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A Potential Klebsiella Bacteriocin with Efficacy Toward the Enterbacteriaceae Family

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A Potential *Klebsiella* Bacteriocin with Efficacy Toward the Enterobacteriaceae Family

by

Kasey Barber

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Abstract

Drug resistance is unfortunately becoming a prevalent issue in the course of patient treatment, ranging from chemotherapy resistance to antimicrobial resistance. The Centers for Disease Control and Prevention (CDC) estimated in 2016 that at least 23,000 people die every year in the United States from an infection with an antibiotic-resistant organism (Munita, et al, 2016). Carl Friedlander was the first scientist to describe *Klebsiella pneumoniae* in 1882 as an encapsulated bacillus after isolating the bacterium from the lungs of patients who had died from pneumonia (Ashurst and Dawson, 2022). *Klebsiella pneumoniae* is the type species for the *Klebsiella* genus and is the bacterium of interest for this project. It is one of the very few Gram-negative bacilli that can cause primary pneumonia, commonly affecting patients with compromised immune systems, alcohol use disorder, or diabetes mellitus (Ristuccia and Burke, 1984). However, microbes are able to produce a wide range of microbial defense systems including classic antibiotics, metabolic byproducts, and lytic agents. Bacteriocins are some of the most common defense mechanisms produced, which are different from antibiotics in that they have a narrow killing spectrum and are toxic only to bacteria that is closely related to the strain that is producing it. It has been estimated that 99% of all bacteria possibly make a minimum of one bacteriocin (Riley and Wertz, 2002). Because of the rapidly growing number of infections that are caused by antibiotic-resistant bacteria along with the harm that broad-spectrum antibiotics can cause to the human microbiome, these bacteriocins are being studied as potential alternatives to tradition antibiotics. In this study, we will assess and characterize a *Klebsiella* bacteriocin that may work synergistically with antibiotics so that antibiotic dosage might be reduced. In this study, we have isolated the plasmids from a possible *Klebsiella* bacteriocin and transformed them into *E. coli* to characterize the plasmid. This potential bacteriocin demonstrates efficacy towards *Citrobacter*, *Enterobacter*, and *Klebsiella* species and could offer an alternative treatment option for the highly drug resistant *Enterobacteriaceae* family.

Acknowledgements

First and foremost, I would like to thank Dr. Sean Fox from the Health Sciences department for his help, guidance, and expertise, as well as for allowing me to jump headfirst into one of his projects and contribute. I so enjoyed my time spent working with him, as well as learning techniques from him. Additionally, I would like to thank Dr. Erik Petersen from the Health Sciences department and Dr. Victoria Palau from the ETSU Bill Gatton College of Pharmacy department of Pharmaceutical Sciences for agreeing to be on my committee and for their efforts and input in helping me to make my thesis the best it could be. Finally, I would like to thank the Honors College at East Tennessee State University for the opportunity to be able to contribute to relevant research in my field of interest. Writing this thesis and gaining lab experience has been a fun and hands-on way to learn more about antimicrobial development and resistance, and I am immensely grateful to have had the opportunity.

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Introduction

Drug and Antibiotic Resistance

Drug resistance is unfortunately becoming an extremely prevalent issue in patient treatments, ranging from chemotherapy resistance to antimicrobial resistance. Specifically, antimicrobial resistance to bacterial pathogens has been associated with high morbidity as well as high mortality (Frieri, et al, 2017). The Centers for Disease Control and Prevention (CDC) estimated in 2016 that, at the very least, 23,000 people die every year in the United States from an infection with an antibiotic-resistant organism. According to another report, antibiotic resistance is estimated to cause roughly 300 million premature deaths before 2050 (Munita, et al, 2016). This issue is worsened by the fact that there are multiple drug resistance patterns in both Gram-positive and Gram-negative bacteria, which are extremely difficult to treat and may even become untreatable with our current antibiotics (Frieri, et. al, 2017). In addition to the fact that our standard antibiotics are becoming less and less effective, there are also very few alternative therapies that can be used to treat bacterial infections, few successful methods of prevention, and very few new antibiotics. These factors require that new and innovative treatment options become available to treat these bacterial infections, as well as better and more effective prevention methods. All these factors combined leave clinical practitioners virtually no options to treat these infections (Frieri, et al, 2017). Not only are these MDR (multi-drug resistant) organisms present inside of a hospital setting, but evidence suggests that there are also antibiotic-resistant bacteria in community settings as well. These MDR organisms are causing what health professionals are deeming a major public health threat, which will affect humans worldwide (Munita, et al, 2016).

The response of the bacteria to the strike of the antibiotic is a great example of adaptation and "survival of the fittest". This is a consequence of a large genetic plasticity of bacterial pathogens that trigger certain responses that can result in mutational adaptations, acquirement of new genetic material, or a change in gene expression; these genetic changes can produce resistance to nearly all of the antibiotics that are currently clinically available (Munita, et al, 2016). In order to combat this problem, it is first necessary to have the background knowledge in the biochemistry and genetics that is used in the accumulation and procurement of resistance. Furthermore, it is also necessary to create plans that antagonize MDR organisms in order to therapeutically treat patients (Munita, et al, 2016).

Polymicrobial Interactions

Microorganisms coexist in an intricate environment consisting of bacteria, fungi, archaea, and viruses that are either on or within the human body, often as intricate polymicrobial biofilm communities at mucosal sites and on abiotic surfaces (Peters, et al, 2012). Very little is known about interactions that occur between microorganisms during polymicrobial growth and their roles in human disease. Microbes very rarely, if ever, exist as single-species, free-living forms; thus, they are most often found thriving in these polymicrobial biofilm communities that are attached to both biotic and abiotic sites (Peters, et al, 2012). The human gastrointestinal tract and oral cavity include a large amount of microbial diversity, which is where an estimated 600 to 1,000 unique bacterial species have been identified as living on these human mucosal sites either as permanently or transiently (Peters, et al, 2012). Because of this large margin of variety and concentration of microbes in these sites, coupled with the limited space that is available, there are a multitude of species-specific physical and chemical interactions that have developed over

time. Many microbes have developed mutualistic as well as synergistic relationships to maintain cohabitation on epithelial surfaces. Furthermore, others have developed competitive and antagonistic approaches during the process of cohabitation (Peters, et al, 2012).

Earliest microscopes revealed that there is colonization of multispecies communities on human tissues; however, there is still not a solid understanding of how these multispecies interactions administrate the progression, range, and severity of human disease. Furthermore, even less is known about how the human host responds to polymicrobial infection as opposed to monomicrobial infection. Previously, it was believed that a single virulence factor facilitated the disease caused by a single organism (Peters, et al, 2012). Although this is true for some human infections, immunization against these single virulence factors of other organisms such as *Staphylococcus aureus* has shown to be far more difficult. Put more simply, just as some virulence cannot be associated with a single virulence factor for many organisms, some diseases can also not be defined as infection by a single species (Peters, et al, 2012).

In addition, biofilms are microbial communities that are embedded in an extracellular matrix which form a highly organized structure that usually causes many human infections. For example, dental caries (more commonly known as tooth decay) is a polymicrobial biofilm disease which is caused by the diet and microbiota-matrix interactions occurring on solid surfaces. Sugars initiate the development of pathogens, aid in assembling the matrix, and acidify the biofilm environment. These steps encourage ecological changes and efforts by multiple species, which further lead to acid damage of tooth tissue that has been mineralized (Bowen, et al, 2018).

Klebsiella

Carl Friedlander was the first to describe *Klebsiella pneumoniae* in 1882 as an encapsulated bacillus upon isolating the bacterium from the lungs of patients who had died from pneumonia (Ashurst and Dawson, 2022). In 1886, the bacterium was named *Klebsiella*. *K. pneumoniae* is a Gram-negative and non-motile bacterium that is found in the environment. The *Klebsiella* genus is found in human intestines, and it the second most populous genus found there (Ristuccia and Burke, 1984). The genus contains four separate species that are recognized by the Centers for Disease Control (CDC): *K. pneumoniae* (which is the type species)*, K. ozaenae, K. rhinoscleromatis, and K. oxytoca* (Ristuccia and Burke, 1984).

Klebsiella pneumoniae, which is the type species for the Klebsiella genus as well as the bacterium of interest for this particular project, is one of the very few gram-negative bacilli that can cause primary (caused by the virus itself) pneumonia (Ristuccia and Burke, 1984). It is most often an opportunistic pathogen, which usually affects those with weakened immune systems, having the tendency to further cause nosocomial infections. It commonly affects patients with alcohol use disorder or diabetes mellitus (Ashurst and Dawson, 2022). Another classification (or subset) of the hypervirulent *K. pneumoniae* strains have elevated capsule polysaccharide production, which can unfortunately cause previously healthy persons to obtain life-threatening community-acquired infections. These infections can result in meningitis, necrotizing fasciitis, endophthalmitis (infection of the tissues or fluids inside of the eyeball), pyogenic (pus-producing) liver abscesses, and severe pneumonia (Lei, et al, 2014). *Klebsiella pneumoniae* has a variety of virulence factors in its arsenal, most commonly capsule polysaccharide, lipopolysaccharide, and fimbriae. *K. pneumoniae* can also utilize outer membrane proteins and determinants to gain iron and utilize nitrogen sources (Lei, et al, 2014).

Bacteriocins

Microbes are able to produce a wide range of microbial defense systems, including classic antibiotics, metabolic by-products, lytic agents, and more. Among some of the most common of these defense mechanisms are bacteriocins, which differ from antibiotics in that they have a narrow killing spectrum and are only toxic to bacteria that is closely related to the strain that is producing it (Riley and Wertz, 2002). Bacteriocins are defined as "ribosomally synthesized antimicrobial peptides" (Chikindas, et al, 2018). Practical applications of these antimicrobial peptides are most commonly in food preservatives, although they are also being used in new research, such as this project, as a possible component of a new group of antibiotics. Furthermore, bacteriocins coupled with new genetic and nanotechnology developments can prove to be beneficial in not only antibiotic applications, but also in new delivery systems and cancer treatments. Additionally, bacteriocins have also been found to regulate quorum sensing, which could open up a very wide range of new applications (Chikindas, et al, 2018).

Bacteriocins have been identified in all major bacterial lineages and have recently been described as universally produced by some members of archaea. It has been estimated that 99% of all bacteria possibly make a minimum of one bacteriocin. The reason that many of them are unknown and have not been isolated is due to lack of research in that particular area (Riley and Wertz, 2002). Related to *K. pneumoniae*, our bacterium of interest, bacteriocins isolated from gram-negative bacteria seem to be created through recombination of bacteriocins that are already in existence.

Because of the rapidly growing number of infections that are caused by antibiotic-resistant bacteria, as well as the degree of harm that broad-spectrum antibiotics can cause to the human microbiome, these bacteriocins are being studied as potential alternatives to traditional

antibiotics. Because both broad- and narrow-spectrum bacteriocins exist and they have low toxicity and are extremely potent against other bacteria (which also include antibiotic-resistant strains), they have the potential to be incredibly beneficial to this area of medicine (Cotter, et al, 2013). They can also be classified based on how they target the infection: by either targeting the cell membrane, or functioning within in the cell by targeting DNA, RNA, and/or protein metabolism (Cotter, et al, 2013). Interestingly, bacteriocins can even be produced in the human gut by probiotic bacteria to counteract intestinal infections (Cotter, et al, 2013). The effects of bacteriocins on pathogens could also further be modified to be made more effective by coupling individual bacteriocins with antimicrobials or membrane-active substances (Cotter, et al, 2013). This thesis aims to identify the inhibitory mechanism at work here in order to hopefully lessen antibiotic resistance in the future.

Methods

Strains, Media, & Growth Conditions

Klebsiella pneumoniae (#13883), Klebsiella pneumoniae (#9997), Klebsiella pneumoniae (clinical UTI strain), Enterobacter cloacae (#23355), Enterobacter aerogenes (#13048), Citrobacter freundii (#8090), Citrobacter koseri (#25408) and *Escherichia coli* strains were sub-cultured from freezer stocks on lysogeny broth (LB) agar and grown overnight at 37ºC. From isolated colonies, fresh overnight LB liquid bacterial cultures (37ºC, shaking 155rpm, 24 hours) were used for all experiments. When required, kanamycin (50ug/ml) or Streptomycin (100ug/ul) was added to LB broth or agar plates for bacterial selection.

Transposon Insertion for Mutant Development

Plasmid DNA was isolated from *Klebsiella pneumoniae #13883* using a Promega Wizard plasmid isolation kit and the Lucigen EZ-Tn5 <R6K*γori*/KAN-2> transposome was used to produce the EZ-Tn5 transposon insertion plasmid library needed for cloning into *E. coli* to create random transposon insertion strains of the plasmid mediated *Klebsiella pneumoniae* antimicrobial protein. This is done through a 2-hour *in vitro* reaction which will randomly insert the transposon into the target DNA and electroporating into competent *E. coli* cells. Utilizing the transposon's kanamycin resistance marker, only *E. coli* clones that contain the *Klebsiella* plasmid DNA containing the transposon will grow during the reaction. The clones were plated on a kanamycin plate to determine which of the clones possessed the kanamycin-resistant transposon. Clones were then screened on a lawn of *C. freundii* to observe zones of inhibition, the plasmid DNA extracted from the clones of interest, and bidirectionally sequenced from primer binding sites within the transposon. Sanger sequencing was performed by the James H. Quillen College of Medicine's Molecular Core facility. The sequences were then analyzed by the National Center for Biotechnological Information (NCBI) genomic (BLAST) and protein (blastx) database and compared to other known *Klebsiella* sequences.

Agar Spot Test

To observe zones of inhibition of the wild-type *K. pneumoniae* (KP_{wt}) and mutant strains ($KP\Delta_{P1\text{-}A3}$ and $KP\Delta_{P1\text{-}A7}$), an agar spot test was performed. Overnight cultures of KP_{wt}, $KP\Delta_{P1\text{-}A3}$, $KP\Delta_{P1\text{-}A7}$, *C. freundii* (CF), and *E. cloacae* (ECL) were each grown in 5mL of LB broth for ~48 hours. EA, CF, ECL, or KP #9997 were spread on separate LB plates to create bacterial lawns. 25µl of each of KP_{wt}, $KP\Delta_{P1\text{-}A3}$, and $KP\Delta_{P1\text{-}A7}$ were spotted onto the bacterial lawns. These LB

plates were incubated overnight at 37ºC and observed for the presence or absence of zones-of-inhibition.

Co-cultures test for antimicrobial production

The following procedure was performed to quantify and compare the inhibitory effect of KP_{wt} with that of transposon insertion mutants $KP\Delta_{P1\text{-}A3}$ and $KP\Delta_{P1\text{-}A7}$. Overnight cultures of the KP strains and CF were created as previously described. The antimicrobial proteins from each KP strain were isolated by centrifugation (3 minutes at 14,000rpm), and the resulting supernatant of each was passed through a 0.22uM filter syringe in order to remove the cellular components and harvest the antimicrobial proteins. This enabled the creation of four test conditions outlined in Table 1. These control and experimental tubes were placed at 37ºC with shaking for ~48 hours. The tubes were serially diluted, spread on LB agar plates to determine the growth of the wild-type and mutants in comparison to the control mixture. Colony-forming units (CFUs) were counted in order to quantify the growth of the CF and ECl with each of the mutants and KP wild-type.

	Amount of CF	Fresh LB	Supernatant LB
Tube 1: Control	25ul	5ml	0ml
Tube 2: Control	25ul	2.5ml	2.5ml KP_{wt}
Tube 3: Experimental	25ul	2.5ml	2.5ml $KP\Delta_{P1\text{-}A3}$
Tube 4: Experimental	25ul	2.5ml	2.5ml $KP\Delta_{P1\text{-}A7}$

Table 1: Test conditions for antimicrobial production by wild type and mutant plasmid strains.

Results and Discussion

K. pneumoniae #13883 Produces Zones of Inhibition on Enterobacteriaceae lawns

To determine if the antimicrobial effect requires the presence of *K. pneumoniae* cells or is a secreted factor, cell and cell-free supernatant (CFS) of *K. pneumoniae* #13883 (hereafter referred to as KP13) were spotted onto bacterial lawns of *K. pneumoniae* #9997, *C. freundii*, *C. koseri*, *E. aerogenes*, *E. cloacae,* and a clinical strain of *K. pneumoniae* from a patient with a KP UTI (hereafter referred to as KP99, CF, CK, EA, ECl, and KPuti respectively) as shown in **Figure 1** below. To create the CFS, overnight cultures were centrifuged and passed through a 0.22uM syringe filter leaving only the cell-free supernatant that contained metabolites that resulted from microbial growth, as well as the residual nutrients from the medium used, which in this case was LB broth. Aliquots containing cells were also incubated on the plates along with the CFS. These bacterial lawn plates were incubated overnight at 37C and monitored for zones of inhibition (ZOI).

On all bacterial competitors tested, the cellular spot of KP13 produced ZOI, however the degree of ZOI varied between the different competing bacterial species and strains. Of the competitors tested, KP99, CF, and ECl had the largest zones of inhibition, producing large and long lasting ZOI (**Figure 1A, 1B, 1C**). The remaining competitors tested, KPuti, CK, and EA also had ZOI, but were very small, indicating KP13's ability to inhibit or kill competitors is both species and even strain specific (**Figure 1D, 1E, 1F**).

KP13 CFS was only able to produce ZOI on KP99, CF, and ECl lawns with a complete absence of ZOI on KPuti, CK, and EA lawns (**Figure 1**). This also indicates KP13's inhibitory ability is species and strain dependent or would need to be produced in a constant amount by

KP13 cells. Taken together, the results from both the KP13 cells and KP13 CFS indicate that the antimicrobial effect KP13 is an, as of yet, unidentified secreted factor.

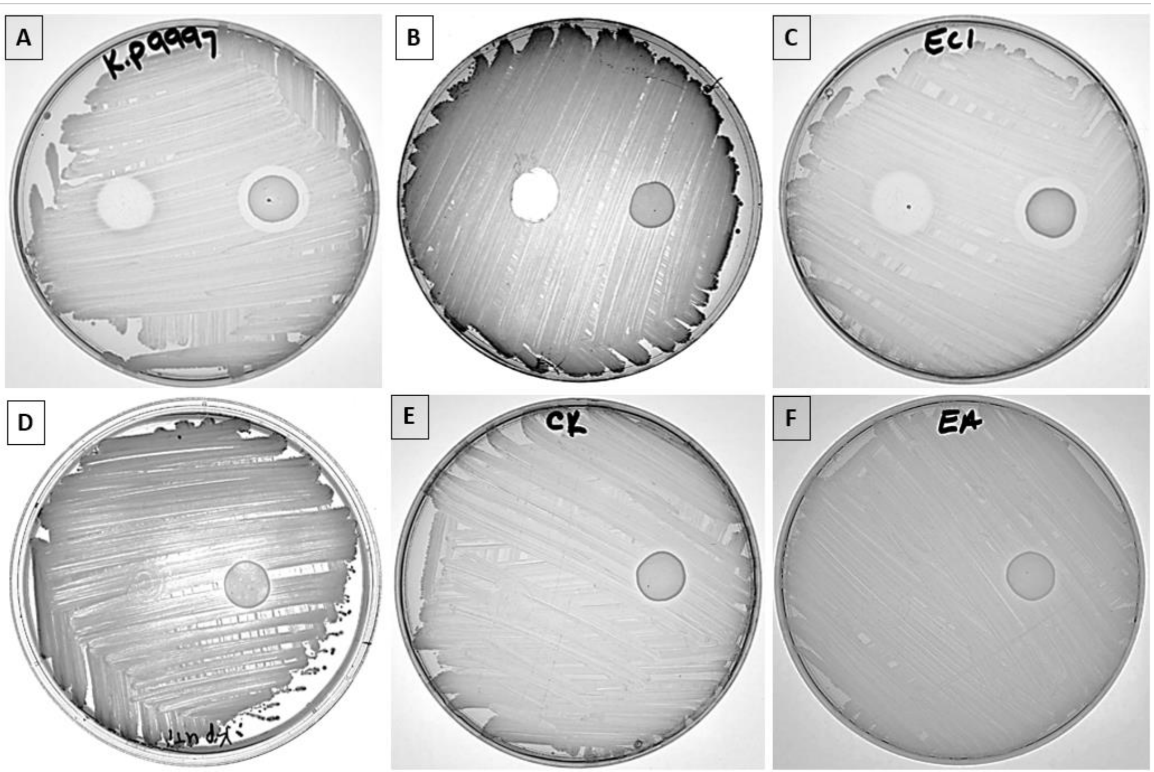

Figure 1: Representative photos of *K. pneumoniae* 1337 cells and CFS grown on competitor bacterial lawns. (A) *K. pneumoniae* 9997, (B) *Citrobacter koseri*, (C) *Enterobacter cloacae*, (D) a clinical strain from a patient with a *K. pneumoniae* UTI, (E) *Citrobacter koseri*, (F) *Enterobacter aerogenes*. The CFS (cell free supernatant) is on the left of each plate and the cells are on the right of each plate.

<u>Transposon Insertion for Mutant Loss-of-Function Identification of Antimicrobial Genes</u>

Our interests then turned to identifying what genetic elements or genes are responsible for the production of this inhibitory secreted molecule or compound. Previous work in our laboratory indicated the secreted factor is a protein, is pH and temperature tolerant, between 10-15 kDa, and, genetically, is located on a KP13 plasmid rather than being chromosomally

encoded. To identify the genes responsible for this protein, we created a large library of transposon insertion KP13 plasmids. **Figure 2A** as well as the Methods section outlines the methods utilized to create this loss-of-function library. Briefly, a plasmid extraction was performed on KP13 and mixed with the transposon EZ-Tn5 (Epicenter). Of note, the transposon contains a Kanamycin resistance gene and the R6Kγ*ori* origin of replication in *E. coli.* These plasmids were then electroporated into *E. coli* and screened on LB-Kanamycin plates for incorporation of the plasmid containing the transposon (**Figure 2B**). Upon creating the loss-of-function library, the *E. coli* containing transposon plasmids were screed on lawns as previously discussed in the agar spot tests, to identify candidates that either gained the function of the antimicrobial protein (create ZOIs) or loss-of-function candidates (do not create ZOIs) (**Figure 2C**).

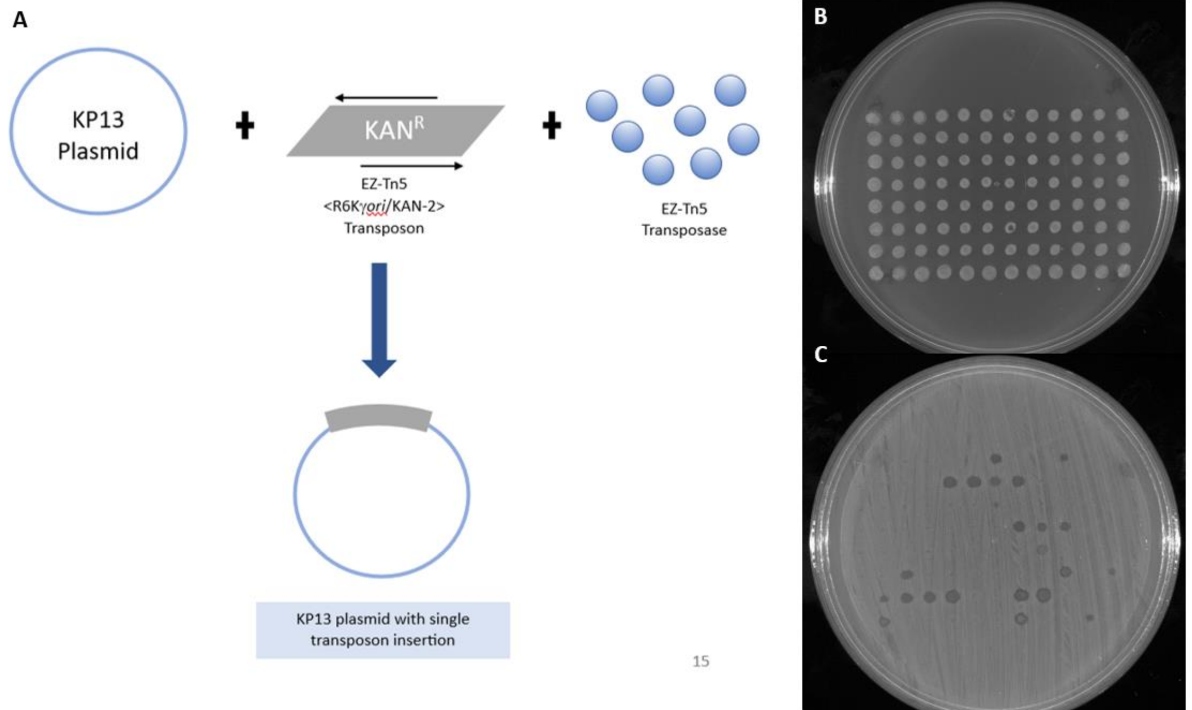

Figure 2: Visual process of creating plasmid transposon insertion library (2A), screening method to confirm transposon plasmid introduction into *E. coli* (2B), screening of transposon candidates on bacterial lawns to identify loss or gain of function mutants (2C).

Agar spot test of selected transposon insertion candidates

Two particular loss-of-function candidates were selected for further investigation. To identify loss-of-function, the mutants were spotted onto bacterial competitor (EA, ECl, CF, KP99) lawns on agar plates using the wild-type KP13 as a control for the presence of ZOIs. (**Figure 3**). As seen in the previous tests, the control, KP13, produced large ZOIs on lawns of ECl, CF, and KP99 (top Figure 3A-C), but minimal ZOI on EA (Figure 3D). The two transposon plasmid mutants, $KP\Delta_{P1\text{-}A3}$, $KP\Delta_{P1\text{-}A7}$, produced no ZOIs on any of the bacterial lawns (bottom Figure 3A-D). This indicates that the plasmid introduced into the *E. coli* containing a transposon

insertion disrupted a genetic element important for the production and secretion of this antimicrobial protein.

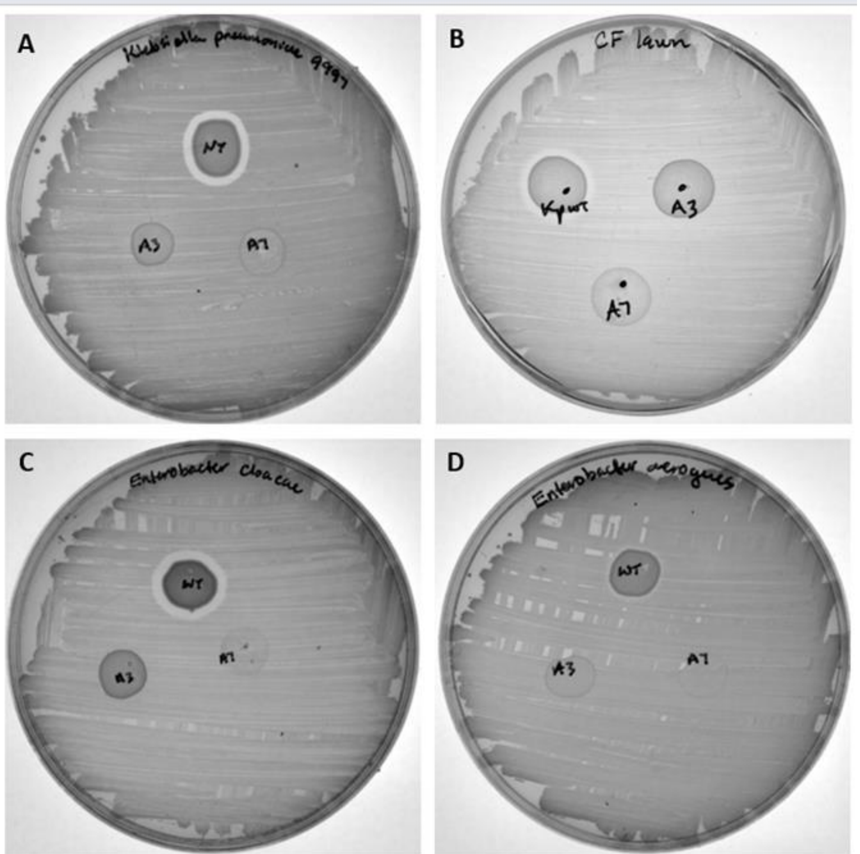

Figure 3: Agar spot test for ZOIs for KP_{wt}, $KP\Delta_{P1\text{-}A3}$, and $KP\Delta_{P1\text{-}A7}$ on competitor lawns of (A) *K.pneumoniae 9997*, (B) *Citrobacter freundii*, (C) *Enterobacter cloacae*, and (D) *Enterobacter aerogenes*.

<u>Characterization of the transposon insertion *Klebsiella* plasmids</u>

Utilizing primers directed toward the transposon insertion, we were able to sequence the interrupted genetic element from the two loss-of-function candidates. The NCBI blast and blastx programs were used to further identify the alignment of nucleotides and proteins respectively of our mutant to other known species of *Klebsiella pneumoniae* (**Figure 4**). The NCBI blast

similarity of the DNA sequence of our mutants most closely matched to the *Klebsiella pneumoniae* strain FDAARGOS_775 with a 97.9% percent identity. 698/713 nucleotides matched with only 6 gaps in the sequence (**Figure 5**). As for the protein alignment, it linked our mutant most closely to an uncharacterized *Klebsiella* protein with a 98.91% identity. 91 of the 92 identities matched in range 1-92, 85 of the 86 matched in range 91-176, and 12 of 22 matched in the range 175-196. None of the ranges had any gaps in protein sequence (**Figure 6**).

```
TCTCTGAGTAGTCGACATGCAGGCATGCAAGCTTCAGGGTTCGAGATGTGTATAAGAGAC
AGAGCTCGGGGAGGAATAACAGCACCGGCATGGCCGGTGCTGGCGTTTATTTTTTGCCGG
ACGGCCACCACCGGCAGGCGGATTTGTCCTCCGGCAGTTCAGGCACCGGCACGTTAAAAT
AGCGGCTGTTATATTCCCGCATCAGCTCCGCTTTCAGCTTCTCGTTCACCGCGCTTTCCC
CGTGCCGCTCGCTGACCACCTCAATCATCGCGCTAATCTGCTCCGGTGAGCACTTCCCGG
CATCAACCACCTGCACACCGTTCTCGATGGCGAACCGCTCCACCTTGCGATAGAGGTAAT
CACACATGTCTTCATTCCTGCGGGTTTGTCCCGTTCCCATTGCGGATTTTCACCGGTGAA
AAAGTACGTGGCTTTTTCGTCGCTGTTGAGCCGCTCGTCATACATCCCGATGGATTTCAT
CATCGCCAGATGCTGGGCCCGGTCGACAAAACAGAAAACATACGCCCCGGTCTGCCAGGC
GAGCACCACCATATCTTCAAGATGCTCGCTGGTCGGCATGATGCAGCGCTCGCCGTCCCG
GTCCTCCGCATCTTCAATAAATACCATGTACAGCGCCATAATCCCTCACTCCGGTGGTCA
TAATTTTAAAGCACGCAAACAGCGGTTCACGATCCAGCTCGCCCGGTTTGCGGGCCCCGC
CGGGTCTGTGGATCACGAATTCATTCACCGACAGGATCCCCGCTGCACCCTGAA
```

Figure 4: DNA sequence of our mutant provided by Sanger sequencing performed by the James H. Quillen College of Medicine's Molecular Core facility.

Klebsiella pneumoniae strain FDAARGOS_775 plasmid unnamed1, complete sequence

Sequence ID: CP040992.1 Length: 26723 Number of Matches: 1

Range 1: 10285 to 10993 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1229 bits(665)	0.0	698/713(98%)	6/713(0%)	Plus/Minus

```
Query  63     AGCTCGGGGAGGAATAACAGCACCGGCATGGCCGGTGCTGGCGTTTATTTTTTGCCGGAC  122
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10993  AGCTCGGGGAGGAATAACAGCACCGGCATGGCCGGTGCTGGCGTTTATTTTTTGCCGGAC  10934

Query  123    GGCCACCACCGGCAGGCGGATTTGTCCTCCGGCAGTTCAGGCACCGGCACGTTAAAATAG  182
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10933  GGCCACCACCGGCAGGCGGATTTGTCCTCCGGCAGTTCAGGCACCGGCACGTTAAAATAG  10874

Query  183    CGGCTGTTATATTCCCGCATCAGCTCCGCTTTCAGCTTCTCGTTCACCGCGCTTTCCCCG  242
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10873  CGGCTGTTATATTCCCGCATCAGCTCCGCTTTCAGCTTCTCGTTCACCGCGCTTTCCCCG  10814

Query  243    TGCCGCTCGCTGACCACCTCAATCATCGCGCTAATCTGCTCCGGTGAGCACTTCCCGGCA  302
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10813  TGCCGCTCGCTGACCACCTCAATCATCGCGCTAATCTGCTCCGGTGAGCACTTCCCGGCA  10754

Query  303    TCAACCACCTGCACACCGTTCTCGATGGCGAACCGCTCCACCTTGCGATAGAGGTAATCA  362
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10753  TCAACCACCTGCACACCGTTCTCGATGGCGAACCGCTCCACCTTGCGATAGAGGTAATCA  10694

Query  363    CACATGTCTTCATTCCTGCGGGTTTGTCCCGTTCCCATTGCGGATTTTCACCGGTGAAAA  422
              |||| | |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10693  CACA-G-CTTCATTCCTGCGGGTTTGTCCCGTTCCCATTGCGGATTTTCACCGGTGAAAA  10636

Query  423    AGTACGTGGCTTTTTCGTCGCTGTTGAGCCGCTCGTCATACATCCCGATGGATTTCATCA  482
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10635  AGTACGTGGCTTTTTCGTCGCTGTTGAGCCGCTCGTCATACATCCCGATGGATTTCATCA  10576

Query  483    TCGCCAGATGCTGGGCCCGGTCGACAAAACAGAAAACATACGCCCCGGTCTGCCAGGCGA  542
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10575  TCGCCAGATGCTGGGCCCGGTCGACAAAACAGAAAACATACGCCCCGGTCTGCCAGGCGA  10516

Query  543    GCACCACCATATCTTCAAGATGCTCGCTGGTCGGCATGATGCAGCGCTCGCCGTCCCGGT  602
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10515  GCACCACCATATCTTCAAGATGCTCGCTGGTCGGCATGATGCAGCGCTCGCCGTCCCGGT  10456

Query  603    CCTCCGCATCTTCAATAAATACCATGTACAGCGCCATAATCCCTCACTCCGGTGGTCATA  662
              ||||||||||||||||||||||||||||||||||||||||||||||||||| || |||||
Sbjct  10455  CCTCCGCATCTTCAATAAATACCATGTACAGCGCCATAATCCCTCACTCCGTTG-TCATA  10397

Query  663    ATTTTAAAGCACGCAAACAGCGGTTCACGATCCAGCTCGCCCGGTTTGCGGCCCCGCCG  722
              | ||||||||||||||||||| |||||||||||||||   ||| |||||||||| |||||
Sbjct  10396  AATTTAAAGCACGCAAACAGCCGTTCACGATCCAGCTCCGCCGTTTTGCGGCCTCGCCG  10337

Query  723    GGTCTGTGGA-TCACGAATTCATTCACCGACAGGATC-CCCGCTGCACCCTGA  773
              | ||||||||| |||||| ||||| |||||||||||||| |||||||||||||||
Sbjct  10336  G-TCTGTGGATTCACGATTTCATCCACCGACAGGATCGCCCGCTGCACCCTGA  10285
```

Figure 5: Nucleotide alignment of our mutant provided by NCBI's nucleotide BLAST database.

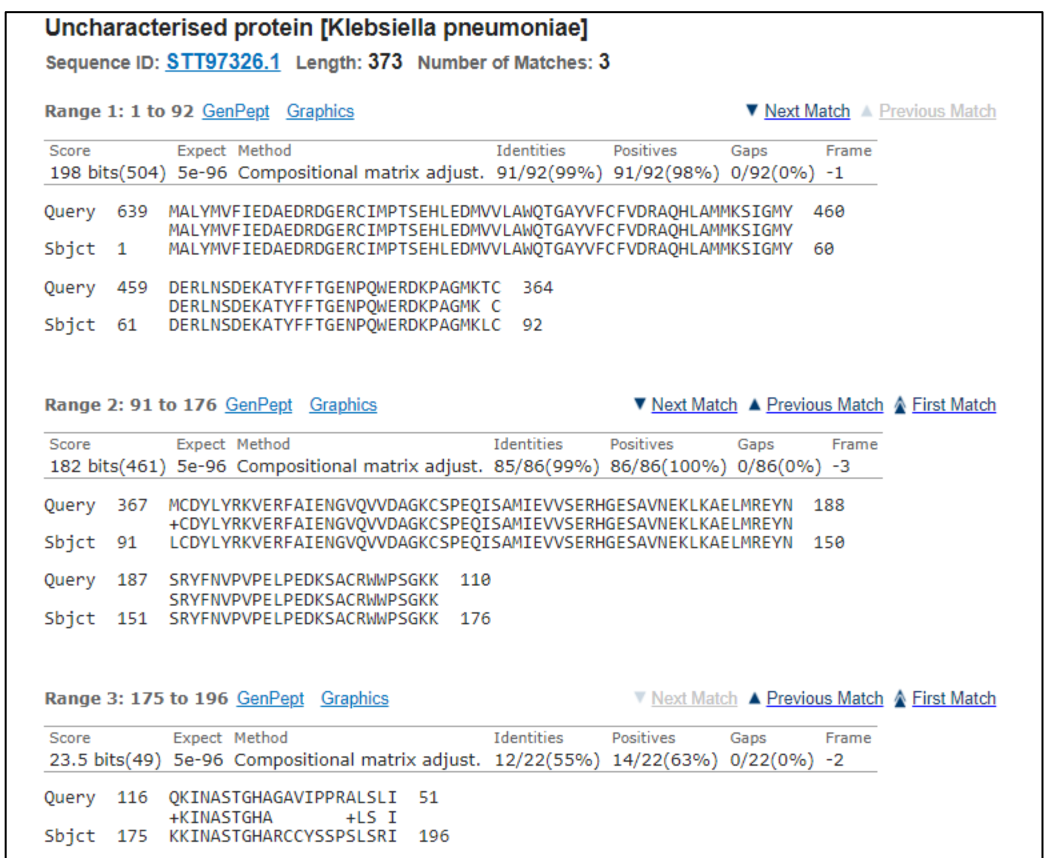

Figure 6: Protein alignment of our mutant provided by NCBI's blastx database.

Planktonic Co-Culture Interactions

Thus far, the *K. pneumoniae* antimicrobial protein has demonstrated an effective inhibitory ability on agar media, but we then wanted to examine if this same effect was seen in free floating, planktonic, bacterial growth. This method allows us to examine both the effects of the KP13 antimicrobial on competing bacteria, as well as, the effects of the plasmid transposon loss-of-function candidates. The viable cell counts of the colony forming units (CFUs) on the LB agar plates from planktonic cultures were enumerated to compare the inhibition of *E. cloacae* growth (Figure 7). When E. cloacae was grown alone, we see the typical strong growth of the bacterium. When growth in the presence of the CFS of the wild-type KP13, there was a 45%

decrease in growth which is indicative that the antimicrobial protein is effective in both colony agar and free-floating planktonic growth. When the CFS of the KP$\Delta_{P1\text{-}A3}$ and KP$\Delta_{P1\text{-}A7}$ loss-of function mutants was used in the growth of *E. cloacae*, the growth increased towards E. cloacae only growth. KP$\Delta_{P1\text{-}A3}$ and KP$\Delta_{P1\text{-}A7}$ only inhibited *E. cloacae* by 31% and 13% respectively. This indicates the interruption of this uncharacterized *Klebsiella* protein could be instrumental in the antimicrobial protein's production and effectiveness of inhibiting *E. cloacae*.

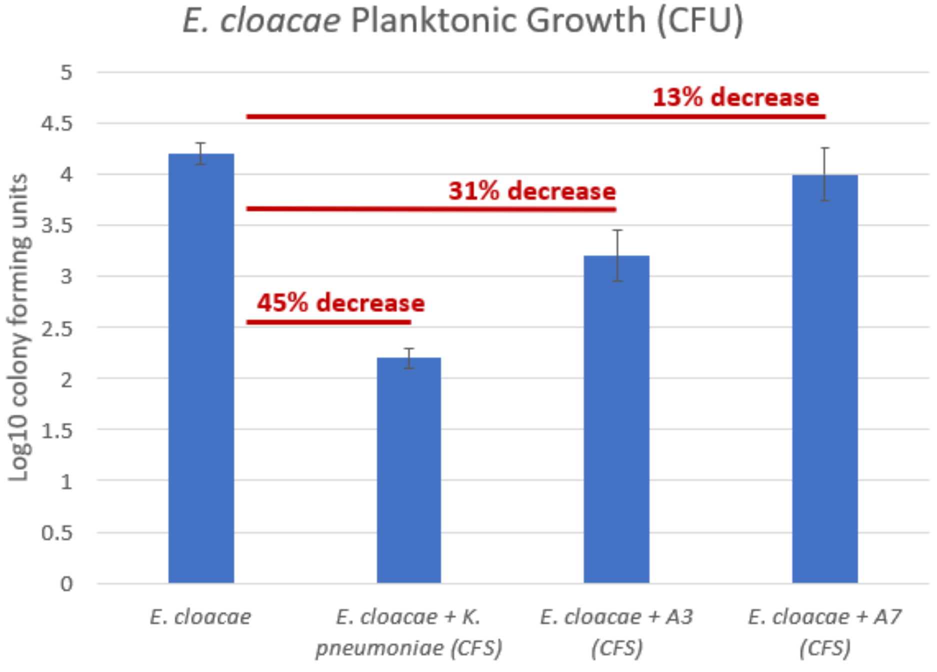

Figure 7: *E. cloacae* 24h growth (CFU's) either alone, with the CFS of wild-type KP13 or the CFS of loss-of-function plasmid transposon candidates.

To support the planktonic CFU data, we also looked at cell density using OD_{600} readings of planktonic growth with a spectrophotometer (Figure 8). The OD_{600} values recorded in Figure 8 showed a decrease of roughly 2/3 when we added our KP_{wt} from our control tube containing only *E. cloacae* bacteria. Our mutants (KP$\Delta_{P1\text{-}A3}$ andKP$\Delta_{P1\text{-}A7}$) also showed a decrease in optical density when measured with a spectrophotometer. These values overall show that addition of our

mutants KPΔ$_{P1-A3}$ and KPΔ$_{P1-A7}$ caused a lesser extent of inhibition of the growth of *E. cloacae* than comparted to KP13 and were trending back towards the growth rates of *E. cloacae* alone without any CFS.

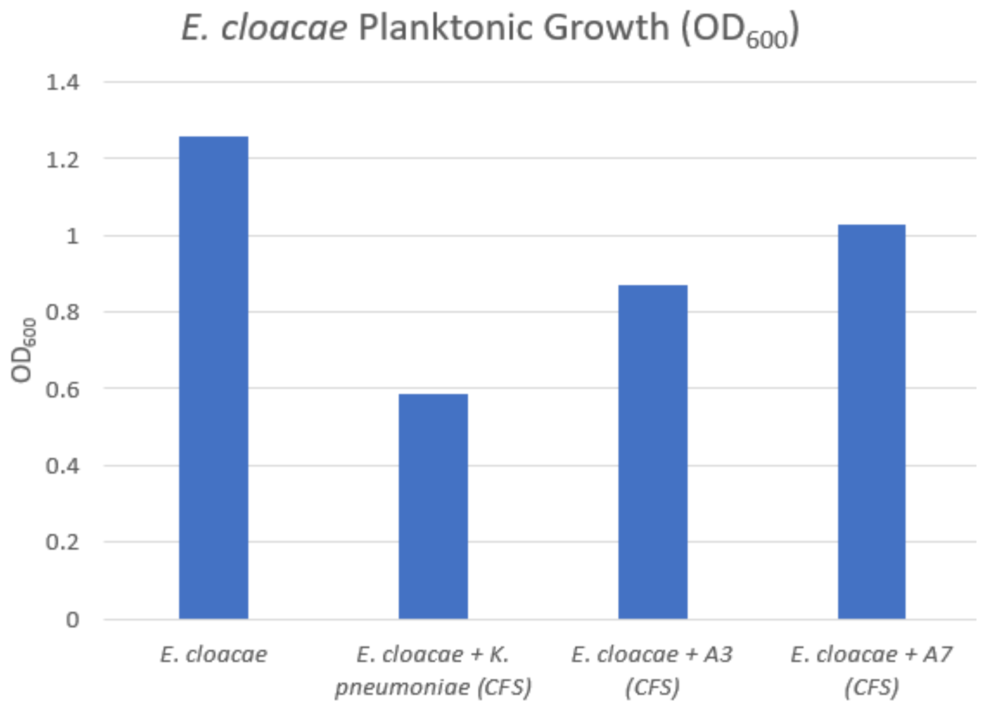

Figure 8: Co-culture data based on viable cell counts and absorbance of each tube sample in a spectrophotometer at 600nm.

Conclusions

Drug resistance, particularly antibiotic resistance, is proving to be a lethal problem. Bacterial and fungal infections are unfortunately inevitable, which leads to an excess of unnecessary deaths caused by the drug resistance we are facing. Interactions such as these discussed previously can play an integral role in how these infections are treated going forward.

Bacteriocins can prove very helpful in clinical settings. This project builds on previous findings that *Klebsiella pneumoniae* produces a potential bacteriocin with efficacy toward *Citrobacter*, *Enterobacter*, and *Klebsiella*.

The *K. pneumoniae* mutants were created via transposon mutagenesis, amplified, and sequenced. The purpose of sequencing the mutants was to identify the genetic elements that were related to the *K. pneumoniae* inhibitory mechanism. Planktonic growth trials were then conducted with our KP transposon mutants. $KP\Delta_{P1-A3}$ and $KP\Delta_{P1-A7}$ along with the KP_{wt}, which allowed us to see the inhibitory affects *in vitro* by counting the colony forming units and using spectrophotometry to observe absorbance. The values obtained from the co-culture test indicated that there are definitely inhibitory affects at work and that the uncharacterized *Klebsiella* protein plays a role in this inhibition.

Further research will focus on gaining more insight into the genetic components of this inhibitory mechanism and continuing to study the genetic components already known in order to apply them to more polymicrobial interactions with a wider variety of microorganisms that are known to cause sometimes devastating bacterial infections. Additionally, we hope to purify this protein to determine its identity and to potentially pair synergistically with antibiotics.

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