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Genetic Characterization of a Klebsiella pneumoniae Secreted Anti-Microbial Protein

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Genetic Characterization of a *Klebsiella pneumoniae* Secreted Anti-Microbial Protein

By

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ABSTRACT

Antimicrobial-resistant bacteria are a major source of ailment in modern-day nosocomial settings, with numerous risks including leading to possible further drug resistance or spreading to those who cannot fight off the infection due to immune suppression or dysfunction. Previous work in our laboratory has determined that *Klebsiella pneumoniae* possesses inhibitory effects on the growth of a variety of bacteria that contain antimicrobial-resistant properties in the Enterobacteriaceae family, a major source of nosocomial antimicrobial-resistance. This novel property of *K. pneumoniae* inhibits the growth of *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae* through an anti-microbial protein. The antimicrobial protein secreted from *K. pneumoniae* has been shown to reduce the density and growth of *C. freundii*, *E. aerogenes*, and *E. cloacae* in both biofilm and planktonic forms. The work performed in this thesis has shown that the antimicrobial protein is plasmid mediated by introducing a transposon (Tn5) to the plasmid to provide resistant selection and a possible way to create a mutant knockout to find the exact location of the gene in the plasmid. Upon transposon mutagenesis of the plasmid, it was electroporated into Rec- *E. coli*. The *E. coli* were then able to produce the antimicrobial protein allowing the formation of zones of inhibition when screened on *C. freundii*, *E. aerogenes*, and *E. cloacae* lawns. Upon confirmation that the plasmid mediates the anti-microbial protein, the plasmid was sent for sequencing to further characterize the gene responsible for coding the anti-microbial protein. This novel antimicrobial protein has high sequence similarity to bacteriocins and, thus far, is a novel and uncharacterized protein of plasmid origin found in only in this particular strain of *K. pneumoniae*. Further research involving this new bacteriocin could aid in the development of treatments for the highly drug resistant Enterobacteriaceae family members.

ACKNOWLEDGEMENTS

I cannot thank Dr. Sean Fox enough for the opportunity to be a part of his lab and his guidance. In doing so, Dr. Fox has not only shown me how to conduct research, but also how to be cordial in my everyday life. He sets the example both inside and out of the laboratory about how to live for his colleagues as both a great mentor and a great father. I also want to thank Donald Fowler for his contribution and help with my lab work, which would not have been possible without him. I also want to thank my amazing friends Pamela Avendaño-Rubí, Culton Hensley, Paulina Ramiez, and Shivam Patel for making sure I was focused during the rigors of having to deal with COVID-19 and for being great friends to me. The people I want to thank the most are my mom, dad, and brother, Owen. You all are the reason I strive to do the things I do every day and why I am the way I am. Thank you for teaching me the things I needed to get a quality education in an environment where I could thrive.

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INTRODUCTION

Historical and Modern Significance of Antimicrobial-Resistance

Shortly after the first introduction of the first antimicrobials to combat microbial infections came the emergence of antimicrobial-resistant (AMR) bacteria, Thusly, this resistance has been the focus of health-related research due to its threat to human health and wellbeing. In the past, researchers would attempt to find novel, new antimicrobials to render the AMR bacteria ineffective by targeting different physiological or structural systems of the bacterium. Inevitably, this caused selective pressures for bacteria to evolve to becoming resistant to not just one, but many different antimicrobials leading to multi-drug resistance (MDR) and threaten human health to greater extents.

According to the World Health Organization's (WHO) list of bacteria with the highest priority of health-risk, there seems to be little hope with outpacing the rate of AMR due to several factors. The WHO states that, "The decreased interest in antibiotic research and development of pharmaceutical companies in the past few decades is probably related to difficulties in clinical development and scientific, regulatory, and economic issues" [1]. Given the WHO's statement of the current atmosphere of antibiotic research and the eminent threat of AMR organisms, humans soon may face the very real threat of AMR organisms returning as the number one cause of morbidity and mortality in human health.

AMR organisms are currently a moderate threat in the world but will soon become a major threat without advancements in identifying new alternative forms of antimicrobials. In

2019, there were 4.95 million deaths associated with bacterial AMRs [2]. To put into perspective of how vast the death toll is every year from AMRs, from the start of the COVID-19 pandemic to current time, which has spanned three years during the writing of this thesis, 5 million deaths have occurred compared to the 4.95 million per year for AMRs in 2019 [3]. The current pandemic has magnified the need for alternative and novel areas to be explored in controlling not only MDR infections, but also emerging and re-emerging pathogens that threaten public health.

Klebsiella pneumoniae

Klebsiella pneumoniae was first identified by Carl Friedlander in 1882 from the lungs of a patient that had passed away from pneumonia, and has since been known to be active in hospital settings due to its mucus-like capsule layer and its increased likelihood to be an AMR organism [4]. In development of *K. pneumoniae*'s AMR is due to the development of carbapenemases, a β-lactamase enzyme that has versatile hydrolytic capabilities to antibiotics [5]. In fact, *K. pneumoniae* is so effective in producing carbapenemases that, "...of the 9000 infections reported to the Centers for Disease Control and Prevention (CDC) due to carbapenem-resistant *Enterobacteriaceae* in 2013, approximately 80% were due to *K. pneumoniae*" [4]. Although *K. pneumoniae* is viewed as a problematic infection to human health, it may also have properties that will aid in combating other infections. Polymicrobial interactions are a recent area of scientific research that explore the natural synergistic, antagonistic, and mutualistic relationships that microbes encounter in the environment and in the human body. The trillions of microbes in the human body all compete for the same space, resources, nutrients, and niches. By observing these interactions and both the physical methods and molecular methods in which they interact, we may find new mechanisms for microbial infection inhibition. Our laboratory has previously shown that a particular *K. pneumoniae* strain secretes a previously uncharacterized

antimicrobial protein. This antimicrobial protein has shown to be most effective with Enterobacteriaceae family members, which is a common source of infection critical to human health and the number one bacterial family of the World Health Organization's priority list [1].

Enterobacteriaceae Family

The Enterobacteriaceae family is part of the domain: Bacteria, phylum: Proteobacteria; class: Gammaproteobacteria; and order: Enterobacteriales [6]. In addition, Enterobacteriaceae contain some of the most formidable bacteria to threaten human health and include numerous AMR bacteria. The Enterobacteriaceae family contains, but is not limited to: *Klebsiella pneumoniae, Escherichia coli, Salmonella enterica, Enterobacter aerogenes,* and *Proteus vulgaris.* Of the 120 species that Enterobacteriaceae encompasses, less than 25 species of Enterobacteriaceae contain 95% of all clinically significant bacterial strains related to AMR [6]. Most infections result in urinary tract infections (UTIs), gastrointestinal disease with symptoms such as diarrhea, or can spread to the bloodstream resulting in life-threatening complications and sepsis [7]. In addition, the Enterobacteriaceae family can be quite dangerous, especially beta-lactamase-producing Enterobacteriaceae. Many "beta-lactamase-producing Enterobacteriaceae also is on the rise, causing 197,400 infections and 9,100 deaths a year," which is a significant amount for only one family of bacteria [8].

Bacteriocins

For years, scientists have been searching for alternative ways to kill and inhibit microbes, especially with the continuous rise of antimicrobial-resistance (AMR). In 1925, André Gratia reported that an antibiotic, of protein nature, had bacteriolytic properties from *E. coli* "V" [9]. *E. coli* "V" produced anti-microbial proteins, due to a colicin, which are bacteriocins exclusive to *E. coli*. Since Gratia's discovery of bacteriocins, research on bacteriocins has expanded greatly.

Currently, there are many different categories of bacteriocins, which include, but are not limited to: microcins, colicin-like bacteriocins, tailocins, and Gram-positive classes I-IV [10].

Bacteriocins are a kind of antimicrobial peptide produced by bacteria, which can kill or inhibit bacterial strains closely related or non-related, but will not harm the bacteria themselves by specific immunity proteins [11]. Although what defines a bacteriocin is restrictive, the mechanism of their function can be quite diverse. Bacteriocins can, but are not limited to, being pore forming, being nucleases, being peptidoglycanases, inhibit DNA replication, and protein synthesis [11].

Currently, bacteriocins have many uses in everyday life. They are used for "food preservation, diverse therapeutic purposes such as treatment of peptic ulcer, spermicidal agent, and woman care, anticancer agents, veterinary use, skincare, and oral care, and also for plant growth promotion in agriculture" [12]. While the current uses are impressive, there is also, "The possibility of developing bacteriocins into next generation antibiotics, accompanied with the rapid development in genetics and nanotechnology, paves the way to even more fascinating applications such as novel carrier molecules (delivery systems) and the treatment of cancer" [13]. The potential uses of bacteriocins could greatly enhance our ability to treat AMR bacteria, work synergistically with combined antibiotic therapy, or offer new targets for development of novel therapeutics.

Klebsiella pneumoniae Bacteriocins

Currently, there are many different types of *Klebsiella* bacteriocins. There are so many *Klebsiella* bacteriocins that, in fact, they have their own names: klebicins. Klebicins can be pore-forming, have nuclease activity, and peptidoglycan-degrading activity [14]. In addition, there are many colicin-like plasmids, bacteriocins exclusive to *E. coli*, that have similar mechanisms to

colicins [14]. In addition to targeting bacteria, klebicins have also been, "identified, cloned, [and] expressed in plants and characterized nine pore-forming and peptidoglycan-degrading bacteriocins from different *Klebsiella* species" [14]. The fact that klebicins can influence plants as well is significant.

Our lab has previously identified a strain of *Klebsiella pneumoniae* that produces an uncharacterized antimicrobial protein that inhibits planktonic and biofilm growth of *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*. This protein was shown from previous work in our lab to be between 12 kDa and 20 kDA and 128 and 181 amino acids, respectively [15]. Numerous properties of the anti-microbial protein have been elucidated, but little is known about the genetic components of the genes responsible for production of this protein, let alone the identity of the protein, has been explored yet.

MATERIALS AND METHODS

Bacterial Strains & Growth Conditions

Unless otherwise stated, all bacterial strains used in this study (Table 1) were routinely cultured on Luria-Bertani (LB) broth and agar plates. Overnight cultures were made by inoculating isolated colonies into LB broth and grown at 37^{o}C for 18 hours with shaking (160rpm). When necessary, kanamycin (50μg/ml) was added to LB agar plates or broth for selective growth of *K. pneumoniae* mutants. Yeast-Nutrient Broth (YENB) was used to facilitate making *K. pneumoniae* cells electrocompetent.

Strains	**Characteristics**	**References**
K. pneumoniae 13883	Used for mutagenesis, plasmid isolation	ATCC

E. aerogenes 13048	Used for inhibition assays	ATCC
C. freundii 8090	Used for inhibition assay	ATCC
E. cloacae 23355	Used for inhibition assays	ATCC
K. pneumoniae 9997	Comparison studies	ATCC
Clinical *K. pneumoniae*	Comparison studies	ATCC

Table 1. List of bacterial strains implemented in this study.

Transposon	**Characteristics**	**References**
pRL27	Conjugative Tn5 transposon delivery vector; Kan^R	Yale *E. coli* Stock Center
EZ-Tn5	Used for chromosomal transposon delivery; Kan^R	Lucigen
EZ-Tn5<R6Kyori>	Used for plasmid transposon delivery; Kan^R	Lucigen [16]

Table 2. List of transposon systems implemented in this study

Transposon Mutagenesis of *Klebsiella* Cells

K. pneumoniae cells are notoriously known for having a thick sticky capsule that inhibits electroporation coupled with the difficulty of maintaining electrocompetence during the normal procedures for doing so. Thusly, we developed electrocompetent *K. pneumoniae* cells for transposon mutagenesis by streamlining a same-day use method in combination with addition of EDTA for inhibition of polysaccharide production from two previously published protocols [17]. A single isolated colony of *K. pneumoniae* was inoculated into YENB broth and grown overnight at 37°C with shaking. The next day, the stock culture was used to inoculate a new tube 1:100 with fresh YENB media with 7 µL if 1M EDTA (pH 8) for a final EDTA concentration of

70 mM in Yeast Extract Nutrient Broth. The culture was grown 37°C with shaking until the mid-exponential phase was reached at an OD_{600} reading of 0.04-0.06. The culture was aliquoted into 1.5 mL microcentrifuge tubes and centrifuged for 5,000rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in 1 mL of nuclease free distilled water, centrifuged at 5000rpm for 5 minutes and discard the supernatant and this washing step was repeated once more. On the final supernatant removal step, the pellet was resuspended with 30uL of nuclease free distilled water. Cells were used for electroporation immediately after and are not stable for freezing for future use.

The electrocompetent *K. pneumoniae* cells were added to a Gene Pulser cuvette with a 0.1cm gap (BioRad) and 1μL of transposon-vector plasmid (pRL27) or 1μL of EZ-Tn5 was added. This mixture was allowed to incubate at room temperature for 10 minutes and then electroporated at 2500V with 1 mL of 37°C SOC media added immediately added. The transformed cells were incubated at 37°C for an hour with shaking. After incubation,100μL of the electroporated cells were serially diluted and spread on LB plates containing Kanamycin and grown at 37°C overnight.

Transposon Mutagenesis of *Klebsiella* Plasmid

An overnight culture of *K. pneumoniae* was grown in LB broth at 37°C with shaking and plasmid DNA was isolated using a PureYield miniprep kit (Promega), run on a 1% agarose gel, and the resulting bands cut out and purified with a PureLink Gel Extraction Kit (Invitrogen). From this plasmid extraction, 0.2 μg of plasmid DNA was combine with 1μL 10X reaction buffer, 0.2 μg of the <R6Kγ*ori*/KAN-2> transposon, 1 μL of transposase, and nuclease free water. The mixture was incubated at 37°C for 2 hours followed by 1 μL of stop solution and

incubated at 70°C for 10 minutes. The plasmid/transposon mixture was combined with 25μL TransforMax™ EC100™ electrocompetent *E. col* of the competent cells and a Gene Pulser cuvette with a 0.1cm gap (BioRad) was used to electroporate at 2500V with 1 mL of 37°C SOC media added immediately added. The transformed cells were incubated at 37°C for an hour with shaking. After incubation,100μL of the electroporated cells were serially diluted and spread on LB plates containing Kanamycin and grown at 37°C overnight.

Screening for Antimicrobial Protein Production

Potential transposon insertion mutants from both the chromosomal library and plasmid library were patch plated onto LB + Kanamycin for a secondary screening and confirmation. Overnight cultures of *C. freundii* were used to make confluent lawns on LB agar plates and mutants were transferred to the competitor lawns, incubated overnight at 37°C and inspected for the presence or absence of the zone of inhibition wild-type *K. pneumoniae* typically displays. For those mutants that lacked a zone of inhibition, the resulting plasmid or chromosome was isolated, and sent for sequencing using primers directed towards the ends of the transposon. The resulting sequences were analyzed by NCBI BLAST.

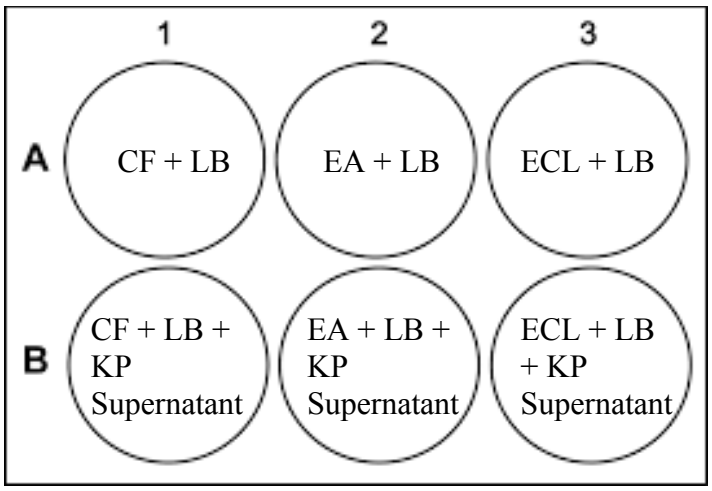
Biofilm Inhibition with *K. pneumoniae* Protein and Mutant *K. pneumoniae* 13 Vector Protein

Cultures of wild-type *K. pneumoniae*, *E. coli* harboring the *K. pneumoniae* plasmid with the added transposon, *C. freundii*, *E. aerogenes*, and *E. cloacae* were all inoculated into LB broth and incubated overnight at 37°C with shaking. The wild-type *K. pneumoniae* and *E. coli* harboring the *K. pneumoniae* transposon plasmid cultures were centrifuged at 10,000rpm for 3 minutes and the resulting supernatant of *K. pneumoniae* and *E. coli* transposon plasmid was filtered through 0.2 μm syringe filters to remove any remaining cells from the supernatant. The

E. aerogenes, *E. cloacae*, and *C. freundii* cultures were standardized using a spectrophotometer to an OD_{600} reading of 0.01 (~$1x10^6$ cells/ml). To two 6-well plates, coverslips were added to the bottom and the following combinations were added (see Figure 1):

Well 1: LB broth with *C. freundii* only
Well 2: LB broth with *E. aerogenes* only
Well 3: LB broth with *E. cloacae* only
Well 4: ½ LB broth & ½ *K. pneumoniae* supernatant with *C. freundii*
Well 5: ½ LB broth & ½ *K. pneumoniae* supernatant with *E. aerogenes*
Well 6: ½ LB broth & ½ *K. pneumoniae* supernatant with *E. cloacae*

In the second 6-well plate the procedure was repeated but substituted the *E. coli* transposon plasmid spent media in place of the wild-type *K. pneumoniae* spent media. These plates were incubated at 37°C for 24 hours. Coverslips were mounted on microscope slides, washed, heat fixed, and stained with crystal violet to observe biofilm growth.



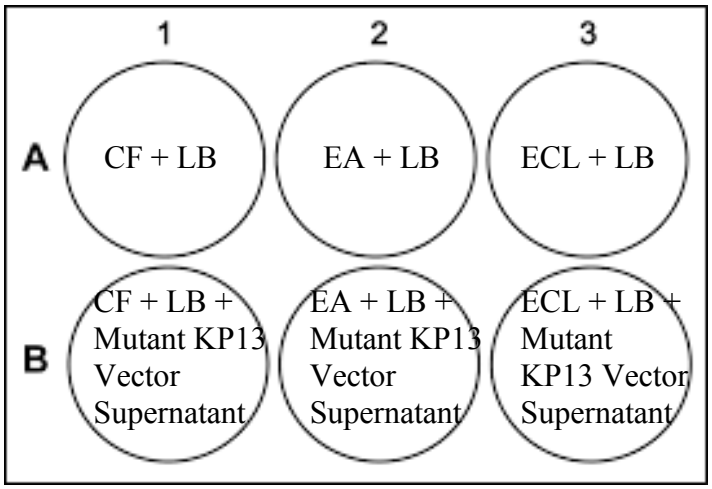


Figure 1: Representation of 6-well plate configuration for biofilm comparison

RESULTS AND DISCUSSION

Klebsiella pneumoniae's Inhibition of *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*

Previous work in our lab has shown that *K. pneumoniae* contains a protein that exhibits antimicrobial behavior towards members in the Enterobacteriaceae family, specifically, *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*. As shown in Figure 2, *K. pneumoniae* cells and *K. pneumoniae* spent cell free supernatant both produce zones of inhibition on *C. freundii*, *E. aerogenes*, and *E. cloacae* bacterial lawns. When quantitating the zones of inhibition (Figure 3), *K. pneumoniae* 13 creates different sized zones of inhibition on *C. freundii*, *E. aerogenes*, and *E. cloacae* with average zone of inhibition sizes of 2.5 mm, 1.5 mm, and 3.25 mm in *C. freundii*, *E. aerogenes*, and *E. cloacae*, respectively. Interestingly is the stark difference between the zone of inhibitions of *K. pneumoniae* exerts upon *E. aerogenes* and *E. cloacae*. While both belong to the Enterobacteriaceae family, *E. aerogenes* has increased genetic

similarity with *K. pneumoniae* than *E. cloacae*. A possible explanation for this difference in zones of inhibition could be that the antimicrobial protein could recognize *E. aerogenes*'s similarities to *K. pneumoniae* verses the differences it recognizes with *E. cloacae*.

Figure 2: Representative photo of *K. pneumoniae* inhibition of *C. freundii*

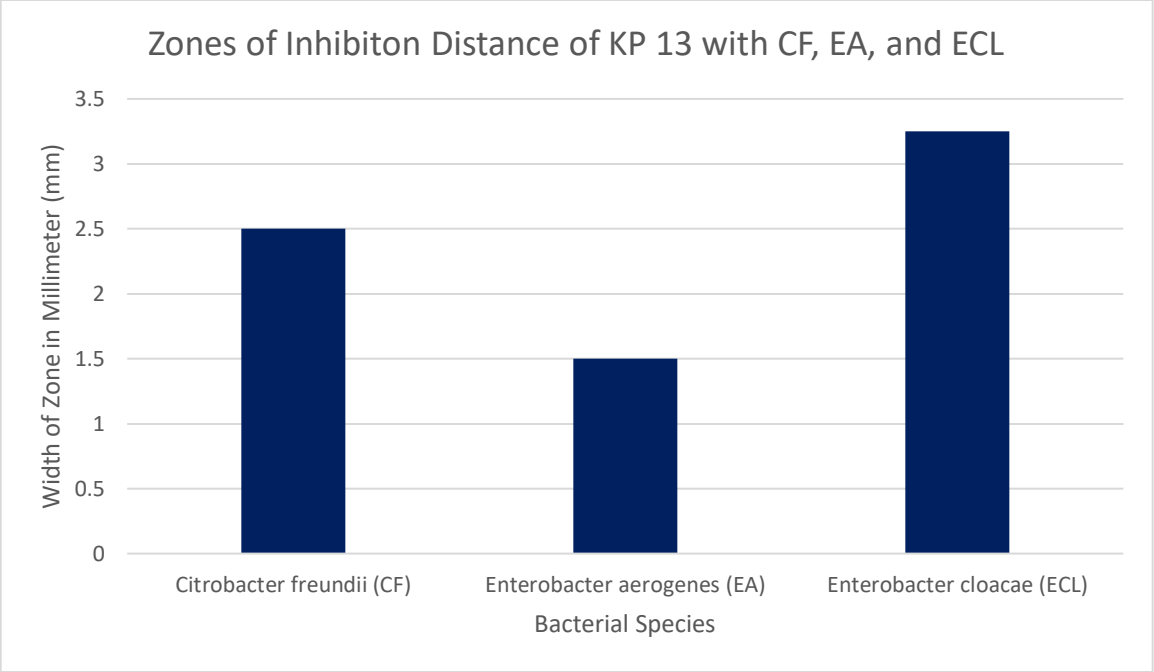


Figure 3: Spot Test Width of Zones of Inhibition of *K. pneumoniae* 13 with *E. aerogenes*, *C. freundii*, and *E. cloacae*

Transposon Mutagenesis of *Klebsiella* Chromosomal DNA

Upon demonstrating that *K. pneumoniae* possesses an antimicrobial protein, we then turned our attention to discovering the genetic elements important for the production of this protein in hopes of elucidating the identification of the protein. In the beginning, it was originally believed that the gene responsible for producing the antimicrobial protein was a part of *K. pneumoniae* chromosomal DNA. To target this hypothesis, we implemented an EZ-TN5 transposon system electroporated into competent *K. pneumoniae* cells to create a knockout mutant library through random transposon mutagenesis (Figure 4). These random transposon mutants were screened against *E. aerogenes*, *E. cloacae*, and *C. freundii* bacterial lawns for those mutants that had a loss-of-function phenotype resulting in a lack of zone of inhibitions. After

multiple attempts, the mutant screen was unsuccessful in producing any mutants. We believe it was due to *K. pneumoniae*'s thick polysaccharide capsule, and therefore needed to find a new way to insert the transposon. We found that two things improved the efficiency of transforming the *K. pneumoniae* bacterial cells: making the cells electrocompetent with EDTA that inhibits polysaccharide production and making fast, ready-to-use competent cells at room temperature. Unfortunately, after transforming approximately thirty 96-well plates, roughly 3,000 independent mutants, no mutants with the correct phenotype were found. A different transposon delivery system, pRL27, using conjugation was also attempted (Figure 5 and 6). This too resulted in no mutants with the correct phenotype being found.

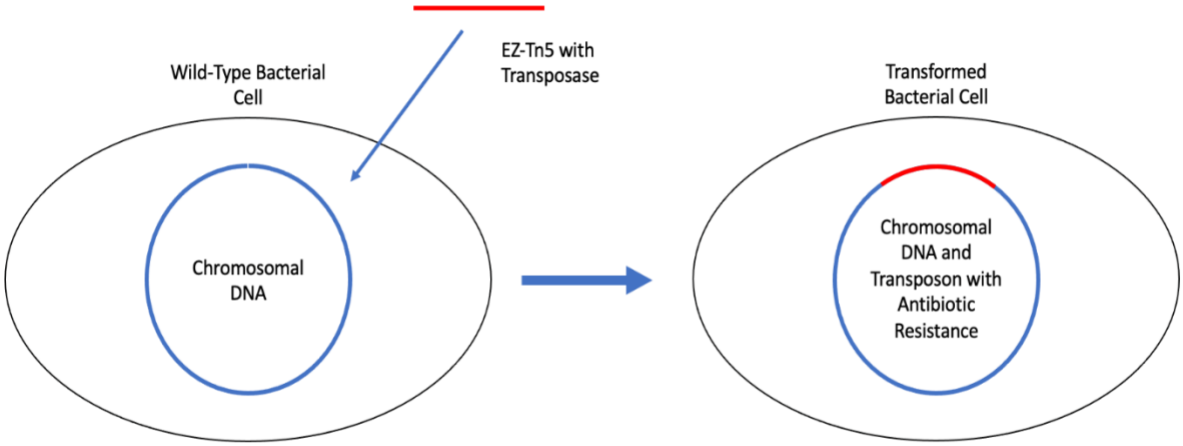


Figure 4: Simplified Mechanism of Transposon Mutagenesis with EZ-Tn5

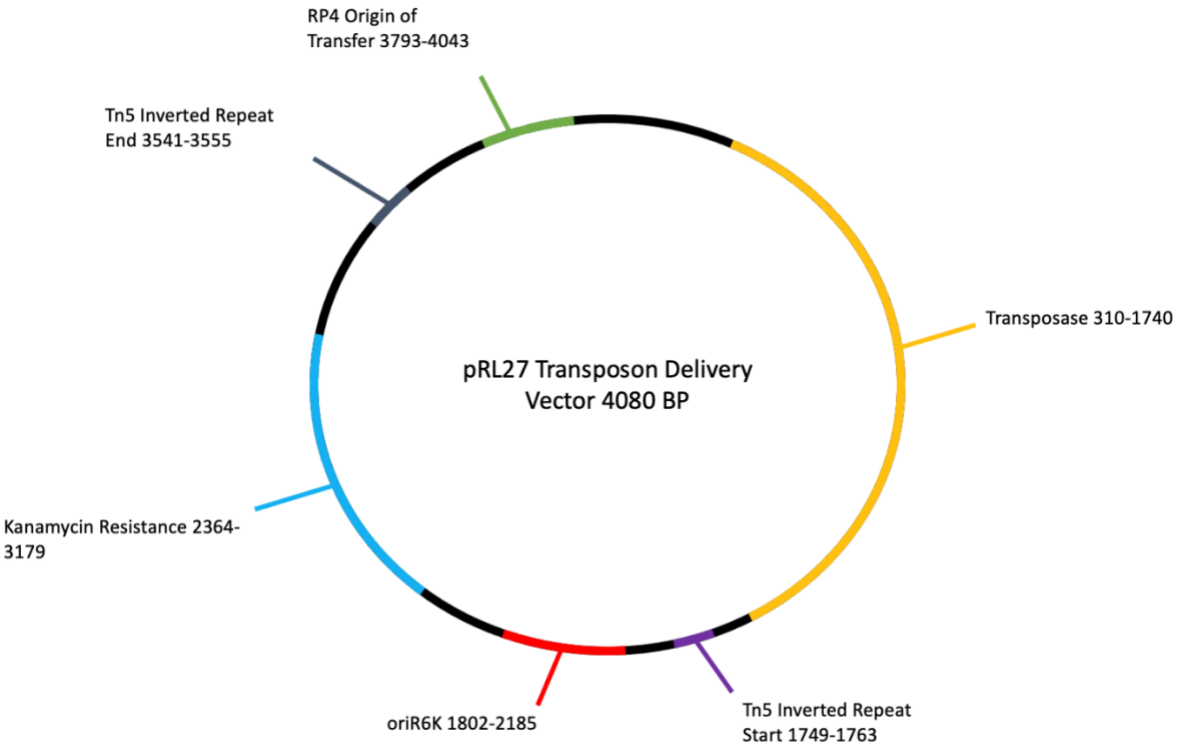


Figure 5: Anatomy of the pRL27 Transposon Delivery Vector

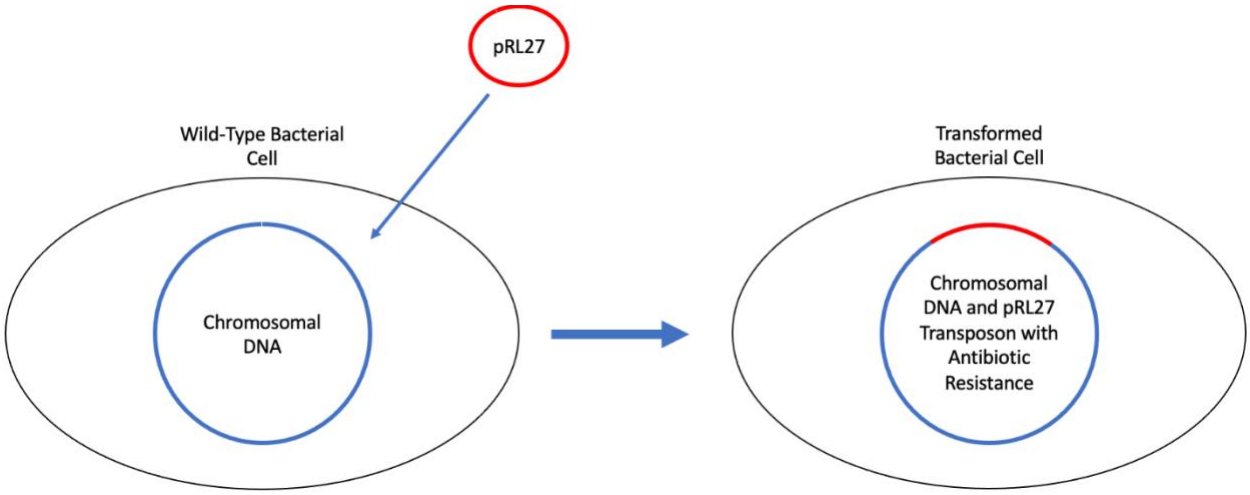
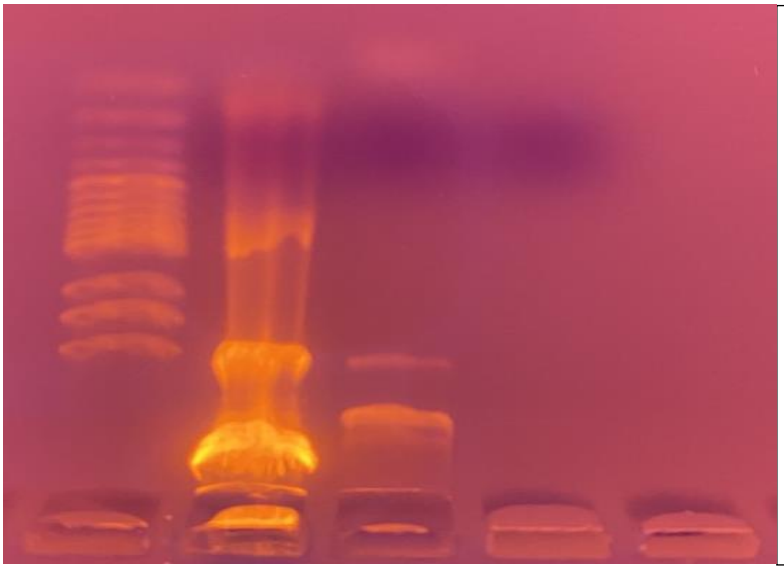


Figure 6: Simplified Mechanism of Vector Mediated Transposon Mutagenesis



Well Contents (Left to Right)
1. 100 Base Pair Ladder
2. Lambda Hind iii ladder
3. KP 13883
4. KP 9997
5. KP UTI

Figure 7: *K. pneumoniae* Plasmid Extraction Comparison in a Gel Electrophoresis to Verify that It Contained a Plasmid

Transposon Mutagenesis of Plasmid

Our last method we decided to determine if *K. pneumoniae* contained any plasmids to determine if the antimicrobial was plasmid mediated. Upon performing a plasmid isolation and running on a gel for visualization we found that *K. pneumoniae* 13 does indeed contain a plasmid (Figure 8). We attempt to deliver a transposon into the plasmid and express the plasmid in *E. coli.* This allowed the opportunity to investigate the plasmid for its ability to possibly produce the antimicrobial protein. This was done by exposing the plasmid to EZ-TN5 <R6Kγ*ori*/KAN-2> for in-vitro transposition (Figure 9). The plasmid was electroporated into TransforMax™ EC100™ Electrocompetent *E. coli* as a vector for the transformed plasmid.

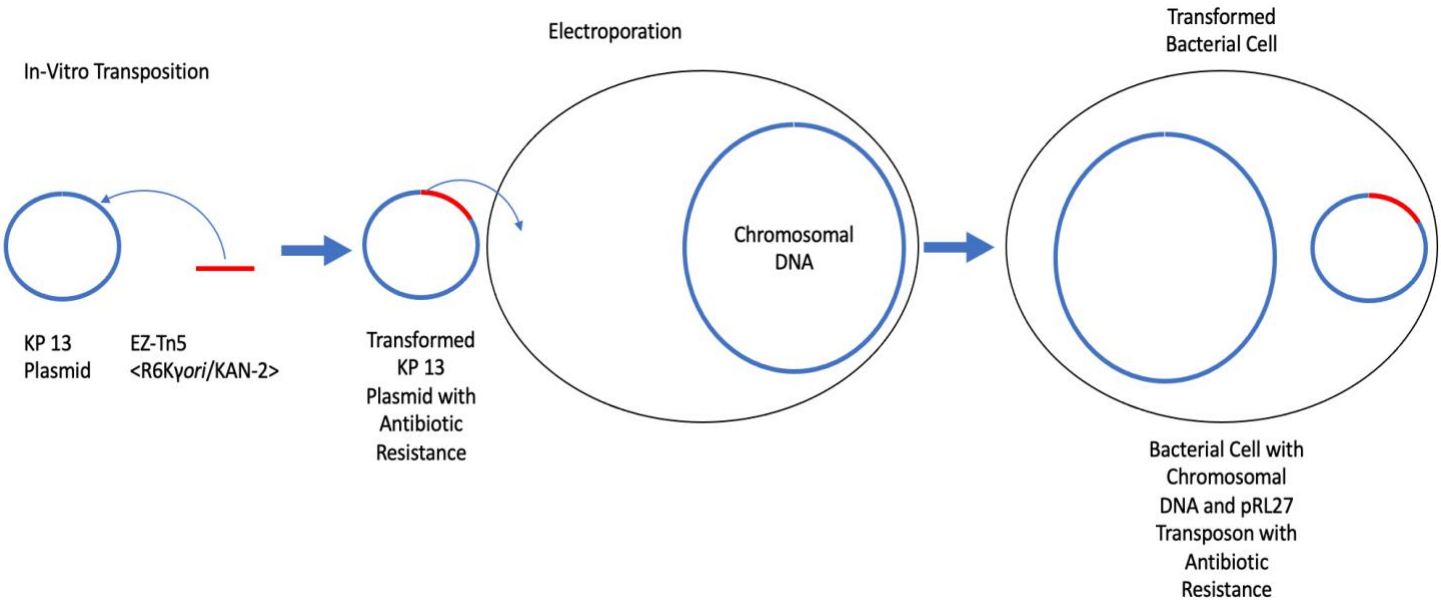


Figure 8: Process of In-Vitro Plasmid Mutagenesis to Transform a Bacterial Cell

Subsequently, cells grew on kanamycin agar and where spot tested with *Citrobacter freundii*, *Enterobacter aerogenes*, and *Enterobacter cloacae* to conclude if the transformed Mutant *K. pneumoniae* 13 plasmid could produce the protein and create zones of inhibition like *wild-type K. pneumoniae.* The transformed Mutant *K. pneumoniae* 13 plasmid in *E. coli* did produce the zones of inhibition (Figure 10), signifying that the plasmid was responsible for the antimicrobial protein. These zones of inhibition s were calculated and show that while there is reduced zones of inhibition from wild-type *K. pneumoniae*, the *E. coli* are now able to produce the antimicrobial *K. pneumoniae* protein. The sole exception of this inhibitory zones of inhibition towards *E. aerogenes*, where *E. coli* harboring the *K. pneumoniae* plasmid do not produce zones of inhibition.

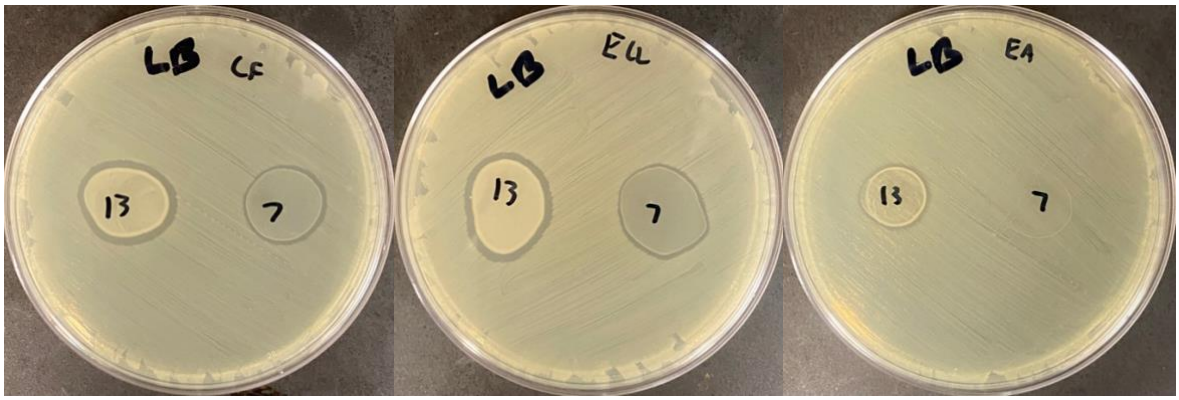


Figure 9: LB Plates Showing Zones of Inhibition with Lawns of *C. freundii*, *E. cloacae*, and *E. aerogenes* with *K. pneumoniae* 13 (13) and Mutant *K. pneumoniae* 13 Vector (7) Spot Tests

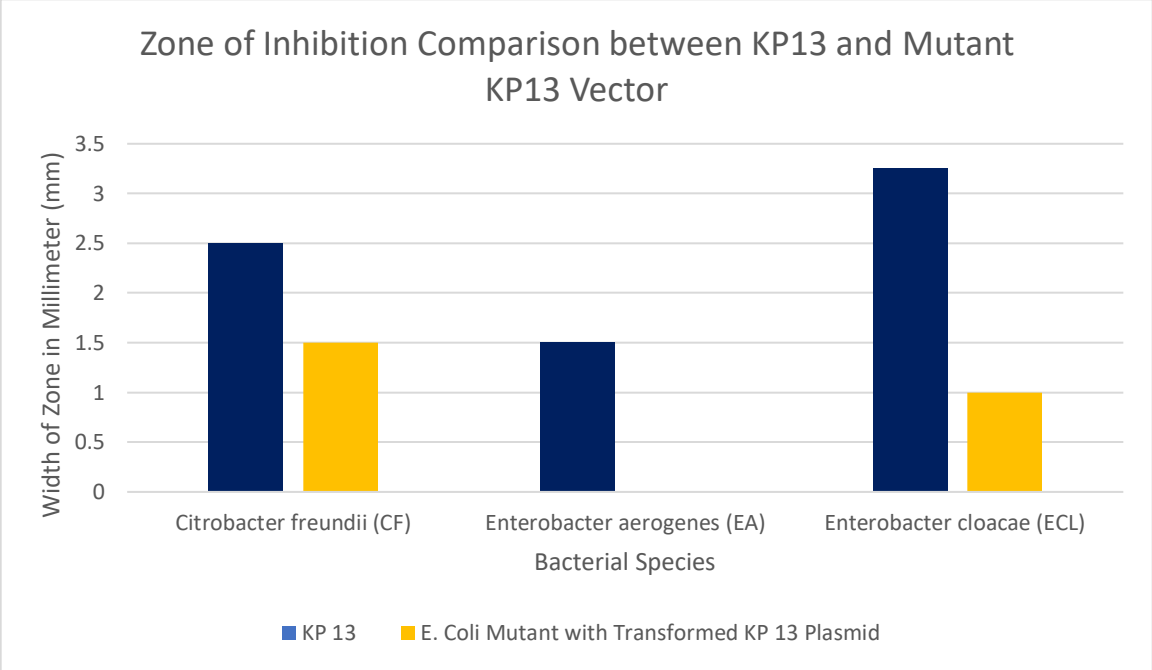
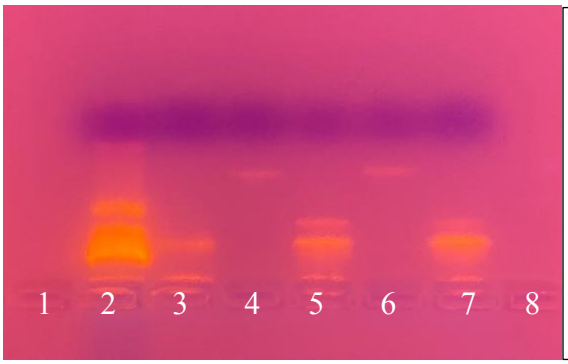


Figure 10: Comparison of Zone of Inhibition Width between *K. pneumoniae* 13 and Mutant *K. pneumoniae* 13 Vector with *E. aerogenes*, *C. freundii*, and *E. cloacae*

As shown in graph 2, there were are differing results regarding the size of the zone of inhibition. For *Citrobacter freundii*, the wild-type *K. pneumoniae* created a zone approximately

2.5 mm compared to the 1.5 mm of the Mutant *K. pneumoniae* 13 Vector. For *Enterobacter aerogenes*, which was the most surprising, *K. pneumoniae* 13 created a zone of inhibition of 1.5 mm and the Mutant *K. pneumoniae* 13 Vector did not create one at all. For *Enterobacter cloacae*, created a zone approximately 3.25 mm compared to the 1mm of the Mutant *K. pneumoniae* 13 Vector.

After this confirmation that the Mutant *K. pneumoniae* 13 Vector created zone of inhibitions, we created a mutant *K. pneumoniae* 13 vector library. Through screening, many different mutant *K. pneumoniae* 13 vectors exhibited different characteristics in wells 4 and 6 of Figure 7. Wells 4 and 6, respectively named samples A6 and A8, do not produce the zone of inhibition and are not similar in size to the wild-type *K. pneumoniae* 13 plasmid. Due to the sizes of samples A6 and A8 and their dissimilarity to the wild type, we thought that samples A6 and A8 would not be worth investigating further.



Well Contents (Left to Right)
1. NA
2. Lambda/Hin 3 Ladder
3. Wild-Type *K. pneumoniae* 13 Plasmid
4. Colony with no anti-microbial protein production (A6)
5. Colony with protein production (A7)
6. Colony with no anti-microbial protein production (A8)
7. Colony with protein production (A10)
8. NA

Figure 11: Gel Electrophoresis of Plasmids that had Undergone In-Vitro Transposon Mutagenesis

Biofilm Inhibition

To further test the efficacy of the mutant *K. pneumoniae* transposon plasmid within *E. coli*, we used a biofilm formation test. Biofilms were grown of *C. freundii*, *E. aerogenes*, and *E. cloacae* in 6-well plates in the presence of LB only, LB + wild type *K. pneumoniae* cell free broth, or LB + *E. coli* containing the *K. pneumoniae* plasmid cell free broth. Coverslips were added to the bottom of the wells so that we could examine the effects microscopically. Sample A7 (*E. coli* containing the *K. pneumoniae* plasmid) compared to the wild-type *K. pneumoniae* against *C. freundii* biofilms is shown in *C. freundii* in Figure 13, against *E. cloacae* biofilms in Figure 14, and against *E. aerogenes* biofilms in Figure 15. In all three bacteria tested, the controls in LB only have robust biofilm growth. When exposed to the antimicrobial protein supernatants of wild-type *K. pneumoniae* or the *K. pneumoniae* protein expressed from *E. coli*, the biofilms of *C. freundii*, *E. aerogenes*, and *E. cloacae* all decrease dramatically.

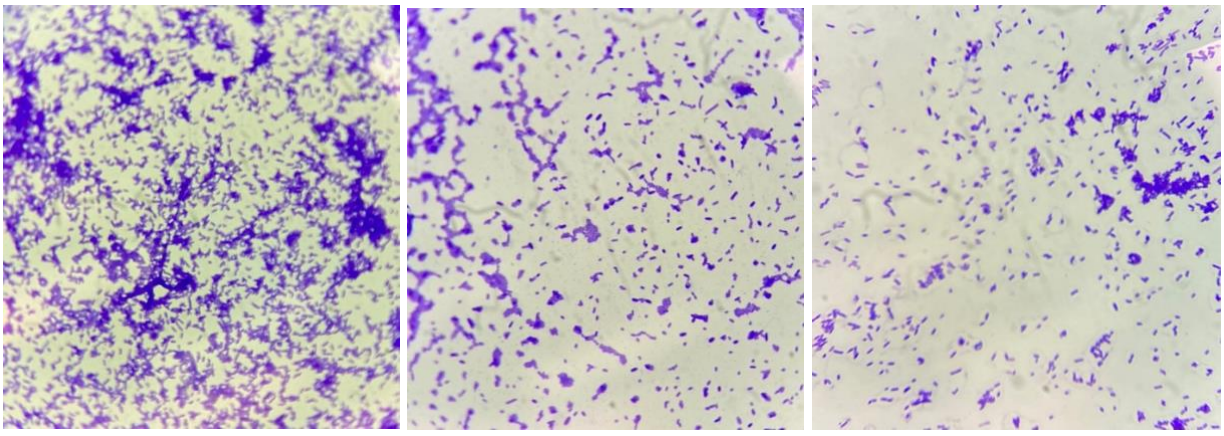


Figure 12: Biofilm Production of *C. freundii* with LB (Left), LB + *K. pneumoniae* 13 Protein (Middle), and LB + *E. coli* containing the *K. pneumoniae* plasmid (A7) (Right)

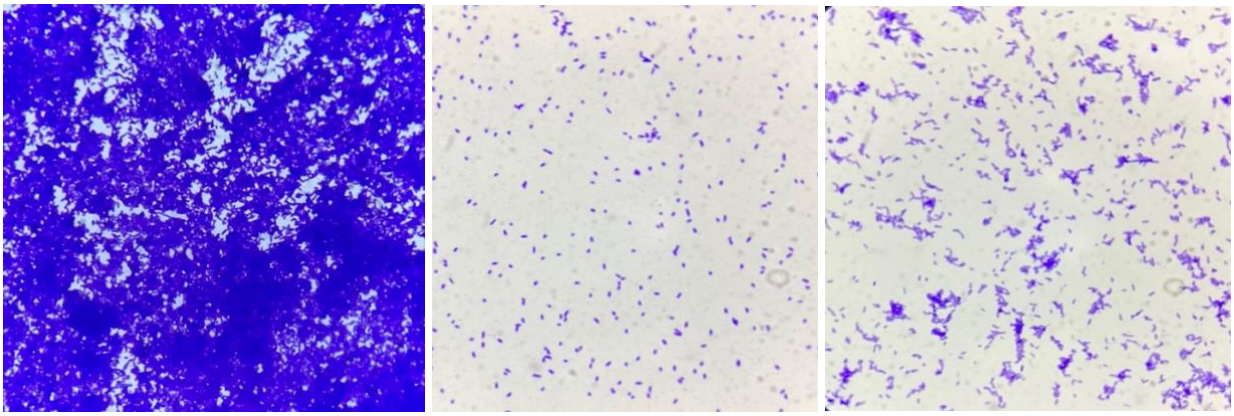


Figure 13: Biofilm Production of *E. cloacae* with LB (Left), LB + *K. pneumoniae* 13 Protein (Middle), and LB + *E. coli* containing the *K. pneumoniae* plasmid (A7) (Right)

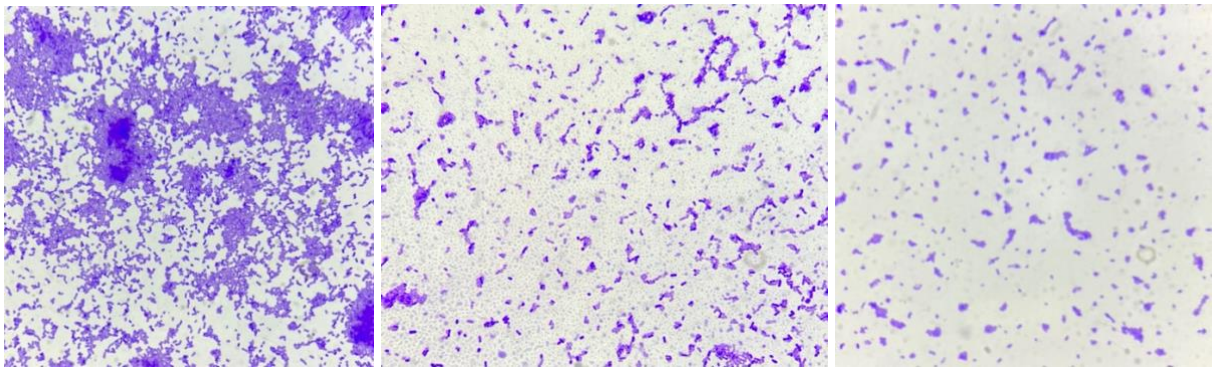


Figure 14: Biofilm Production of *E. aerogenes* with LB (Left), LB + *K. pneumoniae* 13 Protein (Middle), and LB + *E. coli* containing the *K. pneumoniae* plasmid (A7) (Right)

Plasmid Sequences

Since the known sequence of the transposon is now inserted into the plasmid and has verified phenotypic production of the antimicrobial protein, we can use the transposon to develop primers for sequencing the insertion site of the transposon and the gene disrupted. Two samples were sequenced, A7 and A10, and the resulting sequences were analyzed by NCBI BLAST

comparison. Below is the nucleotide sequence alignment of plasmid mutant A10 (Figure 16) and the protein alignment of A10 (Figure 19) and nucleotide sequence alignment of plasmid mutant A7 (Figure 18) and protein alignment of A7 (Figure 20).

EB13 Sequence

Query 1-50	TCTCTGAGTAGTCGACATGCAGGCATGCAAGCTTCAGGGTTCGAGATGTG
Query 51-100	TATAAGAGACAGAGCTCGGGGAGGAATAACAGCACCGGCATGGCCGGTGC
Query 101-150	TGGCGTTTATTTTTTGCCGGACGGCCACCACCGGCAGGCGGATTTGTCCT
Query 151-200	CCGGCAGTTCAGGCACCGGCACGTTAAAATAGCGGCTGTTATATTCCCGC
Query 201-250	ATCAGCTCCGCTTTCAGCTTCTCGTTCACCGCGCTTTCCCCGTGCCGCTC
Query 251-300	GCTGACCACCTCAATCATCGCGCTAATCTGCTCCGGTGAGCACTTCCCGG
Query 301-350	CATCAACCACCTGCACACCGTTCTCGATGGCGAACCGCTCCACCTTGCGA
Query 351-400	TAGAGGTAATCACACATGTCTTCATTCCTGCGGGTTTGTCCCGTTCCCAT
Query 401-450	TGCGGATTTTCACCGGTGAAAAAGTACGTGGCTTTTTCGTCGCTGTTGAG
Query 451-500	CCGCTCGTCATACATCCCGATGGATTTCATCATCGCCAGATGCTGGGCCC
Query 501-550	GGTCGACAAAACAGAAAACATACGCCCCGGTCTGCCAGGCGAGCACCAC
Query 551-600	CATATCTTCAAGATGCTCGCTGGTCGGCATGATGCAGCGCTCGCCGTCCC
Query 601-650	GGTCCTCCGCATCTTCAATAAATACCATGTACAGCGCCATAATCCCTCAC
Query 651-700	TCCGGTGGTCATAATTTTAAAGCACGCAAACAGCGGTTCACGATCCAGCT
Query 701-750	CGCCCGGTTTGCGGGCCCCGCCGGGTCTGTGGATCACGAATTCATTCACC
Query 751-774	GACAGGATCCCCGCTGCACCCTGAA

Figure 15: Nucleotide sequence of Transformed *K. pneumoniae* Plasmid A10

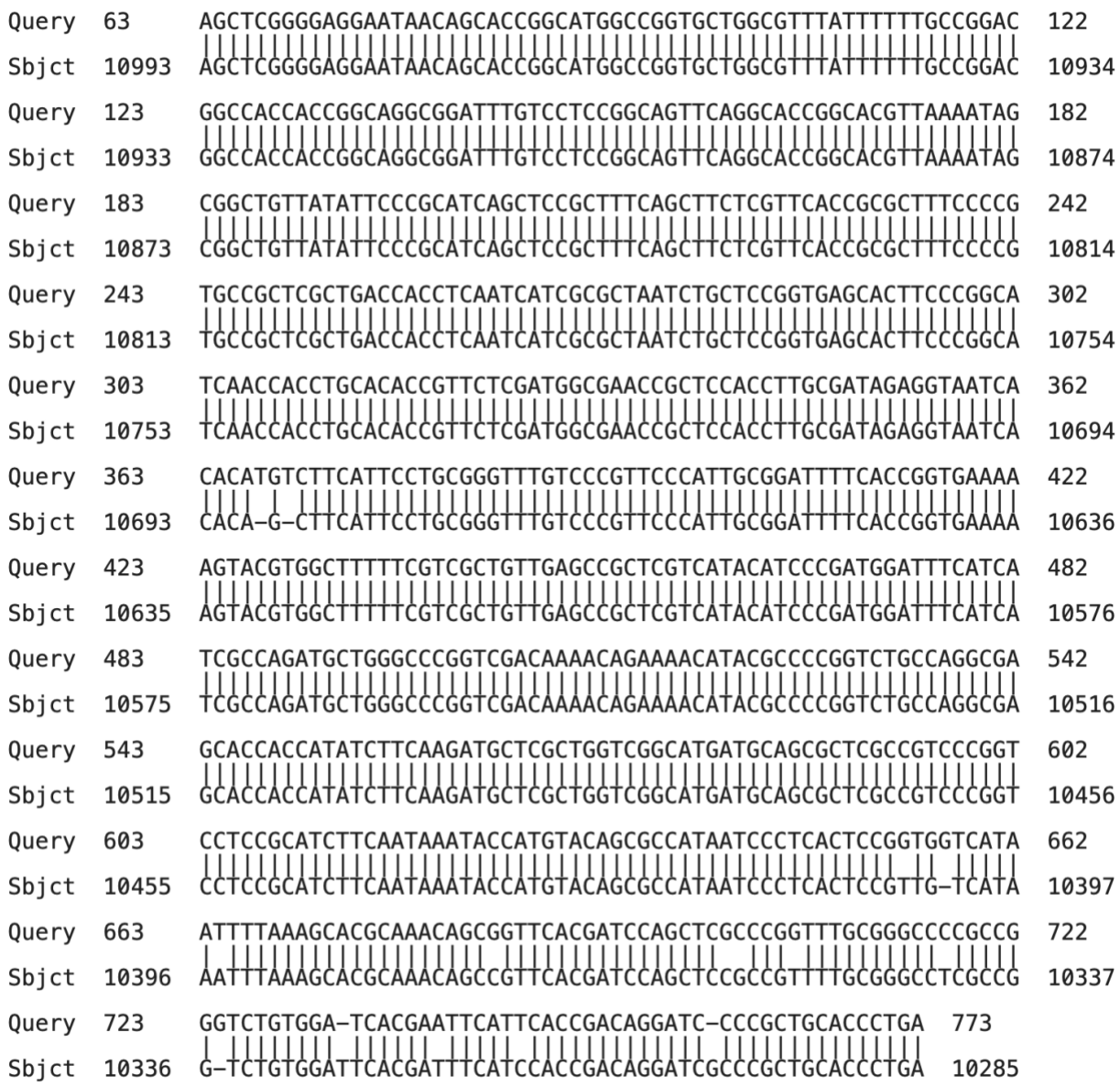


Figure 16: Nucleotide sequence alignment of A10 *K. pneumoniae* plasmid from NCBI BLAST comparison to the published *K. pneumoniae* plasmid sequence [18]

Using the BLASTN software, it was found that "*Klebsiella pneumoniae* strain FDAARGOS_775 plasmid unnamed1, complete sequence" has a 98% similar identity to EB13 with only 6 gaps [18].

EB73 Sequence

Query 1-50	AGTAGTCGACACTGCAGGCATGCAAGCTTCAGGGTTGAGATGTGTATAAG
Query 51-100	AGACAGTCCACGTTCCCCGCCTCCCGGCTGGCCCGGCGGCGCTCCACGTC
Query 101-150	CACCCGGATCTCCCCGGTCTGGCCCGCCGCCCACTGCGCGGACTCCTGGC
Query 151-200	TGGCCGCGCGATAGAACCAGCGGATCGGCGTGTTATCCAGTACCGTGGTT
Query 201-250	TTCACAAAATCAGCGGGCAGATCCTGGCCGCACTGGCCGAAGTCGCCCAG
Query 251-300	CGACTGGTGCGCGAGCAGCAGGTTGCAGTCCCGGCTGCGCAGCGTCCCCA
Query 301-350	GCGCGTTCAGCACGTATTTCGACAGCAGATATTTAATCTCATCGAGCATG
Query 351-400	ATGCTCGCGTGCGGCCACATCCGGAACTCGTCCCGCGCAATGATGATTTG
Query 401-450	CGCGCAGCGGGCAAACAGCATCTTCTGCACCCGGATCACCGCCTCATCGT
Query 451-500	CCATCGAGCCGATGACATACAGGCAGCCGCCGCCGTTGATTATCCCCTGC
Query 501-550	CACGTCGATGCCGCTGTCCGTCTGCAGGACGCTCAGGTCGGCCACGTTCT
Query 551-600	CCAGCCTGGNGATCAGCCCCTTCACGTTCTTTTTCAAGGGTCTCCGGCAG
Query 601-650	CGCGTACGCCGCCGCCAGCAACTGGCTGAATGTTTGCCCCCCTGCGGAAA
Query 651-700	CTGCTCCGCCAATCANCCTTCNCCGCTTTTTGCTCCCTGATACGGGATNT
Query 701-741	GTTTCCCACGCTTCCCGGTTGACCTCAGGTTAAATCTNGAC

Figure 17: Nucleotide sequence of Transformed *K. pneumoniae* Plasmid A7

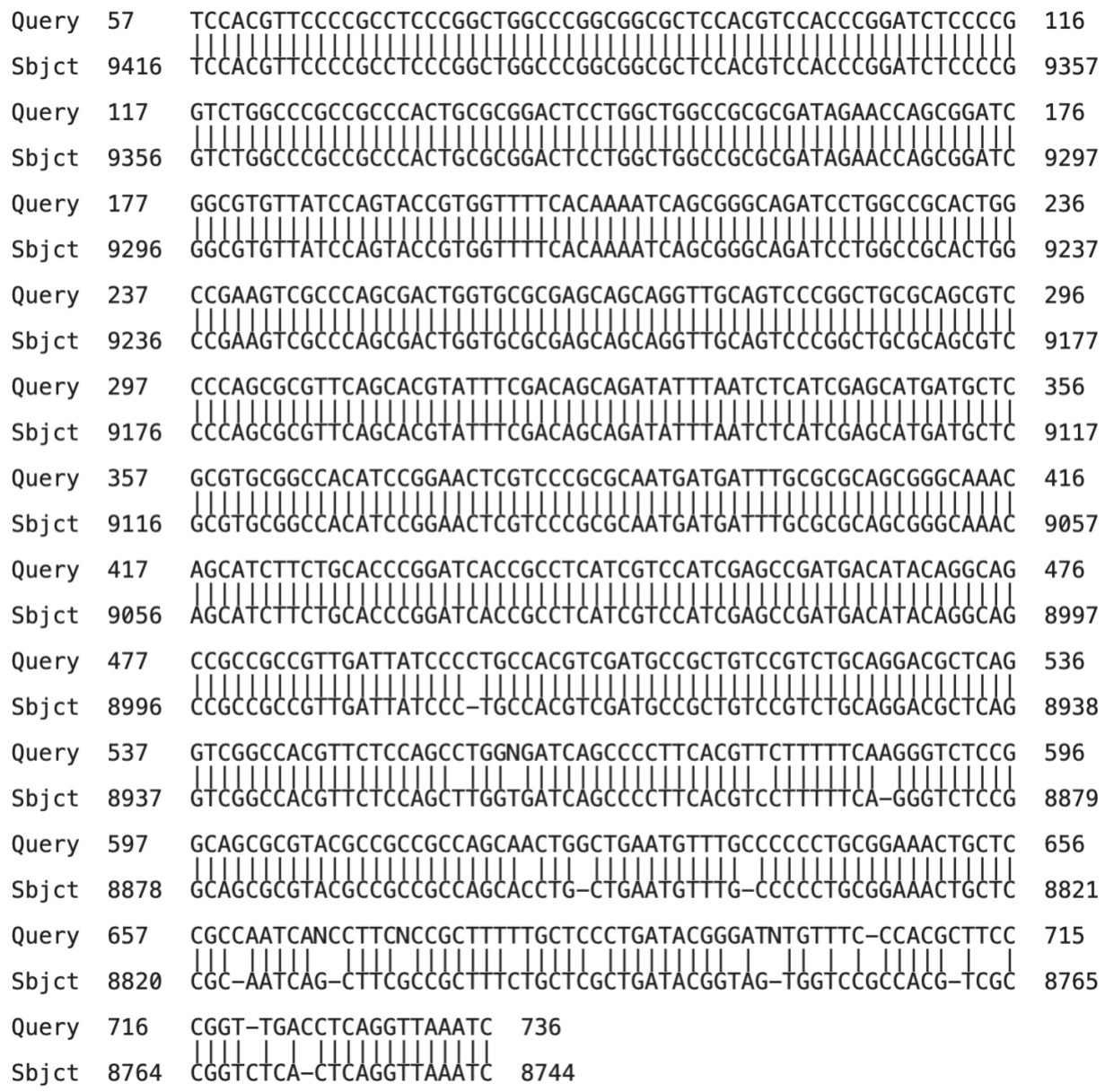


Figure 18: Nucleotide sequence alignment of A10 *K. pneumoniae* plasmid from NCBI BLAST comparison to the published *K. pneumoniae* plasmid sequence [18]

Using the BLASTN software, it was found that the same genetic code of "*Klebsiella pneumoniae* strain FDAARGOS_775 plasmid unnamed1, complete sequence" has a 96% similar identity to EB73 with only 11 gaps [18].

Possible Amino Acid Sequence of the Anti-microbial Protein

EB13

```
Query  639  MALYMVFIEDAEDRDGERCIMPTSEHLEDMVVLAWQTGAYVFCFVDRAQHLAMMKSIGMY  460
            MALYMVFIEDAEDRDGERCIMPTSEHLEDMVVLAWQTGAYVFCFVDRAQHLAMMKSIGMY
Sbjct  1    MALYMVFIEDAEDRDGERCIMPTSEHLEDMVVLAWQTGAYVFCFVDRAQHLAMMKSIGMY  60

Query  459  DERLNSDEKATYFFTGENPQWERDKPAGMKTC  364
            DERLNSDEKATYFFTGENPQWERDKPAGMK C
Sbjct  61   DERLNSDEKATYFFTGENPQWERDKPAGMKLC  92


Query  367  MCDYLYRKVERFAIENGVQVVDAGKCSPEQISAMIEVVSERHGESAVNEKLKAELMREYN  188
            +CDYLYRKVERFAIENGVQVVDAGKCSPEQISAMIEVVSERHGESAVNEKLKAELMREYN
Sbjct  91   LCDYLYRKVERFAIENGVQVVDAGKCSPEQISAMIEVVSERHGESAVNEKLKAELMREYN  150

Query  187  SRYFNVPVPELPEDKSACRWWPSGKK  110
            SRYFNVPVPELPEDKSACRWWPSGKK
Sbjct  151  SRYFNVPVPELPEDKSACRWWPSGKK  176
```

Figure 19: BLASTX Comparison of Sequenced Plasmid to "Uncharacterised protein [*Klebsiella pneumoniae]"* [19]

Using BLASTX, when the DNA sequence of EB13 was inputted, it found that "Uncharacterised protein [*Klebsiella pneumoniae*]" had a 98.91% similar identity with 0 gaps [19].

EB73

```
Query  721  QPGSVGNXSRIREQKAXKXDWRSSFRRGANIQPVAGGGVRAAGDP*KRT*RG*SPGWRTW  542
            + G V +  RI EQKA K      F +GANIQ V      A  +  K+  +G
Sbjct  22   ETGDVADHYRISEQKAAKL-IAEQFPQGANIQQVLAAAY-ALPEALKKDVKGLITKLENV  79

Query  541  PT*ASCRRTAASTWQGIINGGGCLYVIGSMDDEAVIRVQKMLFARCAQIIIARDEFRMWP  362
               +  +  +     GIINGGGCLYVIGSMDDEAVIRVQKMLFARCAQIIIARDEFR WP
Sbjct  80   ADLSVLQTDSGIDVAGIINGGGCLYVIGSMDDEAVIRVQKMLFARCAQIIIARDEFRRWP  139

Query  361  HASIMLDEIKYLLSKYVLNALGTLRSRDCNLLLAHQSLGDFGQCGQDLPADFVKTTVLDN  182
            HASIMLDEIKYLLSKYVLNALGTLRSRDCNLLLAHQSLGDFGQCGQDLPADFVKTTVLDN
Sbjct  140  HASIMLDEIKYLLSKYVLNALGTLRSRDCNLLLAHQSLGDFGQCGQDLPADFVKTTVLDN  199

Query  181  TPIRWFYRAASQESAQWAAGQTGEIRVDVERRRASREAGNVD  56
            TPIRWFYRAASQESAQWAAGQTGEIRVDVERRRASREAGNV+
Sbjct  200  TPIRWFYRAASQESAQWAAGQTGEIRVDVERRRASREAGNVE  241
```

Figure 20: BLASTX Comparison of sequenced plasmid to "TraM recognition domain-containing protein, partial [*Klebsiella pneumoniae*]" [19]

Using BLASTX, when the DNA sequence of EB73 was inputted, it found that "TraM recognition domain-containing protein, partial [*Klebsiella pneumoniae*]" had a 73.87% similar identity, 78% positive match with 2 gaps [19].

Given. The information of both BLASTXs, there could be a possible concluded size of the anti-microbial protein that is produced by the *K. pneumoniae* 13 plasmid. The average amino acid is 0.11 kDa [20], and given the information that EB13 has, there is a possible 174 amino acids and EB73 has a possible 241 amino acids. It calculates weights of 19.14 kDa and 26.51 kDa, respectively.

CONCLUSION

The conclusions from these results changed the aim of the project drastically. Our lab had preconceived notions that the gene responsible for producing the anti-microbial protein was integrated into the chromosomal DNA, but with the confirmation of a plasmid changed the process for characterizing the genetic components of the antimicrobial protein.

Subsequently after finding the plasmid, our lab had to ensure that it exhibited the gene responsible for the antimicrobial protein. To indicate a proper plasmid insertion, the isolated plasmid was exposed to a transposon to allow for integration into the plasmid and disrupting the gene sequence and antibiotic selection to differentiate proper electroporation and insertion. After the confirmation of the zones of inhibition, our lab wanted to compare the mutant *K. pneumoniae* 13 vector with the wild-type *K. pneumoniae* 13. As shown in Image 7, Image 8, Image 9, Image, 10, and Image 11, there are few differences between the mutant *K. pneumoniae* 13 vector and the wild-type *K. pneumoniae* 13, with the exception of the mutant *K. pneumoniae* 13 vector not able to inhibit *E. aerogenes* on a LB plate, but it could inhibit *E. aerogenes* biofilm growth.

Subsequently, two mutant *K. pneumoniae* 13 vector colonies were isolated and sequenced to get EB13 and EB73 shown in Sequence 1 and Sequence 2, respectively. Of course, these are only estimates and inferences of base pairs length and similarities to other sequences. In addition, from Sequence 1 and Sequence 2, the primary structure of the amino acid sequence can be inferred from the forementioned sequences downstream of the transposon insertion site, as shown in Possible Translation 1 and Possible Translation 2.

Possible future work could include, but not limited to, obtaining more sequences from isolated mutant *K. pneumoniae* 13 vectors, co-cultures comparisons between the wild-type *K. pneumoniae* 13 and vector *K. pneumoniae* 13 with *C. freundii*, *E. cloacae*, and *E. aerogenes*, and making a mutant *K. pneumoniae* plasmid that has the gene for the antimicrobial protein knocked out. In addition, there is also looking to understand the method by which the antimicrobial protein inhibits growth of other bacterial species. Since the *K. pneumoniae* 13 plasmid inhibits growth of *C. freundii*, *E. cloacae*, and *E. aerogenes*, which are all species genetically similar to *K. pneumoniae*, there could be more tests with *K. pneumoniae* 13 with other strains of *K. pneumoniae* and other species of bacteria to gain an understanding of similarity between affected species.

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