IN VIVO TRANSPOSON MUTAGENESIS OF STREPTOMYCES SP. MG1

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

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Members of the genus *Streptomyces* are filamentous and spore forming bacteria that live in soil and aqueous environments. Within these environments, *Streptomyces* as well as other bacteria play a large role in their environment through their ability to interact by producing various chemical signals. To understand what type of chemical signals bacteria produce in these environments and how their signals affects other bacteria, we have established a model system using two soil bacterium: *Streptomyces* sp. Mg1 (*S.* Mg1) and *Bacillus subtilis*. When wild-type *S.* Mg1 and *B. subtilis* are cultured together on an agar surface, noticeable lysis and degradation of the *B. subtilis* colony is observed. We propose a transposon mutagenesis to identify genes in *S.* Mg1 related to its ability to lyse and degrade *B. subtilis* colonies.

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CHAPTER I
INTRODUCTION

Bacteria are all around us. As human beings, it is estimated that bacteria outnumber the total number of cells in our bodies by a factor of 10 to 1 (1). Furthermore without these bacteria to carry out vital functions in our bodies, our physiology would be compromised (1). These bacteria survive in an environments where they constantly compete for nutrients and space. To better aid themselves, some bacteria produce secreted products to compete against other bacteria. We know some of these products as secondary metabolites. Additionally, bacteria can also face nutrient poor environments and may undergo transition to dormant states for long term survival, such as sporulation. These spores are dormant cells which upon encountering favorable conditions can germinate and propagate their lineage

Streptomyces carries out a large secondary metabolism that allows it to produce typically at least twenty secondary metabolites per strain (2). Within the interaction between *Streptomyces* sp. Mg1 (*S.* Mg1) and *B. subtilis*, we hypothesize that *S.* Mg1 secretes a molecule that actively lyses cells and degrades the colonies of *B. subtilis*. The *S.* Mg1 genome is approximately 8.7 megabases and we estimate it contains around 8700 genes. We hypothesize various gene products working together may be responsible for the ability of *S.* Mg1 to lyse *B. subtilis.*

To identify the genes encoding these products, we use transposon mutagenesis to create mutants of *S.* Mg1 which will be screened in search of non-lytic inducing colonies. A transposon is a piece of DNA that has the ability to change its position within a genome, randomly. Transposon mutagenesis, when combined with a genetic screen, is a forward genetic approach that we use to identify genes that are responsible for particular phenotypical characteristics. To carry such an

experiment out, we introduce a non-native transposon to *S*. Mg1. The introduction of this transposon is facilitated via a plasmid contain the respective transposon. pHTM, shown in Fig. S1 is a plasmid that contains a thiostrepton-inducible *Himar1* transposon, which contains two antibiotic markers to test for transfer of the plasmid during transformations and conjugation, as well as identification of mutagenized cells. pHTM has been previously used in successfully mutagenize *Streptomyces albus* and *Streptomyces coelicolor* (3). Then through a screen that I have designed, we will be able to screen for non-lytic mutants. As there are many various bacterial interactions in the environment, it is vital to be identifying mutants of the correct interaction as many interactions may result in observation of similar phenotypes. For example, in my project, even though the interaction of growth inhibition and the interaction for cell lysis share a very similar phenotype, the biochemical pathways are highly different. The adjustments to my screen have been appropriated to fit these conditions, such that the interaction that will be visible will be that of cell lysis. Through identification of non-lytic mutants we can identify genes responsible for the lytic activity and gain knowledge that will allow us to identify biological components underlying this interaction.

CHAPTER II
METHODS

The bacterial strains and plasmids that were used in this experiment are shown in Table 1.

Table 1. Table of strains and plasmids used

Strains	**Relevance**
Streptomyces sp. Mg1	Native isolated strain of *Streptomyces* that shows lytic degradative activity of *Bacillus subtilis*
E. coli JV36	Used to perform transfer of pTSR001 and pHTM plasmid into *S.* Mg1 through conjugation
E. coli DH5α	Used to obtain greater quantity of pTSR001 plasmid
Bacillus subtilis 3610	Wild type; used to screen for non-lytic mutants of *S.* Mg1
Plasmids	
pDR183	Contains the bla gene; provides ampicillin resistance
pHZ1358	Contains the tsr gene; provides thiostrepton resistance
pSET152	Plasmid used as the backbone for the construction of pTSR001
pHTM	Contains the thiostrepton inducible *Himar1* transposon with the antibiotic resistant markers of apramycin and hygromycin
pTSR001	Plasmid constructed in this paper; contains pSET152 backbone with antibiotic resistance genes of ampicillin (*bla*) and thiostrepton (*tsr*)

To effectively use the transposon mutagenesis as a method to introduce mutations in the *S.* Mg1 genome, it was vital to test the sensitivity of *S.* Mg1 to the chemical inducer, thiostrepton. We found that wild type *S.* Mg1 was extremely sensitive to thiostrepton (Figure 1: Picture of Thiostrepton sensitivity). Therefore to carry out further experiments, it is necessary to introduce thiostrepton resistance to *S.* Mg1.

Enzymatic assembly of plasmid pTSR001 conferring Thiostrepton resistance

Plasmid maps of pDR183, pHZ1358 and pSET152 were analyzed to design pTSR001, a plasmid that confers resistance to ampicillin and thiostrepton. Primers were designed to amplify gene products of plasmids pDR183 (*bla* gene), pHZ1358 (*tsr* gene) and pSET152 (backbone). Primers were also designed to include overhanging regions of homology between the resulting polymerase chain reaction (PCR) products. Resulting fragments were then assembled into pTSR001, using the Gibson assembly protocol. The assembly of pTSR001 was confirmed via gel electrophoresis (Figure 2).

Accumulation of plasmid pTSR001

Assembled plasmid, pTSR001 was transferred into chemically competent cells of a conjugative strain *Escherichia coli* DH5α. Transformation of plasmid was confirmed by selection on antibiotic resistance marker encoded by the plasmid on Luria-Bertani agar (LB: 10 g/L tryptone, 5 g/L yeast extract and 5 g/L salt) with the addition of ampicillin at 100 µg/mL. A single surviving colony was picked and grown in an overnight liquid culture of LB with the addition of ampicillin at 100 µg/mL. Resulting culture was concentrated and a plasmid purification procedure (5) was conducted to increase the amount of plasmid pTSR001 available for further experimentation.

Introduction of TSR001 to *Streptomyces* sp. Mg1

The plasmid, pTSR001 was transferred into chemically competent cells of a conjugative strain *Escherichia coli* JV36. Plasmid transfer was confirmed by selection on antibiotic resistance marker encoded by the plasmid. Single transformed colony was picked and grown in LB liquid culture under selection of ampicillin (100 µg/mL). Resulting culture was diluted to 1:10. 100 µL of 10^8 spores/mL of *S.* Mg1 was intergenically conjugated with 2 µL of the diluted overnight

culture of *E. coli* JV36 pTSR001 plated on AS-1 agar at 30 °C. Plates were flooded with antibiotics of ampicillin (100 µg/mL) and thiostrepton (50 µg/mL), 15 hours later to select for *S.* Mg1 exconjugates that contain the plasmid pTSR001. Exconjugants were passaged onto 30 mL agar plates of maltose-yeast extract-malt extract (MYM: 4 g/L maltose, 4 g/L yeast extract, and 4 g/L malt extract) with the addition of the antibiotics, naladixic acid at 30 µg/mL, ampicillin at 100 µg/mL and thiostrepton at 50 µg/mL, to confirm transfer of pTSR001 into *S.* Mg1 and to inhibit further growth of the donor *E. coli*. Resulting growth of the *S.* Mg1 exconjugants containing the plasmid were concentrated and sporulated on MYM plates. The collected spore stock of *S.* Mg1 pHTM was stored at 4 °C.

CHAPTER III
RESULTS

If successful in randomly mutating the *S.* Mg1 genome and isolation of non-lytic mutants, inverse PCR experiments would be completed and insertion loci would be identified. Through experiments done by previous members in the lab, the expression of the *S.* Mg1- pk1 gene cluster constitutes a large role in the production of the LDA conferring metabolite. The genes clusters that might be found through the transposon mutagenesis project could be other genes related in the biosynthesis of LDA or genes encoding transporter proteins aiding in the secretion of LDA metabolite in the surrounding media. These newly discovered genes may or may not be linked to the *S.* Mg1- pk1 gene cluster, thus identification of these genes may also result in the discovery of profound anabolic pathways that can be pursued as areas of further research in understanding the interactions between *S.* Mg1 and *B. subtilis*.

In addition to performing the transposon mutagenesis within the *S.* Mg1 genome and isolating mutants, the act of transposon mutagenesis itself is an accomplishment. The establishment of a viable transposon mutagenesis system in *S.* Mg1 in combination with a specific phenotypic screen will allow members of the lab, as well as other labs, to select for mutants of interest in the *S.* Mg1 genome. By doing so, we will be able to appropriate gene functions to genes scattered through the *S.* Mg1 genome, further elucidating how *S.* Mg1 interacts with its environment.

CHAPTER IV
CONCLUSION

Bacteria produce small molecules to interact with their environments. In the interaction between *Streptomyces* sp. Mg1 and *Bacillus subtilis*, we hypothesize that *S.* Mg1 produces some molecule that causes the degradation of *B. subtilis.* To study the lytic ability of *S.* Mg1, we use a genetic approach of transposon mutagenesis in conjunction with an appropriate screen, to understand this chemically mediated phenotype.

Establishment of transposon mutagenesis system in *Streptomyces* sp. Mg1

The purpose of the transposon mutagenesis is to randomly introduce mutations in *S.* Mg1 and effectively generate a library of mutants. There are many advantages to using transposon mutagenesis to generate mutants over other approaches, such as spontaneous mutation. We use a nonreplicative transposon contained on pHTM to conduct the transposon mutagenesis. In theory, the nonreplicative transposon will allow us to introduce a single mutation within one *S.* Mg1 colony and if the mutagenesis is random, each of these mutants should contain changes within their DNA different from the other. Another advantage is the sequence of the transposable element is known, thus identifying the location loci of the mutation is much easier. Through the establishment of this mutagenesis system, I, as well as future members of the lab, will be able to study any genes of interest in the *S.* Mg1 genome.

Screening and identification of non-lytic inducing mutants

The transposon mutagenesis protocol will effectively enable me to generate a library of *S.* Mg1 mutants. To be able to identify mutants of interest, we make use the genetic tool of a screen. Through a screen that I have designed a screen to identify non-lytic *S.* Mg1 mutants, I can effectively study genes responsible for the lytic activity of *S.* Mg1. Two components are needed

for an effective screen. The first is to be able to isolate single colonies of non-lytic *S*. Mg1 as lysing colonies produce halos that encompass larger area than the colony itself and will overshadow the non-lytic phenotype. However if a too low density of *S.* Mg1 mutants are plated, the length of the screening step may be doubled or tripled, therefore an optimal density of mutants must be plated to allow for efficient and accurate screening. By using wild type *S*. Mg1 and a mutant of *S*. Mg1 unable to lyse *B*. *subtilis*, I have determined the optimal density to be around 50 to 60 mutants a plate. This concentration will allow me clearly to isolate and identify the ability of single *S.* Mg1 colony to lyse *B. subtilis.* The second component of the screen is to make sure that the density of *B. subtilis* in the agar overlay will be able to identify non-lytic/lytic mutants. This component is vital to make the difference between lysis and growth inhibition. The phenotype the lab observes when plating *S*. Mg1 with *B. subtilis* on an agar surface, is of lysis. To replicate these conditions within a screen, *B. subtilis* must be grown to a lawning concentration before interacting with *S.* Mg1. If a too low density of *B. subtilis* is plated, the screens may result in false positives, however if a too high density is plated, then the *B. subtilis* cells might overrun *S.* Mg1 by greatly outnumbering them. Therefore an optimal density should not only be obtained for *S.* Mg1 mutants plated, but also for the amount of *B. subtilis* overlayed on top. I have determined that the optimal concentration of *B. subtilis* used for an overlay, is at a cell culture OD of .8 to .9.

Future endeavours

Currently, I have successfully given thiostrepton resistance to *S*. Mg1 through conjugative experiments. I will perform conjugation experiments to introduce the nonreplicative transposon harbored by pHTM to the *S.* Mg1 pTSR001 cells. Then I will induce the transposon to mutate *S*. Mg1. Next, I will apply the screen I have developed to isolate non-lytic colonies. Then I plan

to take the non-lytic colonies and perform an inverse PCR protocol to identify the insertion loci of the transposon, thus determining the genes responsible for lytic activity. These genes will be areas of further research to characterize the pathway of the lytic molecule's synthesis and activity.

Conclusion

By understanding the function of small molecules in microbial interactions, we may not only better understand our environment, but also learn how to predict and control the outcome of these interactions as well. While in this thesis, I focus on the genetic aspects of the interaction; many other approaches can also be used to study the lytic activity of *S.* Mg1 such as chemical analysis of the small molecule released, or the molecule's interaction with *B. subtilis.* Through the study of this molecule's production, release and interaction with other organisms, downstream applications may include aiding rationale drug design by discovering new targeting pathways of these therapeutics.

REFERENCES

1. **O'Hara AM, Shanahan F**. 2006. *The gut flora as a forgetten organ.* EMBO. 7(7): 688-693.
2. **Procopio RE, Silva IR, Martins MK, Azevedo JL, Araujo JM.** 2012. *Antibiotics produced by Streptomyces.* Science. 16(5): 466-471
3. **Bilyk B, Weber S, Myronovskyi M, Bilyk O, Petzke L, Luzhetskyy A**. 2013. *In vivo random mutagenesis of streptomycetes using mariner-based transposon Himar1.* Appl Microbiol Biotechnol. **97**:351-359