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Synergistic Inhibition of Resistant Enterobacteriaceae Using a Possible Klebsiella Secreted Bacteriocin with Broad-Spectrum Antibiotic

Andrew Robbins

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by

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Abstract

Due to the increasing prevalence of multi-drug resistant (MDR) bacteria, it is now important to begin the search for novel means of defending against such resistant infections. Enterobacteriaceae is a clinically relevant family of bacteria that has shown extensive resistance to many antibiotics, especially after biofilm formation. Inhibitory poly-microbial interactions within this family have been observed. It is known that *Citrobacter freundii* (CF) growth is significantly inhibited by *Klebsiella pneumoniae* (KP) through a secreted protein. In this study, the potential KP bacteriocin was screened for its inhibitory effects on CF at various phases of biofilm development. The suspected KP bacteriocin was also tested for its ability to decrease the dosage of antibiotics necessary to inhibit CF growth. Using spectrophotometric analysis, it was shown that the combined treatment of streptomycin and the KP protein allowed a decrease in the minimum inhibitory concentration of streptomycin needed from 50 μM to 32 μM. The combined treatment also yielded increased inhibition at the initial attachment phase of CF infection, as well as after biofilm development. The study uses the secreted KP protein to show the use of poly-microbial interactions within clinical applications. Future projects concerning this KP molecule can pursue the use of a *C. elegans* model to determine its efficacy in vitro.

Acknowledgements

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Introduction

Bacterial Disease:

Throughout the history of human civilization, thousands of different infectious diseases have caused widespread morbidity and mortality. Such diseases are caused by what we call pathogens, a term used to describe the millions of infectious organisms that can cause human disease. Pathogens are primarily bacteria and viruses, but also include parasites and fungi (1). These microorganisms are almost always unseen by the naked eye, but are universally found in our environment. Bacteria belong to the domain eubacteria, alongside archaea and eukaryotes, which comprise all other organisms. There are over 30,000 formally named species of bacteria, and this number continues to grow at a rapid rate (2). Due to their particular importance in medicine, such bacteria are being taxonomically classified which new emerging biotechnologies using genome phylogenies (3). The vast majority of bacteria are nonpathogenic towards humans, meaning they cause no detectable disease upon exposure. The relatively few bacterial species that are known to cause human disease also present a wide range of differences among the species, such as their reservoir(s), pathogenicity, and overall virulence. Disease may result from acute exposure to the bacterial species, or even from an imbalance of an individual's pre-existing microbiome, such as seen in the human digestive tract (4). The vast extent of morbidity and mortality caused by communicable bacterial diseases necessitates continued research in the field of pathogenic bacteriology and the search for effective treatments against such diseases.

Antibiotic resistance:

Antibacterial drugs, commonly known as antibiotics, are naturally or synthetically synthesized compounds that are selectively toxic towards bacterial cells, meaning they harm bacterial cells while having no effect on our human cells and tissues. German immunologist Paul Ehrlich described antibiotic compounds as "magic bullets", that are highly bactericidal yet

maintain host cell integrity (5). Antibiotic resistance refers to the ability of certain bacterial species to withstand the effects of certain antibiotic drug therapies. Different bacteria have exhibited resistance since the creation of some of the first antibacterial therapies during Erhlich's time. Penicillin, one of the most well-known antibiotics, was developed by Alexander Fleming in the 1940s. Despite the Nobel Prize winning drug being incredibly effective at killing a majority of bacterial species, Fleming soon observed strains that were no longer harmed by penicillin as early as 1945 (6). As the demand for such antibiotics drugs rose, the appearance of resistant strains began to increase. The mechanisms of how antibiotic resistance spreads is an area of ongoing research, but many strains are thought to have acquired resistance to a particular antibiotic drug through horizontal gene transfer. One common example is through conjugation or environmental uptake of a cellular plasmid which contains a gene that confers resistance to a given antibiotic (7).

In 2018 the Center for Disease Control and Prevention (CDC) reports that more than 2 million US residents will acquire a drug-resistant infection, and more than 23,000 may die from such infections (8). There are several factors contributing to the spread of antibiotic resistance, including the overuse of antibiotic drugs from healthcare professionals, patient non-compliance to the drug protocol, and the lack of monetary incentives for established pharmaceutical companies to dedicate research and development funding to discovering new antibiotic compounds. Due to the ever-increasing presence of resistant bacterial pathogens, other modalities of searching for bacteriostatic and bactericidal compounds needs to be considered. One such model, as presented in this research, is from an inhibitory molecule seen from the bacterial species *Klebsiella pneumoniae* (KP).

Enterobacteriaceae:

Enterobacteriaceae is a taxonomic family classification of bacteria. Within the *Enterobacteriaceae* family, there are approximately 200 species. These species share many common characteristics, such as their Gram-negative cell wall and their rod-shaped morphology, known as bacilli (9). Bacteria of the *Enterobacteriaceae* family are commensal symbionts, meaning they exist in our environment (or even on/within our body) and cause no detectable disease in humans. However, there are a particular handful of *Enterobacteriaceae* species that are widely recognized for their devastating contagious diseases they have caused throughout history. Such notable species include those of *Escherichia*, *Salmonella*, *Shigella*, *Citrobacter*, *Klebsiella*, and *Yersinia* (10). The degree of morbidity and mortality caused by the virulence of each of the *Enterobacteriaceae* species differs drastically. For instance, certain *Escherichia* and *Salmonella* species are known to cause thousands of cases of acute food poisoning each year, while *Yersinia pestis*, the causative agent of the plague, is noted to have a case fatality of 50% or more if left untreated (11).

In modern medicine, the *Enterobacteriaceae* family is of particular importance for its causation of several nosocomial infections. Nosocomial infections, also referred to as hospital-acquired infections, are those which originate from healthcare facilities, such as intensive care units within hospitals or other outpatient care facilities. The economic burdens of nosocomial infections are astronomical and continue to rise quickly. It has been cited that within the United States alone, nosocomial infectious collectively cost hospitals nationwide upwards of 50 billion dollars annually (12). Such nosocomial infections are often highly resistant to several common antibiotic drug therapies, further increasing their clinical and financial burden.

One notable genus within the *Enterobacteriaceae* family is *Citrobacter*. There are now more than 15 different species identified within the *Citrobacter* genus, many of which are found

throughout the environment in the soil and wastewater, and even within the human gut (13). Although the majority of the species are noninfectious to humans, species such as *C. koseri* (*CK*) and *C. freundii* (*CF*) are opportunistic infections, causing meaningful disease states in immunocompromised individuals. Among neonates, such infections can result in sepsis or meningitis. Among the elderly, immunocompromised, or immunosuppressed, *Citrobacter* infections can be present within the urinary tract, respiratory tract, wounds, bone, peritoneum, endocardium, meninges, and bloodstream (14). The most common among these infections are urinary tract infections (UTIs). There is no current vaccine therapy for infectious *Citrobacter* species. The majority of *Citrobacter* infections exist within a bacterial biofilm, where aggregates of bacteria exist within a complex carbohydrate structure that lends a greater defense against human immune cells, and better adherence to tissues. Similar to many other bacteria within the Enterobacteriaceae family, *Citrobacter* infections are often noted for their resistance to common antibiotic therapies. *C. freundii* often exhibits a higher degree of drug-resistance in comparison to *C. koseri* (15). A majority of *C. freundii* strains are resistant to antibiotic drugs such as ampicillin, carbenicillin, and cephalothin. In recent years, *C. freundii* has acquired resistance to newly developed drug treatments such as piperacillin, third-generation cephalosporins, and monobactams (16). β-lactamase production is also common among *Citrobacter* pathogens, further limiting the availability of drug choice for patients. It has even been observed that *C. freundii* encodes site-specific recombinases that utilize specific integrases to incorporate genes into their own chromosome that allow resistance to various drugs (17). Although many *Citrobacter* species contain gene sequences in the parental chromosome that confer resistance to certain antibiotics, such as the stated β-lactamase production, *Citrobacter* drug-resistance has spread and become common via the mechanism of horizontal gene transfer between bacteria (18). Horizontal gene

transfer involves the exchange of DNA between two, or more, bacteria within the same generation through conjugation, transformation, or transduction. *C. freundii* maintains a relatively high copy number of plasmids that lend further resistance to other drug therapies, making certain strains multi-drug resistant (MDR) or extensively-drug resistant (XDR) (19). Upon infection with a MDR *Citrobacter* species, such self-transmissible plasmids can be transferred throughout the interspecies population of infection via conjugation from donor to recipient cells, or through environmental uptake of the plasmid via transformation. The nosocomial pathogen's combination of a lack of effective drug treatments and preventative vaccines, alongside its high capacity for virulence among susceptible populations, demands ongoing and urgent investigation into the mechanisms of its drug resistance and development of new effective therapies to combat such infections.

Within the *Enterobacteriaceae* family is *Klebsiella,* which contains particular species that are of clinical significance, such as *Klebsiella pneumoniae*. *Klebsiella* species are found throughout nature, as well as part of the normal human flora of the nose, mouth, and intestines (20). Despite being a part of the normal human flora, *K. pneumoniae* also functions as an opportunistic pathogen, causing disease among very young, very old, or populations with pre-existing conditions. Many of these cases result from a hospital-acquired infection, as with other *Enterobacteriaceae* bacteria (21). A notable characteristic of *K. pneumoniae* is its ability to produce a heavy extracellular polysaccharide, which also lends to its smooth, shiny appearance upon culturing. The presence of its capsule plays a key role in its virulence, allowing it to adhere to many tissues and avoid phagocytosis by human immune cells such as neutrophils and macrophages (22). The sticky carbohydrate capsule lends *K. pneumoniae* the ability to adhere to many abiotic surfaces as well, seen clinically in the infection of medically-implanted devices. As

with many of *Enterobacteriaceae* species, *Klebsiella* infections are often present as a biofilm, where the capsule helps the bacterial population aggregate together to better evade immune cells and shield antibiotic targets (21). This tendency towards biofilm formation is an important virulence property exhibited in Enterobacteriaceae as well within multiple other pathogenic bacteria. Other clinical manifestations of *K. pneumoniae* infections may result in UTIs, pneumonia, diarrhea, meningitis, or sepsis (23). Currently, there is no vaccine for *Klebsiella* infections. Similar to *Citrobacter*, many *Klebsiella* species exhibit various degrees of resistance to common antibiotic drug therapies. Nosocomial *K. pneumoniae* infections are often cited as drug-resistant by mechanism of their drug-efflux pumps (24). Therefore, *Klebsiella* is another example of a genus within the *Enterobacteriaceae* family that indicates the need for better treatment against nosocomial Enterobacter infections.

Bacterial Biofilms:

Bacterial infections often exist within a "biofilm". A bacterial biofilm is a given population of bacteria, comprised of either the same or different species, growing on a living or inert surface or tissue (25). Planktonic bacteria are individual cells that are free-floating and unattached to any surface or other cells. This bacterial biofilm is the result of an aggregation of hundreds or thousands of planktonic bacteria coming together. The presence of the biofilm population, in comparison to individual planktonic cells, can greatly change many of the characteristics of the population, including its adherence to various surfaces and tissues, ability to evade host immune defenses, and heightened resistance to antibiotics. Recent analyses reveal that upwards of 80% of bacterial infections are the result of biofilm formation (26).

Biofilms formed from aggregated planktonic bacteria are highly complex and organized, leading to many emerging properties that benefit the entire population. It has been shown that when enough planktonic cells aggregate onto the same surface, gene expression changes among

certain bacteria within the population that leads to the production of sticky extracellular polysaccharides and other adhesive proteins (28). Such adhesive compounds result in a highly viscous and adherent biofilm. Biofilms have the ability to enter into a dormant state, where they are not able to be cultured yet remain viable, and will reactivate the population's metabolism upon the detection of a more favorable environment. Biofilms have been observed to exhibit motility, as they detach collectively or in clumps in order to further spread infection to other tissue or seek more favorable environments after a period of dormancy (29). Due to the highly viscous matrix surrounding established biofilms, host immune cells, particularly neutrophils and macrophages, are far less efficient at digesting the bacterial cells via phagocytosis. For instance, human macrophages preferentially digest planktonic *E. coli* cells with far greater efficiency than compared to *E. coli* cells that have aggregated into a biofilm (30). Likewise, the ability to tightly adhere to human tissues further compounds the ability to establish persistent, or chronic, infections. One notable example is the persistent *Pseudomonas* infection present in patients with Cystic Fibrosis. *P. aeruginosa* biofilms produce an alginate matrix, combined with other proteins and DNA, that is highly viscous and attached to the host's lungs (31). The strong adherence to the tissues in the lungs, along with the host's suppressed ability to combat the bacterial infection, facilitates a highly chronic infection that requires extensive therapies. Other common bacterial infections that are due to biofilm formation are infections present within dental caries, urinary tract infections, kidney stones, and endocarditis (32).

The greatest emergent property of bacterial biofilms, in terms of clinical significance, is their augmented resistance to existing antibiotic therapies. Even bacterial strains that have high susceptibility to certain antibiotics will become less susceptible when grown within a biofilm state (33). The bacterial population within a particular biofilm will be heterogeneous in terms of

their growth state, with cells ranging from exponential growth to cells within a stationary phase. Therefore, many antibiotic drugs that are growth-dependent will not be effective on the population within a stationary phase (34). Reports indicate significantly increased rates of such horizontal gene transfer within the biofilm, leading to the spread of plasmid or chromosomal DNA that will lend resistance to antibiotics (35). There are also several other notable methods through which antibiotic resistance is exhibited and spread throughout a biofilm that are beyond the scope of this paper. The ongoing clinical manifestations of resistant biofilms has led to ongoing research looking into novel therapies against such infections.

Klebsiella and *Citrobacter* exhibit high degrees of biofilm formation that plays a key role in their virulence. The capsule polysaccharide of *C. freundii* is essential to its ability to adhere to many tissues and implanted devices, resulting in various nosocomial infections that are often highly resistant to antibiotic therapies. Nosocomial *K. pneumoniae* infections are also due to bacterial populations within a fully established biofilm at the site of infection, further complicating treatments options (36). Therefore, investigation is needed to evaluate new methods of treating biofilm-based infections among species within the *Enterobacteriaceae* family.

Bacteriocins:

Bacteria often exist in hostile environments that only provide a limited amount of a space and resources for growth. Therefore, bacteria have evolved a variety of methods to outcompete their bacterial neighbors for the vital resources present within the surrounding environment. One effective method to compete for resources is the production of bacteriocins. Bacteriocins are polypeptides produced by the bacterial ribosome that inhibit the growth of closely related bacterial cells within the surrounding environment (37). Nearly all bacterial species produce a variety of bacteriocins. These toxic proteins have different bactericidal mechanisms, such as

being a potent nuclease, or a lytic peptidoglycanase, or others (38). Due to their high bactericidal nature, bacteriocins are continuing to be investigated for their potential applications in clinical medicine. Bacteriocins may have fantastic clinical results against bacterial infections due to their highly specific host range, or the cells they can inhibit, which mimics the selective toxicity of many natural antibiotic compounds. The high bactericidal activity and surprising stability of these secreted enzymes may lend a new alternative mechanism to fight against bacterial infections that are growing in their resistance to normal antibiotic treatments.

The current literature supports that isolates of *Klebsiella pneumoniae* infections usually exhibit a high degree of bacteriocin production. More specifically, it was found that these *Klebsiella* bacteriocins were highly effective against *E. coli* and *Shigella* strains, both of which are of the Enterobacteriaceae family (39). It may be possible that specific bacteriocins of *K. pneumoniae* have the ability to recognize and kill *Citrobacter* cells. This postulation aligns with the existing understanding of bacteriocins and the infectious nature of *Klebsiella* and *Citrobacter*, both of which would be competing to form biofilms at a site susceptible to infection. Crude extracts of Enterobacteriaceae infection sites have been shown to result in the lysis of many related Gram-negative species, thus it may be possible to isolate a specific bacteriocin peptide that has a narrow-spectrum effectiveness against *Citrobacter* (40). It has also been observed *in vivo* that combining both existing antibiotic drug treatments along with bacteriocin treatment has the ability to produce a synergistic effect in inhibition of certain bacterial strains. For example, it has been shown that lower doses of a broad-spectrum antibiotic drug are needed to inhibit *Staphylococcus aureus* strains, when used in conjunction with a known bacteriocin of *Staphylococcus* species (41). Therefore, following this same model,

utilizing selectively toxic *Klebsiella* bacteriocins may be useful in limiting the antibiotic dosage necessary to inhibit *Citrobacter* due to a synergistic bactericidal mechanism.

Streptomycin, a Broad-Spectrum Antibiotic:

Antibiotic drugs are broadly categorized as either being a broad or narrow-spectrum drug compound. This classification is based upon the specific bactericidal or bacteriostatic mechanism of the drug. Narrow-spectrum antibiotics are those which have a mechanism that specifically inhibits a certain structure within the bacterial cell (42). For instance, penicillin is a narrow-spectrum antibiotic which is used against Gram-positive bacterial infections, as its mechanism inhibits cell wall synthesis, resulting in lysis of the cell (43). Narrow-spectrum antibiotics are used when the causative bacterial species is known. The use of a narrow-spectrum antibiotic agent is desirable over a broad-spectrum drug in that it will only inhibit the growth of specific bacteria, and limit the amount of harm done to existing bacterial populations within the human microbiome. Electing for the use of a narrow-spectrum drug can also help lessen the spread of antibiotic resistant pathogens by eliminating the propagation of persistent cells that would survive the effects of a broad-spectrum drug (44). Broad-spectrum antibiotics work by inhibiting a structure or process that is common to nearly all bacterial cells. Consequently, these drugs are also highly effective in killing billions of other resident bacterial cells within our body. Those they do not kill are then highly resistant to the given drug. Therefore, it is extremely important to steward the use of broad-spectrum antibiotics in order to slow the expansion of bacterial resistance.

Streptomycin is one example of a broad-spectrum antibiotic drug, and the drug to be particularly investigated within the study. Streptomycin is a broad-spectrum drug in the sense that it inhibits the function of the bacterial ribosome, therefore interrupting the cell's ability to synthesize the proteins it needs for growth and metabolism. More specifically, Streptomycin

functions by binding to the 16s rRNA in the 30s subunit of the bacterial ribosome, thereby not allowing the initiator tRNA (N-formyl-methionine) from binding at the initiation step of translation (45). Resistance to Streptomycin has been observed by mutations within the gene for the S12 protein of the 16s rRNA, which make the rRNA strand of the ribosome no longer recognizable as the target compound by the Streptomycin molecule (46). Various species within the Enterobacteriaceae family have exhibited extensive resistance to Steptomycin. The typical prescribed treatment of Streptomycin costs more than $200 (47). In order to better steward the use of broad-spectrum antibiotics, here Streptomycin is used as a model drug to investigate whether dosage can be reduced by utilizing a potential KP bacteriocin in order to reduce the spread of resistant bacterial strains, preserve the patient's existing microbiome, and save money.

Present Work:

The main objectives of this research project are to determine the extent of CF inhibition yielded by the combined treatment of the KP molecule alongside streptomycin, and observe the synergistic effect of this combined treatment compared to streptomycin treatment alone. Another objective was to observe if it was possible to decrease the MIC of streptomycin when used in conjunction with the KP molecule, thereby limiting the selective pressure fueling spread of resistant bacteria. Since many of these resistant bacteria often exist within biofilms, it also tests the combined treatment's potential use even after a bacterial biofilm has been established. Ultimately, the study serves as a model for the characterization of other potential poly-microbial interactions that could be used clinical settings to combat resistant infections.

Materials and Methods

Microbial Growth and Standardization:

Klebsiella pneumoniae ATCC® 13883™ was the main strain used throughout the study to investigate the bactericidal effects of supernatant protein(s) present. *Klebsiella pneumoniae*

ATCC® 9997™ was used a control strain. These were tested against *Citrobacter freundii* ATCC® 8090™. KP and CF subcultures were both derived from refrigerated, pre-existing solid LB stocks. Both KP and CF were inoculated into liquid LB for growth.

Obtaining KP Supernatant and Molecule:

The compound of interest, the bactericidal protein, from the KP 13883 strain resides within the supernatant of the freshly grown cells. Aliquots from the fresh KP culture, were centrifuged at 10,000 rpm for 10 minutes in order to pellet out the live cells and leave the supernatant present in suspension. The supernatant was poured into a filtered 0.22-micron filter syringe that functioned to remove any unwanted cellular debris left over from the centrifugation process. After filtration, only the desired supernatant remained and was stored. The KP supernatant could be refrigerated at 4°C and still retain its bactericidal properties.

Initial Screening of Inhibition

From a fresh, overnight LB culture of CF, 10^6 cells were taken from each in order to standardize the concentration of the live cells present. These aliquots were measured for their optical density by using a spectrophotometer (OD_{600}). Based upon the given measurement for turbidity, the volume of the culture that contains 1 million (10^6) CF cells was normalized to OD_{600}= 0.01. This was repeated with each new trial to obtain 1 million cells.

For an initial screening of the inhibitory effects of the KP supernatant, a solid LB agar plate was inoculated with a lawn of CF. KP supernatant was tested for its inhibition by applying a 20μL spot to four various regions on the CF lawn and then incubated overnight at 37°C.

Kirby-Bauer Disk Diffusion for Susceptibility:

A traditional Kirby-Bauer disk diffusion protocol for determining a strain's susceptibility to a particular antibiotic compound was performed. The protocol involved growing a duplicate of

solid LB agar plates with a lawn of CF, and then the same for two plates with KP 13883 lawns. Antibiotics disks were aseptically placed onto the agar plates. There were four such antibiotic disks on each plate to avoid any interference from drug interactions. The antibiotics disks used were as followed: penicillin (P10), chloramphenicol (C30), bacitracin (B10), ampicillin (AM10), tetracycline (TE30), streptomycin (S10), erythromycin (E15), and kanamycin (K30). These four plates were allowed to grow overnight in the 37°C incubator. Pictures were taken and zones of inhibition of each disk, measured for their diameter.

Initial Screening for Effective Streptomycin Dosage against CF:

In order to observe if there was any synergistic effect of inhibition between the Streptomycin antibiotic drug and the KP molecule, it was necessary to determine the minimum inhibitory concentration (MIC) of the Streptomycin drug. Knowledge of the MIC of streptomycin would lend a general idea of what dosages would be effective at inhibiting CF growth alone, and if these could be improved by addition of the KP molecule to the treatment group. An initial screening for an estimate of the inhibitory concentration of streptomycin was conducted by placing a 20 μL aliquot of a 24-hour culture of CF into five x5mL. Doses of streptomycin were added to each tube at a concentration of 10, 20, 40, 60, and 80 μM, respectively. After overnight incubation, tubes were observed for turbidity and photographs were taken. From the tubes, 100 μL. of each was plated onto a LB agar plate and allowed to grow overnight at 37°C. The next day photographs of the plates were taken.

Determination of Efficacy via Plating Serial Dilutions and Colony Forming Units:

The increased efficacy due to the addition of the KP molecule to the streptomycin treatment was also examined by using plated serial dilutions of each of four groups: a control of only CF, CF with the addition of standard dosing of streptomycin, CF with both streptomycin

and the KP molecule, and also CF with the KP molecule alone. Each of the test groups were from a test tube containing 5 mL of fresh LB, and 1 million CF cells were added to each of the four groups. The second test group had 300 microliters of a 50 μg/mL streptomycin stock added, resulting in a 50 μM concentration. The third test group had the same concentration of streptomycin present, but also 2 mL of KP molecule added. The fourth test group had only the 2 mL of KP molecule added, and no streptomycin was present. After 24-hour growth, these cultures were then qualitatively assessed for turbidity, and comparative photographs were taken. A series of serial dilutions were performed in order to determine and compare countable, isolated colonies between each of the four test groups. This was done by taking 100 microliters of the culture and diluting into 900 microliters of PBS buffer (10^{-1}). 100 microliters of this dilution level was then plated onto a LB plate. This was then repeated nine times as 100 microliters of each dilution level was withdrawn and diluted into 900 microliters. 100 microliters of every other dilution level (10^{-3}, 10^{-5}, 10^{-7}, 10^{-9}) was plated onto a fresh LB plate. After 24-hour growth, these five serial dilution plates were then counted for isolated colony forming units present. Any plate with global growth present (200+ isolated colonies) was considered too many to count (TMTC). Comparative photographs were taken of each of the serially diluted plates.

Determining Minimum Inhibitory Concentration of Streptomycin and KP molecule:

Determination of minimum inhibitory concentration (MIC) of the treatments allowed for later comparison of the efficaciousness of singular drug treatment, or drug treatment alongside the KP molecule/bacteriocin. The determination of the MIC was performed on a 96-well microtiter well plate with decreasing volumes of streptomycin added from left to right, and decreasing amounts of the KP molecule added from top to bottom. After addition of 1 million CF cells to each of the 96 wells the KP molecule was added in decreasing volumes from top to

bottom. These volumes were decreasing increments of 25μL ranging from 200μL in row A to 50μL in row G, with row H serving as a control. Once the appropriate volume of the KP molecule was added to the rows of the plate, decreasing doses of streptomycin was added to these columns from left to right as to half the molarity of streptomycin in each column. All cells were volumized to 250 mL with LB. After 24-hour growth, the turbidity and number of live cells present in each well was measured using spectrophotometry. For determination of the turbidity of the well plate, OD readings were taken at a wavelength of 595 nm. Pictures were also taken. Since spectrophotometers simply measuring turbidity will be influenced by left over cellular debris from dead cells (such as those killed by the KP molecule, streptomycin, or both), it was necessary to determine a procedure to measure only the live cells present within the cell by the use of an MTT assay. Upon 24-hour growth of the plate, 20 μL of MTT was added to all wells present on the plate. After addition of MTT, the plate was placed into 37°C incubation for 30 minutes to allow the reduction of the MTT to occur by viable cells present. After incubation, 200μL of acidic isopropanol was added to all wells in order to dissolve the formed precipitate, thus forming the purple-colored solution from the formazan. After the addition of the acid, an OD reading was taken at 450 nm. Pictures were also taken after the MTT procedure.

Comparing Inhibition of Planktonic and Biofilm Populations of *Citrobacter*:

A protocol was performed to investigate the ability of the KP molecule to inhibit CF growth at different stages of biofilm development. This 2-day protocol compared the growth of CF on a control plate having only streptomycin added, and the other plate having both streptomycin and the KP molecule added. 100μL of fresh LB was added to all the wells of the plate, other than row B. 1 million CF cells was added to all wells in the plate. After addition of both the CF and the streptomycin, the control plate was volumized the addition of 1250μL of

fresh LB in order to determine the inhibition of growth by streptomycin alone. The experimental plate was instead volumized using 1250μL of the KP molecule. These plates were then allowed to grow for 24 hours in 37°C incubation. KP molecule and streptomycin added upon initial inoculation tested the efficacy at attachment. In order to investigate the efficacy of inhibition of the streptomycin and KP molecule after the CF biofilm phase, it required that the LB and 1 million CF cells were added to the well plate and then allowed to grow in incubation overnight. Once both the planktonic and the biofilm attachment phase plates had undergone 24-hour growth, pictures were taken to qualitatively assess turbidity. OD readings were taken using three methods per plate for both the planktonic and biofilm growth plates. For columns 1-4 of the plate, a simple OD reading at 595 nm was taken to assess the turbidity of the wells in these columns. For the columns 5-8 of the plate, another MTT procedure was performed by the addition of 10μL of 0.5mg/mL MTT and incubation in 37°C for 10 minutes. After incubation, 200μL of DMSO was added in order to dissolve the precipitate. After the MTT protocol was performed, an OD reading was taken at 450 nm, which allowed for the measurement of the live CF cells present within the well. In order to visualize the biofilm development present in the wells, a staining procedure involving 0.001% aqueous crystal violet (CV) stain was used in columns 9-12. Before addition of CV stain, the liquid was removed from the wells so that only the attached biofilm was left within the well. PBS was used to rinse unattached cells, and then 200μL of CV was then added and allowed to sit for 5 minutes. The CV stain was then removed, followed by a similar PBS rinse to remove unattached dye. This PBS was removed and the well was allowed to dry. Finally, glacial acetic acid was added to the well in order to solubilize the stain attached to cells within the biofilm. After this staining and washing, an OD reading was

taken at 595 nm. Pictures were taken both before and after the addition of the visualization reagents.

Biofilm Growth and Inhibition Imaging:

In order to visualize the growth of the CF biofilm present on a surface, a staining protocol was followed to allow for subsequent imaging of the growth. In the same testing groups as previously used, CF cells were exposed to treatment groups at both the attachment phase and the biofilm phase of growth. A cover slip was used to provide a surface for biofilm development. A Gram stain procedure was used to stain the bacteria. After drying, pictures were taken at 1000x magnification to help visualize the inhibition of the CF lawn.

Results and Discussion

Initial Screening of Inhibition:

Figure 1: Screening KP cell, KP molecule from supernatant, and lyophilized KP on a CF lawn

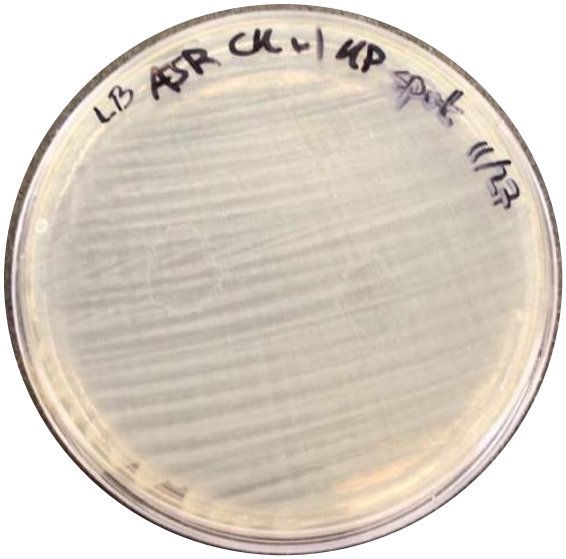


Figure 2: Screening of KP molecule on CK lawn showing no inhibition

Seen above are aliquots of the KP intact cell, the KP supernatant, and the KP lyophilized KP placed on a CF lawn (Figure 1). This figure shows screening for the efficacy of all three of the forms of the KP molecule. It was known that a secreted protein of the KP supernatant, referred to as the KP molecule, effectively inhibited the growth of certain species within the Enterobacteriaceae family. You can observe the increased ability to inhibit the CF when in the

supernatant or lyophilized forms. This supports the notion that this inhibitory compound is indeed a molecule housed within the KP cell, rather than the intact cell itself. However, certain species within the family were not sensitive to the KP molecule (Figure 2). The KP molecule lends no inhibition of *Citrobacter koseri*, a similar relative to *Citrobacter freundii*. Much of the antimicrobial range was established by Seth Jewett, a fellow ETSU Honors-in-Discipline lab mate, under the direction of Dr. Sean Fox. From his experimentation, Jewett observed that *C. freundii* and *E. cloacae* were highly sensitive to the KP molecule. *C. freundii* was confirmed to be highly sensitive to the KP molecule (Figure 1). Therefore, further investigation was pursued using *C. freundii* as the model organism for subsequent testing. As Jewett had also noted, *C. koseri* expressed no sensitivity to the KP molecule. Thus, investigating the action of KP molecule against *C. freundii* compared to its lack of inhibition against *C. koseri* may lead to further understanding the mechanism of the secreted protein.

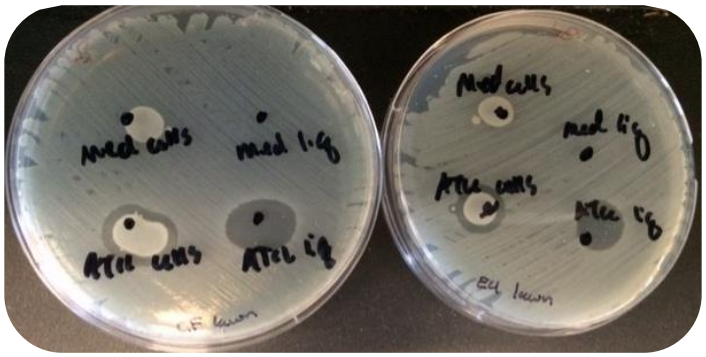


Figure 3: Comparisons of KP strain 13883 (bottom spots) and KP 9997 (top spots)

KP strain 13883 demonstrates the ability of the to inhibit the growth of both CF and *Enterobacter cloaceae* (EC) lawns (Figure 3). It shows that the ability to inhibit the growth of these species is specific to the 13883 strain, as the KP 9997 showed no inhibition of either species. Therefore, we knew that it was not a random mutagen within our laboratory that had caused this said inhibitory effect. Furthermore, it is interesting to observe how the 13883 strain is able to inhibit both CF and EC, yet not be able to inhibit *C. koseri* (CK). After genetic

investigation, it is known that CF and EC are genetically more similar to KP than CK is. This is further reason to believe that the KP molecule is likely to be a bacteriocin, as it inhibits those species that are most similar to itself. Therefore, it is logical to postulate that the KP molecule may be recognizing the genetically similar CF and EC cells by some shared recognition factor. However, the CK cells would not contain the said recognition factor, and thus would not be recognized as a competitor cell and there would be no inhibitory effect observed from the KP molecule/bacteriocin.

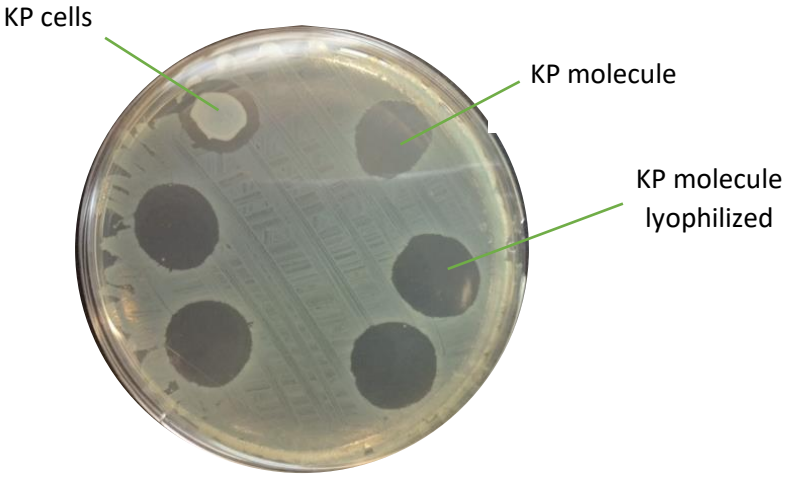


Figure 4: KP cells (top left), KP molecule (top right), and KP molecule lyophilized (bottom)

Various forms of the Klebsiella show differences in their inhibition of CF (Figure 4). The top left shows simply the intact KP cells which give a modest zone of inhibition. The top right shows the increased extent of inhibition whenever the KP cell is taken out through a centrifugation and filtering procedure to simply receive the supernatant (see methods). The supernatant of the KP has a heightened ability to inhibit the CF lawn (Figure 4). As we had suspected the KP molecule to be a bacteriocin, we were curious as to whether taking the KP supernatant through a lyophilization procedure would increase the inhibition of CF since the liquid portion of the KP supernatant would be removed. The lyophilized KP yielded even greater inhibition compared to both the KP cells and the KP molecule within the supernatant (Figure 4).

The observation of increased inhibition while within the supernatant form, and even more when lyophilized (liquid of supernatant removed leaving only solid molecules present) lends further evidence to the hypothesis that the KP molecule is indeed a bacteriocin.

Kirby-Bauer Disk Diffusion for Susceptibility:

Citrobacter freundii	
Antibiotic	**Zone of Inhibition (mm)**
Penicillin (P10)	0
Chloramphenicol (C30)	1.8
Bacitracin (B10)	0
Ampicillin (AM10)	0
Tetracycline (TE30)	1.6
Streptomycin (S10)	1.7
Erythromycin (E15)	0
Kanamycin (K30)	1.8

Table 1: Kirby-Bauer Results showing zones of inhibition for CF

Klebsiella pneumoniae	
Antibiotic	**Zone of Inhibition (mm)**
Penicillin (P10)	0
Chloramphenicol (C30)	1.8
Bacitracin (B10)	0
Ampicillin (AM10)	0
Tetracycline (TE30)	1.5
Streptomycin (S10)	1.6
Erythromycin (E15)	0
Kanamycin (K30)	2

Table 2: Kirby-Bauer Results showing zones of inhibition for KP

The intent behind performing a Kirby-Bauer test for antibiotic sensitivity/susceptibility was to establish which drug(s) would be beneficial to test for potential synergistic properties with the KP molecule. The drugs that would be potential candidates for further research are those which exhibit a slight zone of inhibition that indicates their susceptibility, but not large enough where the drug is already highly effective against the bacteria. Furthermore, it was important that CF was slightly susceptible to the drug so that a synergistic effect could be observed alongside of

the KP molecule. The antibiotic profile of KP was also obtained in order to ensure any results that were contrary to expectations were not due to a given antibiotic causing some sort of inhibition of the KP molecule itself. As observed from Table 1 and Table 2 (p.27), four such drug candidates resulted, chloramphenicol, tetracycline, streptomycin, and kanamycin. From the performed Kirby-Bauer test, each of these drugs exhibited a zone of inhibition that was greater than 0 mm yet still less than or equal to 2 mm. This measurement indicated their present, yet incomplete, inhibition of both CF and KP. Streptomycin was chosen as the drug to be tested for its potential synergy alongside the KP molecule into the inhibition of CF. The decision to test streptomycin as the drug of choice was due to knowledge of reported clinical cases of Enterobacteriaceae species, such as CF, having resistance to streptomycin (see more within introduction, pp. 14, 15).

Screening for Effective Streptomycin Dosage against CF

Figure 5: Determining Effective Streptomycin Dosage

In order to test whether addition of the KP molecule to a treatment protocol would allow for a decreased dosage of streptomycin, it was first necessary to determine the minimum inhibitory concentration (MIC) of streptomycin that is needed to inhibit the growth of a standard culture of *C. freundii*. After a review of current literature regarding inhibition of Enterobacteriaceae, we began by testing various concentrations of streptomycin dosages ranging from 10 μM to 80 μM followed by plating (Figure 5). As seen by the plating, there was an

obvious decrease in the growth of CF present on the 60 μM dosage compared to the 40 μM dosage.

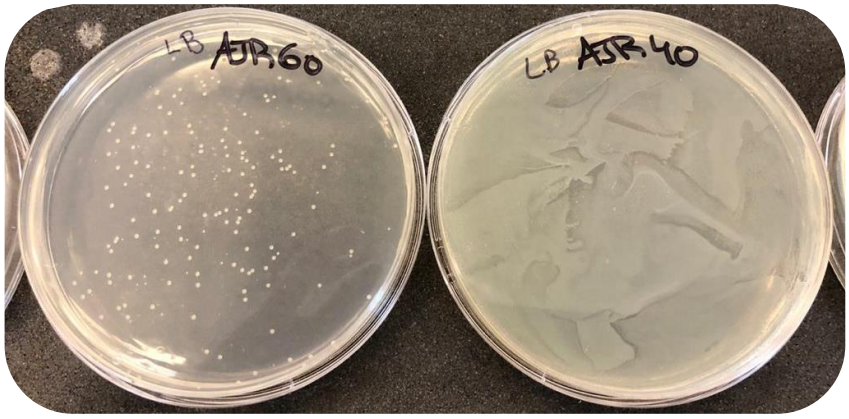


Figure 6: Plating Showing Estimation of Streptomycin MIC

There was a significant increase in the inhibition of CF as the streptomycin dosage was increased from 40 to 60 μM (Figure 6). Therefore, the test was effective at providing an estimation of the MIC for the streptomycin at 50 μM. This is an estimation of the minimum concentration of streptomycin in a dose that could provide effective inhibition against a standard culture of CF. This value could then be used a reference to see if the addition of the KP molecule to the treatment could decrease the concentration of Streptomycin needed within an effective dose and also lend an increased inhibitory effect through a synergistic mechanism.

Determination of Efficacy via Plating Serial Dilutions of Treatment Groups:

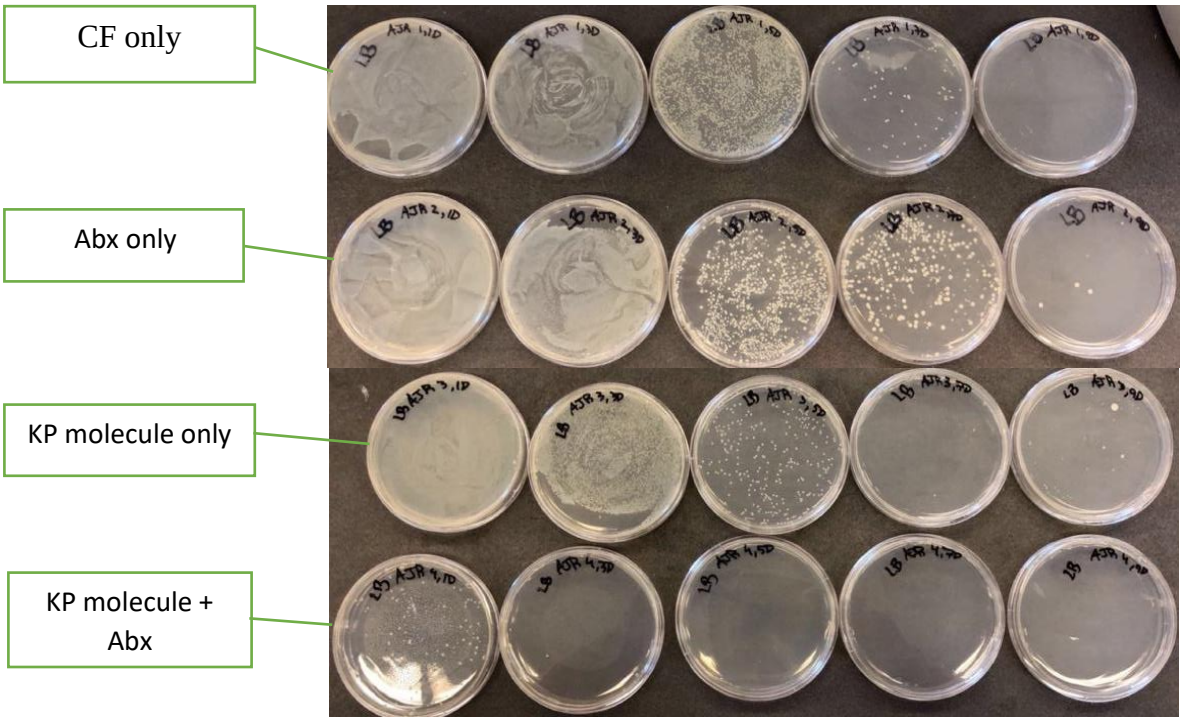
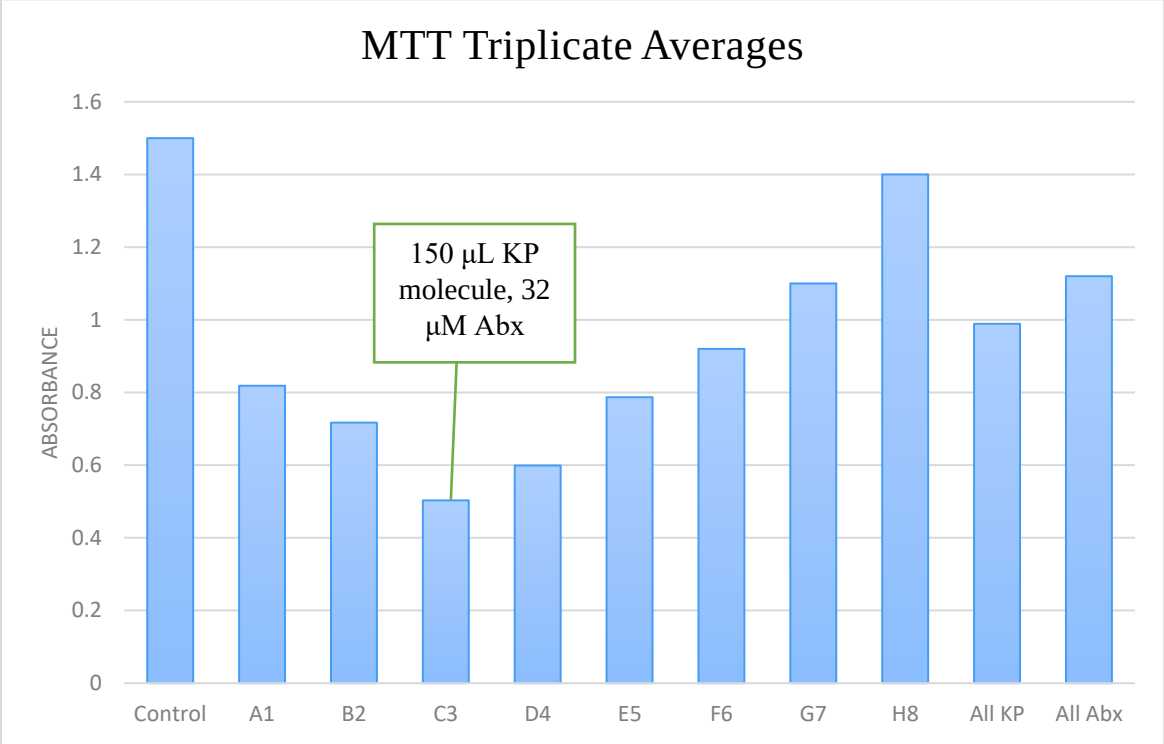


Figure 7: Comparing inhibition of KP + Streptomycin with KP alone and Streptomycin alone

The treatment of KP and streptomycin together is synergistic in some way, and yields a greater extent of inhibit of the CF present (Figure 7). The trials performed in Figure 7, such as the one seen above, had LB plates diluted out seven times in order to find countable colonies of CF. As you can see, the control with CF alone yielded colonies that were too high to count out to the fifth dilution at 1:10000, and created a solid lawn at the first dilution. The treatment of streptomycin alone and the KP molecule alone both also acted in a similar manner, still resulting in a dense lawn of growth at the first dilution level. However, when the treatment of KP molecule and streptomycin is combined, there is a drastic decrease in the growth of CF even at the first dilution level. Compared to the lawn of CF growth under the treatments of KP molecule and CF alone, the KP + Streptomycin yielded a heightened ability to inhibit the growth of CF. This was observed by having less than 100 countable CF colonies on the first dilution level (Figure 7). Therefore, such plating trials qualitatively demonstrate the ability of the KP molecule

and Streptomycin to have a synergistic ability to inhibit the growth of CF. The term “synergistic” inhibition is used as the combined treatment yields a greater extent of inhibition in comparison to each individual treatment by itself. Although these trials do not explain the mechanism of the inhibition, it clearly indicates its efficacy in vitro.

Spectrophotometric Analysis of Effective MIC of Streptomycin and KP molecule



Graph 1: Spectrophotometric reading following MTT procedure to find MIC

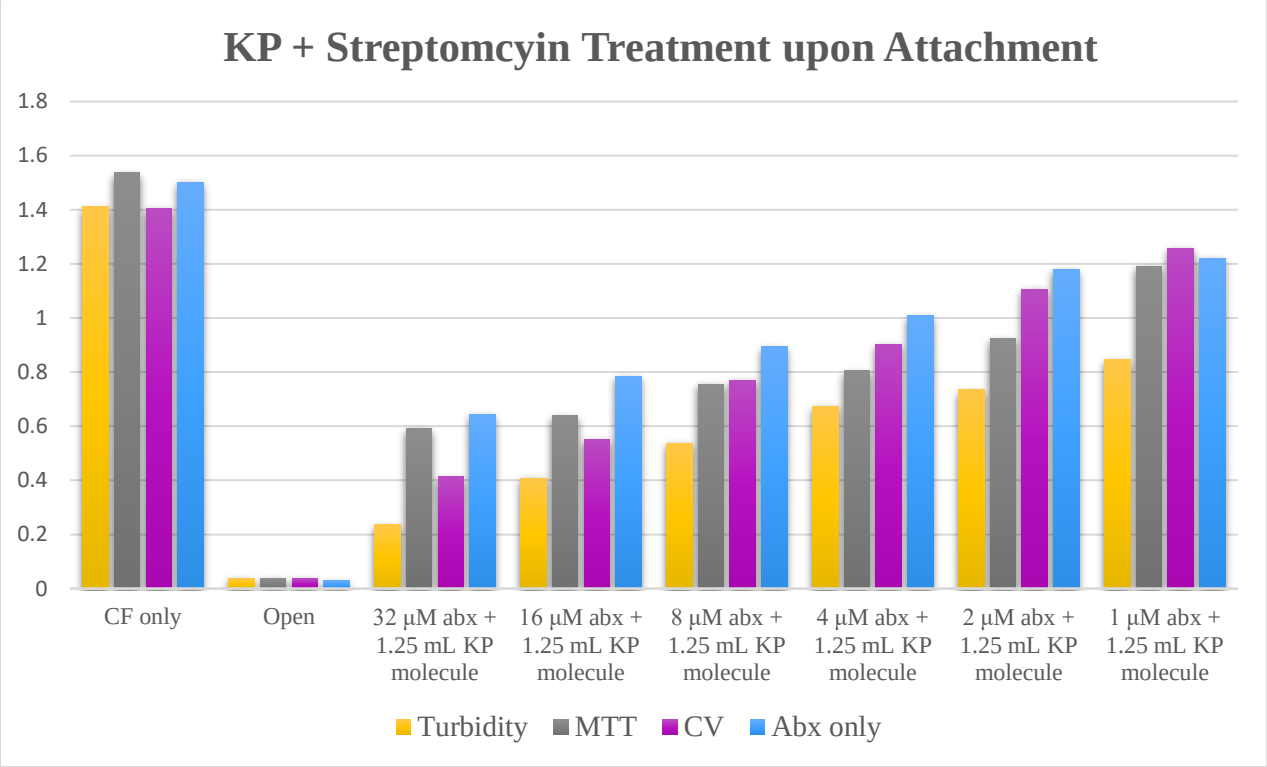
The minimum inhibitory concentration (MIC) is a general measurement of how much of a particular antimicrobial agent is needed to inhibit the visible growth of a given organism after an overnight incubation. Therefore, a MIC value that is lower indicates that a particular antimicrobial agent is effective at inhibiting the growth of that particular test organism. These values help clinicians better assign an appropriate dosage of antibiotics while treating a patient with a given infection. Failure to assign a dosage that at least meets the MIC for the particular drug and bacteria can expose the bacterial population to selective pressure that results in the hastening of the progression of persistent bacterial populations that are resistant. However, many

MIC's have not been established for given antibiotic/bacteria interactions. After observing that the combined treatment of KP molecule + Streptomycin was more effective than each of these treatments individually, it was necessary to determine the MIC of them combined.

Graph 1 shows a distinct decrease in the growth of CF in the fourth column labeled "C3". The absorbance from spectrophotometric analysis gave an absorbance value of approximately 0.5 on average, which is nearly less than half of the absorbance values given with KP only and Streptomycin only treatments (approx. 1.0). Recall that these trials tested the growth of CF at various combinations of KP molecule + Streptomycin combined treatment. Therefore, the absorbance value at the C3 indicates that there is some middle ground in the effectiveness of the combined treatment where it is more effective than there being a greater majority of nearly all KP molecule or nearly all Streptomycin in the treatment. C3 had a combined treatment of 150 μL of KP molecule and a streptomycin concentration of 32 μM. Columns A1 and B2 have a greater amount of KP molecule added than C3 but less streptomycin added, while columns D4-H8 have less KP molecule than C3 but more streptomycin added. Note that the combined treatment allows the MIC of streptomycin needed to inhibit the growth of CF drops from approximately 50 μM from initial screenings of streptomycin, down to at least 32 μM or less when combined with the KP molecule in an appropriate dosage. This indicates that CF is most effectively inhibited while the treatment cocktail is composed of moderate amounts of both KP molecule and streptomycin rather than a dosage of only one or the other. Furthermore, it is advantageous to limit the dosage of antibiotic drugs, such as streptomycin, needed to inhibit a bacterial infection. As seen from these trials, compounds such as the KP molecule can be effective in allowing a decrease in the MIC of certain antibiotics, thereby limiting the development of persistent multi-drug resistant bacterial populations.

This colorimetric MTT assay procedure was performed after initial readings of turbidity, as it is an irreversible end-point reaction that reflects the number of viable cells present. MTT (IUPAC name 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) is a yellow-colored tetrazole that is reduced to a purple-colored compound called formazan within viable cells. This reduction is carried out by oxidoreductases present in viable cells. Upon formation of the insoluble purple formazan, an acidic reagent is added to the well to dissolve the precipitate, allowing for photometric reading the purple-colored solution present.

Comparing Inhibition of Planktonic and Biofilm Populations of *Citrobacter*:

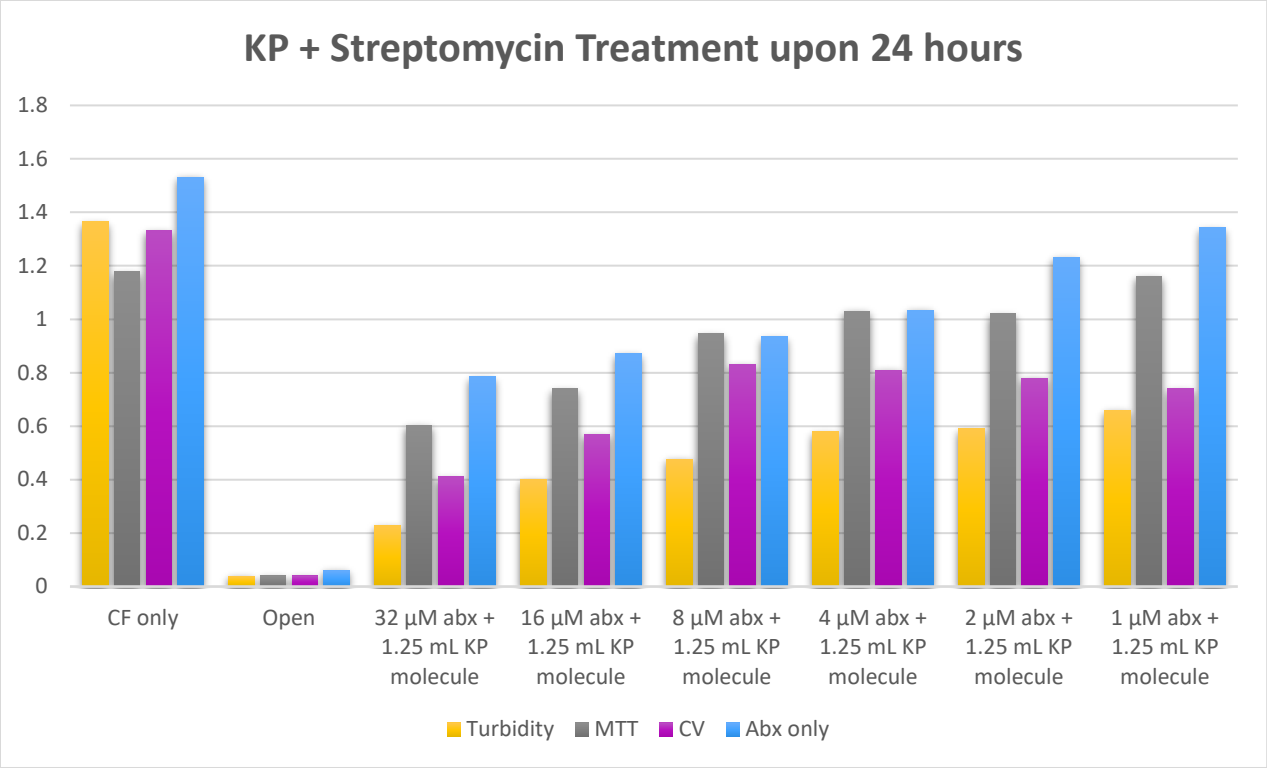


Graph 2: Measuring CF growth after KP + Streptomycin treatment at Attachment Phase

Graph 2 gives a graphical representation of the absorbance readings at various treatment levels with increasing amounts of Streptomycin concentration when treated at attachment phase of CF growth. The turbidity readings served to show the overall cell density of the CF growth, which includes both viable and dead cells. From Graph 2, the combined treatment of KP

molecule and streptomycin decreases the turbidity readings by more than half at the MIC value of 32 μM. The trend of decreases in turbidity at each treatment level follows this pattern even as the concentration of streptomycin used is decreased, although the magnitude of difference in turbidity between combined treatment and streptomycin treatment alone does decrease as the streptomycin concentration decreases. The MTT readings serve as an indicator of the amount of viable cells left remaining in the culture, as the MTT reagent is a substrate that only metabolically active cells react with, resulting in a distinct color that is measureable on the spectrophotometer. At the MIC of streptomycin (when in combined treatment with KP molecule) at 32 μM, the MTT reading is only approximately a third of the control reading. This indicates that not only does the combined treatment of KP molecule and streptomycin decrease the overall turbidity of the CF culture, it also decreases the proportion of these cells that are viable within the culture. This means that a significant amount of the cell density, measured as turbidity, is resulting from dead CF cells. The crystal violet (CV) staining procedure allowed a measurement of the estimated cellular mass accrued as the CF culture grew. It is known that CF infections often exist in a biofilm state, which increases the overall cell mass as exopolysaccharides are produced by cells within the developing biofilm. The CV stain was taken up by the cells and their cell wall and then rinsed, and then expelled through the lysing steps, which enables the extent of the CV stain released to be an indicator of the overall cell mass present within the culture. There were significantly decreased absorbance readings after CV staining under the combined treatment in comparison to the individual streptomycin treatment (Graph 2). This indicates the combined treatment is effective as inhibiting CF's ability to initially form a biofilm. The inhibition of biofilm formation is important clinically, as it allows the bacterial infection to

remain more susceptible to other antibiotic treatment options and be less adherent to other internal body surfaces or implants.



Graph 3: Measuring CF growth after KP + Streptomycin treatment at 24 hours

Graph 3 shows the same measurements as Graph 2, except the CF culture was exposed to the combined treatment of the KP molecule and the Streptomycin after 24 hours of growth. This 24 hours of undisturbed CF growth allows the bacterial populations to aggregate and form dense biofilms (see Figure 8a), which lends increased resistance to antibiotics and ability to resist human immune defenses. In a similar manner to the combined treatment in the attachment phase, the combined treatment after 24-hour CF growth yielded a significant decrease in turbidity when the MIC of streptomycin was administered (32 µM). When compared to the control, the 32 µM streptomycin and KP molecule treatment yielded a decrease in absorbance from approximately 1.3 to 0.2. Likewise, the combined treatment helped to decrease the density of the established CF biofilm. At higher dosages of streptomycin (closer to the MIC value) within the combined

treatment, the MTT readings of the CF growth indicated that a lesser amount of the cell density present was derived from viable cells. The CV staining procedure also yielded absorbance readings that indicate the biofilm that had been established during the initial 24 hours of growth was reduced in mass. However, the CV staining procedure is rather technique sensitive, as removing the staining and rinsing agents without touching the biofilm of the CF can be quite difficult. Further experiments investigating the effects of the combined treatment on established CF biofilms is needed to fully understand its extent and mechanism of inhibition. Despite technique limitations, the combined treatment of the KP molecule alongside an appropriate dosage of streptomycin does seem to effectively inhibit CF growth, even after the bacterial population has been able to establish a biofilm. The observed efficacy of the combined treatment makes the KP molecule an obvious avenue for further research into its interaction with other antibiotics, as well as its efficacy within an animal model.

Biofilm Growth and Inhibition Imaging:

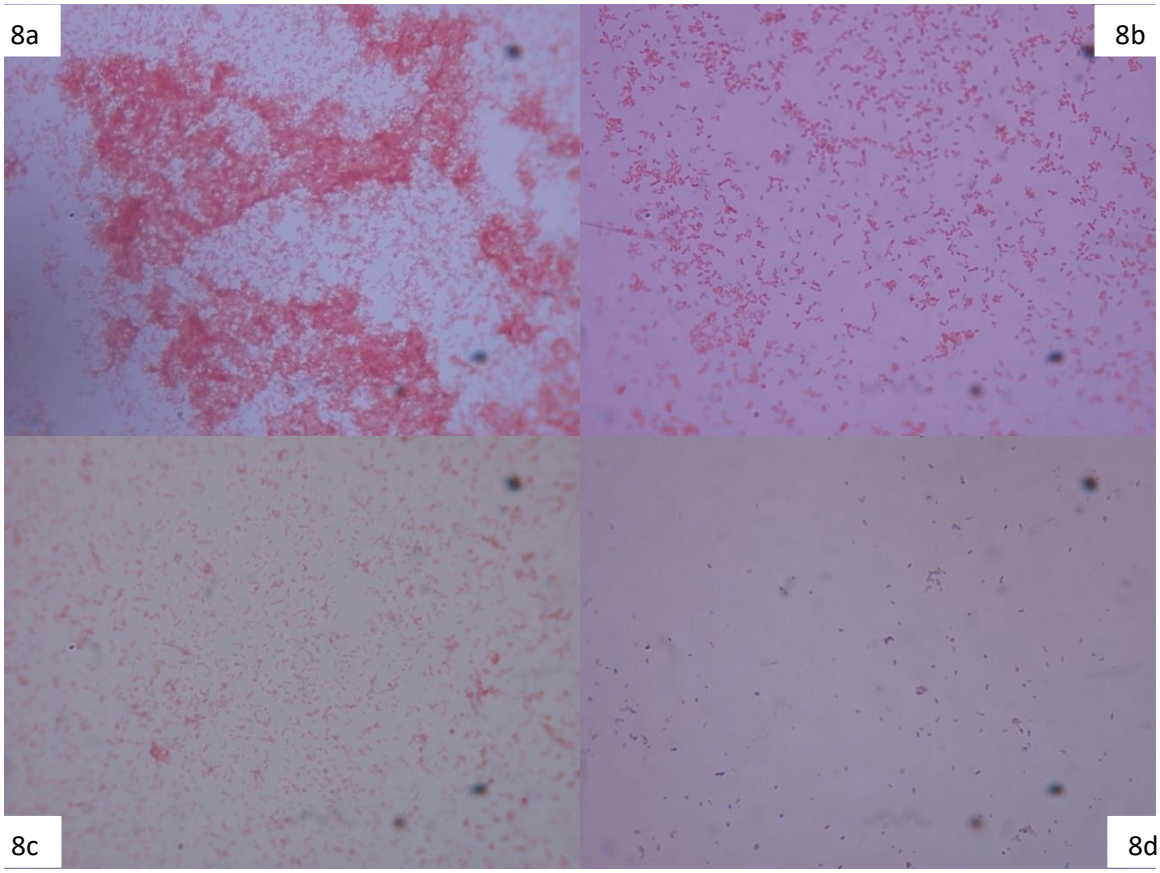


Figure 8: Microscopy revealing treatment outcomes after 24-hours CF biofilm growth; 8a) Control CF only; 8b) Streptomycin only; 8c) KP molecule only; 8d) KP molecule+Streptomycin

Light microscopy imaging was used to help visualize the increase in inhibition when there is CF exposed to a combined treatment of both KP molecule and a dosage of streptomycin (Figure 8). The CF lawns grown were allowed to grow undisturbed for 24 hours to allow the formation of a dense biofilm. The biofilm of uninhibited CF is seen well in 8a, where there are dense populations of aggregated bacterial growth. It is known these dense biofilms are of clinical importance, as they make the bacterial populations more resistant to antibiotics and other means of traditional treatments. Figure 8b (Streptomycin only) shows the moderate inhibition of the CF biofilm, while 8c (KP molecule) shows an even more pronounced inhibition than does the use of Streptomycin. However, 8d shows the nearly complete inhibition of the CF biofilm when the

bacterial population is exposed to a combined treatment of both KP molecule and Streptomycin. Figure 8 serves as a visualization aid to the data presented in Graph 3, which highlights the increased inhibition of CF biofilms when exposed to both the KP molecule and Streptomycin, as opposed to just one individual treatment. The combined treatment's ability to inhibit CF growth even after the infection has had adequate time to develop a dense biofilm further solidifies its potential as a valuable clinical treatment modality.

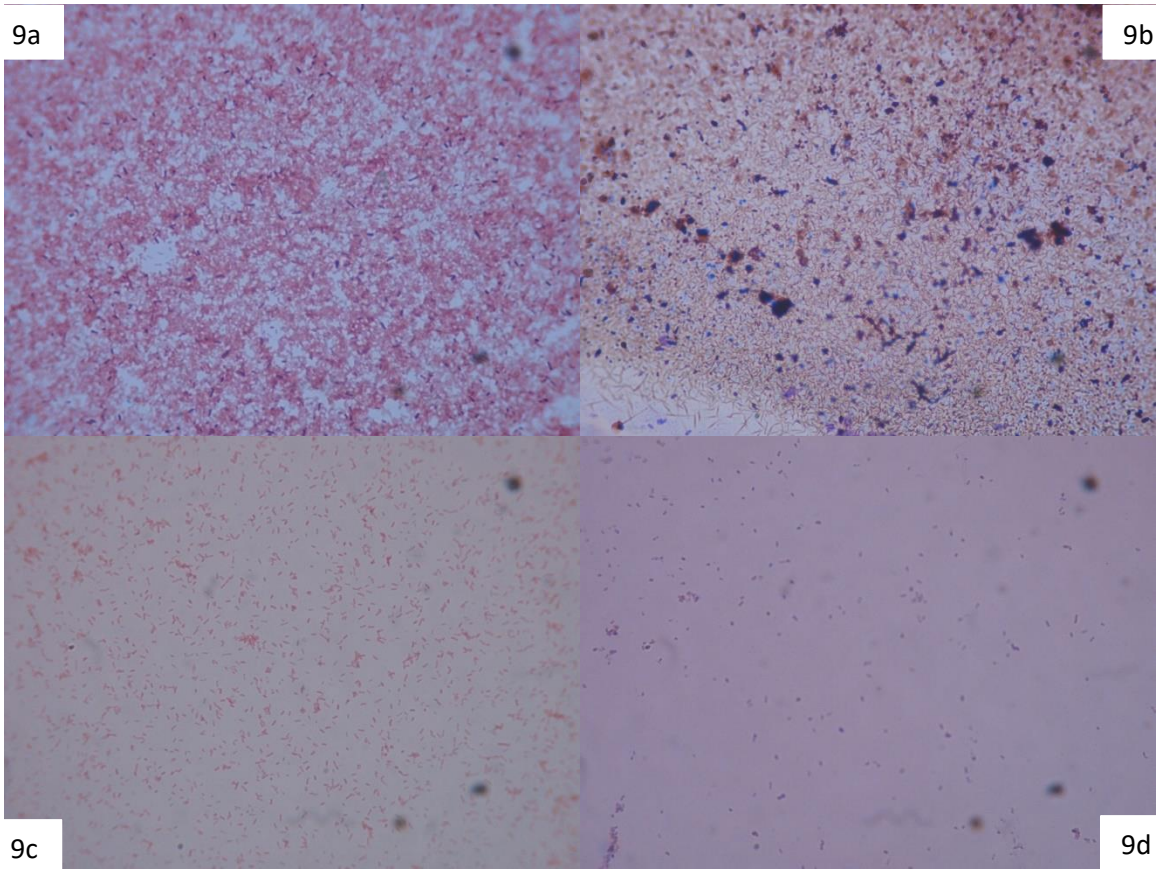


Figure 9: Microscopy revealing treatment outcomes at CF attachment phase; 9a) Control CF only; 9b) Streptomycin only; 9c) KP molecule only; 9d) KP molecule + Streptomycin

Light microscopy imaging was used again to help visualize the inhibitory effect of the combined treatment of the KP molecule and Streptomycin against CF at attachment phase (Figure 9). Figure 9a shows CF growth that has yet to develop dense biofilm populations. 9b (Streptomycin only) and 9c (KP molecule only) show the moderate ability of both Streptomycin

and the KP molecule to individually inhibit CF. 9d shows the nearly complete inhibition of the CF growth when the CF is exposed to the combined treatment of the KP molecule and Streptomycin together when used at the attachment phase of CF growth. Imaging supports the combined treatment's potential efficacy in clinical scenarios at multiple stages of CF infection, both before and after initial CF biofilm formation, as examined within this experiment.

Conclusions

Citrobacter freundii is simply one species among hundreds of pathogenic bacteria that continue to evolve greater resistance to several previously reliable antibiotic medications. Novel antibacterial compounds and treatment modalities must urgently be researched and developed to combat the effects of such resistant strains. Within this research presented, one means of combating resistant bacteria may be to incorporate antimicrobial compounds derived from competitor species. The research presented exhibits the use of a suspected bacteriocin from *Klebsiella pneumoniae* as one such inhibitory compound. Future needed research endeavors include investigation into the mechanism of the KP molecule and its efficacy within an animal model, as well as the search for similar inhibitory compounds from other bacterial species.

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