

**ENGINEERING DELETIONS IN THE MG1-PK1 GENE CLUSTER OF  
*STREPTOMYCES* sp. MG1 TO ABOLISH PRODUCTION OF THE MG1-  
PK1 METABOLITE**

An Undergraduate Research Scholars Thesis

by

SABRINA MOISAN

Submitted to Honors and Undergraduate Research  
Texas A&M University  
In partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by  
Research Advisor:

Dr. Paul D. Straight

May 2014

Major: Molecular and Cell Biology

# TABLE OF CONTENTS

	Page
ABSTRACT.....	1
ACKNOWLEDGEMENTS.....	2
CHAPTER	
I        INTRODUCTION .....	3
II        METHODS .....	6
Bacterial strains and preparation of genomic DNA .....	6
Library construction.....	7
Probe design.....	7
Screening the Cosmid Library .....	8
III        RESULTS AND DISCUSSION .....	10
Building the cosmid library .....	10
Cell lysis and transfer of DNA to nylon membranes.....	10
Hybridization .....	11
Duplicating the library .....	13
Hybridization of new membranes.....	14
IV        CONCLUSION.....	16
REFERENCES .....	17

## ABSTRACT

Engineering Deletions in the Mg1-Pk1 Gene Cluster of *Streptomyces* sp. Mg1 to Abolish Production of the Mg1-Pk1 Metabolite. (May 2014)

Sabrina Moisan  
Department of Biology  
Texas A&M University

Research Advisor: Dr. Paul D. Straight  
Department of Biochemistry and Biophysics

Many species of bacteria produce secondary metabolites in response to competitor species and changes to their local environment. *Streptomyces* sp. Mg1 (*S. Mg1*) produces the polyketide metabolite Mg1-Pk1, which participates in the defense of *S. Mg1* when challenged with *Bacillus subtilis*. When the biosynthetic genes of the Mg1-Pk1 gene cluster are deleted, the resulting mutant strain, *S. Mg1*- $\Delta$ 37, is hypersensitive to growth inhibition by *B. subtilis*. However, deletion of the Mg1-Pk1 biosynthetic genes required removal of ~80 kb from the gene cluster, a perturbation too large for genetic complementation. We aim to introduce a deletion in a single open reading frame that abolishes production of the Mg1-Pk1 metabolite and complement it for rigorous genetic analysis. In preparation, we have constructed and are currently screening a cosmid library in *E. coli* into which gene deletions will be engineered using the lambda red recombineering system and introduced into *Streptomyces* sp. Mg1 through homologous double recombination.

## **ACKNOWLEDGEMENTS**

I would like to thank my research advisor Dr. Paul Straight and my mentor, Dr. Chris Hoefler for guiding me in every aspect of this research project. Another thank you goes to all the members of the straight lab; Carol Vargas Bautista, Reed Stubbendieck, Catalina Mira, Roosheel Patel, and Emma Howard not only for their continuous support but for all the laughs and great memories. Finally, a special thank you to Dr. Jim Hu; I would not have had this opportunity to work in this lab without him.

I would also like to thank Rose Thomas, Alison Pain, and Devin Leslie for their important contributions to this project.

Finally, for welcoming me into their lab and generously lending me their equipment I thank Dr. Craig Kaplan, Dr. Mary Bryk, and Dr. Michael Polymenis.

# CHAPTER I

## INTRODUCTION

In their natural environment, bacterial species constantly interact and compete with other species. Much of the current microbial research has been carried out with bacterial monocultures in which the complexities of these polymicrobial interactions cannot be observed. Bacterial species such as *Streptomyces coelicolor* produce various secondary metabolites, small biologically active molecules that are not essential to the growth and development of the organism,<sup>1</sup> when interacting with other microorganisms.<sup>2</sup> These metabolites might be antagonistic to competing species or may play defensive roles.

To study bacterial competition, our lab has constructed a model system where two competing organisms, *Streptomyces* sp. Mg1 and *Bacillus subtilis*, are cultured together (Fig. 1a). We can use this system to ask questions about the roles of specific secondary metabolites in bacterial competition. Using MALDI imaging mass spectrometry (IMS) we can profile the distribution of secondary metabolites in a co-culture. For IMS, bacteria cultured on a thin agar surface are transferred to a MALDI plate and inserted into the mass spectrometer. The spectrometer is programmed to collect spectra at predefined points in an x-y grid across the sample. The spectra can then be used to construct colorized images.<sup>3</sup> Some metabolites, such as chalcomycin produced by *S. Mg1*, are secreted (Fig. 1b) while others, such as Mg1-Pk1, are associated with the cell envelope (Fig. 1c).<sup>4</sup> When *S. Mg1* is cultured with *B. subtilis*, a region of growth inhibition at the interface between the two organisms can be observed (Fig. 2a). When the biosynthetic genes of Mg1-Pk1 are deleted, *S. Mg1* becomes hypersensitive to the antagonistic

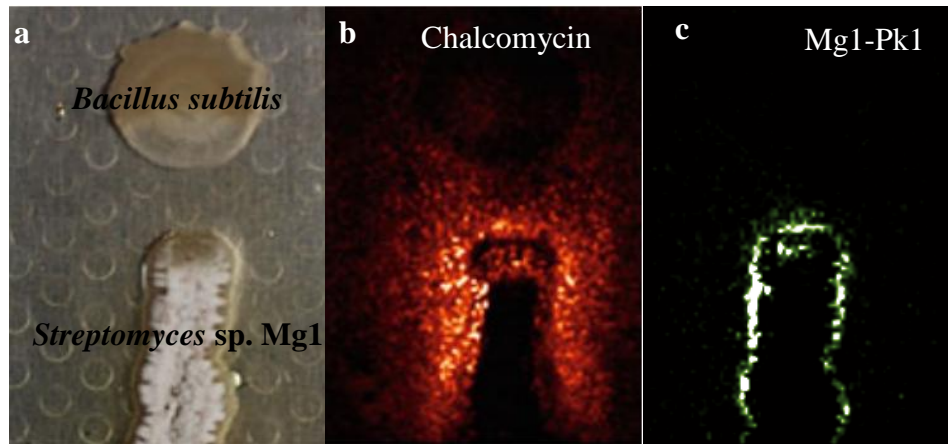


Figure 1. Profiling the distribution of secondary metabolites in a bacterial co-culture (1) (a) Culture of *Bacillus subtilis* and *Streptomyces* sp. Mg1. Using MALDI Imaging Mass Spectrometry we can make colorized images of the distribution of secondary metabolites. Chalcomycin (b) is secreted out into the media while Mg1-Pk1 (c) is cell-associated.

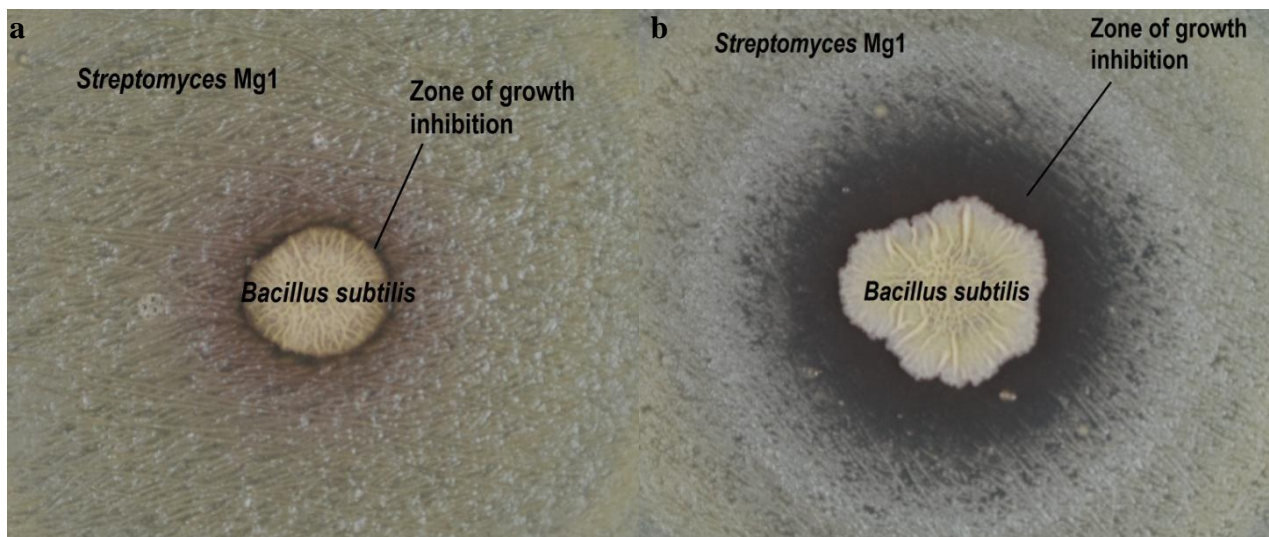


Figure 2. Competition between *Streptomyces* sp. Mg1 and *Bacillus subtilis*. (a) When *B. subtilis* and *S. sp. Mg1* are cultured together a zone of growth inhibition is observed at the interface between the two organisms. (b) When production of the Mg1-Pk1 metabolite in *S. sp. Mg1* is abolished a larger zone of growth inhibition is observed.

effects of *B. subtilis* (Fig. 2b). It appears Mg1-Pk1 assists in the defense of *S. Mg1* against competing organisms.

The current deletion within the Mg1-Pk1 gene cluster is too large to be complemented. Complementation is necessary to affirm that hypersensitivity to *B. subtilis* is a direct result of loss of the Mg1-Pk1 metabolite. To engineer a smaller, targeted, deletion of Mg1-Pk1 biosynthesis, we will use the lambda red recombineering system.<sup>5</sup> This requires the generation of a cosmid library by shearing the *S. Mg1* genome into 40 kb fragments, ligating those fragments into a cosmid vector, and inserting them into *E. coli* by transduction. The library is screened for clones encompassing the Mg1-Pk1 gene cluster by hybridization using probes designed to anneal to different regions of the gene cluster. Deletions are then generated on the *S. Mg1* genomic fragments in *E. coli* using the RED genes from bacteriophage lambda. The mutations are then introduced into the *S. Mg1* genome by homologous double-crossover recombination.

The precise mechanisms of bacterial competition are complex and multi-faceted.<sup>6</sup> We aim to gain an understanding of how the Mg1-Pk1 metabolite serves to defend *S. Mg1* against the antagonism of *B. subtilis* without killing or inhibiting its growth. Our goal is to gain a greater understanding of the means by which bacteria compete in their natural environment. When elucidated, the underlying general mechanisms of bacterial competition may be exploited for the production of next-generation therapeutics.

## CHAPTER II

### METHODS

#### **Bacterial strains and preparation of genomic DNA**

The strain used for this project was *Streptomyces* sp. Mg1 (PSK0558). For preparation of genomic DNA, 50 ml sterile Tryptic Soy Broth (Oxoid) was inoculated with  $10^9$  spores of *Streptomyces* sp. Mg1 (PSK0558) and incubated with shaking (250 rpm) at 30°C for 16 hours. Cells were harvested by centrifugation (3220 x g, 10 min, 25°C) and the pellet was washed twice with 10 mL genomic lysis buffer (50 mM EDTA, 0.1 M NaCl, pH 7.5). The pellet was resuspended in 1 mL genomic lysis buffer with 10 mg/mL lysozyme (amresco) and 1 mg/mL proteinase K (NEB) and incubated at 37°C for 10 minutes. RNase A (Invitrogen) was added to a concentration of 0.1 mg/mL and the mixture was incubated at 37°C for 20 minutes. N-lauroylsarcosine was added to a concentration of 1.4% and the mixture was incubated at 37°C for 15 minutes. The lysate was extracted twice with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol (Invitrogen) with the assistance of Phase Lock Gel (5 Prime). To the aqueous phase, NaCl was added to a final concentration of 0.8 M followed by 0.1 volumes of CTAB/NaCl (10% CTAB, 0.7 M NaCl, 65°C). The mixture was incubated at 65°C for 10 minutes and then extracted with an equal volume of 24:1 chloroform:isoamyl alcohol (Sigma). The CTAB extraction was repeated once more followed by a final phenol:chloroform:isoamyl alcohol extraction. The DNA was precipitated with 0.7 volumes isopropanol and washed sequentially with 70% and 95% ethanol. The dried pellet was dissolved in TE buffer and stored overnight at 4°C. Purity and concentration were determined by UV and agarose gel electrophoresis.



## **Library Construction**

A cosmid library of the *Streptomyces* sp. Mg1 genome was constructed using the pWEB™ system from Epicentre. Briefly sheared genomic DNA was end-repaired and size-selected by pulse field gel electrophoresis (120°, 6 V/cm, switch time 5-8 sec, 14°C, 15 hours total run time, 1% agarose in TBE). DNA fragments co-migrating with a 40 kb standard were excised from the gel and purified. The fragments were ligated with linearized cosmid, packaged into phage, and transfected into *E. coli*. A total of 1536 ampicillin-resistant clones were arrayed into sixteen 96-well plates containing 200 ul of LB [1% (w/v) Tryptone (BD Bacto™), 0.5% Yeast Extract (BD BBL™), 0.5% NaCl] supplemented with 100 µg/ml ampicillin. The library was amplified by incubating the plates at 37°C with gentle agitation for 3 hours. To ease library screening, sets of 4 library pools were constructed by mixing clones from each ¼ of the library maintaining the order of the wells (Fig. 3). Glycerol was added to each well to a 20% final concentration and the plates were stored at -80°C.

## **Probe design**

The Mg1-Pk1 gene cluster sequence was used to create 400-bp probes designed to anneal to various regions of the gene cluster. The probes were generated by PCR with primers annealing at the gene cluster coordinates designated in Table 1. Care was taken to select regions of the gene cluster outside of repeat stretches.

Table 1. Primers used to generate probes. \*bp coordinates are relative to start of the gene cluster

Probe	Forward Primer	Reverse Primer	bp Coordinates*
B	ACGGTCACCCACTTCGTGAT	CCAGTCGTGGGAGGAGGTC	297-691
1	ACGGCGTCGGTGAAGAAGTA	CACCATTTTCGCGAGGAGAC	16984-17437
2	CAGCTCGGTGAGCATCTCCT	GCCGTACCGATCGTCTTCAT	49756-50235
3	TCGGCATCCCACTCAACAC	TACCGGACCTGGTCTTCGTC	78614-78976
4	GGCCGAACTTCTTGTCGATG	AGCGGACCGTACTGAACCTG	107270-107713
5	GATTCGATGGCGCTCTCCT	GACCCTGCTCGGTCTGGTC	414612-142087
6	GACGAGGGAGGAGACCCAGT	TCTCTGCACGGAGTGATGGA	171938-172237
E	TGCGTACATCGAGCCACATC	CTTCTCGACCCGGTTCACAC	185420-185890

### Screening the cosmid library

To screen the library for clones containing Mg1-Pk1 gene cluster fragments, DNA was extracted from the pooled plates and bound to membranes as follows. The pool was amplified by inoculating a new 96-well plate containing 50 ul of LB ampicilin. The plate was incubated at 37°C for 20 hours. The plate was centrifuged and the cells were lysed by alkaline treatment [resuspend pellet in 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA (50 ul); add 0.2 N NaOH/1% SDS (100 ul); halt lysis after 2 minutes with 3M KOAc (50 ul)]. The plate was centrifuged 5 minutes and the lysate was transferred to a 96-well PCR plate. The DNA was denatured at 100°C for 5 minutes, rapidly cooled on ice for 5 minutes, and applied to an Amersham Hybond-N<sup>+</sup> nylon membrane (GE Healthcare) with a dot blot apparatus. The membrane was rinsed with 2X SSC (8.8% w/v Trisodium citrate, 1.7% NaCl, pH 7.5), dried, and the DNA was cross-linked using a UVC-515 UV multilinker with an exposure of 89,000 uJ.

*In situ* hybridization was performed using the designed probes labeled with the Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare) following the protocols included (Amersham).

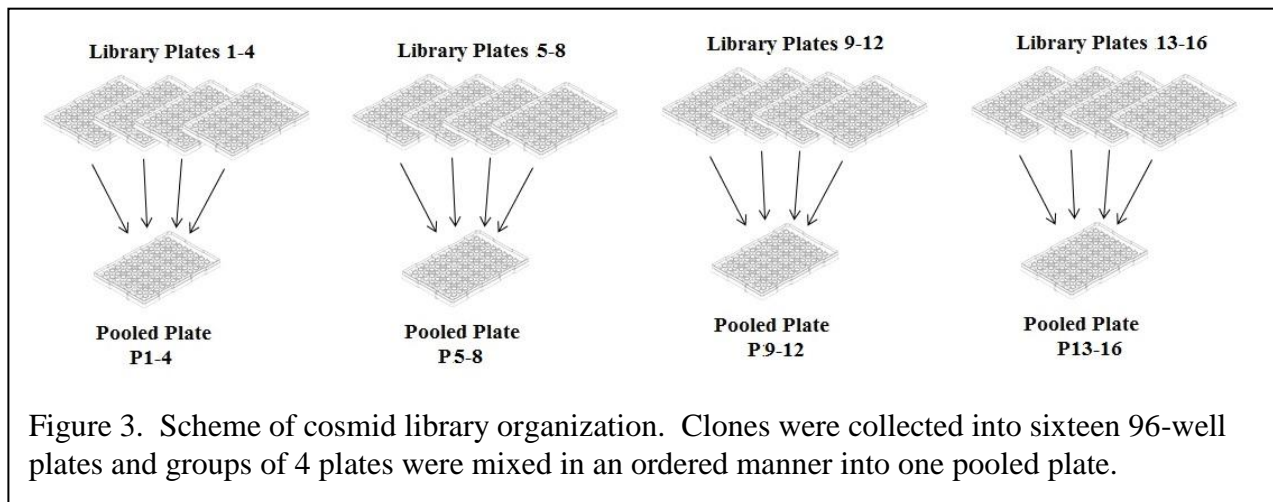
Membranes were stripped between sequential rounds of probing as follows. Blots were rinsed in distilled water and washed twice with 0.2 M NaOH and 0.1% SDS at 37°C for 20 minutes. The blots were rinsed in 2X SSC and dried.

## CHAPTER III

### RESULTS AND DISCUSSION

#### Building the Cosmid Library

Building the cosmid library required shearing *S. Mg1* genomic DNA and isolating 40 kb fragments. These fragments were then ligated into the cosmid cloning vector, packaged into phage, and transduced into *E. coli*. A total of 1536 single clonal colonies of *E. coli*, each containing a different cosmid, were arrayed into sixteen 96-well plates. To make screening easier, pooled plates were made as shown in Fig. 3. The result was 20 plates total: 16 library plates and 4 pooled plates.



#### Cell Lysis and Transfer of DNA to Nylon Membranes

To screen the library for cosmids encompassing the *Mg1-Pk1* gene cluster, the DNA had to be applied to nylon membranes. We tried three methods for fixing the DNA to the membranes. The first two methods were unsuccessful; all three are described here.

First, the clones were grown directly on the membranes. A membrane was placed on an LB agar plate supplemented with ampicillin and the 96-well plates were replica plated on top. After overnight incubation the membranes were lifted off the plates and the colonies were lysed by alkaline treatment. The membranes were washed in 2X SSC to remove cell debris and leave only the DNA bound to the membrane. This step was unsuccessful because the cell debris was tightly bound to the membrane and could not be washed away.

Second, to reduce the likelihood of the cells adhering tightly to the membranes, we decided to grow the clones on agar plates first and lift the colonies with the membranes. The 96-well plates were replica plated on the agar plates and, after overnight incubation, the colonies were lifted off the plate onto a membrane. Although this method allowed for cell debris to be washed away in the SSC wash, not all colonies could be successfully transferred to the membrane in the lift.

Third, we extracted the DNA from clones in a 96-well plate and applied the DNA directly to the membrane with a dot blot apparatus. Using this method, we started with cells in each well of the plate and no cell debris was left behind after the SSC wash. We concluded this was the best method for transferring the DNA to the membranes but only performing a round of hybridization would reveal if it was successful.

## **Hybridization**

Initial rounds of hybridization at 55°C resulted in high background making it difficult to distinguish positive from background signal. This led us to optimize the hybridization protocol

before continuing with library screening. A membrane was constructed using different concentrations (1 pg – 100 ng) of the 8 probes as target DNA (Fig. 4). Before blotting, the probes were mixed with *E. coli* lysate (*E. coli* overnight culture lysed by alkaline treatment) to control for the *E. coli* DNA present in the library. Two different concentrations of *S. Mg1*

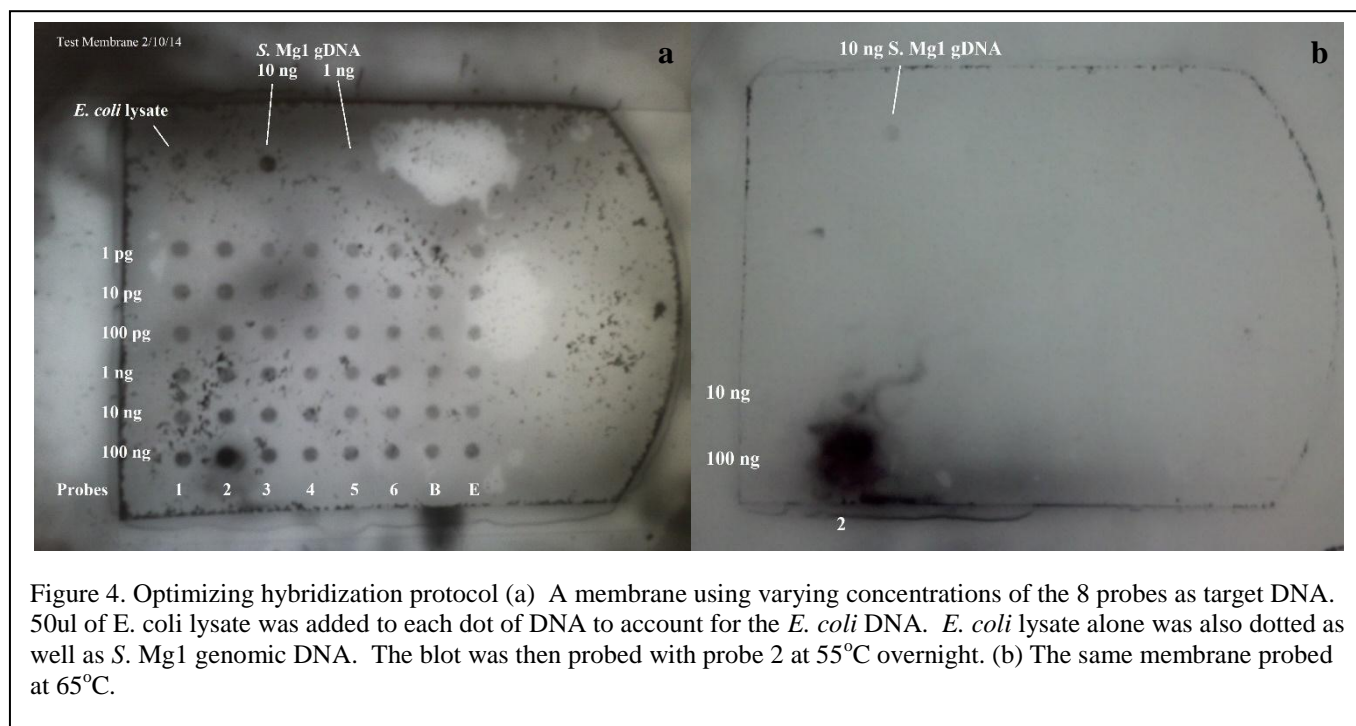


Figure 4. Optimizing hybridization protocol (a) A membrane using varying concentrations of the 8 probes as target DNA. 50ul of *E. coli* lysate was added to each dot of DNA to account for the *E. coli* DNA. *E. coli* lysate alone was also dotted as well as *S. Mg1* genomic DNA. The blot was then probed with probe 2 at 55°C overnight. (b) The same membrane probed at 65°C.

genomic DNA as well as *E. coli* lysate alone were also blotted as controls. A first hybridization using probe 2 at 55°C gave high background signal (Fig. 4a). At a DNA concentration of 100 ng it is possible to distinguish the distinctly darker positive signal from the background signal. At 10 ng or less of target DNA the positive signals in the probe 2 column are undistinguishable from the background signal in the other columns. We can also see a significant signal from the *E. coli* lysate.

We determined stringency of the hybridization needed to be increased. Hybridization with probe 2 was performed at 65°C resulting in no background signal (Fig. 4b). At this increased

temperature the limit of detection is 10 ng of target sequence DNA and signal is no longer detected from the *E. coli* lysate or other DNA sequences. Hybridization was repeated at 60°C with similar results leading us to select this as a sufficiently restrictive temperature.

We used this new temperature (60°C) to perform additional hybridizations of the membranes but no signal was detected. This may have been due to a probe not hybridizing to any of the target DNA on the membrane or having too low a concentration of target DNA. To test this low concentration hypothesis, we streaked a few clones from the frozen library plates onto LB ampicillin agar plates and, after incubation, observed a very low cell density. We concluded the lack of signal from the hybridizations was most likely due to a low DNA concentration on the membranes due to a low initial cell density.

### **Duplicating the Library**

After determining that our initial library pools had insufficient cell density, we reconstructed the pools and included a library amplification step. The library was duplicated by transferring 20 ul from each well of the initial library to 180 ul of LB broth with 100 µg/ml ampicillin in new 96-well plates. Cells were allowed to grow overnight to increase cell density. The optical density of each well was estimated to be at least an average of 0.50 (SD 0.17). We can estimate a cosmid yield for these cultures to be approximately 5-10 ng of cosmid DNA per clone. The plates were pooled as before and the 16 new library plates were centrifuged and the supernatant removed. The cells were resuspended in 200 ul of 20% glycerol and stored at -80°C. New membranes were made using the pooled plates and replacing the DNA in one of the wells with

10 ng of *S. Mg1* genomic DNA as a positive control. With this positive control we can be certain that, if no positive signal is detected, it is not due to a failure in the hybridization itself.

### **Hybridization of new membranes**

Hybridization of the new membranes was performed at 60°C using probe 4. We have identified a positive signal on the membrane containing DNA from the pool of plates 1-4 (Fig. 5). This signal corresponds to four potential clones containing a gene cluster fragment. We will grow the four clones individually under selection, extract the DNA, and attempt to amplify the target sequence by PCR. This will allow us to identify which of the four clones contains the target sequence. We will end-sequence its insert to map its location in the Mg1-Pk1 gene cluster.



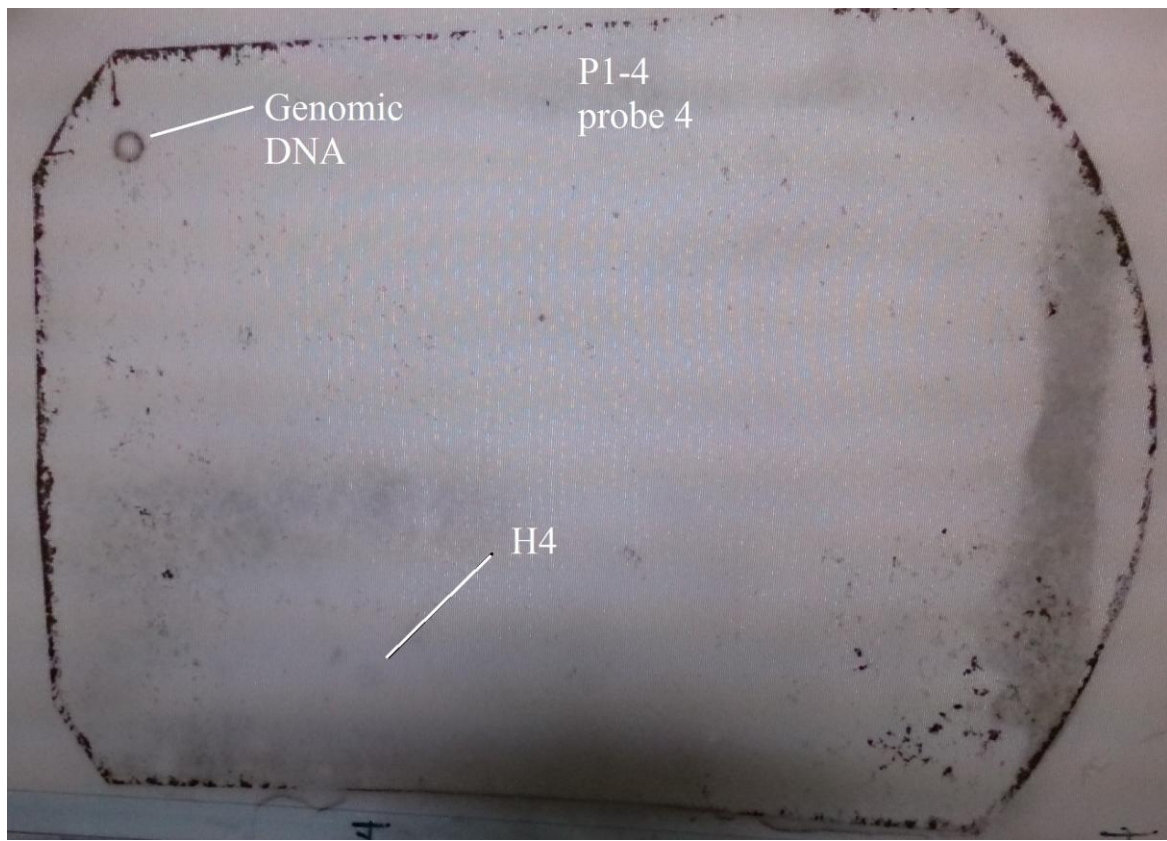


Figure 5. Hybridization of membrane from pooled plates 1-4. Hybridization of the membrane with probe 4, performed for 20 hours at 60°C. A positive signal is seen from the genomic DNA and a faint positive can be seen at a spot corresponding to well H4 on the 96-well plate.

## CHAPTER IV

### CONCLUSION

Genetic manipulation of *Streptomyces* is challenging. A system shown to work well with *S.coelicolor* and other streptomycetes makes use of lambda red-mediated recombination developed in *E. coli*. To make use of this system in *S. Mg1*, we have constructed a cosmid library containing the *S. Mg1* genome. We have determined the optimal hybridization temperature and have identified a potential clone containing an Mg1-Pk1 gene cluster fragment. Once amplified by PCR, the insert will be end-sequenced to map its location within the Mg1-Pk1 gene cluster. We will continue to screen the remaining  $\frac{3}{4}$  of the library to identify approximately 15 clones containing fragments encompassing the Mg1-Pk1 gene cluster.

Once the library has been screened and clones encompassing the gene cluster have been identified and mapped, we will be able to engineer various deletions in the Mg1-Pk1 gene cluster. Culturing engineered mutants with *B. subtilis* will allow us to observe the effects of a particular mutation on *S. Mg1*'s ability to defend itself against *B. subtilis*.

## REFERENCES

1. Straight, P. D. & Kolter, R. Interspecies chemical communication in bacterial development. *Annual review of microbiology* **63**, 99–118 (2009).
2. Traxler, M. F., Watrous, J. D., Alexandrov, T., Dorrestein, P. C. & Kolter, R. Interspecies interactions stimulate diversification of the *Streptomyces coelicolor* secreted metabolome. *mBio* **4**, e00459–13– (2013).
3. Yang, Y.-L., Xu, Y., Straight, P. & Dorrestein, P. C. Translating metabolic exchange with imaging mass spectrometry. *Nature chemical biology* **5**, 885–887 (2009).
4. Barger, S. R. *et al.* Imaging secondary metabolism of *Streptomyces* sp. Mgl during cellular lysis and colony degradation of competing *Bacillus subtilis*. *Antonie van Leeuwenhoek* **102**, 435–445 (2012).
5. Murphy, K. C. Use of bacteriophage lambda recombination functions to promote gene replacement in *Escherichia coli*. *Journal of bacteriology* **180**, 2063–2071 (1998).
6. Gust, B. Chapter 7 Cloning and Analysis of Natural Product Pathways. *Methods in Enzymology* **458**, 159–180 (2009).