GENOME MINING FOR THE BIOSYNTHETIC GENE CLUSTER RESPONSIBLE FOR THE ANTIBIOTIC PROPERTIES OF *PSEUDOMONAS AUERGINOSA* AGAINST PATHOGENIC BACTERIA

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THESIS STATEMENT

Numerous clinically relevant bacteria are found in nature and mostly in the soil. There is a dire need for new antibiotics to be discovered to counter the rising prevalence of antibiotic resistance. The purpose of this research is to determine the biosynthetic cluster gene responsible for antibiotic activity of a selected bacterial isolate using genome sequencing, database comparison, genome mining, and mutation.

ABSTRACT

DEPARTMENT OF BIOLOGY

DANIEL S. OYEKA   **M.S. CLAFLIN UNIVERSITY, 2022**

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Antibiotics as a secondary metabolite that can be produced naturally by bacteria or synthesized have metamorphosed medicine, drastically reduced the death rate of infectious diseases, and saved millions of lives. They are a form of medicine that inhibits the growth of or kills bacteria. The rapid emergence of antibiotic-resistant bacteria happening worldwide is affecting the potency of the drugs available. This issue is complicated by the very low number of novel antibiotics discovered or produced, due to the amount of time, money, and resources required for traditional drug discovery. Therefore, there is a need for novel antibiotic research and with about 75% of antibiotics in clinical use being derived from soil bacteria, the objective of this study was to examine selected environmental bacterial isolates for antibiotic activity, genome mining of an isolate with the aid of DNA sequencing technology and bioinformatics tools to reveal the biosynthetic gene cluster (BGC) responsible for its antibiotic activity and possibly review untapped cryptic biosynthetic gene clusters of the isolate. Nine environmental soil isolates were co-cultured with *Micrococcus luteus, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter aerogenes, Salmonella enteritidis, Streptococcus agalactiae,* and *Serratia marcescens* on Mueller Hinton agar

to determine which isolates produced a zone of inhibition which is indicative of antibiotic production. Six of the isolates produced a zone of inhibition against at least four of the ten test bacteria. To identify the genus and species of the antibiotic-producing bacteria, the 16S rRNA gene was PCR amplified, confirmed, and sequenced. The sequences were compared to the 16S rRNA gene database at the National Center for Biotechnology Information (NCBI) and the identification of the antibiotic-producing bacteria revealed that the two main genera were *Bacillus* and *Pseudomonas*. Genetic mining and analysis of secondary metabolite biosynthetic gene clusters of the isolates required genomic DNA to be isolated, purified, and sequenced. The sequences were compared to the gene database at NCBI via R-console and with the aid of more bioinformatics tools such as Rapid Annotation using Subsystem Technology (RAST), antiSMASH, Integrated DNA Technologies (IDT), the antibiotic core genes of four BGCs for *Pseudomonas aeruginosa* were identified, three were PCR amplified and cloned and two were analyzed utilizing gene knockouts. Mutation analysis revealed that neither the pyoverdine nor the endophenazine A&B is responsible for the antibiotic activity of *P. aeruginosa*.

KEYWORDS AND ABBREVIATIONS

Keywords: Antibiotic activity, Genome mining, Biosynthetic Gene Clusters, *Pseudomonas aeruginosa*, Conjugation, Gene Knockout.

Abbreviations:

DNA	Deoxynucleic Acid
BGC	Biosynthetic Gene Cluster
HGT	Horizontal Gene Transfer
CDC	Centers for Disease Control and Prevention
rRNA	Ribosomal Ribonucleic Acid
LB	Luria Bertani
MH	Mueller Hinton
PCR	Polymerase Chain Reaction
ZOI	Zone of Inhibition
DAP	Diaminopimelic acid
AGE	Agarose Gel Electrophoresis

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BACKGROUND AND LITERATURE REVIEW

INTRODUCTION

For years, mankind struggled with infectious diseases caused by bacteria which was a time by far the leading cause of death in humans. Based on the composition of their cell wall, bacteria are largely divided into the Gram-positive and the Gram-negative bacteria. The Gram-positive bacteria possess a cytoplasmic membrane surrounded by a firm and rigid cell wall, while Gram-negative bacteria consist of a thin cell wall that is surrounded by a lipid membrane called outer membrane (OM) (Kapoor *et al*., 2017). The need to counter the diseases caused by bacteria led to the development of antibiotics.

Antibiotics are secondary metabolites that can be produced naturally by microbes or synthesized that only fight bacterial infections either by killing the bacteria (bactericidal) or inhibiting the growth of the bacteria (bacteriostatic). Antibiotics have been used for millennia to treat infections, although, until the last century, people did not know that the infections were caused by bacteria. In 1910, Paul Ehrlich a German physician discovered the very fight antibiotic, salvarsan. He noticed some chemical dyes did not color some bacteria cells and concluded that, according to this principle, it must be possible to create substances that can kill certain bacteria selectively without harming other cells (Hutchings *et al*., 2019). Alexander Fleming, a Scottish physician-scientist discovered penicillin in 1928 and started the golden age of natural product antibiotic discovery by the 1940s leading to the mass production of antibiotics. The revolution in the "golden era" in the forties and early fifties is when almost all major groups of antibacterial antibiotics were discovered. Antibiotics isolated in this period were primarily obtained from *Streptomyces* species, representing some 70 to 80% of all isolated compounds (Nordenfjäll, n.d., 1996).

Classes of Antibiotics

There are nine major classes of antibiotics: beta-lactams, macrolides, fluoroquinolones, chloramphenicols, aminoglycosides, anti-metabolites, ansaymcins, lipopeptides, and tetracyclines. These antibiotics can be classified based on their mechanism of action on the bacteria.

1. Antibiotics targeting cell wall

The bacterial cell consists of a cell wall that provides shape, rigidity, and protection to the bacteria cell. The cell wall is made up of peptidoglycan consisting of glycan and short peptides. The cell wall is strengthened due to the formation of a 3D structure of the D-alanyl-alanine portion of the peptide chain is cross-linked by glycine residues in the presence of penicillin-binding proteins (PBPs). Antibiotics such as the β-lactam and glycopeptides affect the biosynthesis of the bacteria cell wall. The β-lactams are thought to primarily mimic the D-alanyl alanine portion of the peptide and bind to the PBPs making it unavailable for the cross-linking of the cell wall while the glycopeptides such as vancomycin and teicoplanin (Kahne *et al.*, 2005), prevent binding of this D-alanyl with the PBPs and inhibits the cell wall synthesis.

2. Inhibitors of Protein Biosynthesis

The bacterial 70S ribosome is responsible for the translation process and it is subdivided into two ribonucleoprotein subunits, the 30S subunit that binds to the mRNA to begin initiation and 50S subunit that binds to t-RNA and controls elongation **(**Vannuffel and Cocito**, 1996)**. Antibiotics can inhibit the synthesis of proteins by targeting either the 30S or 50S subunit of the bacterial ribosome. Inhibitor of the 30S subunits includes the aminoglycosides that interact with 16S rRNA of the 30S subunit causing misreading and early termination of the mRNA strand. Also, the

tetracyclines act upon the conserved regions of the 16S rRNA to inhibit the t-RNA to the A site of the ribosome (Kapoor *et al*., 2017).

Inhibitors of the 50S subunits include the Chloramphenicol that binds with the conserved regions of the peptidyl transferase cavity of the 23S rRNA of the 50S subunit stopping the protein synthesis by preventing binding of t-RNA to the A site of the ribosome, while the macrolides, lincosamides, and streptogramins B affect the early stage of protein synthesis, called translocation, by targeting the conserved sequences of the peptidyl transferase center of the 23S rRNA of the 50S ribosomal subunit leading to an early detachment of incomplete peptide chains.

3. Inhibitors of DNA replication

DNA replication is the very key step to gene expression and cell growth. The bacterial enzyme responsible for unwinding the DNA is called the DNA gyrase or topoisomerase IV. The DNA gyrase consists of 2 subunits. Subunit A which makes an incision and reseals the DNA strands while subunit B is responsible for the introduction of negative supercoils. Antibiotics such as the Fluoroquinolones (FQ) bind to the subunit A of the enzyme interfering with the nicking and resealing functions (*Kapoor et al*., 2017).

4. Folic acid metabolism inhibitors

Folic acid is an essential nutrient necessary for protein and nucleic acid synthesis. Folic acid cannot diffuse into the bacterial cell; therefore, it must be synthesized by bacteria from the substrate para-amino-benzoic acid (PABA). Sulfonamides act by competing with PABA as a substrate for the enzyme dihydropteroate synthase, which converts PABA into dihydropteroic acid while antibiotics such as trimethoprim, ormetoprim, and pyrimethamine function by inhibiting

dihydrofolate reductase activity necessary for purine and pyrimidine (building blocks) nucleotide synthesis (Kapoor *et al*., 2017).

5. Increase permeability of the bacterial membrane

The bacterial cell protection layers are the plasma membrane and cell wall. The cell wall consists of peptidoglycan which is found outside the cytoplasmic membrane. It is considered a permeability barrier except for small substrates. The bacteria are negatively charged because of peptidoglycan and lipopolysaccharides (LPS) on the outer membrane; therefore, antibiotics like polymyxins that are positively charged are attracted to the bacterial cell wall. The polymyxins bind to the bacterial cell membrane and increase its permeability. These alterations lead to osmotic imbalance producing an out-flow of cellular molecules, derailing respiration and rapid water intake causing turgidity leading to cell death (Ali *et al*., 2020).

Antibiotic Resistance

Antimicrobial resistance (AMR) can be defined as the ability of microorganisms to survive and thrive under the presence of antimicrobial agents/antibiotics. There are various types of antimicrobial agents such as antibiotics, disinfectants, and food preservatives that can be used against microorganisms to reduce their ability to grow, inhibit their multiplication or even kill them. Bacterial resistance is regarded as a major concern in healthcare organizations with potential impacts globally. As antibiotics are used around the world, the opportunity is given for the bacteria to develop stronger complicated resistance against those antibiotics increases. As a result, some new modified strains appear to reduce the chance for the treatments to be appropriately effective in patients, causing profound consequences leading to clinical complications and death(Ali et al.,

2020). There are 2 major types of AMR and they are natural or intrinsic resistance and acquired or extrinsic resistance Natural resistance may be intrinsic resistance which can be described as a resistant trait ubiquitous within a bacterial species not gotten from horizontal gene transfer and regardless of prior exposure to any antibiotic or induced whereby the genes are naturally occurring in the bacteria, but are only expressed to resistance levels after exposure to an antibiotic (Reygaert, 2018).

There are three major mechanisms by which bacteria mediate intrinsic resistance are differences in membrane permeability and access, efflux pumps, and modification of target molecule. Bacteria are classified into two Gram positive or Gram negative by a process known as Gram staining. This difference in the structure of the cell wall means that Gram-negative bacteria are resistant to vancomycin (a glycopeptide antibiotic) because their extra outer membrane (OM) prevents a large molecule like vancomycin from entering the cell. Gram-negative bacteria and some Gram-positive bacteria also have structures called porins, which act as pores through which molecules including nutrients can pass through the membrane into the cell. In some intrinsically resistant bacteria, their chemical properties or the size of porins exclude certain antibiotics. A lower number of the porins are expressed in the membrane is also thought to contribute to intrinsic resistance.

Another mechanism of intrinsic resistance in Gram-negative bacteria is due to the presence of efflux pumps. These are membrane proteins found in the cytoplasmic membrane that naturally remove antibiotics and environmental toxins from the cell keeping low their intracellular concentrations. Examples of intrinsic genes include *acr*AB/*tol*C in *E. coli*, *nor*A in *S. aureus*, and *lmr*A in *Lactococcus lactis* (Peterson and Kaur, 2018). The efflux mechanism can pump out antibiotics at the same pace at which it enters the cell preventing the antibiotics from reaching their

target site. Efflux pumps are multidrug transporters but can also be specific to antibiotics except for polymyxin and are capable to pump a wide range of unrelated antibiotics such as macrolides, tetracyclines, and fluoroquinolones. This significantly contributes to multidrug resistance naturally existing in organisms (Kapoor *et al.*, 2017).

Natural alterations in the target sites of antibiotics that prevent drug binding is a common mechanism of resistance. Target site changes often come from unpremeditated mutation of a bacterial gene on the chromosome. Since antibiotic interaction with the target molecule is generally quite specific, a minor alteration of the target molecule can have an important effect on antibiotic binding. There might also be cases of the microbial ability of some bacterial species to make enzymes that can inactivate the antibiotics with an example of AmpC β -lactamase in *E. coli* (Ali *et al.*, 2020).

Acquired resistance is also known as extrinsic resistance and is defined as a phenomenon where naturally susceptible bacteria can develop resistance against certain antibiotics by receiving foreign genetic materials that confer that resistance from other bacterial strains (Ali *et al.*, 2020). There are also three major mechanisms of acquired resistance: modification of drug targets, antibiotic inactivation, and plasmid-encoded specific efflux pumps.

There are various sections in the bacterial cell that may be targeted by antibiotics and there are just as many targets that may be modified by the bacteria to confer resistance to these drugs. For example, resistance to the β-lactam drugs used almost primarily by gram-positive bacteria is via alterations in the structure and/or the number of penicillin-binding proteins (PBPs) found as a cross-linking molecule in the cell wall. A change in the number (increase or decrease in PBPs will have a decrease in drug binding ability) of PBPs impacts the number of drugs that can bind to that target. Also, an alteration in structure (e.g., PBP2a in *S. aureus* by the acquisition of the mecA

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gene) may decrease the ability of the drug to bind, or totally inhibit drug binding. Further examples are vancomycin (which works by inhibiting cell wall synthesis) and daptomycin (which works by depolarizing the cell membrane). Resistance to vancomycin is mediated through the acquisition of van genes which gives changes in the structure of peptidoglycan precursors that cause a decrease in the binding ability of vancomycin. Daptomycin requires the presence of calcium serving as a receptor and mutations in genes (e.g., mprF) lead to a change in the charge of the cell membrane surface to positive, inhibiting the binding of calcium. RNA polymerase mutations also induce resistance to rifampicin. Resistance to drugs that target the ribosomal subunits may occur via ribosomal mutation, ribosomal subunit methylation, or ribosomal protection. These mechanisms interfere with the ability of the drug to bind to the ribosome. For drugs that attack nucleic acid synthesis such as the fluoroquinolones, resistance is via modifications in DNA gyrase or topoisomerase IV structures can greatly impede the binding of drugs(Reygaert, 2018).

There are two broad methods by which bacteria inactivate drugs: by actual degradation of the drug, or by transferring a chemical group to the drug. There are three main enzymes that inactivate antibiotics, and they are β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs) (Reygaert, 2018). β-lactamases break up nearly all β-lactams that have ester and amide bonds Examples include penicillins (penicillinase), cephalosporins (cephalosporinases), metallo-β-lactamases and oxacillin hydrolyzing enzymes. About 300 β-lactamases are known to date. β-lactamases are broadly prevalent enzymes that are classified using two main classification systems: Ambler (structural) and Bush–Jacoby–Medeiros (functional) (Kapoor *et al*., 2017).

Aminoglycosides (AGs) are neutralized by specific enzymes: Phosphoryl-transferases, nucleotidyl-transferases or adenylyl-transferases, and AACs. These AMEs reduce the affinity of a

reformed molecule, impede binding to the 30S ribosomal subunit and provide extended resistance to AGs and FQ. AMEs are identified in *S. aureus*, *E. faecalis*, and *S. pneumoniae* strains (Kapoor *et al*., 2017).

Some Gram-positive and Gram-negative bacteria and some of *Haemophilus influenzae* strains display resistance to chloramphenicol, and they possess an enzyme chloramphenicol transacetylase that acetylates hydroxyl groups of chloramphenicol where altered chloramphenicol is unable to bind to a ribosomal 50S subunit properly(Kapoor *et al*., 2017).

Bacteria possess chromosomally encoded genes for efflux pumps. Some are expressed continuously conferring the intrinsic resistance, and others are induced (high-level resistance is usually via a mutation that modifies the transport channel) with an example such as the TetK and TetL of *S. aureus*. This induces an extrinsic resistance that poses a great threat to the clinical field and human health(Bismuth *et al*., 1990).

Intrinsic vs. Acquired Resistance

Intrinsic antibiotic mechanisms are normally chromosome-encoded and include non-specific efflux pumps (which likely evolved as a general response to environmental toxins), or mechanisms that serve as permeability barriers. These processes are fixed in the core genetic make-up of a bacteria cell. A largely examined example of an intrinsic resistance system is the AcrAB/TolC efflux pump in *Escherichia coli*, with very broad substrate specificity and can export different classes of antibiotics, dyes, detergents, and disinfectants at the pace at which they enter the cell. Vancomycin resistance in *E. coli* and other Gram-negative bacteria provides another example of intrinsic resistance, which results from the permeability barrier imposed by the outer membrane. The intrinsic mechanisms are regarded to confer low-level antibiotic resistance in the

original host. The acquired resistance mechanisms, on the other hand, are generally gotten by horizontal gene transfer or plasmid-encoded specific efflux pumps (such as TetK and TetL of *S. aureus*) and enzymes that can alter the antibiotic molecule by transferring a chemical to it or the target of the antibiotic. These mechanisms pose a more serious threat (than the intrinsic mechanisms) to human health because of a change in the context of the resistance determinant from chromosomal to plasmid-mediated, resulting in their enhanced expression and dissemination(Peterson and Kaur, 2018).

Biosynthetic Gene Cluster

Secondary metabolites are derived from primary metabolic pools and central metabolic pathways where acetyl-COAs are critical initial building blocks for the synthesis of terpenes, polyketides, amino acids, and secondary metabolites being used for the synthesis of non-ribosomal peptide secondary metabolites such as penicillin. In contrast to genes that are required for the synthesis of a primary metabolite which are distributed throughout the microbial genome, the genes encoding the enzymatic activities to produce any secondary metabolite are arranged in a connecting fashion known as a biosynthetic gene cluster (BGC) (Keller, 2019). These genes encode proteins that can synthesize the antibiotics and often include genes encoding regulatory elements, transport proteins, resistance factors, or those involved in precursor production (Cimermancic *et al*., 2014; Tietz and Mitchell A, 2015).

Our future goal for this project is to discover a new antibiotic from an environmental bacterium. Freezer stock of environmental bacteria isolates was grown in agar and selected based on their antibiotic activity using the modified Kirby Bauer assay against 10 known pathogenic bacteria isolates. My goal for this project was to determine which biosynthetic gene cluster is

responsible for the antibiotic properties of a selected isolate (*Pseudomonas aeruginosa)* and possibly discover a new biosynthetic gene for a novel natural antibiotic.

BACKGROUND

Adnani *et al.* (2017) experimented on marine bacteria and interdisciplinary technologies enabling biosynthesis and the discovery of new antibiotics. The advancement of both genomics and metabolomics has shown that microbial biosynthetic capacities on Earth exceed earlier expectations. This is due to the realization that many microbial natural product producers have biosynthetic types of machinery not immediately feasible in classical laboratory fermentation conditions. These dormant biosynthetic gene clusters encode for many potential new antibiotics and are very enticing targets for activation under certain laboratory conditions. By co-culturing *Rhodococcus* sp. and *Micromonospora* sp. a new bis-nitroglycosylated anthracycline was made whose mechanism of action seems to derive from other anthracyclines. The structure of keyicin was determined using high-resolution MS and NMR technologies and molecular modeling studies. The structural and genomic comparisons of keyicin biosynthetic gene clusters within the *Micromonospora* genome to other anthracycline-producing systems were possible due to sequencing. The new natural product was shown to be selectively active against Gram-positive bacteria including both *Rhodococcus* sp. and *Micromonospora* sp. *E. coli*-based chemical genomic studies showed that keyicin's mechanism of action does not contain nucleic acid damage compared to other anthracyclines (Adnani *et al.* 2017).

Raja *et al.* (2010) experimented on the screening and isolation of antibiotic-producing psychrophilic actinomycetes against *Streptococcus*. Antibacterial activity was found against *S. mutans* and *S. oralis*. Six actinomycetes were isolated from the soil sample by crowded plate technique and primarily screened and identified as *Intrasporangium* sp., *Dactylsporangium* sp., *Micromonospora* sp., *Streptoverticilium* sp. and two *Streptomyces* sp. The bacterial isolates came from fifty tooth samples collected from the dental hospital and identified as either *S. mutans* or *S.*

oralis. Hemolytic activity on blood agar plates and biochemical testing were used to confirm the identification of test bacteria. *S. mutans* was very sensitive to *Dactylsporangium* sp, while *S. oralis* was sensitive to *Streptomyces.* A pertinacious substance responsible for antimicrobial activity was produced by *Dactylsporangium* sp. This paper reports this is the first time a species other than *Streptomyces* has shown antimicrobial activity against streptococcus-associated dental disease (Raja *et al.* 2010).

Song *et al.,* (2017) performed research on the antibiotic screening of gram-negative bacteria, *Burkholderia gladiol* BCC0238, which was isolated from a cystic fibrosis patient. The purpose of the experiment was to identify a novel bioactive metabolite from the *Burkholderia* genus, and the research led to the discovery of gladiolin, a novel macrolide antibiotic that functions as an RNA polymerase inhibitor, compared with a known antibiotic called etnangien and antimicrobial activity against *Mycobacterium tuberculosis.* Antimicrobial screening of purified against some gram-negative, gram-positive, and the ESKAPE panel of pathogens yielded moderate activity against the gram-negative bacteria while the gram-positive bacteria proved to be resistant at a clinically revelant minimal inhibitory concentration. Using single-molecule real-time (SMRT) sequencing technology yielded a genome of 8,489,985 bases assembled into four contiguous sequences. Targeted mutagenesis and sequence analysis revealed the pathway of the gladiolin biosynthetic gene cluster and BLAST searches showed a high degree of similarity between the PKS subunits involved in gladiolin and etnangien biosynthesis and detailed analysis of the catalytic domains within the PKS subunits of both antibiotics allowed for proposing a plausible pathway for gladiolin biosynthesis. This study, via structural elucidation, provides a stable and yet very potent antibiotic against *M. tuberculosis* (Song *et al.,* 2017).

Ochoa *et al.* (2018) conducted an experiment on the microbiota of marine animals and their ability to show antibiotic activity. Bacterial strains were isolated from samples collected from five marine mammal carcasses. *Micromonospora* strain was identified showing activity against a variety of Gram-positive pathogens (such as *S. aureus*, *E. coli*, *P. aeruginosa*, and *Enterobacter aerogenes*) and no apparent human cytotoxicity. Compound isolation showed a new complex glycosylated polyketide, phocoenamicin, with strong activity against the intestinal pathogen *C. difficile*. *C. difficile* is a bacterium found in hospital settings that are hard to treat. Using BioMAP, an activity-profiling platform, the metabolite was clustered with other known ionophore antibiotics. Fluorescence imaging and flow cytometry were used to show that phocoenamicin was suited to shift membrane potential without damaging membrane integrity. Phocoenamicin has comparable activity to vancomycin, which is the main antibiotic used for *C. difficile* infections. The discovery of phocoenamicin shows the potential of using marine mammal microbiota in drug discovery and host protection (Ochoa *et al.* 2018).

Iqbal *et al.* (2018) conducted an experiment where they screened, characterized, and optimized the antibacterial peptides produced by *Bacillus safensis* strain MK-12. In previous years more bacteria are gaining multidrug resistance to antibiotics out on the market. Therefore, there is a need for new drugs that are effective against these said multidrug-resistant bacteria. Soil was taken from many different locations for antimicrobial metabolite-producing bacteria. During the primary screening, twenty-five percent of the bacterial isolates showed antimicrobial activity. *B. safensis* strain MK-12 characterized through colony morphology, biochemical testing and 16S rRNA gene sequencing was found to have strong antimicrobial activity against Gram-positive and Gram-negative multidrug-resistant clinical strains. This study suggests that collecting and isolating

bacterial isolates from unexplored areas could yield results in the fight against antibiotic resistance (Iqbal *et al.* 2018).

Kudo et al. (2015) conducted an experiment on *Streptomyces scabrisporus* with the strain *S. scabrisporus* JCM 11712 to further study hitachimycin, a macrolactam antibiotic that has shown to be an antiprotozoal and antitumor antibiotic, as well as have a potent antimicrobial activity against *Bacillus subtilis* and *Micrococcus luteus*. The main structural features of hitachimycin are the unique β-phenylalanine (β-Phe) starter unit of its polyketide skeleton and although the mechanism of β-Phe incorporation into the polyketide during hitachimycin synthesis is unclear, it is likely to be related to the biosynthetic pathways for macrolactam antibiotics, including those for cremimycin, incednine, and vicenistatin. Therefore, the study aimed to identify the hitachimycin biosynthetic gene cluster and further the knowledge of β-amino acid biosynthetic machinery and selective incorporation mechanisms. *S. scabrisporus* JCM 11712 chromosomal DNA was extracted by standard phenol/chloroform extraction and automated gene annotation was performed with the MiGAP server. Routine genetic analyses were conducted by using Geneious, and the 80-kb DNA sequence that included the putative hitachimycin biosynthetic gene cluster was found. Selective mutagenesis by gene knockout of the *hitA* gene inserted an apramycin resistance gene cassette where the mutant produced no hitachimycin. Then supplementation with (S)-β- Phe led to the restoration of hitachimycin but not with the (R)-β- Phe showing that not only is the *hitA* gene relevant in the biosynthesis of hitachimycin but the stereo- chemistry of β-Phe is critical for recognition by the β-amino- acid-selective ATP-dependent ligase HitB and other downstream enzymes. In conclusion, the study identified the hitachimycin biosynthesis gene cluster by genome mining which is a novel member of the β-amino-acid-containing macrolactam biosynthesis group, and characterized the (S)- β-Phe-forming PAM (Kudo *et al.*, 2015).

Tyc *et al.* (2014) experimented with interspecific interactions on antimicrobial activity among soil bacteria. Some bacteria species only produce antimicrobial products if in the presence of a competing species. A small amount is known about the prevalence of interaction-mediated induction of antibiotic production in natural soil bacteria samples. A high output method to screen to produce antimicrobial activity was developed by monocultures and pairwise combinations of 146 phylogenetically different bacteria isolated from soil. Antimicrobial activity was monitored by the growth responses of two human pathogenic model organisms, *E. coli* WA321 and *S. aureus* 533R4). Thirty-three percent of isolates showed antimicrobial activity only in monoculture, while forty-two percent showed activity only when tested in interactions. *S. aureus* had more bacterial isolates showing activity against it than *E. coli*. Only six percent of species showed interaction-mediated induction of antimicrobial activity. Twenty-two percent of all interactions shown revealed interaction-mediated suppression of antimicrobial activity. Interaction-mediated induction of antimicrobial activity was more prevalent for the combinations of Flavobacteria and alpha-Proteobacteria, unlike the antimicrobial activity seen in various classes (Tyc *et al.* 2014).

Li *et al*. (2017) executed an experiment on *Pseudomonas acidophila* ATCC 31363 for the discovery of a monobactam antibiotic which is the only β-lactam class of antibiotics that does not fall prey to bacterial antibiotic resistance using β-lactamases, an enzyme that utilizes mostly classical serine hydrolase activity against β-lactam drugs, cleaving the β-lactam ring and thus rendering the drugs ineffective. The metallo-β-lactamases (MBLs) are regarded as broad-spectrum antibiotic-resistant to the producing host and there are currently no clinically approved MBL inhibitors, indicating the level of the challenge these resistance proteins present in treating bacterial infections hence the need for more monobactam antibiotics such as the sulfazecin. And up until

this study, the biosynthesis of monobactams remains poorly understood and BGCs have been identified. Therefore, this study aimed to isolate and characterize the sulfazecin BGC from *P. acidophila* ATCC 31363, demonstrating the reason why the monobactams are not rendered ineffective by β-lactamases. The total genomic DNA was isolated from *P. acidophila* Sul⁻-7 yielding a 490-bp and a BLASTP analysis of the fragmented genome showed homology to NRPSs from several secondary metabolic pathways (61%–78% identity), and nonribosmal peptide synthetases responsible for the biosynthesis of sulfazecin. The single-stranded disruption constructs were delivered into wild-type *P. acidophila* by electroporation and mutant cells were grown for two rounds without antibiotic selection before screening for a double crossover and loss of plasmids resulting in knockout mutants that were confirmed by PCR of the targeted regions followed by DNA sequence analysis showing loss of function in the biosynthetic gene for sulfazecin. The study was also demonstrated that, instead of serine, the β-lactam ring in sulfazecin is derived from L-2,3-diami- nopropionate (L-2,3-Dap), hence the inability of MBLs to use its serine hydrolase activity to cleave its β-lactam ring (Li *et al*., 2017).

MATERIALS AND METHODS

MATERIALS

Bacterial isolates were from soil samples collected in Orangeburg, SC. Mueller Hinton agar, Luria Bertani agar, pKnock plasmid, and *Escherichia coli* strain BW29427 was purchased from the Coli Genetic Stock Center, Yale University (New Haven, CT). pKNOCK-Km was purchased from Addgene (Watertown, MA). Luria Bertani (LB) broth and agar, kanamycin, agarose, diaminopimelic acid (DAP), Rapid DNA Ligation kit, and *Eco*RI were purchased from Fisher Scientific (Pittsburg, PA). The Gel/PCR DNA Fragments Extraction Kit were purchased from IBI Scientific (Peosta, IA). The Qiaprep Spin Miniprep Kit was purchased from Qiagen (Germantown, MD). The NEB One Taq Start 2X Hot Start master mix was purchased from New England Biolabs (Ipswich, MA). The QIAprep Spin Miniprep Kit was purchased from Qiagen (Germantown, MD). The oligonucleotide primers were purchased from Eurofins Genomics (Louisville, KY) (Table 1). 16S rRNA gene was sequenced by Eurofins Genomics (Louisville, KY) and the genomic DNA was sequenced by Microbial Genome Sequencing Center (Pittsburg, PA).

METHODS

Bacterial Culture

Nine unidentified environmental bacterial isolates labeled NKC1, NKC23, ML-15, ML-1-14, G1B-16, ML-16, G4B-5, G3B-7 and ML-3-12 were streaked for isolation from the freezer stocks onto the Mueller Hinton agar plates and the plates were incubated at 30°C overnight for optimal growth.

Table 1. Primers for Amplification of the 16S rRNA Gene and BGC Core Genes

Gene	Seq Name	Seq 5' to 3'
16S rRNA	16S rRNA FBAC	AGAGTTTGATCCTGGCTCAG
	16S rRNA RBAC	ACGGCTACCTTGTTACGACTT
Bicyclomycin	Bic_F	ACGTGAATTCGTGAGCGGTATAAAGCGAAGA
	Bic_R	ACGTGAATTCCCTGAGTGCTCCAGGAAATAG
Pyoluteorin	Pylt_F	ACGTGAATTCCATTCTGGAACCTCCTTCGT
	Pylt_R	ACGTGAATTCTGCACGCCAAGGTGATAG
Endophenazine	Endo_F	ACGTGAATTCGTTTACCGACAACCTGGAATTG
	Endo_R	ACGTGAATTCAATAGCCCTGCGGATACC
Pyoverdine	Pyv_F	ACGTGAATTCCGCCGAACAACTTTCAGACTA
	Pyv_R	ACGTGAATTCGAACGCGAGGAACGACTG

Co-culture Assay

Colonies of the bacterial query isolates suspended in the 750 µl distilled water. The bacterial isolates were spread over the surface of a Mueller Hinton agar plate and colonies of *Micrococcus luteus, Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter aerogenes, Salmonella enteritidis, Streptococcus agalactiae,* and *Serratia marcescens* were toothpicked onto the agar. The co-cultured plates were incubated at 30°C for 48 hours. The plates were examined to find which environmental isolates were producing an antibiotic against the test bacteria by producing a zone of inhibition.

Colony PCR of 16s rRNA gene

A thin-walled PCR tube was obtained and 50 µl of sterile water was added. A small portion of the colony was picked up with a sterile toothpick. The toothpick was placed into the sterile water and the bacteria were recovered by spinning the toothpick in the water. The tube was capped and heated in the thermal cycler at 99°C for 10 min to lyse the bacteria. The fluid was removed and transferred to a 1.5 ml microcentrifuge tube. The tubes were centrifuged at 10,000 rpm for 3 min to pellet the cell debris. Ten microliters of fluid was removed from the tube and transferred into a new 1.5 ml microcentrifuge tube.

16S rRNA gene PCR was set up using the following on each tube PCR Master Mix: 1 µl crude DNA prep, 1 µl 100 µM 16S rRNA FBAC primer, 1 µl 100 µM 16S rRNA RBAC primer, 25 µl 2X Master Mix, and 22 µl H_2O for a total volume of 50 µl. The thermal cycler was set up with the following program: 35 cycles of 94°C for 2 min, 94°C for 20 sec, 55°C for 30 sec, 68°C for 1.5 min followed by 68°C for 5 min. Ten microliters of the PCR sample were removed

and placed into a new 1.5 mL microcentrifuge tube, and the remaining PCR sample was placed into the freezer. Two microliters of the loading buffer were added to the microcentrifuge tube and gently mixed. A 1% agarose gel was prepared in 1X TAE. Each PCR sample was loaded into a well. Ten microliters of DNA ladder were loaded into a separate well on the gel. The samples were electrophoresed at 170V for 45 minutes until the bromophenol blue dye had run about ~2/3 of the length of the gel. A picture of the gel was captured using a Bio-Rad ChemiDoc XRS imager. For each bacterial isolate, the 16s rRNA gene were purified using the Gel/PCR DNA Fragments Extraction Kit according to the manufacturer's instructions and sequenced by Eurofins Genomics LLC.

Genomic DNA isolation

Three milliliters of each bacterial isolate were cultured in LB broth in a 15 ml conical tube at 25°C overnight. The tubes were centrifuged at 3500 rpm for 10 min at 4 °C to pellet the bacteria, the supernatant was discarded, and the bacterial pellet stored at -20°C for 48 hours. The frozen pellet was thawed at room temperature, resuspended in 500 µl lysis buffer (0.1 M Tris-Cl [pH 8], 50 mM EDTA, 1% sodium dodecyl sulfate) and transfer to a 1.5 ml screw-cap microcentrifuge tube. Glass beads were added to cover the bottom of the tube. The tube was vortexed vigorously for 30 sec and 25 µl of 4.5 M NaCl was added to each tube and vortexed again for 30 sec. Three hundred and fifty microliters of phenol/chloroform were added to each tube. The tube was mixed by vortexing for 15 sec and centrifuged at 10,000 rpm for 5 minutes. Using a P1000 set at 300 µl, the tube was tilted at 45° angle, the top (aqueous) layer was pipetted and placed into a new 1.5 ml screw-cap microcentrifuge tube. Three hundred microliters of chloroform was added to the

aqueous layer and mixed by vortexing for 15 sec followed by centrifuging the tubes at 10,000 rpm for 5 minutes and the top layer was transferred to a new 1.5 ml microcentrifuge tube.

One milliliter of cold 100% ethanol was gently layered on the sample and mixed gently by inversion several times until the ethanol was thoroughly mixed. The samples were placed on ice for 15 minutes, centrifuged at 10,000 rpm for 5 minutes and the supernatants were gently removed using a pipettor. One milliliter of 70% cold ethanol was to the DNA pellets. The tubes were inverted three or four times to wash the salt off the pellet and all sides of the tubes which were centrifuged at 10,000 rpm for 5 minutes. The supernatant was gently removed without dislodging the DNA pellets. The pellets were dried and gently resuspended in 100 µl TE (10 mM Tris pH 8, 1 mM EDTA), incubated at 37°C for 15 min and were briefly centrifuged and pipetted a few times to properly mix the samples. Absorbance readings were taken to determine the concentration of the samples using the NanoDrop spectrophotometer. The genomic DNA sent to Microbial Genome Sequencing Center for sequencing using the combination of the Nanopore and Illumina platforms.

Mutagenesis of the Core Genes in BGCs in Pseudomonas aeruginosa NKC1

Sample Preparation and Plasmid Isolation

E. coli carrying the pKNOCK-Km plasmid was streaked for isolation onto LB agar with 25 µg/ml kanamycin and incubated at 37 °C for 24 hours for optimum growth. A colony of the E. coli (pKNOCK-Km) was grown in 3 ml of the LB broth. The tube was incubated with shaking at 200 rpm for 24 hours. The 1 5ml conical tube was centrifuged at 5000 rpm for 5 minutes and supernatant poured off without disturbing the pellet. Plasmid DNA was isolated from the bacteria using the QIAprep Spin Miniprep Kit according to manufacturer's instructions.

Cloning of the Core Biosynthetic Genes

Genomic DNA from *P. aeruginosa* NKC1 was used in a PCR as described above except the extension time was 30 sec to amplify internal fragments of the core biosynthetic genes for bicyclomycin, pyoverdine, pyoluteorin, and endophenzine using primer pairs Bic_F/R, PyvF/R, Pylt_F/R, and Endo_F/R, respectively. Ten microliters of PCR fragments were analyzed by agarose gel electrophoresis as described above and the remaining sample was purified using the Gel/PCR DNA Fragments Extraction Kit. The DNA fragments and pKNOCK-Km were digested with the restriction endonuclease *Eco*RI as shown in Table 2. pKNOCK-Km was analyzed by agarose gel electrophoresis. The DNAs were purified and each internal fragment was ligated to pKNOCK-Km as indicated in table 3 and incubated for 30 minutes at room temperature and then 4ºC overnight.

Three LB/kanamycin/DAP agar plates were prepared for each ligation reaction, plus one plate for determining transformation efficiency. The tubes containing the ligation reactions were centrifuged to collect the contents at the bottom. Two microliters of each ligation reaction were added to a sterile 1.5 ml microcentrifuge tube on ice. Tubes of chemically competent *E. coli* BW29427 were removed from storage and placed in an ice bath until just thawed. The cells were mixed by flicking, and 50 µl of cells was carefully transferred into each 1.5 ml microcentrifuge tube and gently flicked to mix then placed on ice for 20 minutes. Nine hundred and fifty microliters of room temperature SOC medium was added to the tubes containing cells transformed with ligation reactions, and 900 µl to the tube containing cells transformed with uncut plasmid. The tubes were incubated for 1.5 hours at 37 ºC with shaking. One hundred microliters of each

Table 2. *Eco*RI Restriction Digest Reactions

Reaction Component (µl)	Endophenazine (E.P)	Pyoluteorin (P.Y)	Pyoverdin (P.V)	pKNOCK-Km
PCR product	35	35	35	20
10X Buffer	5	5	5	5
*Eco*RI	3	3	3	3
Sterile Water	7	7	7	22

Table 3. Ligation Reactions

Reaction Component (µl)	Endophenazine (E.P)	Pyoluteorin (P.Y)	Pyoverdin (P.V)	Self-Ligation
2X Rapid Ligation Buffer	4	4	4	4
pKNOCK-Km Vector	3.1	3.1	3.1	3.1
PCR product	2.1	1	1.1	-
T4 DNA Ligase	1	1	1	1
Nuclease-free water to a final volume of	9.8	10.9	10.8	11.9

transformation culture was plated onto duplicate the tube containing cells transformed with uncut plasmid. The tubes were incubated for 1.5 hours. The tubes were incubated for 1.5 hours at 37 °C with shaking. One hundred microliters of each transformation culture was plated onto duplicate LB/kanamycin /DAP agar plates. The plates were incubated overnight at 37 °C. Colony from the successfully transformed samples were sub-cultured onto LB/kanamycin/DAP plates and were incubated at 37 °C overnight. Colony from the sub-cultured plates were picked and resuspended in LB/kanamycin/DAP broth tubes, incubated at 37 °C with shaking at 200 rpm overnight.

The transformed *E. coli* colonies underwent plasmid purification, *Eco*RI digest, and agarose gel electrophoresis to confirm if the cloning was successful in each tube. Pictures of the gel were captured.

Conjugation of pKNOCK-Km carrying the BGC Core Genes into P. aeruginosa NKC1 and Screnning of Mutants

pKNOCK-Km carrying either endophenazine or pyoverdine was cultured in LB/kanamycin/DAP broth along with *P. aeruginosa* NKC1 on an LB broth without any supplements. The tubes were incubated at 37 °C with shaking at 200 rpm overnight. An LB/DAP agar plates were dried at 37 °C and to each microcentrifuge tube, 100 μl each of *E. coli* (pKNOCK plasmid) and 100 μl NKC1 were added and mixed gently by pipetting. The tubes were centrifuged at 6,000 rpm for 1 min to pellet the bacteria. With the aid of a micropipette, the supernatants were removed without disturbing the pellet. The pellets were resuspended in 50 μl of fresh LB broth and mixed gently to make a homogenous mixture. On the dried LB/DAP agar plates, the 50 μl was pipetted to the center of the plates and allowed to dry completely into the plates and incubated at 37 °C overnight.

LB/kanamycin plates were dried at 37 °C for 30 minutes. In different 1.5 ml microcentrifuge tubes, 300 μl of LB broth was pipetted and with aid of a wire loop, about 1/2 of the bacteria from the LB/DAP plates was added to each LB broth tube and gently mixed to get a homogenous mixture of bacteria. Seven hundred microliters of fresh LB broth was then added, and the samples were gently mixed. One hundred microliters of the bacterial suspension was placed onto the middle of each of the LB/kanamycin agar plates and the bacteria were spread over the surface of the agar with the spreader. The plates were incubate at 37 °C overnight. A portion of a *M. luteus* suspension in 500 μl of water was spread over Mueller Hinton agar plates. The exconjugates (NKC with EP and PY gene) were cocultured in grids with *M. luteus* along with the NKC isolate as a control. The plates were incubated for 2 days to test for loss of antibiotic activity.

RESULTS

Co-Culture of Bacterial Isolates with Bacterial Pathogens

The 9-query antibiotic-producing bacterial (APB) isolates had their antibiotic activity tested according to the procedures described above where the isolates were co-cultured with bacterial pathogens and screened for antibiotic production as indicated by a zone of inhibition. Figure 1 shows the result of the co-culture experiment of the APBs against *A. baumannii, E. aerogenes, E. faecalis, K. pneumoniae, M. luteus, P. aeruginosa, S. aureus, S. agalactiae, S. enteritidis,* and *S. marcescens.* All isolates except Ml-1-14 formed a zone of inhibition against *M. luteus*. Isolate Ml-15 produced a zone of inhibition against *S. aureus, S. agalactiae,* and *S. marcescens.* Isolate Ml-3-12 produced a zone of inhibition against *E. faecalis*, *S. aureus, S. agalactiae,* and *S. marcescens*. Isolate G1B-16 produced a zone of inhibition against *A. baumannii, P. aeruginosa,* and *E. aerogenes*. Isolates GB-7, G4B-5, and NKC23 produced a zone of inhibition against *S. aureus* and *K. pneumoniae*. The results are summarized in table 4.

16s rRNA Gene PCR and Analysis using Basic Local Alignment Search Tool (BLAST)

Figure 2 shows the electrophoretic gel for the six selected isolates in duplicates after amplification of their 16S rRNA gene. The gel shows that the 16S rRNA gene of the bacterial isolates was successfully amplified in the PCR as the expected band at ~1.5 kb was seen. Comparing the isolates sequences to the 16S rRNA gene database at the National Center for Biotechnology Information (NCBI) revealed that the main genera were *Bacillus* and *Pseudomonas*.

Figure 1. Identifying antibiotic activity of APBs against bacterial pathogens
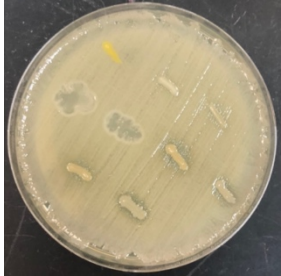
Acinetobacter baumannii

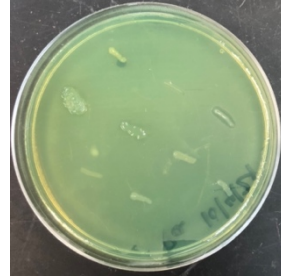*Enterobacter aerogenes*
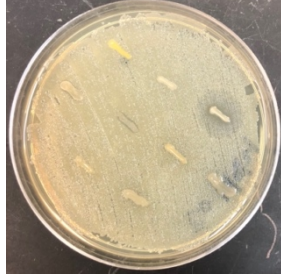
Enterococcus faecalis

Klebsiella pneumoniae

Micrococcus luteus

Pseudomonas aeruginosa

Staphylococcus aureus

Streptococcus agalactiae

Salmonella enteritidis

Serratia marcescens
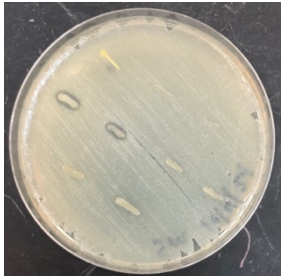
Table 4. Antibiotic activity test of the 9 environmental isolates against 10 pathogenic bacteria

APB	ML	EF	SA	KP	AB	PA	EA	SEN	SAG	SM
Ml-1-14	N	N	N	N	N	N	N	N	N	N
Ml-15	Y	N	Y	N	N	N	N	N	Y	Y
Ml-16	Y	N	N	N	N	N	N	N	N	N
Ml-3-12	Y	Y	Y	N	N	N	N	Y	Y	Y
G1B-16	Y	N	N	N	Y	Y	Y	N	N	N
G3B-7	Y	N	Y	Y	N	N	N	N	N	N
G4B-5	Y	N	Y	Y	N	N	N	N	N	N
NKC1	Y	Y	Y	Y	N	Y	N	N	N	N
NKC23	Y	N	Y	Y	N	N	N	N	N	N

Y indicates a ZOI, N indicates no ZOI. ML-*M. luteus*, EF-*E. faecalis*, SA-*S. aureus*, KP-*K. pneumoniae*. AB-*A. baumannii*, PA-*P. aeruginosa*, EA-*E. aerogenes*, SEN-*S. enteritidis*, SAG-*S. agalactiae*, SM-*S. marcescens*.

Figure 2. AGE PCR of 16S rRNA gene
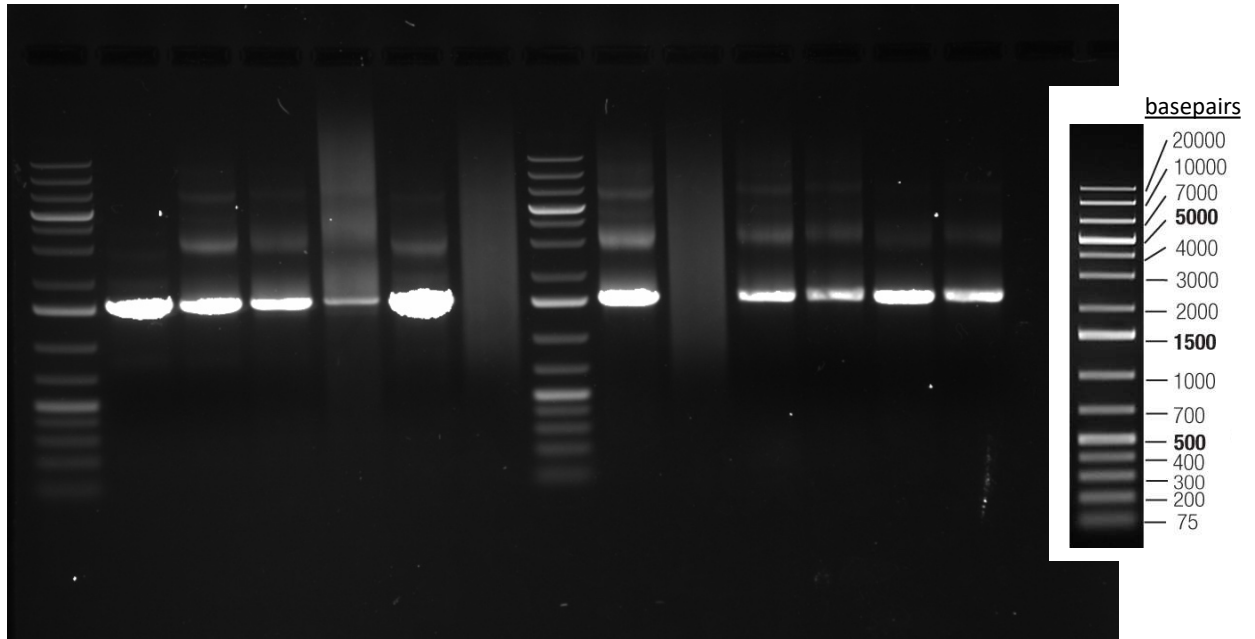
Genomic DNA Sequencing Analysis

The selected isolate NKC1 was identified earlier by 16S rRNA gene BLAST to be *P. aeruginosa.* The selected isolate was further identified by genomic DNA BLAST through the R-console and it gave a 99.5% match to *P. aeruginosa* as shown in table 5.

The sequenced genome of the *P. aeruginosa* was run through the bioinformatics tool antiSMASH to annotate the BGCs present in the genome (Blin *et al*. 2021). Figure 3 shows the overview of the biosynthetic gene clusters present in the *P. aeruginosa* chromosome. The thin line underneath represents the genome and the relative positions of the BGCs identified. The first column on the figure lists the regions, the second column lists the class of the BGC while the third column gives the position of the BGC on the chromosome. The fourth column in the figure shows the similarity of the BGC region based on a known cluster from the MIBiG database while the last column reveals the percentage similarity of the query BGC to the known BGC.

Mutagenesis of BGC Genes

Four BGCs were selected that could be responsible for the antibiotic activity of the *P. aeruginosa* and they included the bicyclomycin, pyoluteorin, pyoverdine and the endophenazine A and B. The core genes for each antibiotic BGC were obtained from the antiSMASH platform and with the aid of another the primer design tool at Integrated DNA Technologies (IDT), the primers to amplify an internal fragment of the core gene of each four BGC was designed. The core genes were amplified by PCR, and the PCR products were analyzed through gel electrophoresis. Figure 4 shows the result of the amplified four core genes in duplicate for the bicyclomycin core gene, the pyoluteorin core gene, the pyoverdine core gene, and the endophenazine core gene. The PCR was successful for pyoluteorin, pyoverdine, and endophenazine.

Table 5. BLAST of *Pseudomonas* Genome

```
All done!
> blast_test
# A tibble: 16 × 21
  query_id    subject_id perc_identity num_ident_match… alig_length mismatches
   <chr>       <chr>         <dbl>          <int>          <int>      <int>
 1 NZ_CP009365… NC_002516…    99.5          9821           9871        40
 2 NZ_CP009365… NC_002516…    99.5          9565           9609        41
 3 NZ_CP009365… NC_002516…    70.2           245            349        93
 4 NZ_CP009365… NC_002516…    65.3           584            894       244
 5 NZ_CP009365… NC_002516…    70.6           127            180        45
 6 NZ_CP009365… NC_002516…    70.6           127            180        45
 7 NZ_CP009365… NC_002516…    97.0            32             33         1
 8 NZ_CP009365… NC_002516…    82.5            47             57         6
 9 NZ_CP009365… NC_002516…    74.2            72             97        16
10 NZ_CP009365… NC_002516…    76.4            55             72        14
11 NZ_CP009365… NC_002516…    78.3            47             60        13
12 NZ_CP009365… NC_002516…    81.6            40             49         9
13 NZ_CP009365… NC_002516…    81.6            40             49         9
14 NZ_CP009365… NC_002516…    74.6            53             71        18
15 NZ_CP009365… NC_002516…    82.6            38             46         8
16 NZ_CP009365… NC_002516…    67.8           101            149        44
# … with 15 more variables: gap_openings <int>, n_gaps <int>, pos_match <int>,
#   ppos <dbl>, q_start <int>, q_end <int>, q_len <int>, qcov <dbl>,
#   qcovhsp <dbl>, s_start <int>, s_end <int>, s_len <int>, evalue <dbl>,
#   bit_score <dbl>, score_raw <dbl>
```

Figure 3. antiSMASH results for the BGCs of *Pseudomonas aeruginosa* NKC1

Select genomic region:

Overview | 1.1 | 1.2 | 1.3 | 1.4 | 1.5 | 1.6 | 1.7 | 1.8 | 1.9 | 1.10 | 1.11 | 1.12 | 1.13 | 1.14 | 1.15 | 1.16

Identified secondary metabolite regions using strictness 'relaxed'

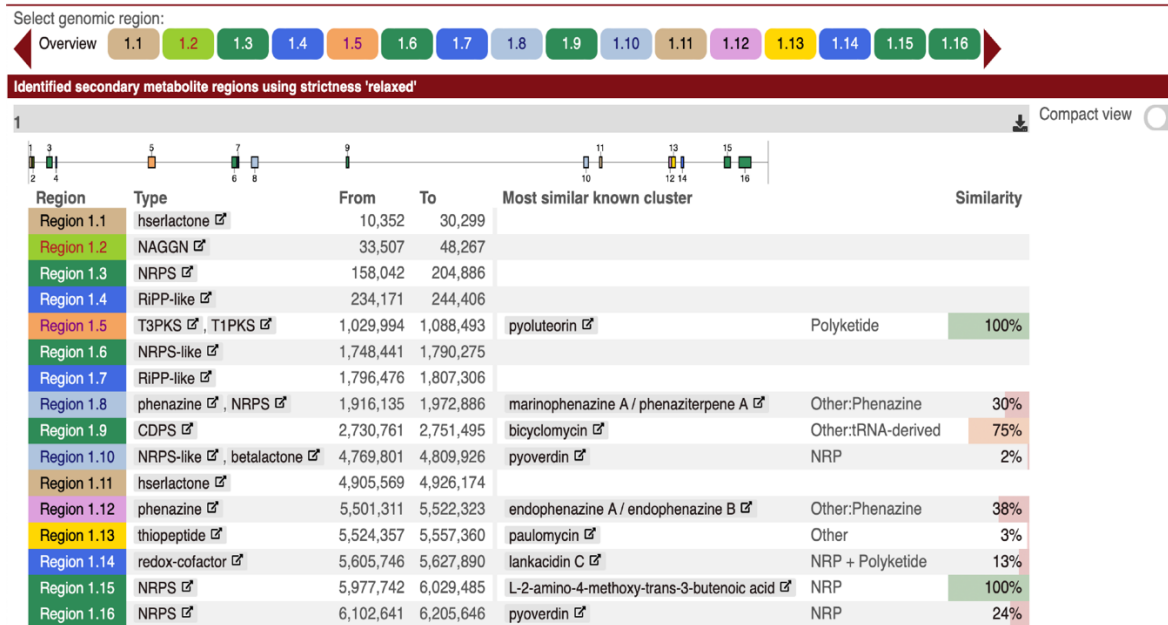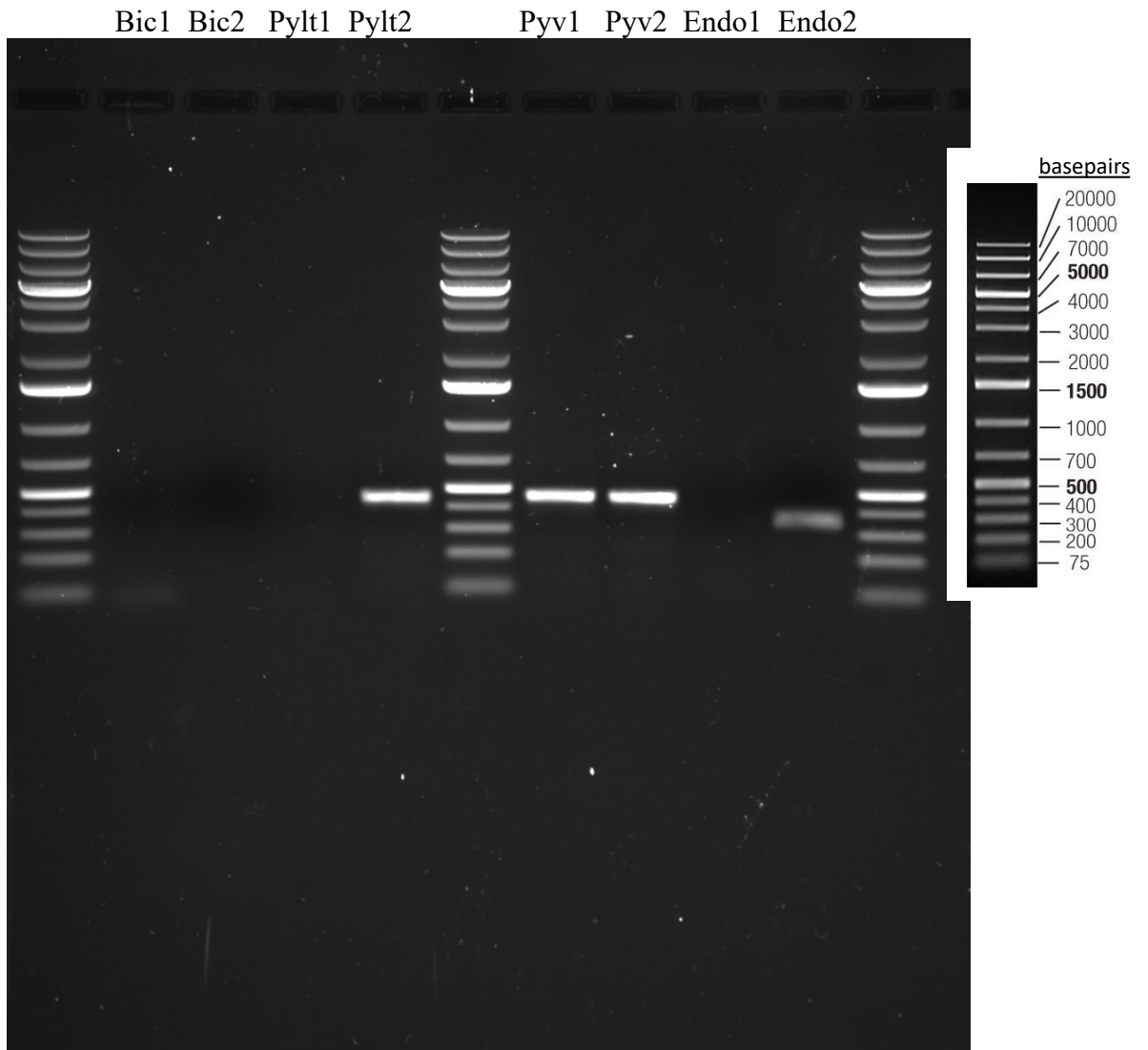| Region	Type	From	To	Most similar known cluster		Similarity
Region 1.1	hserlactone ☑	10,352	30,299			
Region 1.2	NAGGN ☑	33,507	48,267			
Region 1.3	NRPS ☑	158,042	204,886			
Region 1.4	RiPP-like ☑	234,171	244,406			
Region 1.5	T3PKS ☑ , T1PKS ☑	1,029,994	1,088,493	pyoluteorin ☑	Polyketide	100%
Region 1.6	NRPS-like ☑	1,748,441	1,790,275			
Region 1.7	RiPP-like ☑	1,796,476	1,807,306			
Region 1.8	phenazine ☑ , NRPS ☑	1,916,135	1,972,886	marinophenazine A / phenaziterpene A ☑	Other:Phenazine	30%
Region 1.9	CDPS ☑	2,730,761	2,751,495	bicyclomycin ☑	Other:tRNA-derived	75%
Region 1.10	NRPS-like ☑ , betalactone ☑	4,769,801	4,809,926	pyoverdin ☑	NRP	2%
Region 1.11	hserlactone ☑	4,905,569	4,926,174			
Region 1.12	phenazine ☑	5,501,311	5,522,323	endophenazine A / endophenazine B ☑	Other:Phenazine	38%
Region 1.13	thiopeptide ☑	5,524,357	5,557,360	paulomycin ☑	Other	3%
Region 1.14	redox-cofactor ☑	5,605,746	5,627,890	lankacidin C ☑	NRP + Polyketide	13%
Region 1.15	NRPS ☑	5,977,742	6,029,485	L-2-amino-4-methoxy-trans-3-butenoic acid ☑	NRP	100%
Region 1.16	NRPS ☑	6,102,641	6,205,646	pyoverdin ☑	NRP	24%

Figure 4. Agarose gel electrophoresis of the BGC PCR sample

The *Ec*oRI digested PCR fragments were purified, digested with *Eco*RI, and purified again. *Eco*RI digested pKNOCK-Km (Figure 5) was ligated to the fragments and transformed into *E. coli* BW29427. Plasmid DNA was purified from the bacteria, digested with *Eco*RI, and analyzed by agarose gel electrophoresis. Figure 6 shows that the cloning was successful as indicated in columns two and four as the presence of the pyoverdine and endophenazine gene fragments, respectively, are present.

E. coli BW29427 with pKNOCK-Km/Pyv or with pKNOCK-Km/Endo was mixed with the *P. aeruginosa* and plated on LB/DAP plates to aid the growth of the *E. coli* cells and with no kanamycin antibiotic to allow the growth of *P. aeruginosa* in order to conjugate the plasmid from *E. coli* to *P. aeruginosa* (Figure 7).

Figure 8 shows the co-culture experiment of the mutant *P. aeruginosa* in the pyoluteorin core gene or the endophenazine core gene with *M. luteus* on Mueller Hinton agar plates, testing for loss of antibiotic activity by not producing a zone of inhibition. The figure clearly shows zones of inhibition produced by the mutant *P. aeruginosa* across the grids on the MH agar plate meaning the pyoluteorin gene or the endophenazine gene is not responsible for the antibiotic activity of *P. aeruginosa.*

Figure 5. Agarose gel electrophoresis of the *Eco*RI digested pKNOCK-Km and undigested
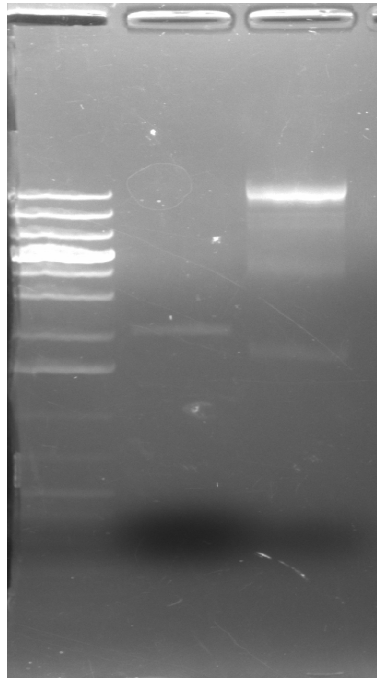
pKNOCK-Km plasmid

Figure 6. Agarose gel electrophoresis *Eco*RI digested pKNOCK-Km(Py) plasmids and
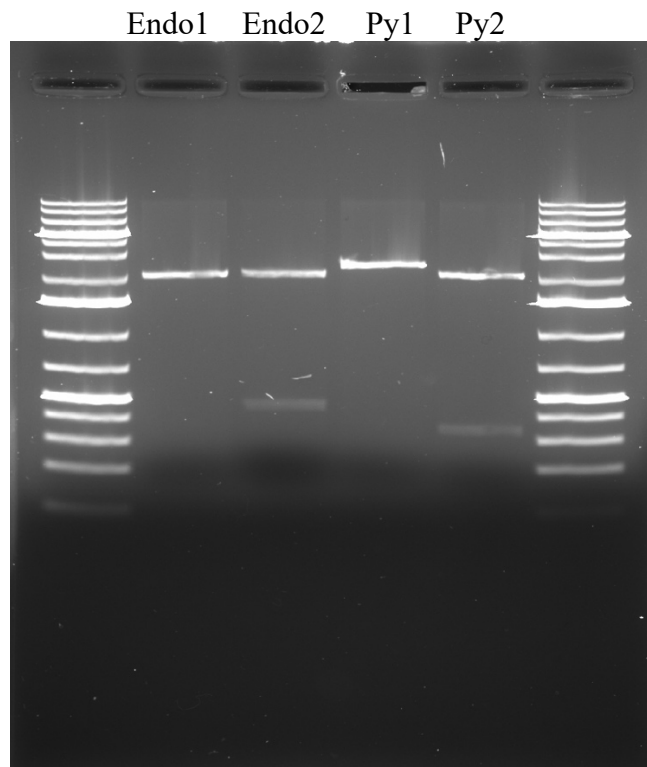
pKNOCK-Km(Endo) plasmids
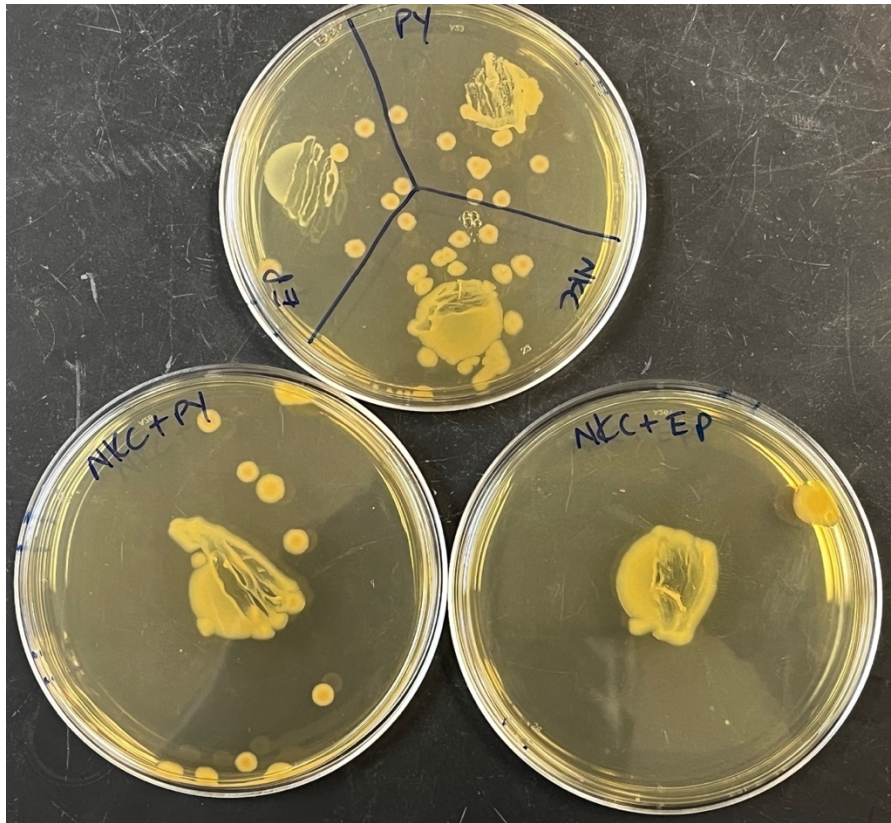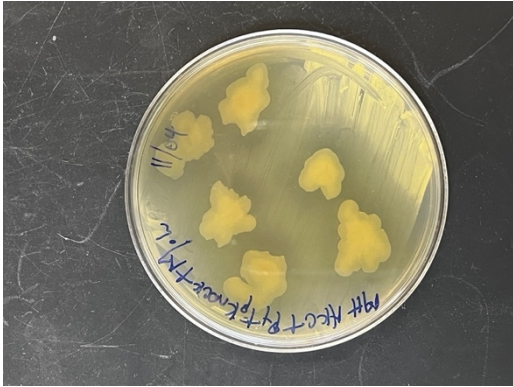
Figure 7. Conjugation Assay

Figure 8. Coculture of *P. aeruginosa* mutants with *M. luteus*

DISCUSSION

The purpose of the co-culture experiment was to identify from 9 environmental isolates which ones were producing a ZOI against the test bacteria *M. luteus, E. faecalis, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. aerogenes, S. enteritidis, S. agalactiae,* and *S. marcescens*. From those nine environmental isolates, it was found that six of them were producing a ZOI against at least four of the test bacteria.

The purpose of the PCR and gel electrophoresis was to amplify and confirm the the 16S rRNA gene PCR product which was sent out for sequencing. The reason for the 16S rRNA gene sequence analysis was to identify the genus and species of the six environmental isolates. Once the data was received from the sequencing laboratory, NCBI-BLAST was used to correctly identify the genus/species of all six bacteria. We carried out genomic DNA isolation and sequencing with the aim of further identification and genome mining. Once the genome sequence was received from the sequencing laboratory, identification was further confirmed by genomic DNA BLAST through the R-console. With the aid of the antiSMASH, we were able to identify the BGCs in *P. aeruginosa*. Using the IDT primer quest tool, we were able to prepare the primers to amplify the core genes BGCs potentially responsible for the antibiotic activity of *P. aeruginosa.*

The purpose the digesting the pKNOCK-Km plasmid and core genes with the same restriction enzyme, *Eco*RI, was to allow easy ligation of both sticky end overhangs during the ligation reaction causing the plasmid to carry our gene of interest (the core genes) to become a recombinant DNA molecule. The *E. coli* with recombinant pKNOCK plasmid was plated on LB/kanamycin/DAP agar plates serving as supplemented selective media with the DAP acting as a peptidoglycan supplement for the *E. coli* and the antibiotic kanamycin as the selective component of the media.

Figure 9 shows the general schematic of the insertional mutagenesis achieved using the pKNOCK-Km vector. During conjugation, *P. aeruginosa* takes up the plasmid from *E. coli* with the core gene fragment and inserts the plasmid into its chromosome at the core gene. The exconjugates are plated on LB/kanamycin plates without DAP supplementation to halt the growth of the *E. coli*, promoting the growth of the now kanamycin-resistant *P. aeruginosa.*

The purpose of the co-culture experiment of the mutant *P. aeruginosa* (with the inserted pKNOCK plasmid) with the test bacterium *M. luteus* was to test for loss of antibiotic activity of the mutant *P. aeruginosa*. Both *P. aeruginosa* NKC1 and the pyoluteorin or the endophenazine mutant produced zones of inhibition against *M. luteus* showing that neither core gene is responsible for the antibiotic activity of *P. aeruginosa.*

Future work includes troubleshooting for the bicyclomycin and pyoverdine genes to identify the BGC responsible for the zone of inhibition and extracting the antibiotic that is being produced by antibiotics produced by *P. aeruginosa*. Once the antibiotic is extracted, work will be done to classify the antibiotic by means of dereplication.

Figure 9 General scheme of insertional mutagenesis using pKNOCK-Km vector
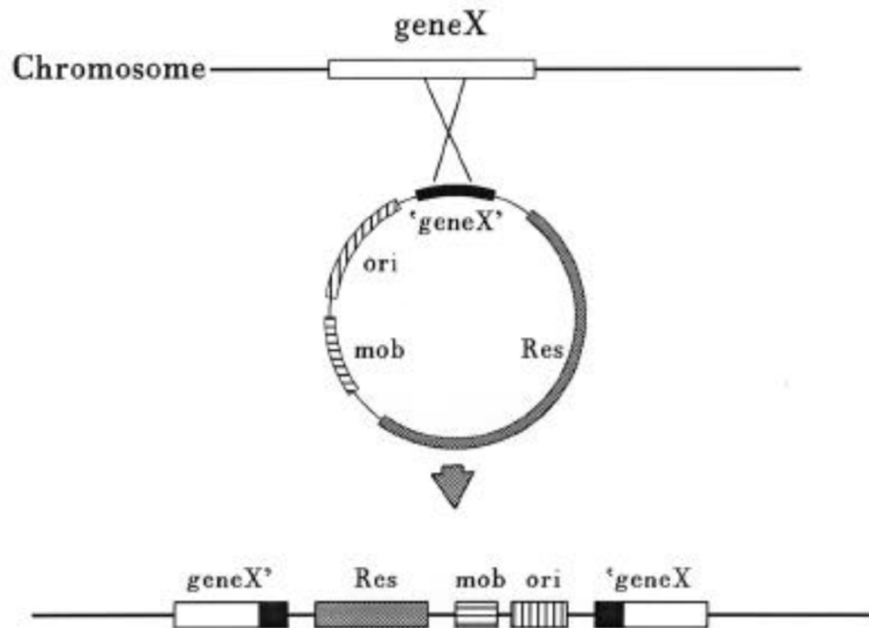
ori: R6K plasmid γ-origin of replication; mob: RP4 plasmid oriT region; Res: kanamycin

resistance gene; gene X: internal fragment for the BGC core genes.

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