* type synthase in the cell will produce an autoinducer molecule called acyl homoserine lactones (AHLs), which will bind to the *luxR* regulator, which will then alter gene expression by either repressing or activating transcription. This system is used to regulate processes such as virulence, motility, sporulation, production of a biofilm, and antibiotic production. Both Gram negative and Gram positive bacteria use QS systems, where AHLs are traditionally produced by the Gram negatives, and processed oligo-peptides are produced as the autoinducer in Gram positive cells. Gram positive bacteria use a two-component system where peptides are secreted by the cell and detected by a sensor kinase within the cell. The sensor kinase will initiate a phosphorylation cascade that will result in the phosphorylation of the response regulator, which can then bind DNA and adjust transcription of the regulated gene. Communication via autoinducers occurs both within and between bacterial species, so bacteria may also alter gene expression based off of signals they receive from another type of bacterium

in their vicinity. Many antibiotics produced by the *Streptomyces* species are known to be controlled under QS systems, so the bacteria only activate expression when they need to compete in their environment (Miller and Bassler 2001).

luxR Solos

The *luxR* gene is usually adjacent to or in the proximity of the *luxI* gene on the chromosome, but some microbes have been characterized to have a *luxR* type regulator with no associated *luxI* gene on the operon. These are known as *luxR* solo regulators, where gene expression is controlled through other signals. These signals may be endogenous, produced by another *luxI* type synthase somewhere else in the genome, or exogenous, received from an external source or a non-AHL signal as shown in figure 2. *luxR* solos have been discovered to be widespread in Proteobacteria, but are not well characterized in other types of bacteria (Hudaiberdiev et al. 2015).

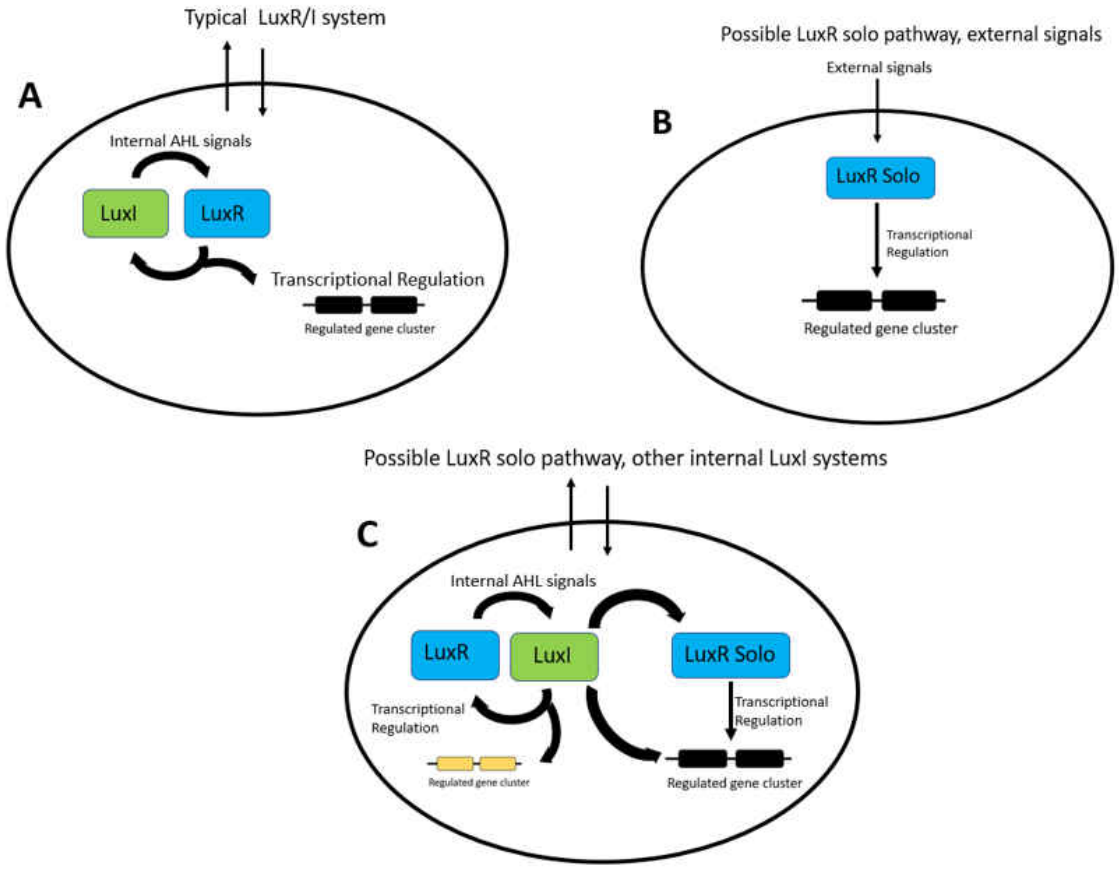


Figure 2: Possible mechanisms of a *luxR* solo regulator. (A) shows a typical *luxR/ luxI* system, (B) and (C) show where a *luxR* solo may receive their autoinducer signals. (Hudaiberdiev et al. 2015)

Rhodococcus

Rhodococcus is a member of the Actinobacteria phylum. It was first identified in 1891 by Zopf. The genus is commonly found in the soil, but it can also be found in other environments such as aquatic environments, in animal dung, and in insects and plants (Bell et al. 1998). Members of the genus are described as being Gram positive, aerobic, nonmotile and is a nocardioform, meaning they lack a set morphology, instead they form hyphae and break off into rods or cocci. The genome of *Rhodococcus* species are typically G-C rich.

Rhodococcus has been shown to produce a variety of bioactive metabolites such as pigments, siderophores, and inhibitory compounds, but remains underexplored as a source of clinically used antibiotics. It is a relative of the *Streptomyces* genus, which also falls under the Actinobacteria phylum, which is known to be a producer of many clinically useful antibiotics. *Rhodococcus* has a diverse range of metabolic abilities such as degrading environmental pollutants and producing intermediates used for synthesizing compounds. Different species may produce different pigments and may have different colony appearances based on the species shown in figure 3.

Figure 3: Strains of *Rhodococcus* on a wheel plate. Pigmentation and texture vary from species to species (Borisova 2011).

Industrial Biotechnology

Rhodococcus is useful for industrial biotechnology due to their ability to produce a variety of compounds such as flocculants, surfactants, amides, and polymers. They can degrade pollutants ranging from simple hydrocarbons to aromatic hydrocarbons, chlorinated polycyclic aromatics, and polychlorinated biphenlys (Bell et al. 1998). *R. rhocochrous* J1 is used in the synthesis of acrylamide, which is a chemical that is used in many industrial processes and is considered one of the most successful applications in using a microbial catalyst. Nitto Chemistry Industry Ltd in Japan uses the strain to produce over 30,000 tons of the chemical every year (Elsayed et al. 2017). Another use of *Rhodococcus* is the desulfurization of fossil fuels, which could improve fuel value and decrease the amount of sulfurous emissions from combustion of coal and petroleum (Bell et al. 1998). *R. erythropolis* is known to produce multiple enzymes that allow them to carry out several types of reactions such as hydrolysis, oxidations, dehydrogenations, desulfurizations, and hydroxylations have made them useful for multiple bioconversions and degradations (de Carvalho and da Fonseca 2005). The *Rhodococcus* genus's diversity and ability to produce a wide variety of enzymes and compounds, as well as carry out a number of reactions make it very useful in the chemical industry.

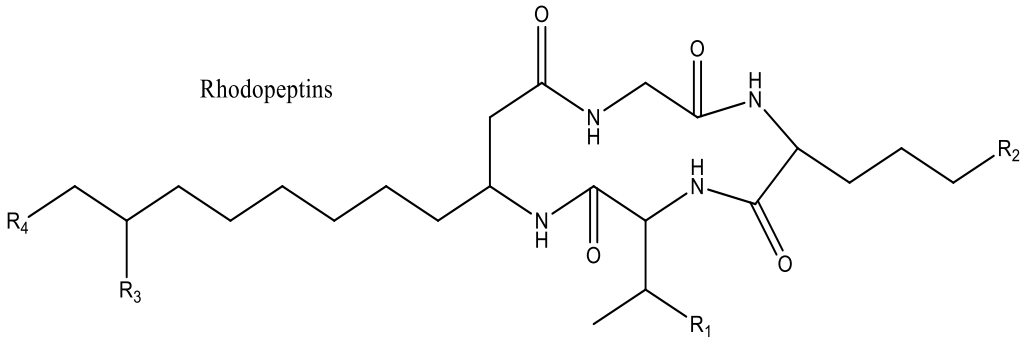
Pathogens

Members of the *Rhodococcus* genus are mostly non-pathogenic with only two strains playing a major role as pathogens in both plants and animals. *R. fascians* is a phytopathogen which is not known to cause human infections, but causes leafy gall disease in plants (Goodfellow, 1989). *R. equi* is the only known animal pathogen in the genus and can infect a

variety of animals but is most known for causing pneumonia in foals and other livestock. *R. equi* is an opportunistic pathogen, and can cause an infection similar to pulmonary tuberculosis in immunocompromised patients. Similar to *Mycobacterium tuberculosis*, *R. equi* infects macrophages, replicates in membrane-bound vacuoles and prevents maturation of the phagosome so the bacteria can proliferate inside of the macrophage (Majidzadeh and Fatahi-Bafghi 2018).

Secondary Metabolites in *Rhodococcus*

Production of secondary metabolites in *Rhodococcus* have been observed. However, based on many newly sequenced genomes, *Rhodococcus* species often contain many different cryptic biosynthetic gene clusters. This strongly suggests that this genus has the genetic potential to synthesize a large variety of secondary metabolites (Ceniceros et al. 2017). The first inhibitory molecule discovered from *Rhodococcus* was an antifungal molecule active against *Candida albicans* and identified in 1999 by Chiba et al. (1999). They identified five novel cyclic tetrapeptides: rhodopeptin C1, C2, C3, C4, and B5 shown in figure 4. These rhodopeptins have no antibacterial activity.



Rhodopeptin	R_1	R_2	R_3	R_4
Rhodopeptin C1	CH_3	NH_2	CH_3	CH_3
Rhodopeptin C2	CH_2-CH_3	NH_2	CH_3	CH_3
Rhodopeptin C3	CH_3	NH_2	H	CH-$(CH_3)_2$
Rhodopeptin C4	CH_3	NH_2	H	CH (CH_3)-C_2H_5
Rhodopeptin B5	CH_3	CH_2-NH_2	H	CH_2-CH $(CH_3)_2$

Figure 4: Structures of the antifungal Rhodopeptins C1, C2, C3, C4, and B5. (Elsayed et al. 2017).

Iwatsuki et al. (2007) discovered the antimycobacterial peptides named laritin A and B. Laritin A was shown to inhibit *Mycobacteruim tuberculosis*. The peptides are produced by *Rhodococcus* sp. K01-B0171 and form a "lasso" structure.

Rhodostreptomycin A and B are two antibiotics that were described by Kurosawa et al (2008). These antibiotics are produced by *Rhodococcus fascians* 307CO after being grown in a coculture with a known antibiotic producer, *Steptomyces padanus*. *R. fascians* does not produce a known antibiotic, but the strain 307CO produces the rhodostreptomycins as a result of horizontal gene transfer from the *Streptomyces* bacteria. The rhodostreptomycin isomers differ from the actinomycins produced by the *Streptomyces*, and are active against a variety of different bacteria

like *E. coli, S. aureus, B. sub*, as well as *S. padanus*. Figure 5 shows the structure of rhodostreptomycin.

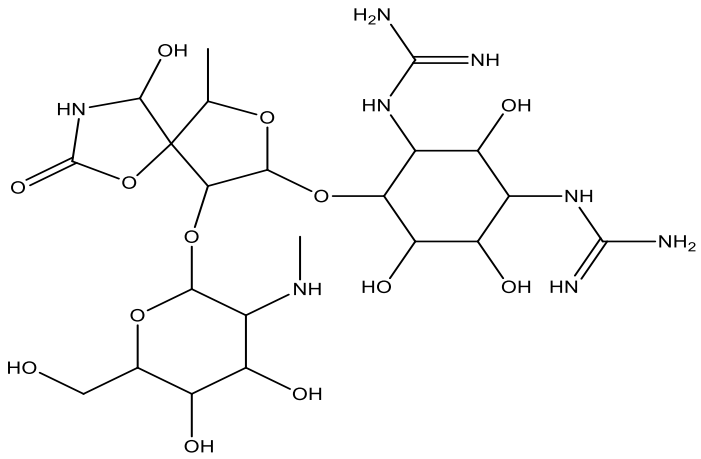


Figure 5: The structure of Rhodostreptomycin (Elsayed et al. 2017).

Rhodococcus sp. MTM3W5.2

Rhodococcus sp. MTM3W5.2 is a strain that was isolated from lawn soil in Morristown, Tennessee in 2011 (Borisova 2011). The bacteria was isolated using an M3 enrichment (heat shock) method and was discovered to produce a potent inhibitory compound active against other *Rhodococcus* species, including the zoonotic pathogen *R. equi*. The inhibitory molecule was discovered using a disk diffusion screen of agar culture extracts. The strain grows large, flat colonies with tan pigmentation at 22°C as shown in figure 6. The sequenced 16S rRNA gene shows a 99% match to that of *R. mannshanesis* (Ward et al. 2018)

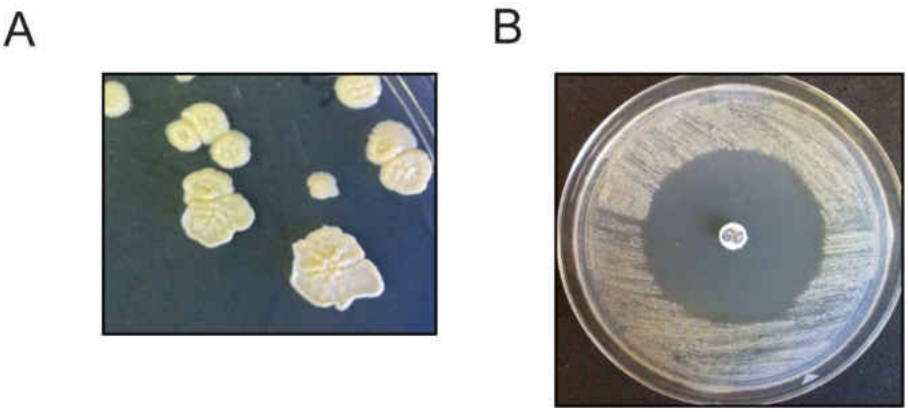


Figure 6: MTM3W5.2 colonies and antibacterial activity. (A) MTM3W5.2 colonies appear large, flat, and have a tan pigmentation. (B) a disk diffusion assay showing the inhibitory molecule against an indicator strain (Ward et al. 2018).

The Inhibitory Molecule

The inhibitory molecule produced by MTM3W5.2 is active against other *Rhodococcus* species and other closely related species, such as *Corynebacterium* and *Gordonia*, but is not active against other Gram positive or negative bacteria. The compound is only produced in stagnant cultures under 22°C. The entire structure of the compound has yet to be determined, but fragments of the compound that have been identified indicate a macrolide-like structure. The compound has a molecular weight of 911.5490 *m/z* and a chemical composition of $C_{52}H_{70}O_{13}$. The gene responsible for this antibiotic was discovered by generating non-producing mutants with random transposon insertional mutagenesis with the transposon pTNR (Ward 2015). Two nonproducing mutants, 2.31 and 77.23 were identified, and the biosynthetic gene cluster responsible for producing the antibiotic was discovered when the entire genome of the producer strain was sequenced. The cluster indicates several type I polyketide synthases that are presumably required to produce the compound shown by the blue arrows in figure 7. Gene

BTZ20_3964, the green arrow in the figure lies at the beginning of the gene cluster, is believed to be the regulator of the cluster.

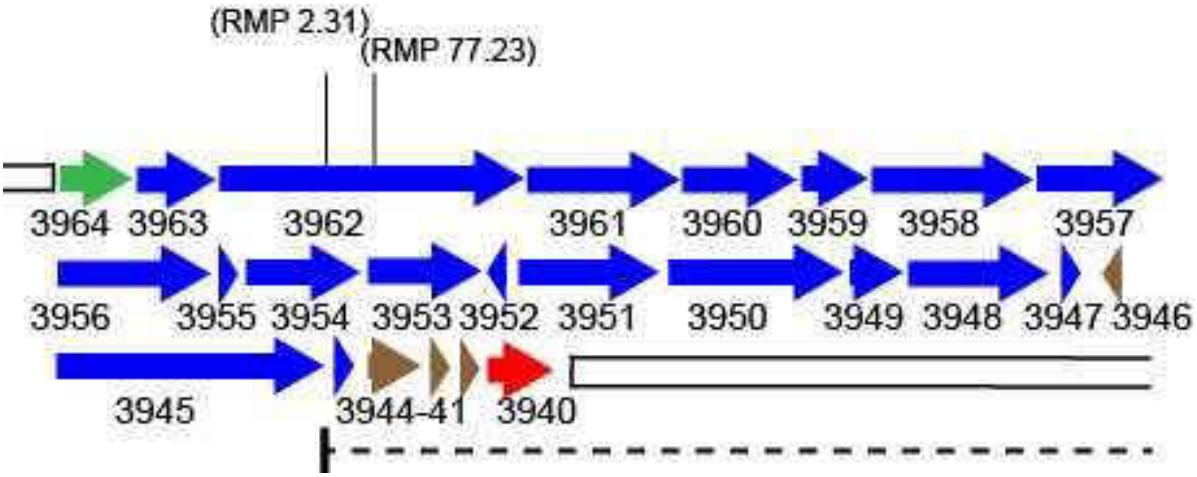


Figure 7: Biosynthetic gene cluster responsible for the inhibitory molecule. Blue arrows are type I PKS genes, green arrow in a transcriptional regulatory gene, red arrows are NRPS type genes, and brown arrows are other gene classes. RMP 77.23 and 2.31 designate the pTNR transposon insertion location to produce a nonproducing mutant. BTZ20_3964 is believed to be a positive regulator of the gene cluster (Ward et al. 2018).

Current Work

Rhodococcus is an underexplored and potential source of new antibiotics. Genomic sequencing indicates that there are a variety of BCGs within the genome of many *Rhodococcus* species, but the function of the majority of these products are unknown and appear to be produced under cryptic pathways. The gene BTZ20_3964 is believed to be a positive *luxR* solo type regulator of the gene cluster. For my project, I hypothesized a deletion of this gene from the genome would render a mutant strain no longer able to produce the inhibitory compound. I also hypothesized that this gene can be cloned into a high expression vector and complement the non-producing mutant, which should restore production of the compound, but may also boost production or awaken other cryptic pathways in the cell.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Growth Media

Lysogeny Broth Medium (LB)

This medium was prepared by combining the following components. The following amounts are for every 500mL of distilled H_2O:

1. Tryptone 5g
2. Yeast Extract 2.5g
3. NaCl 5g
4. dH_2O 500mL
5. Bacto Agar 7.5g

LB plates with 10% sucrose are made by excluding NaCl and adding 50g of sucrose per 500 mL of media.

Rich Media (RM)

This medium was prepared by combining the following components. The following amounts are for every 500mL of distilled H_2O:

1. Glucose (Dextrose) 5g
2. Nutrient Broth 4g
3. Yeast Extract 0.25g
4. Bacto Agar 7.5g
5. dH_2O 500mL

Mueller-Hinton Medium (MH)

MH plates were made by adding 19.5g of Difco™ Mueller0Hinton Agar to 500 mL of dH_2O. The mixture was boiled to mix the ingredients and autoclaved, then cooled in a 55°C water bath. Once cooled, the agar was poured into sterile Petri dishes.

MH broth was prepared by adding 11g of BBL™ Mueller-Hinton Broth to 500 mL of dH_2O. The mixture was boiled to mix and autoclaved.

Bacterial Strains

The strain *Rhodococcus* sp. MTM3W5.2 was used as the wild type strain that produces the inhibitory compound. *E. coli* DH5α was used for plasmid isolation of pEX18km and pEX18kmAD3964 for transformation into *Rhodococcus* sp. MTM3W5.2 to create the merodiploids. *E. coli* DH5α was inoculated on LB medium and incubated at 37°C overnight. *Rhodococcus* MTM3W5.2 was inoculated into RM or LB medium and incubated in a 28°C water bath for 24 hours. Kanamycin was added to the medium at a concentration of 200-400 µg/mL when used for selection. RM slants of the parent strain, merodiploids, and mutant strains were made and stored at 4°C.

Bacterial Seed Cultures

Seed cultures were prepared by inoculating a pure *Rhodococcus* MTM3W5.2 colony in 2mLs of RM broth and incubating in a shaking water bath at 28°C for 1-2 days. Seed cultures were used for genomic DNA isolation, RNA isolation, and preparation of electro-competent cells. *E. coli* seed cultures were inoculated into LB and grown at 37°C overnight.

Genomic DNA Isolation from *Rhodococcus* Cells

A 2 mL LB broth seed culture (with Kanamycin^{200} µg/mL if isolating merodiploids) was prepared and incubated in a 28°C water bath shaking overnight. 0.5 mL of the seed culture was used to inoculate 25 mL of LB broth (with antibiotic if necessary) in a 250mL flask with a cotton stopper and incubated at 28°C in a shaking water bath at least 24 hours. 200 µg of ampicillin from a stock of 50 mg/mL was added to the flask (final concentration around 8ug per mL), and the culture was allowed to incubate in the 28°C water bath while shaking. After 24 hours, 10-15 mL of the culture was placed in a sterile Oakridge tube, and spun to pellet the cells. The supernatant was discarded, and the cells are washed in 10 mL of 1X TE buffer. Cells are pelleted again and supernatant was discarded. The pellet was resuspended in 1 mL of TES buffer and 1 mL of 1X TE buffer with 50 µg of fresh lysozyme. The cells were incubated at 37°C for at least 4 hours while shaking. 500 µL of the mixture was then added to sterile 1.5 mL microfuge tubes. 30 µL of 20% SDS was added to each tube and tubes are inverted to mix. 10 µL of RNase (33 µg/mL) was added to each tube, mixed by inverting, and incubated at 55°C for 15 minutes. 10 µL of proteinase K (20 µg/mL) was added to each tube, mixed by inverting, and incubated at 55°C for 15 minutes. A phenol-chloroform extraction was performed on each tube. 500 µL of

phenol was added to each tube and mixed by inverting, the tubes are spun, and the top layer was collected to a new tube. Two chloroform-isoamyl extractions are performed on each tube by adding 500 µL chloroform-isoamyl (24:1), mixing by inverting, spinning and transferring the top aqueous layer to a fresh tube. To the final tube, add 1 mL of cold 100% ethanol and mix by inverting. The chromosome would then precipitate and clot. The chromosome was spooled with a pipet tip and transferred to a new 1.5 mL microfuge tube containing 400 µL of 70% ethanol. The tube was spun for 1 minute and the ethanol was aspirated. The chromosome is allowed to briefly air dry, then was re-dissolved in 100 µL of 0.1 TE and stored at 4°C.

Genomic DNA Isolation Solutions

1X TE Buffer

5 mL	1M Tris-HCL, pH 7.5
1 mL	0.5 M EDTA, pH 8.0
499 mL	ddH_2O

TES Buffer

9.8 mL	50 mM Tris, pH 8.0
20 µL	0.5 M EDTA
213 µL	40% Sucrose

Deletion of the gene BTZ20_3964

The postulated *luxR* solo gene, BTZ20_3964, was deleted from the chromosome of strain MTM3W5.2 by the method of "overlap extension PCR" (Heckman and Pease 2007). Here a set of four primers; 3964-A, 3964-B, 3964-C, and 3964-D, were designed such that primers 3964-A

and 3964-B amplify about 1119 bp of flanking DNA upstream of the gene BTZ20_3964 and primers 3964-C and 3964-D amplify about 1045 bp of flanking DNA just down stream of the gene. However, primers 3964-B and 3964-C are mutagenic primers that are complementary to each other and can produce a nearly complete deletion of the 3964 gene. The deletion construct, 3964-AD, was produced when the amplified DNAs 3964-AB and 3964-CD are mixed and annealed together at their 3'-ends (their 3'-ends are complementary due to the presence of the B and C primers and can base pair together) (Fig. 8). This produces a template DNA that can be amplified in a final PCR reaction to give the deletion construct 3964-AD. This DNA contains about 1kb of both upstream and downstream flanking DNA sequence, but a nearly complete deletion (only 164 out of 2,790 bp remain) of the BTZ20_3964 gene (Fig. 8).

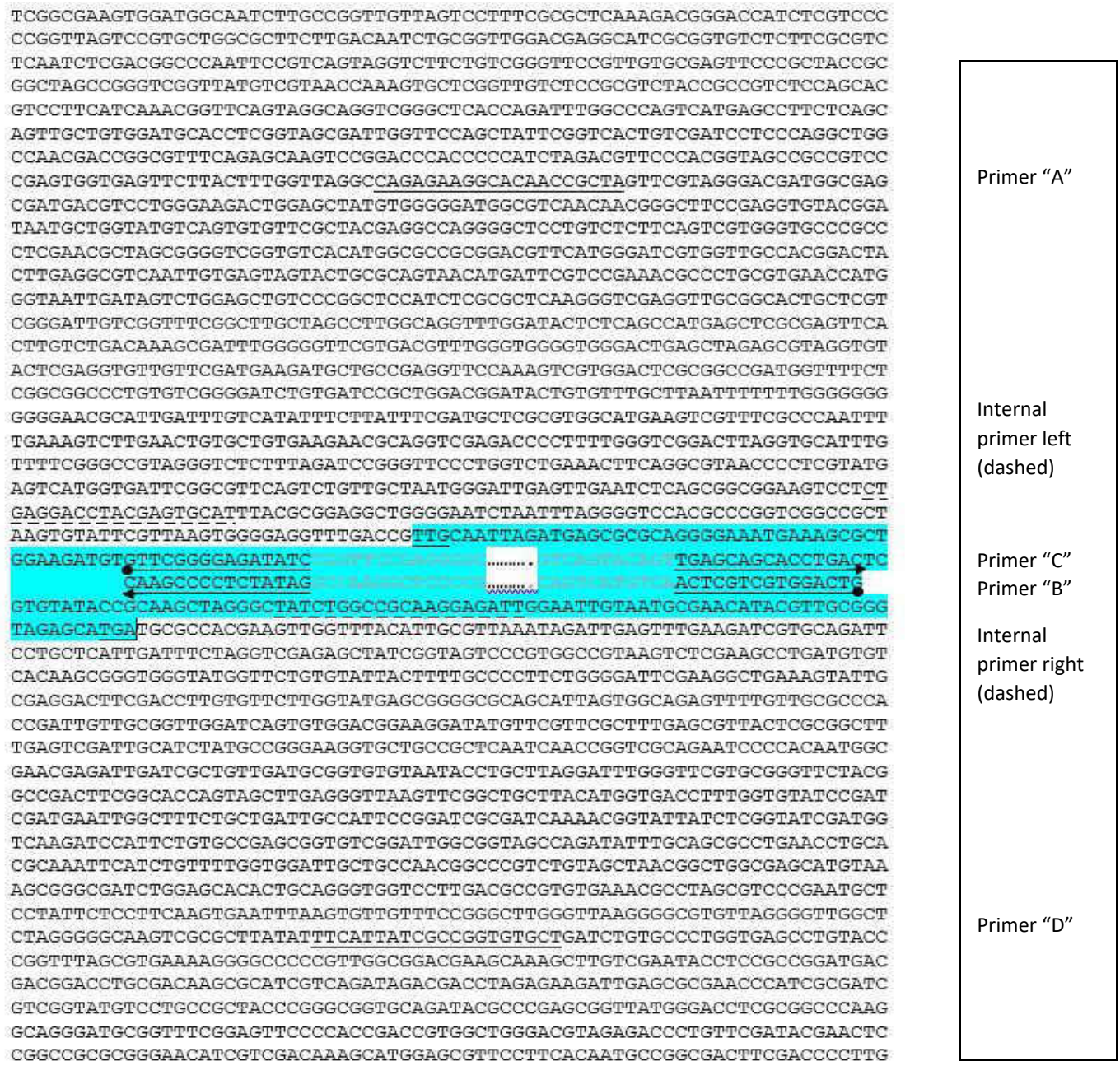


Figure 8. (Continued on next page)

3964-A: 5’- ATGGATCCAGAGAAGGCACAACCGCTA

3964-B: 5’- GTCAGGTGCTGCTCAGATATCTCCCCGAAC

3964-C: 5’-GTTCGGGGAGATATCTGAGCAGCACCTGAC

3964-D: 5’-TCAAGCTTAGCACACCGGCGATAATGAA

Figure 8. Primer design for overlap extension PCR. Blue highlighted sequence is the beginning (TTG start codon) and end (TGA stop codon) of the gene BTZ20_3964 (dots represent large missing region of gene). Solid underlined sequences are primers “A” and “D”. The mutagenic primers “B” and “C” are underlined with a solid line beginning with a black dot and ending with an arrow. Sequences underlined with a dashed line are internal primers used to detect the deleted copy of the 3964 gene in the chromosome of MTM3W5.2.

Creation of Deletion Construct pEX18km3964AD

The suicide vector pEX18Gm as described by Hoang et al. (1998) was used to create the deletion construct. In the plasmid pEX18Gm, the gentamycin resistance marker was replaced with a kanamycin resistance gene at the restriction enzyme sites *Eco*RV and *Ssp*I ,via blunt ligation to create pEX18Km (Fig.14). Both 3964AD (PCR)DNA and pEX18Km plasmid DNA were digested with the restriction enzymes *Bam*HI and *Hin*dIII, and ligated together to create the deletion construct pEX18Km3964AD (Fig.14).

Plasmid Isolation

Plasmids pEX18km, pEX18km3964AD, and pDD57 were transformed into *E. coli* DH5α and isolated using a protocol described by Ward (2015).

Preparation of Electro-Competent Cells

For *Rhodococcus* MTM3W5.2 electro-competent cells, a 2 mL seed culture in RM broth was incubated at 28°C overnight. 1 mL of the seed culture was used to inoculate 50 mL of RM broth in a 250 mL "side arm" flask. The initial density was measured with a Klett colorimeter. The flask was incubated in a 28°C water bath until a Klett reading of around 80 (red filter) was reached; this took about 48 hours. A loop of the culture was streaked out on an RM agar plate and incubated at room temperature for 4 days to check for contamination. The remaining culture was transferred to a 250 mL centrifuge bottle and set on ice for 5 minutes. The culture was centrifuged in a large rotor (FiberliteTM F14-6 x 250y Fixed-Angle Rotor, Thermo Scientific) at 6,000 rpm for 7 minutes at 4°C. The supernatant was discarded and using a pipet, the pellet was resuspended in 30 mL of ice cold 10% glycerol and left to rest on ice for 10 minutes. The centrifuge conditions were repeated and the supernatant was discarded. Again using a pipet, he cell pellet was resuspended in 15 mL of ice cold 10% glycerol and transferred to an Oakridge tube and iced for 10 minutes. The centrifuge was repeated with the same conditions in a small rotor (FiberliteTM F14-6 x 50cy Fixed-Angle Rotor, Thermo Scientific). The supernatant was discarded and the cells are resuspended in 2.5 mL of ice cold 10% glycerol. The centrifuge was repeated, and the supernatant was discarded and the pellet was resuspended in 600 µL of ice cold 10% glycerol. It is important to use a smaller pipette volume here to avoid generating bubbles. 100 µL aliquots of the resuspended cells are transferred to 0.5 mL microfuge tubes and stored at -70°C.

For *E.coli* electro-competent cells, an overnight seed culture of *E. coli* DH5α in 10 mL of YENB broth (0.75% yeast extract, 0.8% nutrient broth) was grown at 37°C. 5-10 mL of the seed

culture to inoculate 250 mL of YENB broth in 500 mL flask and incubated shaking until a Klett reading of 40-45 (red filter) was reached, about 5 hours. Centrifuge conditions are 4000xg for 10 minutes at 4°C. 25 mL for cold sterile water are used for the first two washes, and 5 mL and 1 mL of 10% glycerol are used for the second two centrifuges. Aliquots of 110 µL are transferred to 0.5 mL tubes and stored at -70°C.

Creation of the Merodiploids

Merodiploids of *Rhodococcus* MTM3W5.2 are created by transformation of the deletion construct pEX18km3964AD into electro-competent MTM3W5.2 cells via electroporation. Electro-competent MTM3W5.2 cells and the pEX18km3964AD plasmid prep are chilled on ice to allow to thaw. UV sterilized electroporation curvettes (Fisher Scientific, 2 mm width, 400 µL volume) are set on ice while the cells thaw. 2 µL of the plasmid DNA was added to the competent cells, and the total volume was pipetted into the sterile curvettes, taking care to avoid bubbles. The cells are electroporated at 2,500 volts (V). The pulse time was recorded; between 3-5 seconds is ideal. If the pulse time was outside of this range, or if the cells "pop", the cells are discarded and the electroporation was repeated with new cells. 400 µL of RM broth was added to the transformed cells and transferred to a sterile test tube. The cells are incubated in a 28°C water bath for 3-5 hours while shaking. After incubation, cells are plated undiluted on LB Kan^{200} and LB Kan^{400} plates. The plates were incubated at room temperature until colonies appear, around 5-7 days. Colonies were transferred to wheel plates with the same kanamycin concentration. Controls with cells transformed with no DNA were done, and spontaneous mutants or background colonies were often observed on plates with a kanamycin concentration under 200 µg/mL, so a higher concentration was used for selection. The wild-type gene is 2,793 base pairs.

The amplicon for the internal 3964 primers in the wild-type gene used is about 2,800 base pairs, while the amplicon using the same primers with the fragment AD will be much smaller at about 220 base pairs and should run farther on a gel when used to screen for the deletion construct with PCR.

Mutant Wheels

After possible mutant colonies are plated on a wheel plate (Fig. 9), the plate was allowed to grow at room temperature for at least 2 days. The plate was then stored at 4°C and the strains should remain viable for years if the plate does not dry out. Colonies that produced adequate growth were labeled and used to screen for the presence of the pEX18km3964AD deletion construct.

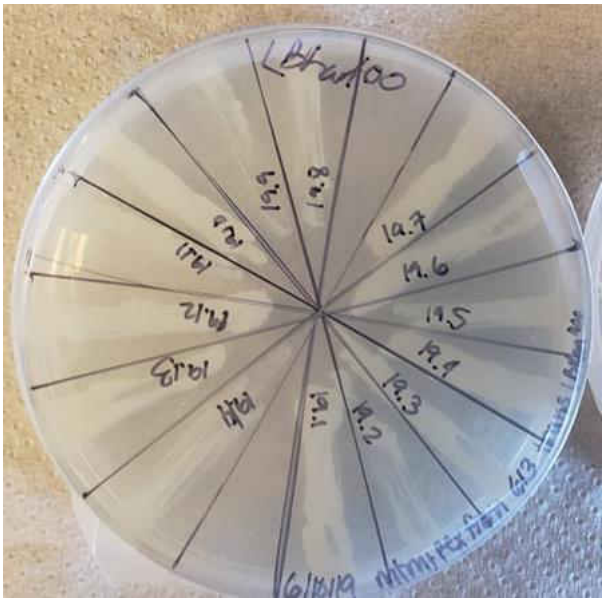


Figure 9: A mutant wheel of transformants. Single colony transformants are streaked on a plate with antibiotic. No more than 16 mutants are placed on a single wheel.

Screening for Merodiploids

Single colony PCR was used to screen for the presence of the pEX18km3964AD construct via a single cross-over recombination with either the upstream or down stream flanking regions of the BTZ20_3964 gene. A single colony (or equivalent cells) was suspended in PCR-H_2O and used as a DNA template for the PCR reaction. The primers pTNRKan left (5'-TAGCTTGCAGTGGGCTTACAT) and pTNRKan right (5'-TTCAGCAATATCACGGGTAGC) were used first to confirm the presence of the kanamycin resistance gene. Any colonies that have a kanamycin resistance gene was then screened with the primers for the s*acB* gene (*sacB* left (forward) 5'GGAAGGCAGTACACCTTGATAG-3',*sacB* right (reverse) (5'GCTCTCCTGAGTAGGACAAATC-3') and internal gene 3964 primers (3964internal left: 5'-CTGAGGACCTACGAGTGCAT-3' and 3964 internal right: 5'-AATCTCCTTGCGGCGGCCAGATA-3'). Alternatively, the Kan gene was screened for by plating potential mutants on LB plates with a 400µg and an 800µg kanamycin disk to look for resistance to kanamycin. Mutants showing resistance to both disks are screened using PCR for the kan gene, s*acB* gene, and the 3964 internal primers. A merodiploid should have 2 bands present for the 3964 internal primers; the larger, wild-type gene 3964 and the smaller, deleted AD3964 from the deletion construct. Colonies that showed all of the desired bands were selected as potential merodiploids and streaked on an RM slant and plated on LB Kan^{200} plates to maintain selection. To determine if the merodiploid was an illegitimate recombinant where a single crossover event has occurred in an area of the chromosome which was not the region of homology adjacent to the gene 3964, 4 primers were created to screen for the location of the inserted plasmid. The primers amplify the regions outside of the flanking regions of gene 3964 and a region internal to the gene. If the plasmid inserts into one of these flanking regions, the

amplicon from these primers should be very large (about 9 Kb) or maybe too large to amplify with the PCR conditions used to screen for the merodiploids. Primer sequences are as followed.

3964newABprimer left 5'-TCTTGCCGGTTGTTAGTCCT-3'

3964newABprimer right 5'-CACCTTACGAGCAATGTCCG-3'

3964newCDprimer left 5'-TGCCACTGAAGGTACGGATT-3'

3964newCDprimer right 5'-GGTTCGCGCTCAATCTTCTC-3'

Sequence around BTZ20_3964 (LuxR solo)

TCGGCGAAGTGGATGGCAATCTTGCCGGTTGTTAGTCCTTTCGCGCTCAAAGACGGGACCATCTCGTCCCCCGG
TTAGTCCGTGCTGGCGCTTCTTGACAATCTGCGGTTGGACGAGGCATCGCGGTGTCTCTTCGCGTCTCAATCTC
GACGGCCCAATTCCGTCAGTAGGTCTTCTGTCGGGTTCCGTTGTGCGAGTTCCCGCTACCGCGGCTAGCCGGGT
CGGTTATGTCGTAACCAAAGTGCTCGGTTGTCTCCGCGTCTACCGCCGTCTCCAGCACGTCCTTCATCAAACGG
TTCAGTAGGCAGGTCGGGCTCACCAGATTTGGCCCAGTCATGAGCCTTCTCAGCAGTTGCTGTGGATGCACCTC
GGTAGCGATTGGTTCCAGCTATTCGGTCACTGTCGATCCTCCCAGGCTGGCCAACGACCGGCGTTTCAGAGCAA
GTCCGGACCCACCCCCATCTAGACGTTCCCACGGTAGCCGCCGTCCCGAGTGGTGAGTTCTTACTTTGGTTAGG
CCAGAGAAGGCACAACCGCTAGTTCGTAGGGACGATGGCGAGCGATGACGTCCTGGGAAGACTGGAGCTATGTG
GGGGATGGCGTCAACAACGGGCTTCCGAGGTGTACGGATAATGCTGGTATGTCAGTGTGTTCGCTACGAGGCCA
GGGGCTCCTGTCTCTTCAGTCGTGGGTGCCCGCCCTCGAACGCTAGCGGGGTCGGTGTCACATGGCGCCGCGGA
CGTTCATGGGATCGTGGTTGCCACGGACTACTTGAGGCGTCAATTGTGAGTAGTACTGCGCAGTAACATGATTC
GTCCGAAACGCCCTGCGTGAACCATGGGTAATTGATAGTCTGGAGCTGTCCCGGCTCCATCTCGCGCTCAAGGG
TCGAGGTTGCGGCACTGCTCGTCGGGATTGTCGGTTTCGGCTTGCTAGCCTTGGCAGGTTTGGATACTCTCAGC
CATGAGCTCGCGAGTTCACTTGTCTGACAAAGCGATTTGGGGGTTCGTGACGTTTGGGTGGGGTGGGACTGAGC
TAGAGCGTAGGTGTACTCGAGGTGTTGTTCGATGAAGATGCTGCCGAGGTTCCAAAGTCGTGGACTCGCGGCCG
ATGGTTTTCTCGGCGGCCCTGTGTCGGGGATCTGTGATCCGCTGGACGGATACTGTGTTTGCTTAATTTTTTTG
GGGGGGGGGGAACGCATTGATTTGTCATATTTCTTATTTCGATGCTCGCGTGGCATGAAGTCGTTTCGCCCAAT
TTTGAAAGTCTTGAACTGTGCTGTGAAGAACGCAGGTCGAGACCCCTTTTGGGTCGGACTTAGGTGCATTTGTT
TTCGGGCCGTAGGGTCTCTTTAGATCCGGGTTCCCTGGTCTGAAACTTCAGGCGTAACCCCTCGTATGAGTCAT
GGTGATTCGGCGTTCAGTCTGTTGCTAATGGGATTGAGTTGAATCTCAGCGGCGGAAGTCCTCTGAGGACCTAC
GAGTGCATTTACGCGGAGGCTGGGGAATCTAATTTAGGGGTCCACGCCCGGTCGGCCGCTAAGTGTATTCGTTA
AGTGGGGAGGTTTGACCGTTGCAATTAGATGAGCGCGCAGGGGAAATGAAAGCGCTGGAAGATGTGTTCGGGGA
GATATCCGGTTCCGAGGGGCGCGTCGTCATAGTAACCGGAGCTTCCGGAGCGGGGAAGACTTCGGTGGTCCAGG
CCTTTGGAGCGCGGCTGCCCGCTGATGTTTGTTTTTGGTCCGCTGTTGGAGCTCGCGCCGAGCGTGACTTGCCT
CTGGGGATACTAGCACAGCTACTTCAACAACAGGCTGTAGATTGTGAGGTGGAATCTCTCTATTACTCCGCACT
CGCCATTCTGAGGGATGCGGAAATGGGGACGGGTGAGGTATTGGCGGGCCCGATGCATAGGCTCGGAGTCGCGA
TCGCGGACATTGCTCGTAAGGTGAAGCTCGTCATCGCAATTGATGATGTGCATTACGCTGACGAGGCTTCACTC
CAGGTCTTGTCCTACGTGCTTCGGCGGTTGGGATCGATTCCGGTGTTGATTGTGGTCACAAGGCCCTTCGGCCC
ATCGATTTCACCGTCGGCATCTTACGCCGAGTTCTCGAGGTGCCCGGGAATACGTCATCTACGGCTGGGTCTGC
TGGGGATTAGCGACATCTATCACATGCTCGTCGGTATCGTGGGCGAGCAATCGGCGGGGCCGCTCTCGGCTGAC
TGTCTCGAGCTGACGGGAGGCAATCCGCGTTTGGTCCGAGCGTTCGCGGAGCAATGTCGCTGGTGGGCCGAGTC
TGGTGACTTGTCGCTTGACGATTGGAACATTCGCCGCATATGTGGGCTTGATTTGGGGCAGGCGGCGCTGGCGT
GCTTGCACAGCATGGACGAGACTGCGGTCCAGCTCGGGACTAGTCTGGCAATACTGGGCGATGAAGCCACCATC
GGCAGGCTTGTACGGTTGACGGGCATCGGGGTCTGCGTTGTGAATGATGTCGTCAGCAGTCTCTCGGCGGCAGG
GCTAGTCGCTTCTGGCAGATTCCGGAATCCGCTGCTGGGTGAGGCGATAGTTGAATGCTTGCCCGTCGCGGAGA
AGATCCAACTGCATCGCAACGTTGCTCGTCTATGTTTCGACGAAGGCAGAGAGCCTTTGGAGGTTGCCCGCTAT
CTGGTGGCCGCAGGTTCGGCGACCGAGCCTTGGGCGGGTCAGGTGCTGCGTGACGCGGCTATCGAAGCGGTTCG
AATTGAGGACAGGCAGCTGGCGATGGAGTGCCTGCAGTTGGCTGAAACTCGATGCGCGAACGAGAGCGATAGGG

Figure 10: Location of new AB primers. Location of primers for amplification of the upstream flanking region of gene BTZ20_3964 for merodiploid legitimacy testing. Yellow highlighted sequences are primers 3964 NewABprimer left and 3964 New AB primer right. Blue highlighted sequence in the 3964 ORF sequence. Underlined sequences are the old primers used to create the AB region of homology for overlap extension PCR.

Figure 11: Location of new CD primers. Location of primers for amplification of the downstream flanking region of gene BTZ20_3964 merodiploid legitimacy testing. Underlined sequences are the primers used to create the CD homology region for overlap extension PCR.

The PCR program (named “Tom1”) was used for Promega’s Gotaq Flexi polymerase and was used to screen for these genes. The program is as follows

1) 95°C 5 minutes
2) 95°C 1 minute
3) 55°C 1 minute
4) 72°C 2 minutes
5) Steps 2-4 repeated (x29)
6) 72°C 5 minutes
7) 4°C Hold

Screening for the Double Crossover Recombination Event

Merodiploids are preserved on plates with kanamycin to maintain selection. A single colony was selected, and the merodiploids are grown in LB broth with no kanamycin to encourage a double crossover event. These cultures were grown shaking in a 28°C water bath for 2 days. Dilutions are plated on LB plates with 10% sucrose and no NaCl added. Dilutions of at least 1/1000 are used to get isolated colonies. Sucrose plates are used to counter-select the *sacB* gene. Colonies are selected from the LB sucrose plates and plated on replica patch plates to screen for the loss of the kanamycin resistance gene. Colonies with the loss of the resistance gene are selected and plated on LB plates and screened again with the same primers, pTNR Kan right and left, *sacB* right and left, and 3964 internal right and left. Double crossovers should retain the smaller deleted band for the 3964 internal primers but should lack all other bands. Double crossovers are screened for antibiotic production via agar extraction.

Cloning BTZ20_3964 into pDD57

The expression vector pDD57 (DeLorenzo et al. 2018) contains a high activity promotor that should allow for high expression of the 3964 gene in *Rhodococcus* and is used for complementing the non-producing mutants. The gene 3964 was amplified by PCR using the program “Phusion1”(for the phusion taq polymerase from New England Biolabs) and the primers BTZ20_3964left680 5’-TTGCTTAATTAACGGAGGCTGGGGAATCTAAT-3’ and BTZ20_3964right3854 5’- TATTGAATTCTTCCGTCCACACTGATCCA-3’ . The PCR reaction was ethanol precipitated and then digested with the restriction enzymes *Eco*RI-HF and *Pac*I for 90 minutes at 37°C. The reaction was heat inactivated at 65°C for 20 minutes. The

expression vector, pDD57 was also digested with these enzymes in these conditions and was then gel purified after gel electrophoresis via electroelution. The digested PCR DNA (gene 3964) was ligated into the digested plasmid and the ligation reaction was transformed into electrocompetent *E. coli* (DH5α). Antibiotic production would be screened with agar extraction and disk diffusion assays (Ward 2015).

"Phusion1": Conditions for NEB Phusion (high fidelity) polymerase

1) 98°C 30 seconds
2) 98°C 10 seconds
3) 67°C 30 seconds
4) 72°C 30 seconds
5) Steps 2-4 repeated x29
6) 72°C 9 minutes
7) Hold at 4°C

Amino Acid Sequence Alignments

An amino acid multiple sequence alignment of the gene BTZ20_3964 was made with other *luxR* solo homologs to show areas of conservation using the program Clustal Omega (Sievers et al. 2011). The reference sequences are those described by Brotherton et al.(2018) and are listed in Table 2.

Table 2. *luxR* Amino Acid Sequences used for the Multi-Sequence Alignment (Brotherton et al. 2018)

Name	Species	Description	Accession Number
Streptomyces	*Streptomyces canus*	Putative LuxR family transcription regulator	AKQ13286.1
traR	*Agrobacterium tumefaciens*	Transcriptional regulator	WP_012478148.1
bjaR	*Bradyrhizobium diazoefficiens* JCM	Transcriptional activator protein	Q89VI3.1
luxR	*Aliivibrio fischeri* ES114	Autoinducer-binding transcriptional activator protein	AAW87995.1
phzR	*Pseudomonas chlororaphis*	Transcriptional activator protein	P54303.1
lasR	*Pseudomonas aeruginosa* PAO1	transcriptional regulator	AAG04819.1

CHAPTER 3

RESULTS

Gene BTZ20_3964 is a Possible *luxR* Solo Regulator

The suspected regulatory locus BTZ20_3694 is a *luxR* solo regulator, meaning it possibly regulates expression of the inhibitory compound produced by MTM3W5.2 without a *luxI* type synthase. A map of the functional domains in the protein is shown in figure 12, part A. Based on a BLAST homology search, only two functional domains were revealed in this large 930 amino acid protein. The first 300 amino acids at the N-terminus show similarity to an ATP binding domain. About the last 300 amino acids at the C-terminus shows similarity to *luxR* regulatory proteins. A multiple sequence alignment of this C-terminal region with other *luxR* solo proteins was constructed using the program Clustal Omega using the reference sequences of a *Streptomyces luxR* solo and other solos from *Proteobacteria* as described by Brotherton et al. (2018). The gene BTZ20_3694 is 930 amino acids long, which is much larger than that of the other reference genes, so the alignment only shows the *luxR* solo region of the protein (Green hatched region, part A, Fig.12), which is composed of roughly the last 300 amino acids. Highly conserved amino acids in the *luxR* solo proteins found among the *Proteobacteria* are highlighted in yellow (part B, Fig.12) and is based on the analysis of Brotherton et al. (2018). The *Rhodococcus luxR* solo is more closely related to the *Streptomyces* regulator than the reference *Proteobacteria* homologs. According to Brotherton et al. (2018), the amino acids at the positions labeled “1” and “2” (part B, Fig. 12) are conserved among all the *Proteobacteria luxR* solos but differ in these proteins found in the *Actinobacteria*. Indeed, the conserved tryptophan at position labeled “1” is instead an alanine in both the *Streptomyces* and *Rhodococcus luxR* proteins.

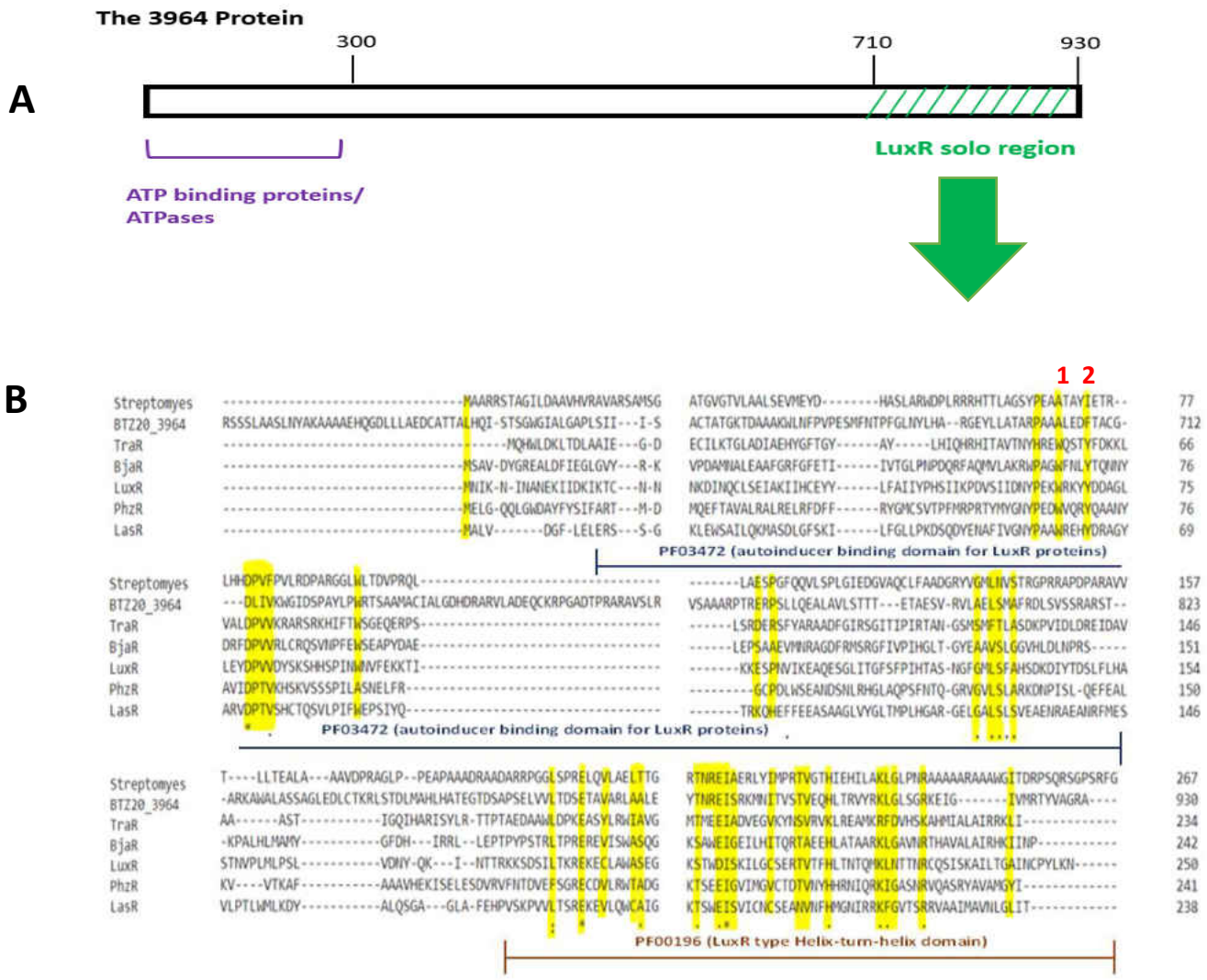


Figure 12:Multiple sequence alignment and map of gene 3964. A: A map of the 3964 protein domains. The *luxR* solo region is shown in green hatching. Green arrow designates region of the protein in the sequence alignment in part B. B: A multiple sequence alignment of the 3964 gene with that of other *luxR* solo homologs made with Clustal Omega. Reference sequences are listed in table 2. Positions 1 and 2 mark the locations where the *Actinobacteria* proteins are described to differ from the *Proteobacteria* (Brotherton et al., 2018). This is within the conserved autoinducer binding domain of *luxR* regulatory proteins.

Creation of the Deletion Construct pEX18Km3964AD

In the first set of PCR reactions, flanking DNA upstream (DNA "AB", Fig.13) and downstream (DNA "CD", Fig.13) were amplified. Then DNAs AB and CD were annealed

together at their 3" ends and extended (Fig. 13). The AD fragment, containing the deleted version of the 3964 gene was cloned into the plasmid pEX18km and the map was show in figure 14. The kanamycin resistant marker can be used to select for the merodiploid and the *sacB* gene can be used for counterselection to obtain a double-crossover mutant.

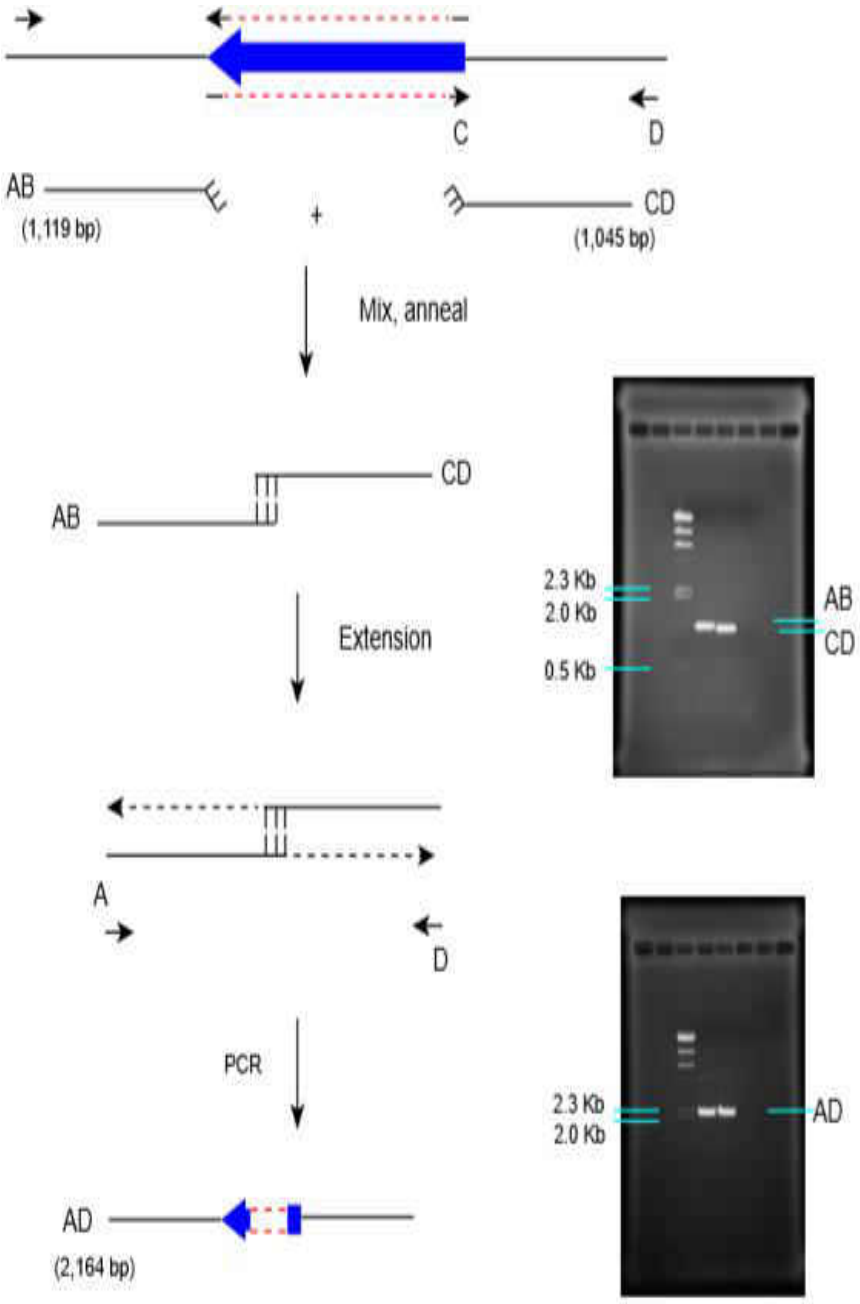


Figure 13: Creation of the 3964AD gene via PCR-overlap extension

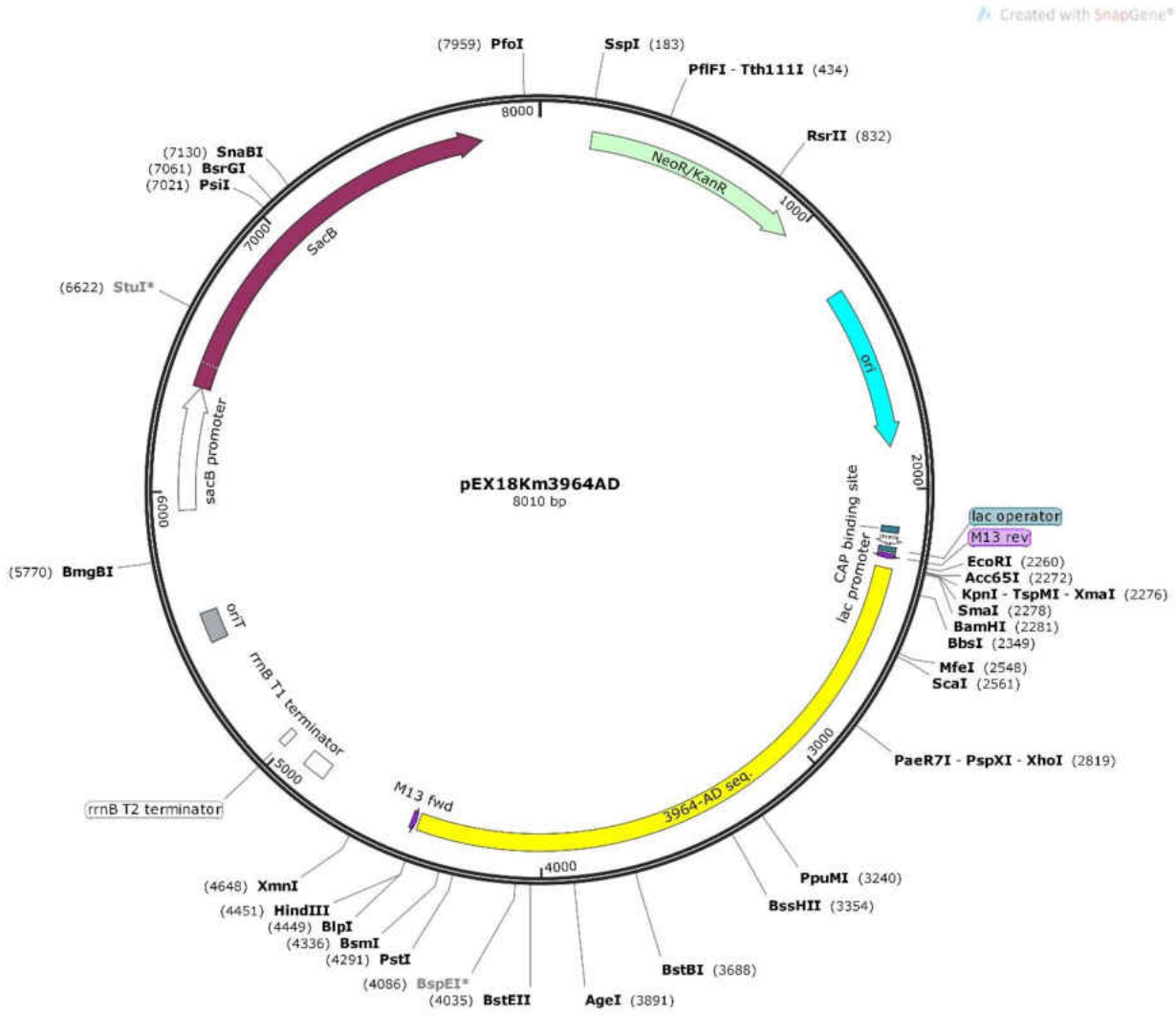


Figure 14: Map of the deletion construct pEX18Km3964AD

Creation of the MTM3W5.2 Merodiploids with pEX18km3964AD

The suicide vector pEX18km was used to introduce the deletion construct 3964AD into the *Rhodococcus* genome in order to generate a merodiploid. The plasmid does not contain an origin of replication for *Rhodococcus*, so it cannot replicate in MTM3W5.2 and allow the bacteria to grow on a kanamycin plate unless a single crossover occurs and the deletion construct

inserts into the genome. Figure 15 part "A": shows the single crossover recombination event to give a merodiploid of the 3964 gene.

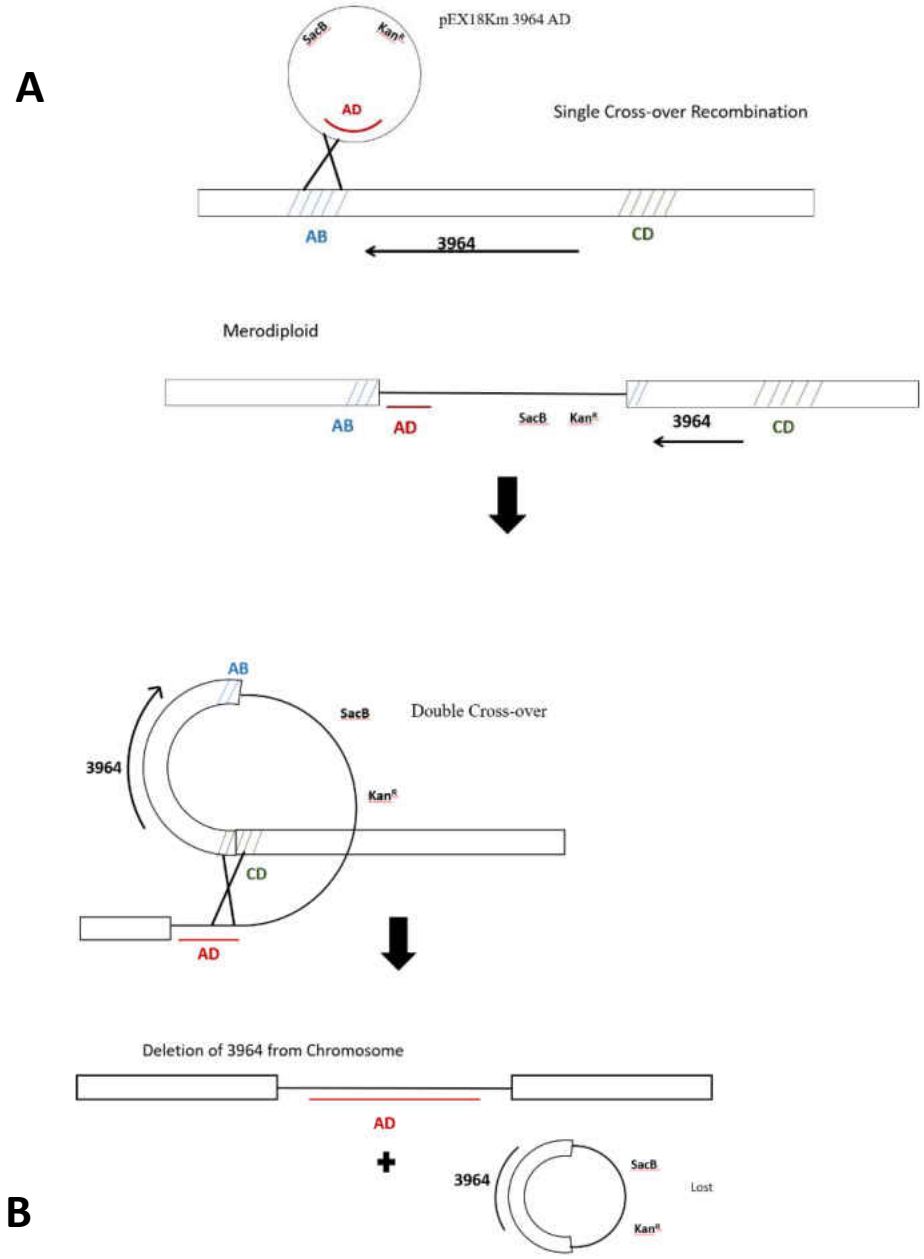


Figure 15: Diagram of the single and double crossover recombination events. In the single crossover "A", the plasmid with the deletion construct inserts into the MTM3W5.2 genome in regions flanking the 3964 gene to make the merodiploid. In part B, a double crossover occurs to replace the functional 3964 gene with the deleted version. The *kan*^{R} gene and *sacB* gene are also lost from the genome.

Plating dilutions of the transformants on LBKan^{50} plates yielded a high number of background colonies that grew on the control plate that had no DNA added to the transformation, so the concentration of antibiotic used for selection was increased. Despite increasing the antibiotic concentration for selection to 400µg/mL, background colonies still appeared on control plates with no DNA after 6-8 days, but the numbers were reduced from the lower antibiotic concentration plates. The parent strain *Rhodococcus* MTM3W5.2 was susceptible to kanamycin at these concentrations, but would, apparently, produce spontaneous mutants that can grow on kanamycin plates. Transformants were plated undiluted on either RM Kan^{400} or LB Kan^{400} plates to increase the yield of merodiploids. Colonies that appeared on plates varied from 3 colonies to 30 colonies per plate and were screened with PCR using the primers to test for merodiploids. Over 200 colonies were screened with the majority of transformants appearing negative for the kanamycin resistance gene. Transformants sometimes tested with a combination of positive results such as having the *kan*^{R} gene, and the s*acB* gene, but only a deleted or functional copy of the 3964 gene, but not both genes (data not shown). Confusingly, some transformant colonies having a *kan*^{R} gene had one or both copies of the 3964 gene but lacked the *sacB* gene. Chromosome DNA was isolated from these mutants to test the PCR again with the same results.

From this transformation, one transformant tested positive for all 4 desired genes, *kan*^{R}, s*acB*, and both the functional and deleted 3964 genes (shown in figure 16, lane 8). This mutant was designated MTM3W5.2_1802 and was used to screen for a double crossover and deletion of the functional BTZ20_3964 gene. In all PCRs where any potential merodiploid was screened and contained both bands for gene 3964, one band was fainter than the other, often the functional 3964 band.

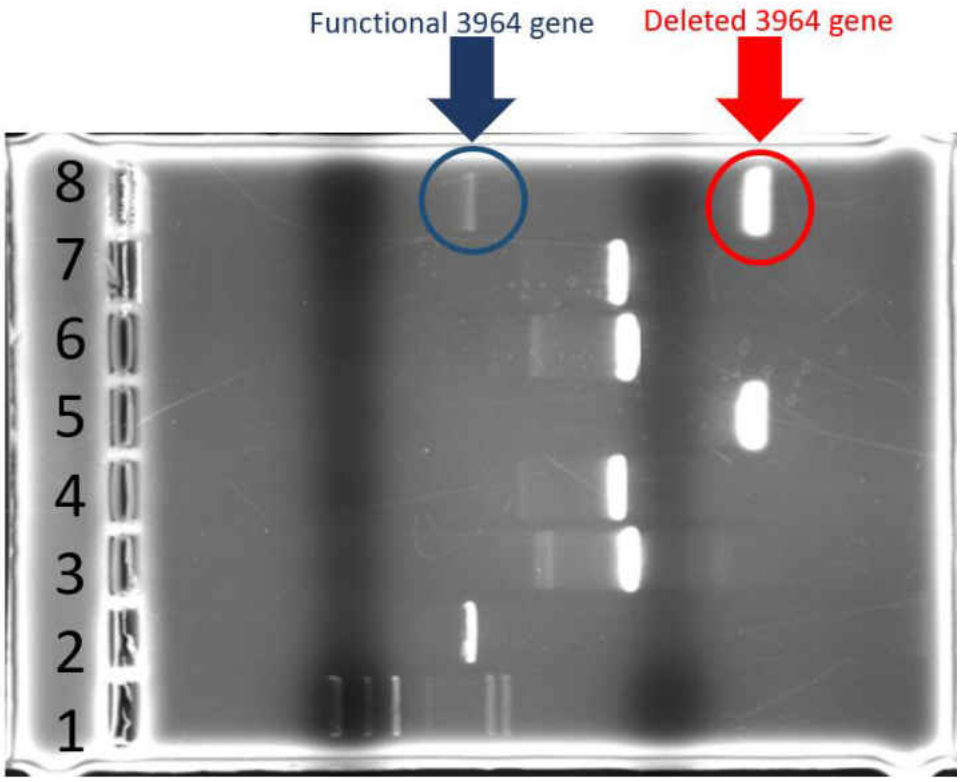


Figure 16: Gel electrophoresis showing primers for merodiploid screening. A gel showing PCR DNA amplicons from the 3 sets of primers, *sacB*, *Kan*^{R}, and the internal 3964 primer. Lane 1, λ*Hin*dIII molecular weight marker. Lane 2, MTM3W5.2 as template DNA plus 3964 internal primers. As expected these primers only amplify the intact gene (~3Kb) but do not amplify the deleted version (~220bp). Lanes 3-5, plasmid pEX18Km3964AD as template DNA with primers for the *sacB* gene (lane 3), the kanamycin gene (lane 4) and the deleted version of the 3964gene (lane 5). Lanes 6-8, MTM3W5.2-1802 strain as template DNA with primers for the s*acB* gene (lane 6), the kanamycin gene (lane 7), and the internal primers for the 3964 gene (lane 8).

Counterselection of Double Crossovers with *sacB*

The pEX18km3964AD plasmid contains *sacB*, which was used for counterselection of mutants that still contain the gene. The merodiploids are grown in non-selective broth to facilitate a double crossover allelic exchange where AD (the deleted version of the 3964 gene) would replace the functional 3964 gene, and the *kan*^{R} and s*acB* genes would also be lost (part "B", Fig.18). Plating on LB + 10% sucrose plates did not show any discernable decrease in

colonies compared to the LB plates, indicating that *sacB* may not be an efficient counterselection method in *Rhodococcus*. Regardless of this, colonies from the LB+ 10% sucrose plates are plated on replica patch plates as shown in figure 17 to screen for the loss of the kan^R gene. Few mutants showing loss of the kan^R gene are observed during the initial round of selection, but selecting a patch from the kanamycin plate in the replica patch plates and growing again for 2 days in non-selective broth would yield more mutants with the loss of the kan^R gene. Screening of these potential double crossovers showed the kan^R gene was lost along with the s*acB* gene, but the deleted 3964 gene construct was also, surprisingly, lost as well, indicating the plasmid was lost from the genome instead of a double crossover to replace the functional gene shown in figure 18. Multiple rounds of this experiment was performed altering the amount of time the bacteria were allowed to grow before plating on the sucrose, but the result was always that the plasmid was lost from the genome but the intact parental copy of the 3964 gene was retained (Fig.18, lanes 5-16).

Figure 17: Patch replica plates for double crossover screening. Patch replica plates on LB and $LBKan^{400}$ from colonies on the LB+10% sucrose plates. Circles patch indicates a colony that appears to have lost the kanamycin resistance gene.

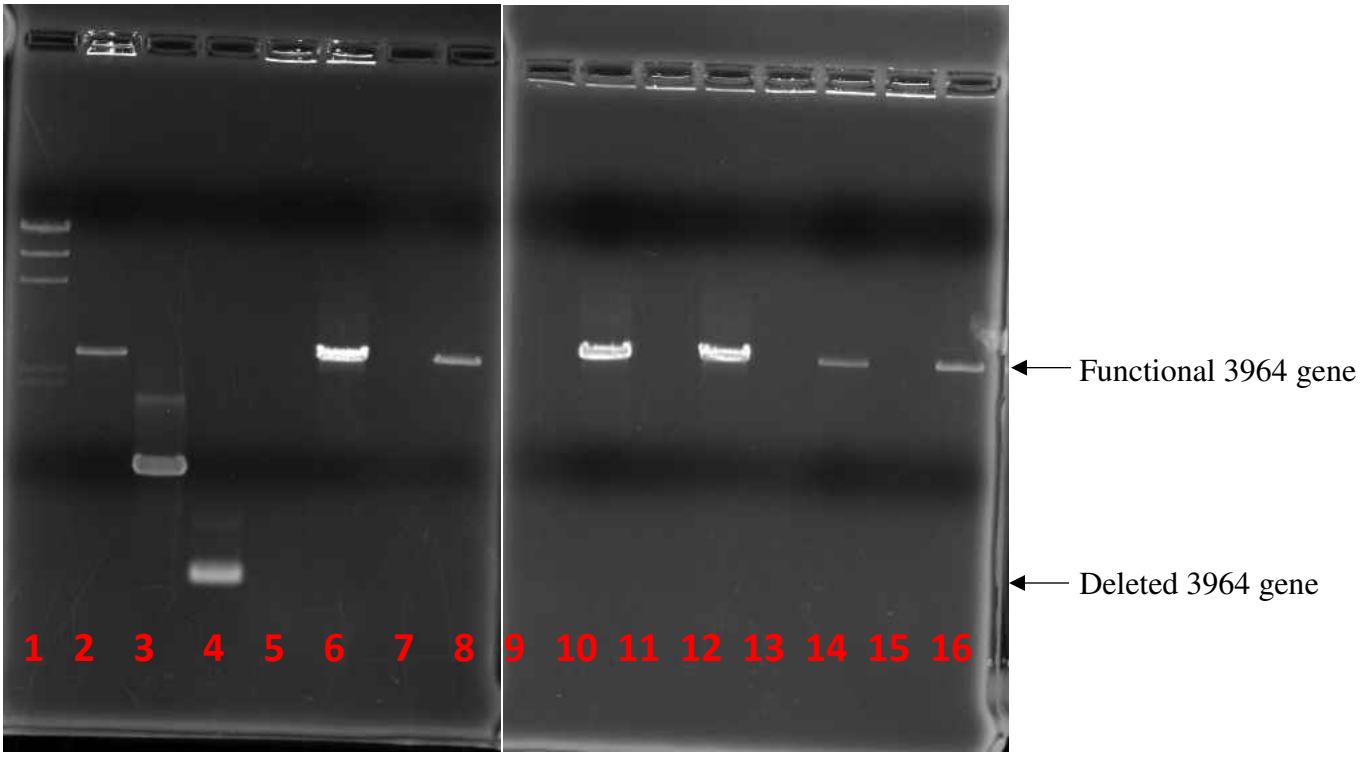


Figure 18: Gel picturing loss of the pEX18km3964AD plasmid from a potential merodiploid. Gel shows the potential merodiploid strain MTMW5.2_1802. PCR reactions used colonies (as DNA template) selected from the LB patch replica plate that has appeared to lose the *kan*^{R} gene. Lanes are as follows: 1: λ*Hin*dIII, 2:MTM3W5.2 colony as a DNA template with 3964 internal primers. 3: pEX18km3964AD plasmid as DNA template with primers for the kanamycin resistance gene. 4: pEX18km3964AD plasmid as template with 3964 internal primers. 5: MTM3W5.2_1802 colony from replica plates as DNA template with primers for the *kan*^{R} gene. 6: MTM3W5.2_1802 colony (replica plate) as template with 3964 internal primers. Lanes 7-16 are additional kanamycin sensitive colonies from the replica plates used as DNA template in similar PCR reactions. Note that the *kan*^{R} gene was indeed lost, but only the functional 3964 gene was retained, suggesting that the plasmid was lost instead of a double crossover taking place

Testing Legitimacy of Merodiploids

Despite the amount of times the replica patch plating was repeated or how many colonies were tested from MTM3W5.2_1802, the deletion construct was always lost instead of a double crossover occurring. There is a possibility of an illegitimate recombination in *Rhodococcus* (Desomer et al. 1991), where the plasmid inserts into the chromosome, but not into the homologous flanking regions of gene 3964. In this instance a double crossover to remove the

parental copy of the 3964 gene would not occur after growth and counter selection on sucrose plates. Primers were designed to amplify a site of the flanking region of gene 3964 and a site internal to gene 3964 to determine if the deletion construct was inserting into the flanking region (Fig. 19). If the plasmid inserts into the flanking region, the amplified region would be much larger (~9Kb) than the parental flanking region, or may be too large to amplify with the normal PCR conditions. The potential merodiploid MTM3W5.2_1802 was determined to be an illegitimate recombinant with these primers, as the plasmid did not appear to insert in the flanking regions of gene 3964 (Fig. 19, lanes 6-14)

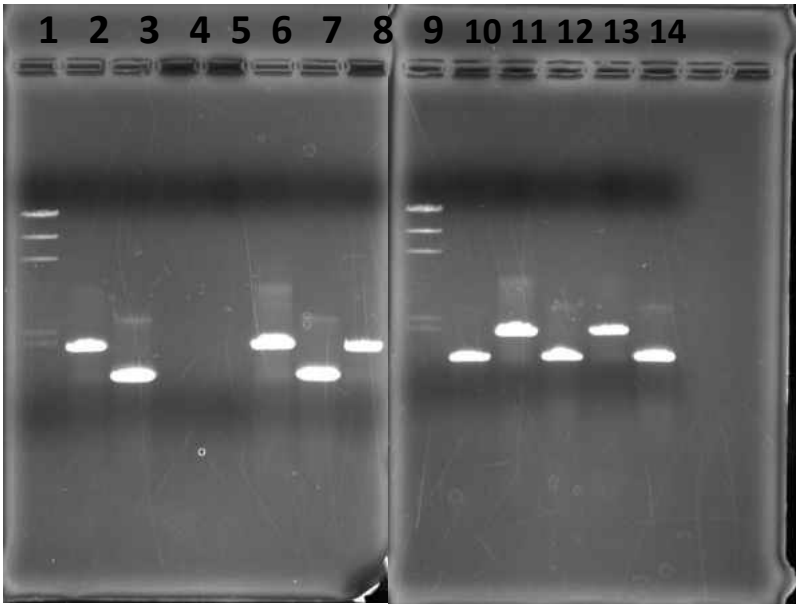


Figure 19: Gel screening for illegitimate recombinants. Gel shows PCR reactions from isolated genomic DNA from potential merodiploids. The flanking region amplicons should be a little over 1kb. If the pEX18km3964AD plasmid inserts into a flanking region, the amplicon should be over 9kb, and would run a little below the second band on the *Hin*dIII marker. The lanes are as followed using a 1/10 dilution of genomic chromosome as the DNA template: 1: λ*Hin*dIII, 2: MTM3W5.2- NewAB, 3: MTM3W5.2-NewCD, 4: pEX18km midi-AB, 5: peX18km midi-NewCD, 6: MTM3W5.2_1800-NewAB, 7: MTM3W5.2_1800-NewCD, 8: MTM3W5.2_1801-NewAB, 9: HindIII, 10. MTM3W5.2_1801-NewCD, 11: MTM3W5.2_1802-NewAB, 12: MTM3W5.2_1802-NewCD, 13: MTM3W5.2_1803-NewAB, 14: MTM3W5.2_1803-NewCD. All potential merodiploids tested did not appear to have the plasmid inserted into the flanking regions.

CHAPTER 4
DISCUSSION

With the rise of multiple antibiotic resistant pathogens, the urgency to discover new antibiotics has increased. Only one new class of antibiotic has been discovered and approved for clinical use in decades, but bacteria are developing multi-drug resistance at an alarming rate. Finding new antibiotics is necessary to treat these pathogens, but is a difficult task due to the low culture rate of microbes and the large number of possibly therapeutic secondary metabolites being produce under a cryptic pathway in vitro. In order to discover new antibiotics and avoid high rediscovery of old antibiotics, underexplored sources need to be examined. *Rhodococcus* is known for its production of bioactive metabolites that have a wide range of activities which makes it useful in the biotechnology industry. *Rhodococcus* is a relative of *Streptomyces*, the biggest producer of our clinically used antibiotics, and has been shown to produce some antimicrobial molecules such as the rhodopeptins, laratins, and rhodostreptomycins. *Rhodococcus* is underexplored as a source of antibiotics, but genomic sequencing of the species indicates a large number of PKS and NRPS genes which could indicate a gene cluster that produces an inhibitory molecule, and *Rhodococcus* remains a potential source of new antibiotics (Ceniceros *et al*. 2017).

Gene BTZ20_3964 as a Possible *luxR* Solo Regulator

Many secondary metabolites may be controlled under quorum sensing systems due to the high cost of many metabolites. Secondary metabolites such as siderophores and inhibitory molecules cost the bacteria a lot of resources to produce, so it is not always efficient to have

these pathways permanently expressed. This is part of the reason why it can be difficult to find new antibiotics because they are often tightly regulated and may be produced poorly under laboratory culture conditions, or not at all. The inhibitory compound produced by *Rhodococcus* strain MTM3W5.2 is only produced in stagnant broths or on agar plates at temperatures below 22ºC (Ward et al. 2018). These strict conditions for expression may be due to the presence of a regulator gene at the start of a biosynthetic gene cluster responsible for the production of the antibiotic. This potential regulator, gene BTZ20_3964, is likely a *luxR* solo regulator. A *luxR* solo regulator lacks a *luxI* type synthase (which normally synthesizes the autoinducer molecule) in the operon. Thus, a *luxR* solo may respond to some other type of signal molecule from internal or external sources (Brotherton et al. 2018). *luxR* regulators are not well defined in *Actinobacteria*, but a multiple amino acid sequence alignment of the BTZ20_3964 protein indicates many areas of conservation with other defined *luxR* solo regulators from *Proteobacteria*, but it shares more aligned amino acids with the *luxR* solo of its phylogenetic relative *Streptomyces* (Fig. 12). The source and nature of the signal molecule for this regulator is unknown, and could be either endogenous or exogenous. There is no *luxI* type synthase located in or close to the gene cluster responsible for producing this inhibitory compound, suggesting that the cluster is controlled by a *luxR* solo regulator.

Transformation of MTM3W5.2 with pEX18Km3964AD

Transformation of *Rhodococcus* MTM3W5.2 proved to be challenging with inconsistent results. The time constant after electroporation was always within the acceptable range, fresh competent cells were regularly used, and incubation time was monitored at the correct temperature, but colony yield always varied. Initially, lower concentrations of Kanamycin (50-

200 µg/mL) were used to select for the merodiploid, but even when plated at dilutions, a high number of spontaneous mutants would appear. Even at $Kan^{200\ \mu g/mL}$, a high number of colonies would appear on the control plates that were electroporated with no added DNA. Similar results are reported in a study performed by Fernandes et al. (2001); antibiotic concentrations under 400 µg/mL yield a high number of spontaneous mutants in *Rhodococcus*. Additionally, the study suggests that plating the transformants immediately on non-selective solid media and adding the antibiotic with a concentration between 400-500 µg/mL after at least 12 hours produced a higher yield of colonies than plating on a selective agar after incubation of the transformants. A soft agar overlay containing the desired amount of antibiotic yielded a higher number of true transformants (Fernandes et al. 2001). These conditions were repeated for MTM3W5.2 and while they did decrease the spontaneous mutants when diluted, potential merodiploids isolated were also deemed illegitimate (data not shown), however the method could be promising for future transformations to create merodiploids.

Insertion of the Deletion Construct into MTM3W5.2

In order to obtain a double crossover mutant where the deletion construct 3964AD replaces the functional BTZ20_3964 copy, the construct pEX18Km3964AD, which lacks an origin of replication in *Rhodococcus* must first crossover into the homologous flanking regions of the functional gene to create the merodiploid. Once the suicide vector recombines into the genome, the merodiploids can be selected for with kanamycin because of the resistance gene contained in the plasmid. Then, the merodiploids are grown in nonselective broth to allow a double crossover recombination event, where the deletion construct 3964AD replaces the functional gene, and the *sacB* and kan^R genes are also lost. Sucrose plates are used to counter

select the s*acB* gene and any cells that have not had the double crossover event should not grow well on sucrose plates, but no significant decrease in the number of colonies on the sucrose plates from the control LB plates was observed. This may indicate that sucrose is not a very efficient counterselection agent for *Rhodococcus* strain MTM3W5.2; the *sacB* protein may not be efficiently expressed or may not be efficient in preventing growth of cells containing the gene in this species. Regardless, colonies taken from the sucrose plates were screened for the loss of the kan^R gene, and it appeared that every loss of the gene indicated the loss of the entire pEX18Km3964AD plasmid, including the deletion construct, from the genome (Fig. 18, lanes 6-16). Despite a double crossover being a rare event, repeating the test should eventually lead to a double crossover mutant, but regardless of how many times the experiment was repeated, the plasmid was always lost. This indicated that the plasmid may not have inserted into the flanking regions of the 3964 gene. In order to test this, primers were created to amplify each flanking region and a region internal to the functional 3964 gene. Insertion of the plasmid into one of the flanking regions should either increase the amplicon size of these primers, or may prevent amplification due to the large size of the amplicon. All potential merodiploids isolated appeared to have intact flanking regions, i.e., the same size as the parental flanking regions (Fig.19, lanes 6-14), meaning the plasmid inserted somewhere else in the genome and would not be able to complete a double crossover to remove the functional 3964 gene from the genome.

Illegitimate Recombination in *Rhodococcus*

Illegitimate recombination of *Rhodococcus* has been observed in previous studies. In a study performed by Desomer et al. (1991), a plasmid that cannot autonomously replicate in *R. fascians* would, instead, stably insert into the bacterial chromosome at, roughly, random

locations. However, the plasmid has a specific DNA sequence site where it recombines with the chromosome; specifically at the plasmid sequence …CCGCANNNGGCGCC… The locations in the chromosome where the plasmid inserts are not related to this plasmid sequence and are thus a form of illegitimate recombination. The DNA sequence "GGCGCC" is found in two locations in the plasmid (pEX18Km3964AD) used for targeted gene deletion in strain MTM3W5.2. One is found within the kanamycin resistance gene and the other is found in the "A" DNA in the upstream flanking region. Neither of these "GGCGCC" sequences are preceded by the conserved "CCGCA" sequence, so it is unknown if this form of illegitimate recombination is occurring here.

Unfortunately, this aberrant recombination event can not be avoided, and in order to find a true merodiploid with the pEX18Km3964AD plasmid inserted into the correct (homologous) region, screening of all merodiploids would have to be done in order to find one with the plasmid inserted into the flanking regions of the functional gene. It is unknown why a varying combination of the genes from the pEX18Km3964 plasmid appears to be inserted into some of the potential merodiploids, such as when the mutant has two copies of the 3964 gene, a kan^R gene, but no *sacB* gene. This may indicate insertion of part of the plasmid, or a recombination event taking place that removes some of the plasmid from the genome. When a gene is missing, but the others are present, the PCR reaction is repeated to confirm the results. If the plasmid inserted into the flanking region of gene 3964, but either the *sacB* or kan^R gene was lost, it would be difficult to select/counter-select for these genes, but it could be possible for a double recombination event to replace the functional 3964 gene to occur, so any of these merodiploids that appears to have the partial plasmid inserted into the targeted region should be saved for later testing.

Future Works

For future works, a true merodiploid must be obtained to facilitate the double crossover event and replace the functional gene 3964 from the genome to determine if the gene is a regulator of the inhibitory compound. Future transformations using the current deletion construct and a soft agar overlay on a nonselective plate may prove promising for recovering a true merodiploid. Alternatively, a process described by Sawitzke et al. (2007) as "recombineering" could be used to create the deletion construct and create a mutant strain of MTM3W5.2 containing a nonfunctional copy of gene 3964. The *in vivo* process uses bacteriophage λ homologous recombination proteins that are collectively known as "Red". The proteins only require about 50 bases of homology for recombination, making it a simple and efficient method and can use linear PCR products or single-stranded oligos. The process requires competent cells that contain the required proteins for the type of recombination, transformation with the DNA construct, and requires a selectable and counter selectable marker, commonly an antibiotic resistance marker and the *sacB* gene. This method could provide a more efficient way to recover true merodiploids in future works, but it would require the λ phage recombination proteins to be expressed in *Rhodococcus* and for the competent *Rhodococcus* cells to be transformed with the linear DNA construct. It is unknown how efficient this process would be in *Rhodoccoccus.* Another possible method for obtaining a mutant strain with a nonfunctional BTZ20_3964 gene would be using the CRISPR-Cas9 system to engineer bacterial genomes as described by Cho et al. (2018). Success in genomic engineering has been observed with members of the *Actinobacteria* including *Corynebacterium* and *Mycobacterium*, but it is unknown how efficient the process would be in *Rhodococcus*.

Once the true merodiploid is obtained, future projects would involve determining if the mutant lacking a functional 3964 gene produces the inhibitory compound. Once this is determined, and if the mutant is nonproducing, restoration of the inhibitory compound would be required to support gene 3964's role as a regulator for the gene cluster. The plasmid pDD57 containing a high activity promotor with the functional gene cloned in should complement the nonproducing mutant and restore production of the compound. Due to the high activity promotor, production of the compound may be increased or become unrestrictive. If higher quantities of the compound can be recovered, the entire structure may be identified. Additionally, it is possible that other cryptic pathways may be awaken by unrestricted production of the regulatory protein, and new novel products with unknown functions may be produced.

An alternative theory to explain why a double crossover was not obtained may be that gene 3964 is an essential gene to *Rhodococcus* MTM3W5.2. Mutations to an essential gene are usually lethal and this may be a reason a mutant lacking gene 3964 was not generated. Secondary metabolites are not required for growth, and the regulator of a secondary metabolite would not normally be an essential gene, but it may be possible that this regulatory gene regulates a separate essential gene cluster located outside of the cluster responsible for producing the antibiotic compound. If a mutant lacking gene 3964 cannot be obtained through this or alternative methods, the role of this regulator as an essential gene may be investigated in the future.

REFERENCES

Arooj M, Koh Y. 2017. Texiobactin, a Potent Killer of Antibiotic Resistant Pathogens. *Journal of Bacteriology and Virology*,47(2), 102.

Bell KS, Philp JC, Aw DW, Christofi N. 1998. The genus *Rhodococcus*. *J Appl Microbiol* 85:195–210.

Berry D, Xi C, Raskin L. 2006. Microbial ecology of drinking water distribution systems. *Curr. Opin. Biotechnol.* 17, 297e302.

Borisova R. 2011. Isolation of a Rhodococcus Soil Bacterium that Produces a Strong Antibacterial Compound [*Thesis*]. [Johnson City (TN)]. East Tennessee State University.

Brotherton CA, Medema MH, Greenberg EP. 2018. *luxR* Homolog-Linked Biosynthetic Gene Clusters in *Proteobacteria*. *MSystems*,3(3).

Ceniceros A, Dijkhuizen L, Petrusma M, Medema MH. 2017. Genome-based exploration of the specialized metabolic capacities of the genus *Rhodococcus*. *BMC Genomics* 18:593.

Center for Disease Control. 2013. Antibiotic Resistance Threats in the United States. Atlanta (GA): Center for Disease Control; http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf.

Chiba H, Agernatu H, Kaneto R, Terasawa T, Sakai K, Kazuyuki D, Yoshioka T. 1999. Rhodopeptins (Mer-N1033), novel cyclic tetrapeptides with antifungal activity from *Rhodococcus* sp. 1. taxonomy, fermentation, isolation, physio-chemical properties and biological activities. J *Antibiot* (Tokyo). 52(8) 695–9.

Cho S, Shin J, Cho B. 2018. Applications of CRISPR/Cas System to Bacterial Metabolic Engineering. *International Journal of Molecular Sciences*, 19(4), 1089.

de Carvalho CCR, da Fonseca MMR. 2005. The remarkable *Rhodococcus erythropolis*. *Appl Microbiol Biotechnol.;* 67:715–726.

DeLorenzo DM, Rottinghaus AG, Henson WR, Moon TS. 2018. Molecular toolkit for gene expression control and genome modification in *Rhodococcus opacus* PD630. *ACS Synth.Biol*. 7:727-738

Desomer J, Crespi M, Montagu MV. 1991. Illegitimate integration of non-replicative vectors in the genome of *Rhodococcus fascians* upon electro-transformation as an insertional mutagenesis system. *Molecular Microbiology*, 5(9), 2115-2124.

Elsayed Y, Refaat J, Abdelmohsen U, Fouad M. 2017. The Genus *Rhodococcus* as a source of novel bioactive substances: A review. *Journal of Pharmacognosy and Phytochemistry*. 6. 83-92.

Fernandes PJ, Archer JA, Powell JA. 2001. Construction of *Rhodococcus* random mutagenesis libraries using Tn5 transposition complexes. *Microbiology*, 147(9), 2529-2536.

Giedraitienė A, Vitkauskienė A, Naginienė R, Pavilonis A. 2011. Antibiotic resistance mechanisms of clinically important bacteria. *Medicina* (Kaunas) 2011;47(3):137–146

Goodfellow M. 1989. Bergey's Manual of Systematic Bacteriology. Philadelphia, PA: Williams and Wilkins. Section 26: nocardioform actinomycetes – Genus *Rhodococcus*. Vol. 4. Baltimore (MD). Wilkins and Williams.

Heckman KL, Pease LR. 2007. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nature Protocols*, 2(4), 924-932.

Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77-86.

Hover B, Kim SH, Katz M, Charlop-Powers Z, Owen J, Melinda A. 2018. Culture-independent discovery of the malacidins as calcium-dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens. *Nat. Microbiol*. 3, 415–422.

Hudaiberdiev S, Choudhary KS, Vera Alvarez R, Gelencsér Z, Ligeti B, Lamba D, Pongor S. 2015. Census of solo *luxR* genes in prokaryotic genomes. *Frontiers in cellular and infection microbiology*, *5*, 20.

Iwatsuki M, Uchida R, Takakusagi Y, Matsumoto A, Jiang C, Takahashi Y, Arai M, Kobayashi S, Matsumoto M, Inokoshi J, Tomoda H, Omura S. 2007. Lariatins, Novel

antimycobacterial peptides with a lasso structure, produced by *Rhodococcus jostii* K01-B0171. *J Antibiot* (Tokyo). 60(6): 357–63.

Kallifidas D, Kang HS, Brady SF. 2012. Tetarimycin A, an MRSA-active antibiotic identified through induced expression of environmental DNA gene clusters. *J Am Chem Soc*. 134:19552-19555

Kapoor G, Saigal S, Elongavan, A. 2017. Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anesthesiology Clinical Pharmacology*,33(3), 300.

Kurosawa K, Ghivirgia I, Sambandan TG, Lessard PA, Barbara JE, Rha C, Sinskey AJ. 2007. Rhodostreptomycins, antibiotics biosynthesized following horizontal gene transfer from *Streptomyces padanus* to *Rhodococcus facians. J Amer Chem Soc*. 130(4): 1126–7.

Lobanovska M, Pilla G. 2017. Penicillin's discovery and antibiotic resistance: lessons for the future*? J. Biol. Med. 2017*, 90, 135–145.

Majidzadeh M, Fatahi-Bafghi M. 2018. Current taxonomy of *Rhodococcus* species and their role in infections. *European Journal of Clinical Microbiology & Infectious Diseases*,37(11), 2045-2062.

Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. *Ann. Rev. Microbiol.* 55, 165–199.

Molloy EM, Hertweck C. 2017. Antimicrobial discovery inspired by ecological interactions. *Curr Opin Microbiol*. 39:121-127

Okada BK, Seyedsayamdost MR. 2017. Antibiotic dialogues: induction of silent biosynthetic gene clusters by exogenous small molecules. *FEMS Microbiol Rev*. 41:19-33

Pruden A, Pei R, Storteboom H, Carlson KH. 2006 Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environ. Sci. Technol*. 40, 7445–7450

Quinn R. 2013. Rethinking antibiotic research and development: World War II and the penicillin collaborative. *Am. J. Public Health* 103, 426–434.

Sawitzke J, Thomason L, Constantino N, Bubunenko M, Datta S, Court D. 2007. "Recombineering": in vivo genetic engineering in *E. coli, S. enterica,* and beyond. *Methods in Enzymology* 421:171-199.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD. and Higgins D.G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol*. 7:539

Singh SB, Barrett JF. 2006. Empirical antibacterial drug discovery—Foundation in natural products. *Biochemical Pharmacology*,71(7), 1006-1015.

Tyc O, Song C, Dickschat JS, Vos M, Garbeva P. 2017. The Ecological Role of Volatile and Soluble Secondary Metabolites Produced by Soil Bacteria. *Trends in Microbiology*,25(4), 280-292.

Walsh CT, Fischbach MA. 2010. Natural Products Version 2.0: Connecting Genes to Molecules. *Journal of the American Chemical Society*,132(8), 2469-2493.

Wade W. 2002. Unculturable bacteria--the uncharacterized organisms that cause oral infections. *Journal of the Royal Society of Medicine,* 95(2), 81–83.

Ward A. 2015. Identification of genes required to synthesize an antibiotic-like compound from the soil bacterium *Rhodococcus* sp. MTM3W5.2. *Electronic Theses and Dissertations*, East Tennessee State University

Ward AL, Reddyvari P, Borisova R, Shilabin A, Lampson B. 2018. An inhibitory compound produced by a soil isolate of *Rhodococcus* has strong activity against the veterinary pathogen *R. equi*. *Plos One*,13(12).

Wright GD. 2018 Unlocking the potential of natural products in drug discovery. *Microb Biotechnol*. ;12(1):55–57.

Xi C, Zhang Y, Marrs C, Ye W, Simon C, Foxman, B , Nriagu J. 2009 Prevalence of antibiotic resistance in drinking water treatment and distribution systems, *Applied and Environmental Microbiology*, 75 (17) 5714-5718;

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