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Identifying Biosynthetic Gene Clusters Involved in Antagonistic Activity in Pseudomonads

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Abstract

Since the discovery of antibiotics, improper usage has led to the evolution and emergence of antibiotic resistant microorganisms. ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* spp.) in particular are among the most deadly due to their unassailable ability to resist antibiotics. To discover new ESKAPE antibiotics and help combat the antibiotic resistance crisis, a molecular strategy was constructed by the educational networks Tiny Earth (in which UNC Asheville is a member) and Small World Initiative. As part of Tiny Earth, 29 soil isolates (SI) were characterized using the 16S rRNA gene and assayed for antibiotic production by Dr. Melinda Grosser's spring 2022 microbiology class (BIOL 339). Three of these isolates (all pseudomonads that exhibited antagonistic activity) as well as two previously isolated pseudomonads (SS 400 and RGRF B10) from Dr. Amanda Wolfe were tested for their amenability to undergo transposon mutagenesis using transposon pBAM1, which was inserted into genomes using biparental mating and conjugation with *E. coli* S17-λpir. This transposon mutagenesis would then allow for identification of a biosynthetic gene cluster involved in antibiotic compound production using replica plate screening. Of the five isolates tested (SS 400, RGRF B10, SI JK, SI JC, and SI RS), SI JK was found to be the most efficient in conjugation and screening. One mutant with loss of antagonistic activity against *Bacillus subtilis* was identified in SI JK. This mutant will go through subsequent whole-genome sequencing to identify a biosynthetic gene locus likely involved in its inhibitory activity.

1. Introduction

From 1940 to 1965, antibiotics were a revolutionary tool in medicine used to decrease morbidity and mortality. Since then, improper use of antibiotics (discontinuous or irregular intake and excessive usage) in clinical and agricultural settings has led to the evolution and emergence of antibiotic resistant microorganisms[1,2]. Increasing trends of antibiotic resistance in the past two decades could potentially be catastrophic for human health, thus being the driving-point for research in microbiological sciences in the last decade[3,4]. Identification of new antibacterial compounds is of great importance due to over 35,000 deaths in the United States each year from antibiotic-resistant infections, and even higher numbers can be seen in low-income countries where high-quality medical care and sanitation is less common[3,5]. Of these deaths, the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* spp.) are of the highest importance, as they utilize various mechanisms to display multidrug resistance. These mechanisms include chemically modifying the antibiotic, modifying the target of the antibiotic, preventing influx of the antibiotic, and expulsion of the antibiotic through efflux[2]. Because of these abilities, ESKAPE pathogens make up the majority of hospital-acquired infections as they spread through person-to-person contact, contaminated equipment, and lack of sterilization[2].

Small World Initiative and Tiny Earth were created as international networks of faculty and students to identify antibiotic producing soil microbes as part of lab curricula and address the issue of antibiotic resistance. One way in which they do this is by implementing crowdsourcing of novel antibiotic discovery, which is the basis for this research[6]. During the 2022 spring semester at the University of North Carolina Asheville (UNCA), students in microbiology class (BIOL 339) isolated 29 soil-derived bacterial strains (which were tested on cetrimide agar plates to reveal that four of these were pseudomonads), characterized these isolates using 16S rRNA gene sequencing, and then tested each for antagonistic activity against "safe" relatives (SRs, non-disease causing strains) of the ESKAPE pathogens.

Transposon vector pBAM1 was chosen due to the fact that it is equipped with genes that allow for a range of genetic manipulations in Gram negative bacteria. Genetic tools, such as plasmids and transposons, can be challenging and do not always work in environmental isolates. For example, plasmids often require the host to produce a specified replication protein, which is uncommon in environmental strains. However, pBAM1 is equipped with R6K, which allows the plasmid to be stably maintained in conjunction with *E. coli* strains that have the *pir* gene in their chromosome (Figure 1). It is also equipped with oriT of RK2, which allows for DNA to be transferred via conjugation. This allows it to be easily mobilized into Pseudomonads[8]. Further, the R6K origin requires pir for replication, so its ability to perform transposon mutagenesis as a suicide vector is notable because after it leaves *E. coli* S17-λpir, it is no longer able to replicate in the recipient strain[9]. Transposon pBAM1 is also equipped with an ampicillin-resistance marker in the backbone, and a kanamycin-resistance marker between the transposon inverted repeats which allows for detection of colonies that contain the transposon (Figure 1).

Three environmental strains in the *Pseudomonas* genus that exhibited high levels of antagonistic activity were selected from this collection for further study, as well as two additional *Pseudomonas* strains previously identified (Amanda Wolfe, unpublished data). The goal of this research was to develop a protocol for performing transposon mutagenesis to generate a collection of insertion mutants in each strain, followed by a screen to identify mutants with a loss in antimicrobial activity. Determining the affected gene in a mutant with loss of inhibitory activity would allow identification of the putative gene region(s) involved in antibiotic production. Understanding the native biosynthetic pathway for an antimicrobial compound can facilitate drug discovery and characterization, and could lead to pipelines for scaling drug production. Additionally, biochemical analysis of extracts of the wild-type strain of an antibiotic-producing isolate relative to a loss-of-function mutant can facilitate antibiotic compound identification and characterization.

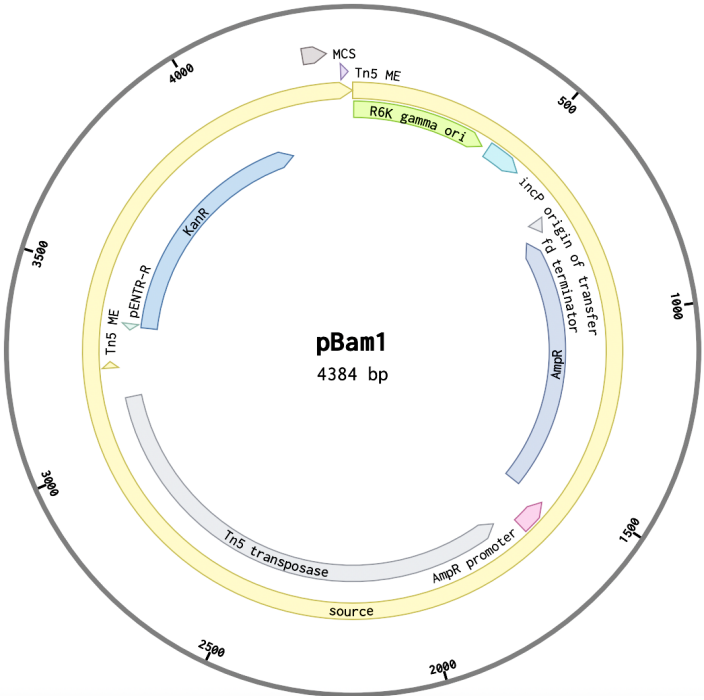


Figure 1. Plasmid map of transposon vector pBAM1.

2. Materials and Methods

2.1 | Strain isolation and growth conditions

UNCA microbiology (BIOL 339) students obtained topsoil samples from the campus of University of North Carolina, Asheville, NC on February 1st, 2022. One gram of soil was suspended in 10 mL sterile water, and then was serially diluted using LB broth from 1:10 to 1:100,000. Then, 100 µl of each dilution was spread plated onto LB agar. Cultures were incubated at 30°C for 48 h. Single colonies from these plates were picked and patched for isolation on LB agar as well as cetrimide agar. All environmental strains were cultured at 30°C. The soil sample from which SI JK was isolated was obtained from red clay in a highly vegetative area where dogs often roam, whereas SI JC and SI RS were isolated from shaded, moist soil. Safe relatives of the ESKAPE pathogens including *Bacillus subtilis, Enterococcus raffinosus, Staphylococcus haemolyticus, Escherichia coli, Acinetobacter baylyi, Pseudomonas fluorescens, and Enterobacter aerogenes* were cultured at 37°C for 20 hr prior to the antagonistic assay. All *Pseudomonas* strains were grown in LB broth at 30°C, *E. coli* S17-λpir (equipped with tra/mob) was grown in LB broth at 37°C. *E. coli* carrying transposon pBAM1 was grown in LB broth with 1 µg/ml ampicillin at 37°C. Since the transposon carries a gene for ampicillin resistance, only the *E. coli* with the transposon were able to replicate.

2.2 | Gene sequencing and phylogenetic analysis

For 16S rRNA gene sequencing, bacterial strains were streaked onto LB and cultured for 2 d at 30°C. Colony PCR was performed by adding a small aliquot of their isolate to 20 µl of sterile H_2O and 30 µl of master mix (containing Q5 polymerase, 10X reaction buffer, $MgCl_2$, forward and reverse primers, dNTPs, and ddH_2O). Students used identical, universal primers targeting the 16S rRNA gene: 27F (27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). To complete the PCR, samples were then placed in a Bio-Rad T100 thermocycler with the following conditions:

Initial denaturation:	98°C for 3 min	
20 cycles of:	98°C	for 10 s
	68°C	for 30 s
	$\Delta T = -0.5^{\circ}$/cycle	
	72°C	for 45 s
15 cycles of:	98°C	for 10 s
	58°C	for 30 s
	72°C	for 45 s
Final extension:	72°C for 2 min	
Hold at 4°C		

Finally, a PCR cleanup was performed to remove extra PCR components using IBI Scientific Kit according to manufacturer instructions. Purified PCR products were sent to Genewiz (South Plainfield, NJ) for Sanger sequencing. Next, 16S rRNA gene sequences from *Escherichia coli, Staphylococcus aureus, Mycobacterium tuberculosis, Bacillus subtilis,* and *Pseudomonas aeruginosa* were used as genomic DNA templates for alignment. Bioinformatic analysis was conducted using the online NCBI Blast tool with the Silvia Wasabi function in order to determine the specific genus of the soil isolates, along with their closest relatives.

2.3 | Antagonistic activity

Environmental strains were streaked on LB agar and SRs were cultured in LB broth overnight for 20 hr prior to the antagonistic assay. To generate a bacterial lawn, 150 µl of a single SR culture was spread plated on LB agar. Single colonies of environmental strains were patched onto the spread-plated SR by picking and streaking onto the plate with sterile toothpicks. Strains were co-cultured overnight at 30°C. Antagonistic activity was assessed by the presence, or absence, of a zone of inhibition.

2.4 | Production of electrocompetent *E. coli* and transformation with pBAM1

The strain *E. coli* S17-λpir can be used as a conjugation donor for delivering plasmids into a wide variety of species. To first insert the transposon vector pBAM1 into this strain, a protocol to create electrocompetent *E. coli* S17-λpir was followed[7]. On the first day, *E. coli* was grown at 37°C on LB agar until a thin lawn was showing. On the second day, a mass of approximately 2 mm was resuspended in 1 ml sterile water and centrifuged at 5000 x g for 5 minutes at 4°C and resuspended in sterile water three times. Then, 1 ug of purified pBAM1 was added to the *E. coli* suspension, transferred into a 0.2 cm gap cuvette and electroporated at 1.8 kV, 25 uF for approximately 5 msec. This suspension was quickly recovered by resuspending in 1 mL LB broth and incubated at 37°C for at least 30 minutes.

2.5 | Transposon mutagenesis with transformation

A protocol for rapid transformation[10] was used to deliver transposon pBAM1 from a plasmid miniprep of *E. coli* S17-λpir to *Pseudomonas* soil isolates. The soil isolates were made electrocompetent by washing them in a 300 mM sucrose solution. Then, 500 µg was mixed with 100 µg of electrocompetent cells and electroporated using a pulse of 25 AF; 200 V; 2.5 kV. Finally, the cells were restored in 1 ml LB broth for 1 hour at 37°C. Each transformed soil isolate was plated onto solid cetrimide agar with 10 µg/ml Kanamycin and incubated for 48 hours to select for mutant pseudomonads.

2.6 | Transposon mutagenesis with conjugation

Biparental mating was used to deliver transposon pBAM1 from *E. coli* S17-λpir to *Pseudomonas* soil isolates[6]. After culturing *E. coli* and recipient *Pseudomonas* strain, 1 ml of each culture was washed and resuspended in 1 ml of 0.85% w/v NaCl and 500 µl of each was mixed in a 1:1 ratio. The mating mixture was vortexed and resuspended in 25 µl 0.85% w/v NaCl. The cell suspension was spotted onto a Durapore membrane filter that was placed onto an LB agar plate and incubated at 30°C upright. Following 48 hr incubation, the filter was placed into a 2 ml microcentrifuge tube and the cells were washed and vortexed off of the filter using 1 ml of LB broth. This solution was diluted 1:1000 (a dilution to result in 30–50 colonies per plate), and plated onto solid cetrimide agar with 10 µg/ml Kanamycin to select for *Pseudomonas* transconjugants, as *E. coli* donor cells are unable to grow on cetrimide. Transconjugants were either replica-plated or spread-patched onto 50 µl of the spread plated-sensitive *B. subtilis* strain, incubated at 30°C for 48 hr, and screened for mutants exhibiting loss of antagonistic phenotype (no zone of inhibition). This process was repeated until a mutant of interest was found.

2.7 | Genome sequencing of *Pseudomonas* strains JC, JK, and 400

Three *Pseudomonas* isolates that showed promise with transposon mutagenesis were selected for whole genome sequencing. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega(R)). Illumina(R) sequencing was performed by SeqCenter, *LLC (Pittsburg, PA)*. The Illumina sample libraries were prepared using the Illumina DNA Prep kit and IDT 10bp UDI indices (to barcode samples), and sequenced on an Illumina NextSeq 2000, producing 2x151bp reads. Demultiplexing, quality control, and adapter trimming were performed by SeqCenter with the Illumina(R) program bcl-convert, which outputs cleaned fastq files organized by barcode. Geneious v10 (Biomatters Inc.) was used to assemble the sequenced genome fragments de novo. The samples were further cleaned and trimmed using the trim ends function as well as the BBDuk plugin. Reads of inserts were filtered by quality 30 and read length 150 bp prior to assembly. Genomes were assembled using close-match pseudomonad genomes available on NCBI using the internal function, allowing assembly to a reference genome. Annotations from the reference genome were then transferred to the assembled soil isolate genome, and these references were used in Anti-SMASH to search for similarity to clusters of genes that are known to make secondary metabolites in other microbes.

2.8 | Replica plating and patching

For each cetrimide and Kanamycin plate that selected mutated soil isolates, one plain LB agar plate was obtained. On this plate, 100 µl of *B. subtilis* (OD_{600} concentration 0.03) was spread using sterile beads. Then, a sterile velveteen square was placed over a disk and pressed into the cetrimide and Kanamycin plate, picking up a fraction of each colony. This square was then imprinted onto the secondary plate, covered in *B. subtilis,* making a replica of the first plate. This allows for easy comparison between the original colony and the colony grown in the presence of a tester strain. Patching was performed in the same manner as the replica plate, except the colonies were manually transferred one by one using sterile pipet tips instead of velveteen squares.

3. Results

3.1 Phylogenetic characterization and antagonistic activity

Prior to the current research study, students in spring 2022 microbiology class (BIOL 339) at UNC Asheville isolated and characterized soil bacteria with antagonistic activity against other microbes, generating a strain collection that was used as source material for the current research. As a means to assess competition, students spread-patched SRs of ESKAPE pathogens and then screened for antagonistic activity. All environmental strains were tested against six SRs including *Bacillus subtilis, Enterococcus raffinosus, Staphylococcus hemolyticus, Escheria coli, Acinetobacter baylyi, Pseudomonas fluorescens, and Enterobacter aerogenes* which resulted in ~900 one-to-one bacterial interactions. Fourteen strains were able to inhibit multiple SRs including both Gram-positive and Gram-negative isolates suggesting a broad range of antagonistic activity. Strains were selected for further study based on their ability to produce antibiotics. SI JK was found to inhibit *S. haemolyticus* and *B. subtilis* (both Gram positive), SI JC inhibited *E. aerogenes, S. hemolyticus, E. coli, P. fluorescens,* and *B. subtilis* (a mixture of Gram positive and Gram negative), and RS only inhibited *B. subtilis*. Students in the UNCA microbiology class examined strain diversity using the 16S rRNA gene. It was amplified, sequenced, and used to construct a neighbor-joining phylogenetic tree using WASABI. The closest relative for SI JK was found to be *Pseudomonas lurida*, SI JC was closest to *Pseudomonas baetica*, and SI RS was closest to *Pseudomonas koreensis*.

3.2 Strategies for Efficient Transposon Mutagenesis

To generate a collection of transposon mutants that could be efficiently screened, a transposition and plating protocol must result in ~30–50 mutant colonies per plate (to avoid overlapping colonies while also minimizing time and materials). To perform transposon mutagenesis in the selected isolates, a transformation protocol was first investigated for its efficiency in delivering the transposon vector pBAM1 into each *Pseudomonas* isolate. A protocol for rapid transformation[10] was used on each *Pseudomonas* species in order to increase efficiency. Soil isolates were made electrocompetent using a sucrose solution and then electroporated with pBAM1. Each transformed soil isolate was then plated onto solid cetrimide agar with 10 µg/ml Kanamycin and incubated for 48 hours. However, only SI JK grew, with 29 colonies. This could be due to the fact that the soil isolates did not produce the correct protein or contain a gene necessary to maintain a transposon. Further, in cases of high-throughput screening, electroporation is expensive and laborious as it requires the preparation of competent cells for each screen[11]. Next, a conjugation protocol using biparental mating to transfer transposon vector pBAM1 from *E. coli* S17-λpir into the soil isolates was investigated. It was concluded that conjugation using *E. coli* S17-λpir not only was more compatible with soil isolates (Table 1), but it was far less laborious, as it only required one round of electroporating pBAM1 into *E. coli* S17-λpir.

Table 1. Conjugation efficiency for each soil isolate at 1:100–1:10000 dilutions, with optimal dilutions highlighted in green

Soil isolate	1:100 dilution	1:1000 dilution	1:10000 dilution
400	no result	no result	no result
B10	no result	no result	no result
RS	1–5 colonies	no result	no result
JK	400–450 colonies	30–40 colonies	3–9 colonies
JC	lawn	300–400 colonies	30–50 colonies

3.3. Strategies for Mutant Screening

Next, an efficient screening technique was optimized to identify mutants that could no longer produce a zone of inhibition against SRs. Replica plating, a technique where colonies on one plate are picked up using a velveteen square and placed identically onto another plate, is typically a much more efficient plating method in comparison to spread-patching. However, many problems were observed with replica plating. The first issue that was encountered was that the velveteen that was used to stamp from one plate to another was not easily sterilized via autoclaving. This could be due to *Bacillus*' ability to sporulate, or that the autoclave did not reach sufficient temperatures or pressures. Another issue was that the thickness of the LB agar plates determined how well the *B. subtilis* was able to grow, which caused a level of variation between plates that made it difficult to determine where zones of inhibition were (Figure 2). Replica plating also requires a lot more materials than spread-patching. For example, if twenty selection plates were used after conjugation, then forty plates would be required for replica plating (one with *B. subtilis*, and one for the replica). On the other hand, if twenty selection plates were used after conjugation, the total colonies could be patched onto around five plates. It was concluded that, although patching is highly inefficient and laborious, the technique led to fewer problems and used far fewer materials.

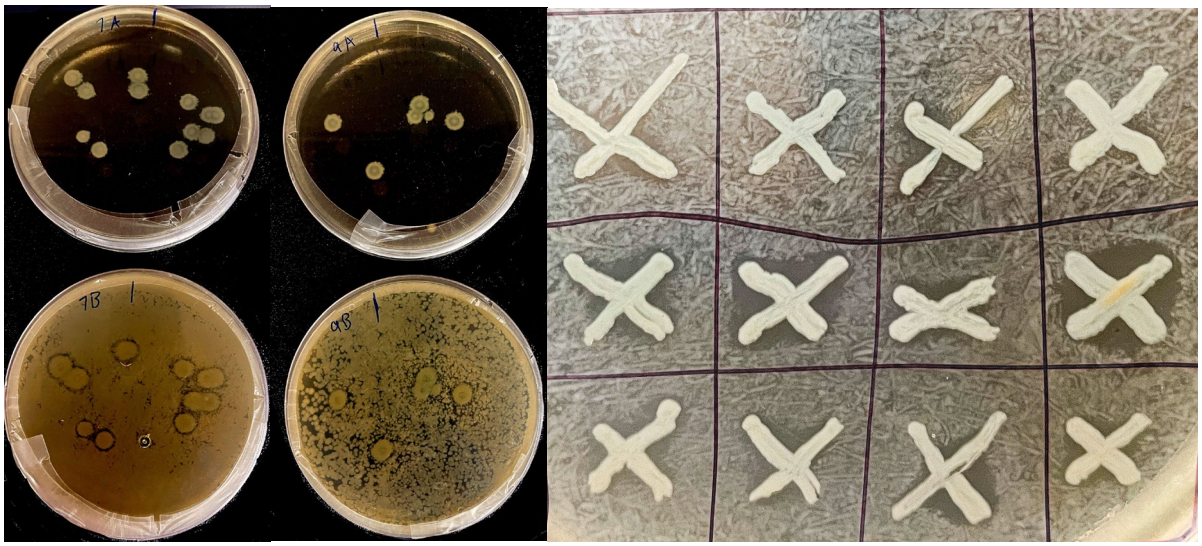
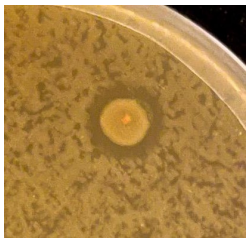
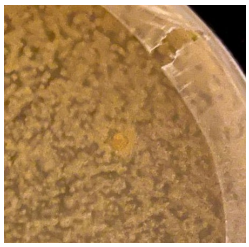
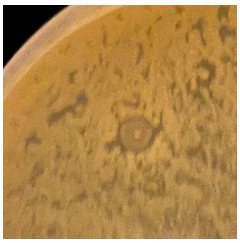
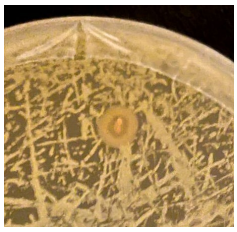
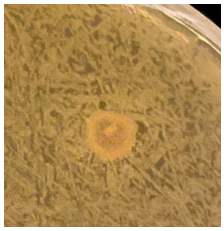
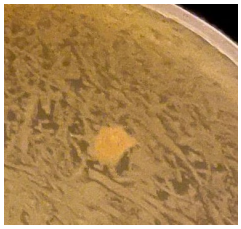
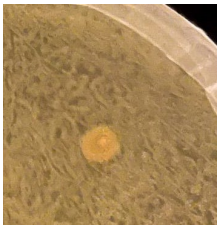


Figure 2. Differences in LB agar thickness creates variation between plates when replica-plating, whereas spread-patching creates uniformity and shows the same zones (or lack thereof) for every colony.

3.4 Choosing the most effective isolate for mutagenesis and screening

While five isolates were originally chosen to be tested (SS 400, RGRF B10, SI JK, SI JC, and IS RS), there were areas where not all isolates were successful. Three isolates, SI RS, RGRF B10, and SS 400, had insufficient success with conjugation (only having success in one to two colonies, if any). JC had the greatest success with conjugation, but did not make consistent zones of inhibition when spread-patched with different tester strains (Table 2). It was concluded that SI JK was the best strain to undergo transposon mutagenesis, and *B. subtilis* was the best tester strain because both SI JK and SS 400 made very clear zones of inhibition in its presence.

Table 2. *Pseudomonas* soil isolates patch tested to test control antagonistic abilities against different ESKAPE SR's

	SS 400	SI JC	SI JK
B. subtilis			
S. saprophyticus			
S. aureus RN4220			

3.5 Whole Genome Sequencing of *Pseudomonas* Isolates JK, JC, and 400

While mutagenesis and screening were in progress, whole genome sequencing was performed for JK, JC, and 400 strains so that we would have reference genomes ready if/when mutants of interest were identified, to facilitate identification of impacted gene clusters. SI JK was the first isolate to be completely assembled and annotated due to its ability to perform under lab conditions (Figure 3). The assembly was submitted to Anti-SMASH to search for secondary metabolites involved in antimicrobial activity. The search showed fengycin and lankacidin C as "most similar known clusters", as these are both compounds known to have antimicrobial activity (Figure 4). This means that there are known biosynthetic gene clusters in other bacteria that produce bioactive compounds fengycin and lankacidin C. The search showed that 13% of the genes in the known fengycin gene cluster have a significant BLAST hit to a cluster of genes in SI JK. This is of great significance, because it means that SI JK could make an antimicrobial that is related in some way to fengycin or lankacidin C. However, it is likely not the same thing because only 13% of the genes in that cluster are similar.

Figure 3. SI JK genome assembled and annotated

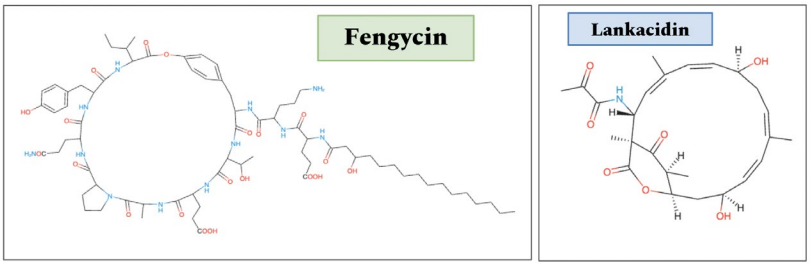


Figure 4. Two antimicrobial molecules that are similar to the molecules encoded by the antagonistic genes in SI JK.

3.6 Identification of a mutant with loss of inhibitory activity

After screening approximately 3,000 mutants, a single mutant was identified with a loss of inhibitory activity against *B. subtilis* (Figure 5). Once it was located, it was restreaked against *B. subtilis* to ensure that the ability to produce antibiotics was completely lost. Experiments are currently underway to isolate transposon junction DNA for sequencing, to identify the disrupted gene.

Figure 5. SS JK transposon mutant with loss of inhibitory activity (outlined in red) patched on LB agar plate with *B. subtilis*.

Discussion

Biochemical analysis of extracts of the wild-type strain of an antibiotic-producing isolate relative to a loss-of-function mutant can facilitate antibiotic compound identification and characterization. This, as well as understanding the native biosynthetic pathway for an antimicrobial compound, could lead to pipelines for scaling drug production. *Pseudomonas* spp. are ideal targets for this due to their ability to inhibit a wide range of both Gram-positive and Gram-negative bacteria including many known human pathogens such as Methicillin-resistant *Staphylococcus aureus* (MRSA)[12] and *Pseudomonas aeruginosa* isolated from cystic fibrosis patients[13]. Outside of medicine, Pseudomonads also have been found to inhibit the growth of pathogenic plant fungi[14] and break down insecticides[15].

Future experiments will begin with extracting and identifying the exact gene that was disrupted by the transposon in the mutant of interest. This will unveil whether or not the gene is related to the fengycin or lankacidin C clusters, or if it is something entirely new. After identifying the gene, the use of mass spectroscopy will help to identify the exact antimicrobial compound produced by the soil isolate. The compound may be similar to fengycin or lankacidin C, but it will likely be novel due to the low similarity score. Further, once the biosynthetic genes needed to make the antibiotic are identified, they could be cloned into *E. coli* and used to produce more of the antimicrobial.

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5. References

1. Robinson TP, Wertheim HF, Kakkar M, Kariuki S, Bu D, Price LB. (2016). Animal production and antimicrobial resistance in the clinic. *Lancet* 387(10014):e1-e3.

2. Jadimurthy R, Mayegowda SB, Nayak SC, Mohan CD, Rangappa KS. (2022). Escaping mechanisms of ESKAPE pathogens from antibiotics and their targeting by natural compounds. Biotechnology Reports 34.

3. Baquero F. (2021). Threats of antibiotic resistance: An obliged reappraisal. International Microbiology : The Official Journal of the Spanish Society for Microbiology 24(4):499-506.

4. Reynolds D, Kollef M. (2021). The Epidemiology and Pathogenesis and Treatment of Pseudomonas aeruginosa Infections: An Update. *Drugs* 81(18):2117-2131.

5. Centers for Diseases Control (CDC) (2019) Antibiotic resistance threats in the United States, 2019. Department of Health and Human Services, CDC. Atlanta.

6. Davis E, Sloan T, Aurelius K, Barbour A, Bodey E, et al. (2017). Antibiotic discovery throughout the Small World Initiative: A molecular strategy to identify biosynthetic gene clusters involved in antagonistic activity. MicrobiologyOpen 6(3).

7. Gonzales MF, Brooks T, Pukatzki SU, Provenzano D. 2013. Rapid protocol for preparation of electrocompetent Escherichia coli and Vibrio cholerae. J Vis Exp. 8;(80):50684.

8. de Lorenzo V, Herrero M, Sánchez JM, Timmis KN. 2006. Mini-transposons in microbial ecology and environmental biotechnology. FEMS Microbiol Ecol (3):211–24.

9. Martínez-García E, Calles B, Arévalo-Rodríguez M, de Lorenzo V. 2011. pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. BMC Microbiol. 11:38.

10. Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. Journal of Microbiological Methods 64(3):391-97.

11. Rodríguez-Beltrán J, Elabed H, Gaddour K, Blázquez J, Rodríguez-Rojas A. 2012. Simple DNA transformation in Pseudomonas based on the Yoshida effect. Journal of Microbiological Methods 89(2):95-8.

12. Farrow JM 3rd, Pesci EC. 2007. Two distinct pathways supply anthranilate as a precursor of the Pseudomonas quinolone signal. J Bacteriol 189(9):3425-3433.

13. Chatterjee P, Davis E, Yu F, James S, Wildschutte J, Wiegmann D, Sherman D, McKay R, LiPuma J, Wildschutte H. 2016. Environmental Pseudomonads inhibit Cystic Fibrosis patient-derived Pseudomonas aeruginosa. Applied and Environmental Microbiology. 83(10).

14. Tran H, Ficke A, Asiimwe T, Höfte M, Raaijmakers JM. 2007. Role of the cyclic lipopeptide massetolide A in biological control of Phytophthora infestans and in colonization of tomato plants by Pseudomonas fluorescens. *New Phytol*. 175(4):731-742.

15. Pinjari AB, Pandey JP, Kamireddy S, Siddavattam D. 2013. Expression and subcellular localization of organophosphate hydrolase in acephate-degrading Pseudomonas sp. strain Ind01 and its use as a potential biocatalyst for elimination of organophosphate insecticides. *Lett Appl Microbiol*. 57(1):63-68.