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Determination of the effects that a previously uncharacterized secreted product from Klebsiella pneumoniae has on Citrobacter freundii and Enterobacter cloacae biofilms

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Determination of the effects that a previously uncharacterized secreted product from *Klebsiella pneumoniae* has on *Citrobacter freundii* and *Enterobacter cloacae* biofilms

By

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Abstract

More so than ever, Multiple Drug Resistant (MDR) bacteria are on the rise due to overuse of antibiotics along with natural selection for adaptations that enhance drug-resistant properties. One particular bacterial family, Enterobacteriaceae, has been problematic, exhibiting several bacterial members that have developed a precipitous resistance to modern antibiotics and are also primary causative agents of nosocomial, or hospital acquired, infections. *Citrobacter freundii* (CF) and *Enterobacter cloacae* (ECL) are two species of the Enterobacteriaceae family causing significant medical concern due to their role in producing numerous opportunistic infections such as bacteremia, lower respiratory tract infections, urinary tract infections, and endocarditis. Adding to the difficulty of this situation is the ability of bacteria to produce biofilms. These biofilms are communities of bacteria that exhibit increased resistance to antibiotic treatment and eradication. Previous work in the laboratory of Dr. Fox at ETSU has identified an uncharacterized product secreted by *Klebsiella pneumoniae* (KP), another member of the Enterobacteriaceae family, which appears to have inhibitory effects toward CF and ECL. The current study was designed to characterize the effects this secreted product has on CF and ECL biofilms. Through a high throughput microtiter plate assay, the effects of this secreted product were examined on CF and ECL phases of biofilm attachment and maturation. Based on our findings, we have concluded that this secreted product can be categorized as a possible bacteriostatic agent against biofilm cell density, biofilm mass, and cell viability for both biofilm

phases of attachment and maturation. These results demonstrate the potential for future antimicrobial applications of this product for CF or ECL infected patients.

Introduction

Bacterial Biofilms

Microorganisms, much like humans, are subjected to the mechanisms of evolution, such as natural selection. As such, over time, there have been many diverse and unique adaptations developed among the microbial community. One such way that bacteria has adapted to survive when conditions are less than favorable is to mass together and form a slimy film on a wide variety of different surfaces. We call this collaborative phenomenon biofilm production.

We can define a biofilm as an assemblage of microbial cells that is irreversibly associated (cannot be removed by gentle rinsing) with a surface and enclosed in a polymeric matrix [1]. Structurally, a biofilm consists of numerous subunits, referred to as microcolonies. These microcolonies reside within a matrix of extracellular polymeric substances (EPS) with a close proximity to each other. This, in turn, provides the bacteria with an ideal environment for the creation of nutrient gradients, gene exchange, and quorum sensing activities [1]. Biofilms are amorphous and dynamic complexes that can also provide a community of bacteria with adaptive resistance to antibiotics [2]. This aspect is especially important due to the worrisome possibility that standard antibiotics that would typically inhibit or kill bacteria in their planktonic, free-swimming state fail to have the same effect once a biofilm is produced [3,4]. Also, when compared to free-swimming bacteria,

biofilms are much better adapted to withstand adverse environmental conditions such as nutrient deprivation, pH changes, oxygen radicals, and even biocides [5]. These adaptive benefits are only possible due to communities of bacteria joining together to form coordinated and cooperative groups that, some say, appear analogous to multicellular organisms [6].

The developed tolerance to antibiotics alone makes it easy to see the evolutionary benefits of biofilm production. The communal setting of a biofilm illustrates the capability of bacteria to function cooperatively and altruistically for their survival. The results of this cooperation prove more efficacious for bacteria's prosperity than simply existing in a planktonic form. As such, these bacterial slime layers are now everywhere. Biofilms have become ubiquitous in natural, industrial, and most worryingly, clinical environments [7]. It is now accepted that, between this ubiquitous nature of biofilms and estimates that approximately 90% of bacteria exist in biofilms, living in groups is critical for a bacteria's ecology and evolution [5,6]. The general competition among bacteria, even of the same species, can often result in a "kill or be killed" methodology. However, in biofilms, it is observed that some bacteria within the biofilms decrease their own reproductive output in order to increase the fitness of other cells. This can thereby benefit the biofilm community as a whole rather than just an individual organism [6].

Bacteria are able to produce biofilms and subsequently work together as a microbial community due to a secretion-detection mechanism called quorum sensing (QS). The process of QS provides a mechanism for self-organization and regulation of microbial cells. This involves an environmental sensing system that

allows bacteria to monitor and respond to their own population densities [3]. Via the production of diffusible organic signaling molecules, known as auto-inducers, bacteria are able to communicate with each other at high enough population densities to result in a change of gene expression [2,3].

Once the QS mediating signaling is achieved, the microbial community can begin the process of biofilm production. At present, processes governing biofilm formation that have been identified include: 1) the pre-conditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface; 2) Transport of planktonic cells from the bulk liquid to the surface; 3) Adsorption of cells at the surface; 4) Desorption of reversibly adsorbed cells; 5) Irreversible adsorption of bacterial cells at a surface; 6) Production of cell–cell signaling molecules; 7) Transport of substrates to and within the biofilm; 8) Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm; and finally 9) Biofilm removal by detachment or sloughing [3]. Simplified, the process can be condensed to 4 main steps: preparation, attachment, maturation, and dispersion. Depending on the bacteria, there are numerous alterations or additions that can be made with each step as well as multiple mechanisms that can aid the bacteria in achieving their optimal conditions for biofilm production. For example, some bacteria have pili or other extracellular filamentous appendages that aid in attachment [3] while others may rely on the assistance of extracellular DNA or lipids to do the same [5].

It is worth revisiting that all of the processes mentioned above, from attachment onward, are occurring embedded in an EPS comprised matrix.

Proportionally in biofilms, the microorganisms account for less than 10% of the dry mass, whereas the matrix can account for over 90% [3]. This conglomeration of different biopolymers and water aids in the cohesion of the cells of the biofilm, adhesion to the surface they are bound to, as well as diffusion of various bacterial products [3,8]. Also, when the surrounding environment's nutrients have been depleted, there are enzymes produced by the bacteria of the biofilm that can break down the biopolymers of the EPS. This provides the bacteria with immediate access to carbon and energy sources when needed [8]. This mechanism of storage can sustain a biofilm rather efficiently when considering the fact that 50-90% of biofilms carbon contents resides in the EPS matrix [1].

The efficiency and resilience that biofilms provide bacteria illustrated thus far effectively shows how they could be highly problematic in a clinical setting. In recent years, there have been growing concerns about the ability of biofilms to adhere and flourish within a patient, either on their internal natural body surfaces, such as the mucosal epithelial linings for many of the body's tracts, or on the abiotic surfaces of indwelling medical devices (IMDs) [4]. With their adapted resistance to many conventional antibiotic treatments, bacterial biofilms are able to survive the standard treatments that physicians traditionally would use. As such, these biofilms can eventually lead to clinical bacteremia or even chronic infection [2,4].

With the expanding research into the capability of biofilms to bind to internal body surfaces, we are now able to identify multiple bacterial pathologies that owe their persistence to biofilm formation. Biofilms have been linked as contributive agents from something as simple as infection of a mild laceration to chronic

inflammatory lung conditions, such as cystic fibrosis [5,7]. Other, more benign, conditions such as urinary tract infections or middle ear infections can also have biofilms contributing to their severity [4].

Due to the numerous resident bacteria within a biofilms' constant exchange of resistance plasmids, biofilm aggregates have greater genetic diversity than standard planktonic bacteria. The secretion of endotoxin achieved by biofilm bacteria aid in making them more virulent. Factor these facts in with the reduced susceptibility to the antimicrobial agents that would traditionally eradicate the planktonic form of the same bacteria, and the treatment of these otherwise brief infections becomes much more difficult [1,7]. There has even been evidence to support the claim that clinical biofilms can even acquire the ability to evade their host's own immune responses [1,2]. Thankfully, these infections are typically confined to a single location, as the biofilm detaching from the surface it's adhered to would usually threaten its own prosperity [7].

In many ways, biofilm infection of IMDs is very similar to the situations as described above. Wherever the device is located is where the biofilm will form and similarly use the surrounding host environment to proliferate. Contrarily, with biofilm infection on the abiotic surface of IMDs within a patient, the option of removal is a perfectly viable treatment plan. This will typically be done in coupling with systemic antibiotic therapy to ensure that any planktonic cells that detach when the biofilm-infected device is extracted are eradicated before causing further infection [4]. For some IMDs, such as urinary catheters, this treatment option is both simple and effective seeing as how they can be easily removed. However, there

are IMDs, like dialysis fistulas or artificial heart valves that, if infected, can lead to severe systemic bacteremia and be extremely expensive, not to mention dangerous, to replace. The best viable approach to the treatment of biofilm-infected IMDs such as these is a combination of surgical removal and antibiotic treatment, just like with simple IMDs. However, these procedures can present much higher risks to the patient and expenditures ranging from $15,000 to $50,000, depending on the IMD and the invasiveness of the surgical procedure required [2,4].

Enterobacteriaceae

Of the known biofilm-producing bacteria relevant in both research and clinical settings, there are numerous members belonging to the bacterial family Enterobacteriaceae. Taxonomically, the bacterial family currently has 53 genera and, of these, 26 are known to be associated with infections in humans [9]. Members of this family share numerous characteristics, such as being typically small, gram negative, non-sporing, straight rods that are facultatively anaerobic and grow well at standard human body temperature, 37°C [9]. There are several clinically significant members of this bacterial family that are causing concern in the medical field, as their rapid adaptations outdo our current treatments. Between their effectiveness and virulence as pathogens and their rapidly evolving tolerance to the most powerful weapons in our antibiotic arsenal, these members of the Enterobacteriaceae family have researchers scrambling for a leg up.

One such genus of concern is *Klebsiella*. All *Klebsiella* species tend to act as opportunistic pathogens, typically only infecting individuals who are immunocompromised or suffering for another chronic illness. They are an

incredibly adaptive class of bacteria, being able to survive in both the environment (water sources, soil, sewage, etc.) as well as the mucosal surfaces of mammals, including humans. As such, the clinical areas of most concern for *Klebsiella* infection are ones such as the urinary, respiratory, or intestinal tracts. This genus' most medically famous species, *Klebsiella pneumonieae* (KP), is the most common cause of community-acquired bacterial pneumonia, particularly among chronic alcoholics. When put into perspective of bacterial species causing gram-negative bacteremia, *Klebsiella* species are second only to the infamous *Escherichia coli*. This genus has begun to exhibit multiple species strains that are increasingly antibiotic-resistant to many conventional antibiotics due to their production of extended-spectrum β-lactamases that cleave the β-lactam structure of said antibiotics [10].

Another tenacious genus within the Enterobacteriaceae family that has caused serious infection in neonates and immunocompromised individuals is *Citrobacter*. This genus raises particular concerns due to its member's ability to cause infections affecting a patient's Central Nervous System (CNS) more severely than simply causing bacteremia or sepsis as other genera do. These bacteria are typically located in the feces of mammals and can then spread through water, soil, and even food [9,11]. If infected with a virulent *Citrobacter* strain, an individual could develop bacterial meningitis. In 80% of these confirmed cases the disease can eventually lead to painful and debilitating CNS abscesses. Signs and symptoms of *Citrobacter* meningitis or sepsis include temperature instability, irritability, decreased oral intake, jaundice, vomiting, lethargy, hypotonia, and possibly seizures [11]. A particular species that contributes to these clinical manifestations is

Citrobacter freundii (CF), which is virtually always resistant to standard bacterial antibiotics like ampicillin or cephalosporins, making it very difficult to treat. It usually takes a varied combination of antibiotics tailored to a specific situation coupled with surgical draining of CNS abscesses to effectively treat such illness presented by this species of *Citrobacter*. Even with these combinations of drugs and surgery, some physicians have referred to the overall effectiveness of this treatment as "generally disappointing." [11]

Another genus of Enterobacteriacae deemed highly important in the realm of causing serious nosocomial, opportunistic infections is *Enterobacter*. These bacteria are a common species that have been linked to serious cases of bacteremia, endocarditis, osteomyelitis, and multiple other chronic diseases. An exemplary species of the whole genus, demonstrating their resilience and pathogenicity, is *Enterobacter cloacae* (ECL). The species normally resides as commensal microflora in the intestinal tracts of humans and other animals. As a pathogen, it is able to create biofilms, secrete cytotoxins, and exhibit multi-drug-resistance. This species is intrinsically resistant to ampicillin, amoxicillin, first-generation cephalosporins, as well as cefoxitin. This remarkable resistance is owed to the bacteria's production of constitutive Amp C β-lactamase. However, in recent years, clinical isolates have been found that have the ability to produce carbapenemase, which can render one of our most powerful antimicrobial drugs useless in treating an infection [12].

As stated earlier, all of these genera mentioned exhibit powerful antibiotic resistance that makes treatment of associated infections highly complicated. The most concerning development in recent years, as mentioned briefly with ECL, is that

some genera of Enterobacteriaceae have developed adaptations against our "antibiotic of last resort": Carbapenems [12]. Every genus mentioned above, not just *Enterobacter*, includes certain species that can produce strains exhibiting this new adaptation of defense. These species make up the classification of Carbapenem Resistant Enterobacteriaceae (CRE). When the first beta lactamases were developed by bacteria to combat penicillin in the 1960s, carbapenems were created as humanity's counter [13]. It is a member of the antimicrobial class of β-lactams and possesses the broadest spectrum of efficacy against both gram-positive and gram-negative bacteria [13]. Carbapenems attack bacteria by entering through their outer membrane proteins and inhibiting the enzymes within the cell responsible for peptidoglycan production via penicillin binding proteins (PBPs). This ultimately results in the weakening and eventual lysis of the cell's protective structure, leading to cell death [13]. Carbapenemases are β-lactamases that CRE can produce specifically to attack the structure of the antibiotic before it ever reaches the PBPs, rendering it useless. There are also other methods, such as efflux pumps and gene mutations that some bacteria can utilize to also prevent carbapenems from performing their function [13]. The best treatments we have for bacterial infections exhibiting such resistance are combination antibiotics, monotherapies, or returning to previously-thought obsolete drugs, such as colistin. At this point, there is no single treatment option that is superiorly effective against CRE [14].

Secondary Metabolites

It has been mentioned previously that bacteria have multiple mechanisms for interacting with one another. These interactions can be either cooperative or

hostile depending on the situations and the interactive tendencies of the particular bacteria involved. What was not directly mentioned, however, were the actual agents responsible for these interactions. The integral agents of the microbial community responsible for these cell-to-cell interactions can simply be referred to as secondary metabolites (SMs).

SMs can be defined as cellular products that do not play an essential role in growth, development, or reproduction of the producing organism. These metabolites are often bioactive compounds and can perform important functions in bacterial defense, competition, signaling, and ecological interactions [15]. These products are not found among bacteria that are still in their primary growth phase (trophophase), but are produced when bacteria enter their subsequent production phase (idiophase) [16]. SM production is typically brought on due to a depletion or exhaustion of an essential nutrient (such as Carbon), the presence of some other inducer, or simply a decrease in growth rate [16,17]. The secondary metabolites are typically produced by modifying primary metabolite synthases that ultimately result in primarily amino acid derivatives that can be used for a wide variety of functions [16,18]. Antibiotics, toxins, pheromones, and even the auto-inducers mentioned above in relation to quorum sensing are all examples of SMs [17].

Clinically, the ability of SMs to have antibiotic properties is the most significant as far as humans are concerned. Infection-preventing antibiotics such as bacitracin or erythromycin are both derived from SMs made by some bacteria with the original purpose to be used against other bacteria [18]. This method of using bacteria's own weapons against them has been the foundation of modern bacterial

infection treatment since Fleming's ingenuity resulting in penicillin. Ultimately, this foundation is all possible due to bacteria's own adaptations for self-preservation against other microbes. The antimicrobial SMs produced can react to other bacteria by inhibiting their growth, which in turn allows the bacteria secreting them more nutrients in their environment. Another possibility, for some, is simply killing rival bacteria outright to achieve the same effect [19,20]. These two methods of microbial warfare are, as such, classified as bacteriostatic (inhibiting) or bactericidal (killing) actions [19].

The current experiment utilizes all of the above information in an effort to determine the effect and efficacy of a currently unknown product secreted by KP. We believe it is likely a SM considering its characteristics of what appears to be competitive inhibition on CF and ECL biofilm formation. Both of these bacterial species have exhibited multiple drug resistance (MDR) to traditional treatments, and as such, new methods of treatment are in demand. This product has been found to be present in the filtered, cell-free spent media from a KP broth culture and appears to produce similar effects in inhibiting these species in this form as it is directly secreted from living cells. Both stages of biofilm attachment and maturation for the two species in question are analyzed when introduced to the filtered KP liquid containing the unknown product. Multiple assays were performed in order to determine optical density, mass, and cell viability of the biofilms produced. Hopefully, with the data and scientific deduction from these trials, a new method of

treating biofilms produced by the growing threat of MDR Enterobacteriaceae may emerge.

Materials and Methods

Strains and Media

Klebsiella pneumoniae (ATCC#13883), *Citrobacter freundii* (ATCC#8090), and *Enterobacter cloacae* (ATCC#23355) stocks were purchased from the American Type Culture Collection. Luria broth (LB), both as a broth and an agar medium, was used to propagate bacterial strains. When necessary, Carbenicillin (10 μg/mL) was added to media for the selection of *K. pneumoniae.*

Preparation of Cell-Free Spent Media

Fresh overnight cultures of KP were used to acquire the inhibitory molecule of interest. Briefly, 5 mL overnight cultures were pelleted by centrifugation and the resulting supernatant was further purified via a 0.22-micron syringe filter. The filtered supernatant was then combined in equal portions with the LB broth and immediately used for biofilm assays.

Zones of Inhibition

To determine an average zone of inhibition (ZOI) for the unknown secreted molecule from KP, overnight cultures of bacteria (LB broth, 37°C) of CF and ECL were used to create bacterial lawns on LB agar plates. 15 μL of overnight KP culture, KP cell-free supernatant, or varying concentrations of Ampicillin were spotted onto

the lawns. Plates were incubated at 37°C for 24 hours and zones of inhibition (millimeters) were measured.

Biofilm Formation

In order to produce biofilms for the experiment, overnight cultures of CF and ECL were adjusted to an OD_{600} reading of 0.01 (~1 x 10^6 cells/mL) in LB broth and seeded into a 96 well microtiter plate. For experiments involving the attachment phase, CF and ECL were seeded either into wells with LB broth or a combination of equal parts LB and KP cell-free spent media. LB (no bacteria) only wells served as a control for background. The 96 well plates were then covered with parafilm and incubated at 37°C for 24h. For experiments involving the maturation phase, CF and ECL were seeded (~1x10^6cells/mL) into the wells with half the amount of LB broth and incubated for 4h at 37°C. After this short incubation to establish bacterial biofilms, fresh LB or KP cell free spent media was added accordingly to control and experimental wells. The 96 well plates were covered with parafilm, and incubated an additional 24h at 37°C.

Assessing Biofilm Cell Density, Biofilm Mass, and Bacterial Metabolism

Biofilm characteristics were assessed by UV/VIS spectral readings using a GENESYS 10S or Thermo Fisher Multiskan microtiter plate spectrophotometer. Each biofilm assay was done in triplicate with each of the three trials having multiple replicated independent samples.

Biofilm Cell Density Assay

After incubation, bacterial biofilms were dislodged from wells and optical density (OD_{600}) was determined. A well containing LB broth only was used as a blank.

Biofilm Mass Assay

The dye crystal violet (CV) was used to assess biofilm mass. Briefly, the liquid portion of the biofilm was removed from each well and wells were washed once with 1X PBS. A 0.1% CV solution was added and incubated at room temperature for ten minutes. The dye was then pipetted off and wells were again washed with 1X PBS and the remaining dye within the biofilm cells was released using 33% acetic acid. Spectrophotometer readings (OD_{595}) were used to assess the amount of released dye.

Biofilm Cell Metabolism Assay

For determining cell viability, this experiment incorporated the use of an MTT assay. Briefly, the yellow colored MTT is reduced to its insoluble formazan, yielding a purple color if the cells were actively metabolizing. To do this, MTT (5mg/mL) was added to the 96-well plate and incubated at 37°C for thirty minutes. Acidic isopropanol was added to resolubilize excess MTT. Spectrophotometer readings (OD_{570}) were used to assess the amount of reduced MTT.

Biofilm Viability

Standard plating and dilution methods were used to determine colony-forming units (CFU) of the biofilms. Wells were serially diluted and plated on LB agar plates, incubated at 37°C for 24h, and enumerated the next day.

Results and Discussion

Inhibition of CF and ECL Biofilm Attachment

Preliminary studies investigating the KP molecule's effect on planktonic culture has shown definitive inhibition of CF and ECL growth in culture. Taking this information into consideration we first tested the ability of this molecule to inhibit the beginning stages of biofilm development, the attachment stage. Early biofilm attachment was assessed for cell density (optical density), biofilm mass (crystal violet staining), and biofilm cell viability (MTT and CFU enumeration). The 96 well microtiter plates were uniformly inoculated with the same amount of CF or ECL (~$1x10^6$ cells/mL) with LB alone or equal amounts of LB and KP cell free spent media. Each of the plates could then be assessed for the four different assays at once (Figure 1A).

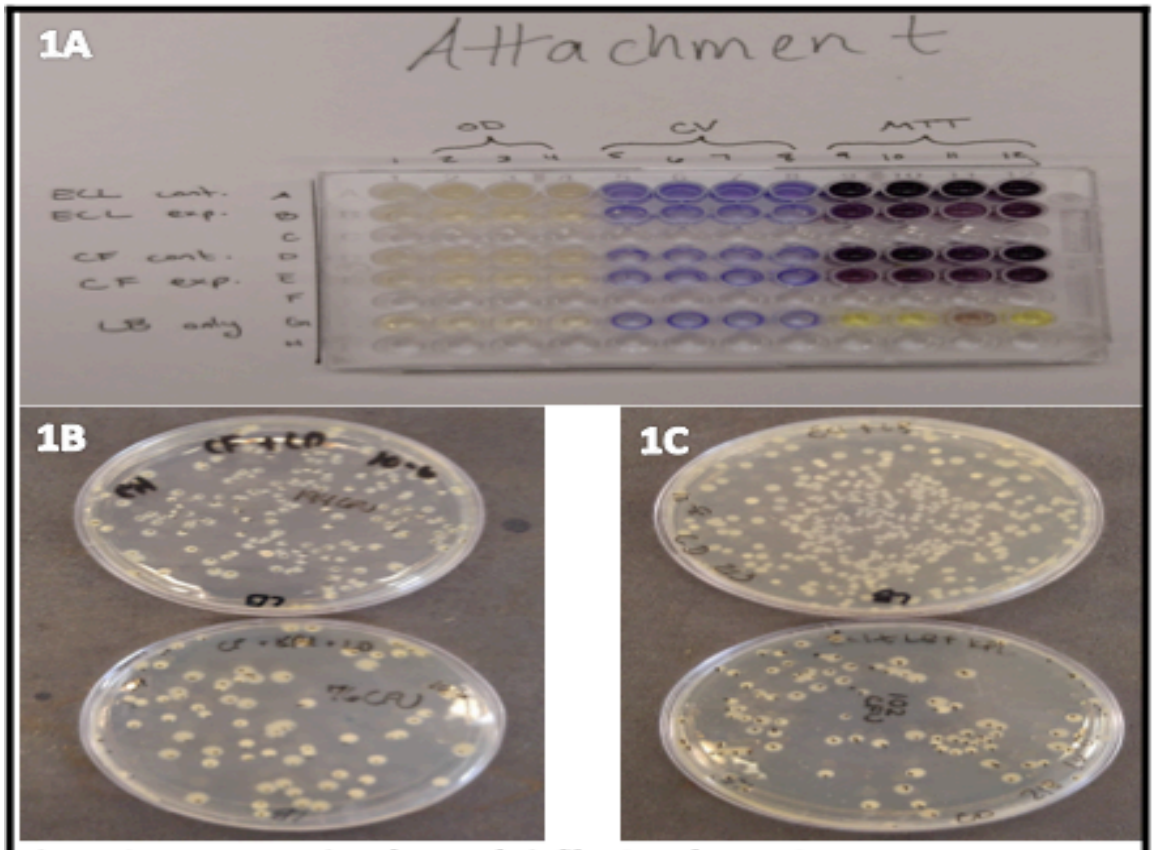

Figure 1: Representative Photos of Biofilm Attachment Assays.
(1A) Representative 96 well microtiter plate with the completed biofilm OD, CV, and MTT assays for the biofilm attachment phase (note the mild contamination in well G11, a control). ***(1B)*** Representative of a reduction of 194 CFUs to 76 CFUs between the CF control and experimental plate at a 10^{-6}dilution. ***(1C)*** Representative of a reduction from >200 CFUs on the ECL only plate down to 102 CFUs on the ECL with KP product plate at a 10^{-8}dilution.

Controls for each assay consisted of LB only wells containing no bacteria. There was a visible decrease in turbidity between the control and experimental wells. Optical density readings were consistent over the three independent trials and showed a 62% decrease for CF biofilms treated with the KP molecule and a 68% decrease in ECL-treated biofilms (Figure 2A/2B). However, thanks to our additional row of wells containing nothing but LB broth, we were able to determine that there was bacterial biofilm development in all wells. This indicates that, at the point of biofilm attachment, the uncharacterized product from KP is inhibitory to attachment and eventual production but is unable to fully prevent attachment of

bacteria. It is important to note that we have thus far not been able to isolate and purify the molecule of interest and so the cell free spent media contains very dilute amounts of the molecule. It is possible that upon isolation and purification of the molecule that a completely inhibitory effect, in regards to attachment, could be possible. Figure 1A shows the microtiter assay plate at the conclusion of all performed assays. Dilution plates for CF (Figure 1B) and ECL (Figure 1C) were also compared to further illustrate the inhibition of both bacteria.

2A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	0.697	0.744	0.819	0.753333333	0.061533189
CF+KP mol	0.275	0.305	0.309	0.296333333	0.018583146
Ecl	0.809	0.793	0.746	0.782666667	0.032746501
Ecl+KP mol	0.207	0.199	0.237	0.214333333	0.020033306

2B

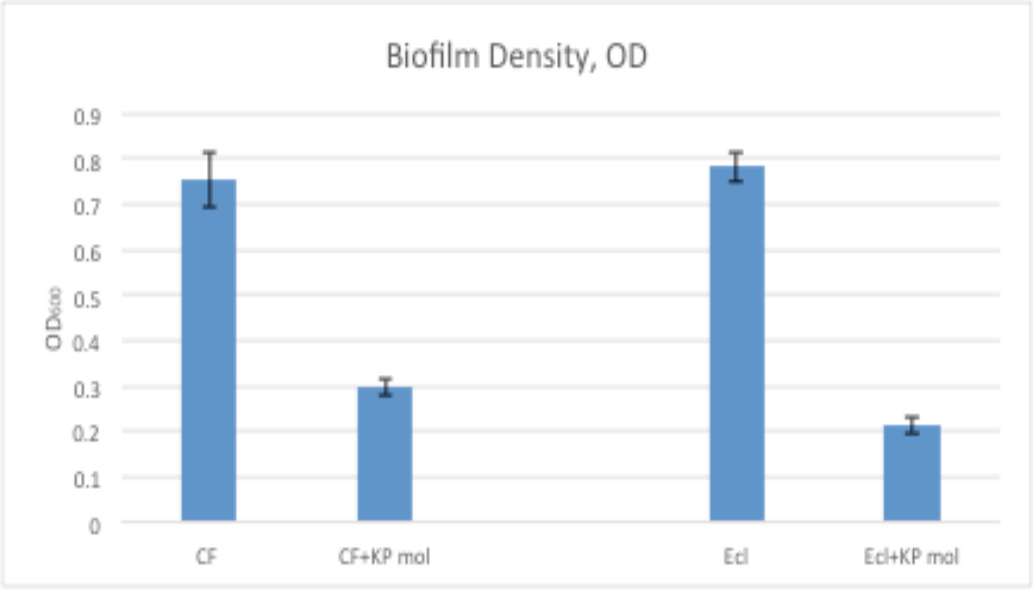

Figure 2: Numerical and Graphical Results of OD Measurements for Attachment Phase Biofilm Density. ***(2A)*** Shows numerical OD values from wells 2-4 in all 3 Attachment trials with calculated overall averages and standard deviations. ***(2B)*** Graphically illustrates the overall OD averages to determine biofilm density for all 3 Attachment trials with confidence interval bars.

For assessment of biofilm mass, CV was used to stain attached cells on the 96 well plates. Cells that take up the CV stain are lysed, releasing the dye, and mass can be calculated as a proportion by absorbance at OD_{595}. When CF was treated with the KP molecule there was a 73% reduction in biofilm mass in comparison to the CF untreated control (Figure 3A/3B). The KP molecule appears to have had an even more prominent reduction of 85% on ECL biofilm mass during attachment phases (Figure 3A/3B). We believe this to be indicative of a major reduction in biofilm mass due to the inhibitory actions of the uncharacterized molecule.

3A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	1.33	1.47	1.52	1.44	0.098488578
CF+KP mol	0.265	0.469	0.414	0.382666667	0.105547777
Ecl	2.14	1.98	2.67	2.263333333	0.361155553
Ecl+KP mol	0.16	0.1588	0.682	0.3336	0.301723847

3B

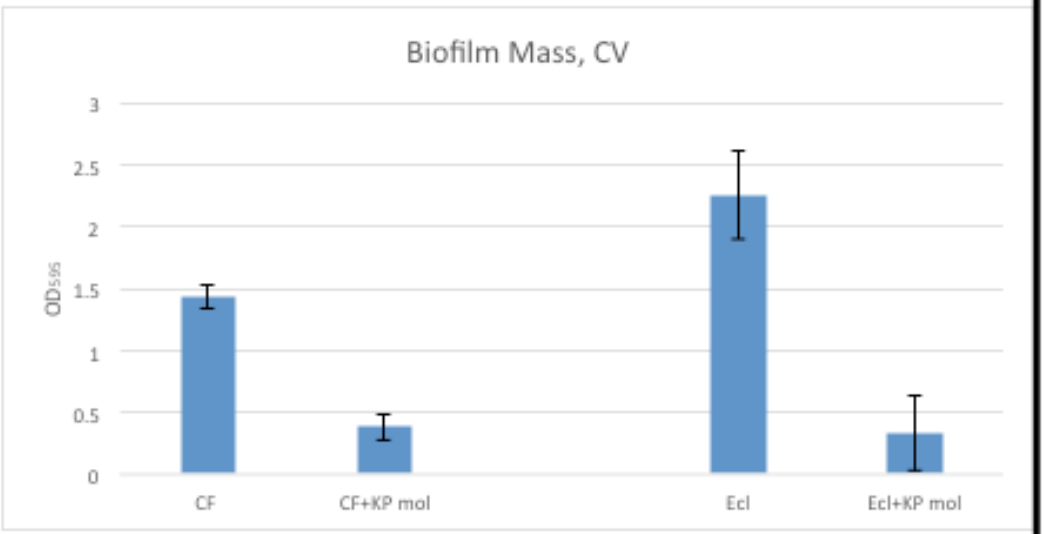

Figure 3: Numerical and Graphical Results of OD Measurements for Attachment Phase Biofilm Mass. ***(3A)*** Shows the OD values of CV wells from all 3 Attachment trials with calculated overall averages and standard deviations included. ***(3B)*** Shows the graphical representations of the overall Optical Density averages for biofilm mass in all 3 Attachment trials with confidence interval bars.

The OD and CV assays only express cells in terms of amount of cells, but are unable to indicate whether those cells are, in fact, alive or dead. To determine if there was a reduction in cell viability we implemented an MTT assay. In this assay the yellow MTT is reduced to a purple formazan product by actively metabolizing bacterial cells. Thus, the amount of reduced product can be determined by spectrophotometer readings (OD_{570}) and the proportion of cells that are viable can be calculated. There was a reduction in both CF and ECL cell viability, though this reduction was not as drastic, as compared to the other OD and CV reductions. CF exhibited a 52% reduction in viable cells between control and experimental wells while ECL revealed a substantially greater 71% reduction between its two conditions (Figure 4A/4B). As such, for both CF and ECL at the attachment stage of

4A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	1.67	2.287	1.97	1.975666667	0.30853903
CF+KP mol	1.03	1.267	0.537	0.944666667	0.37240614
Ecl	2.13	2.401	1.87	2.133666667	0.265518989
Ecl+KP mol	0.54	0.861	0.435	0.612	0.221939181

4B

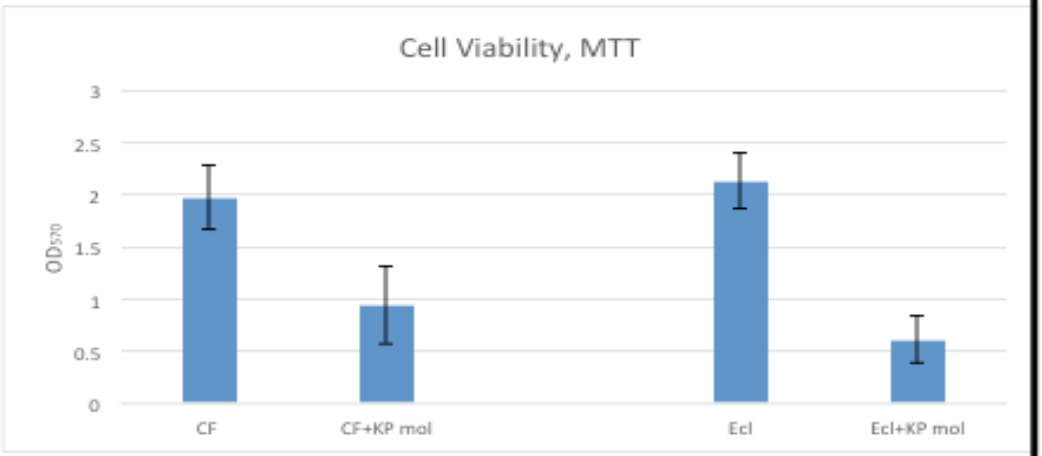

Figure 4: Numerical and Graphical Results of OD Measurements for Attachment Phase Biofilm Cell Viability. ***(4A)*** Shows us the numeric OD values of MTT wells from all 3 Attachment trials with calculated overall averages and standard deviations determining the presence of viable cells in the solutions tested ***(4B)*** Shows, graphically, the overall Optical Density averages of amounts of viable cells for all 3 Attachment trials with confidence interval bars as determined via MTT assay.

biofilm formation, there is a quantifiable decrease in viable cells present in the cultures.

Inhibition of CF and ECL Biofilm Maturation

We then tested the ability of this molecule to inhibit early-stages of maturation, when the biofilms are forming, but not fully mature yet. Similarly to attachment, biofilm maturation was assessed for cell density (optical density), biofilm mass (crystal violet staining), and biofilm cell viability (MTT and CFU enumeration). The 96 well microtiter plates were uniformly inoculated with the same amount of CF or ECL (~1x10^6 cells/mL) and half the amount in each well of LB alone (100 μL). This plate was placed in the 37°C incubator for a total of 4 hours. After this span of time, the remaining amounts of LB broth or KP product were added to their respective wells. Each of the plates were assessed for the four different assays at once, shown in Figure 5A.

Controls for each assay were comprised of wells filled with only LB and no bacteria. There was a visible decrease in turbidity between the control and experimental wells. Optical density readings were consistent over the three independent trials and showed a 56% decrease for CF biofilms treated with the KP molecule and a 65% decrease in ECL treated biofilms (Figure 6A/6B). Both of these values were only slightly (<~7%) less than what we found for the attachment series, indicating a slight lessening in effectiveness of the molecule at inhibiting biofilm formation at the maturation stage.

Due to this experimental series focusing on the biofilm phase of maturation, it was expected that we would see biofilm growth in all wells except our LB only wells. This fact reinforces that, at the dilutions used for this experiment, the KP product in question possesses bacteriostatic rather than bactericidal effects due to pre-formed biofilms still being present, yet inhibited from the point of KP product addition onward. Figure 5A shows the microtiter assay plate at the conclusion of all performed assays. Dilution plates for CF (Figure 5B) and ECL (Figure 5C) were also compared to further illustrate the inhibition of both bacteria.

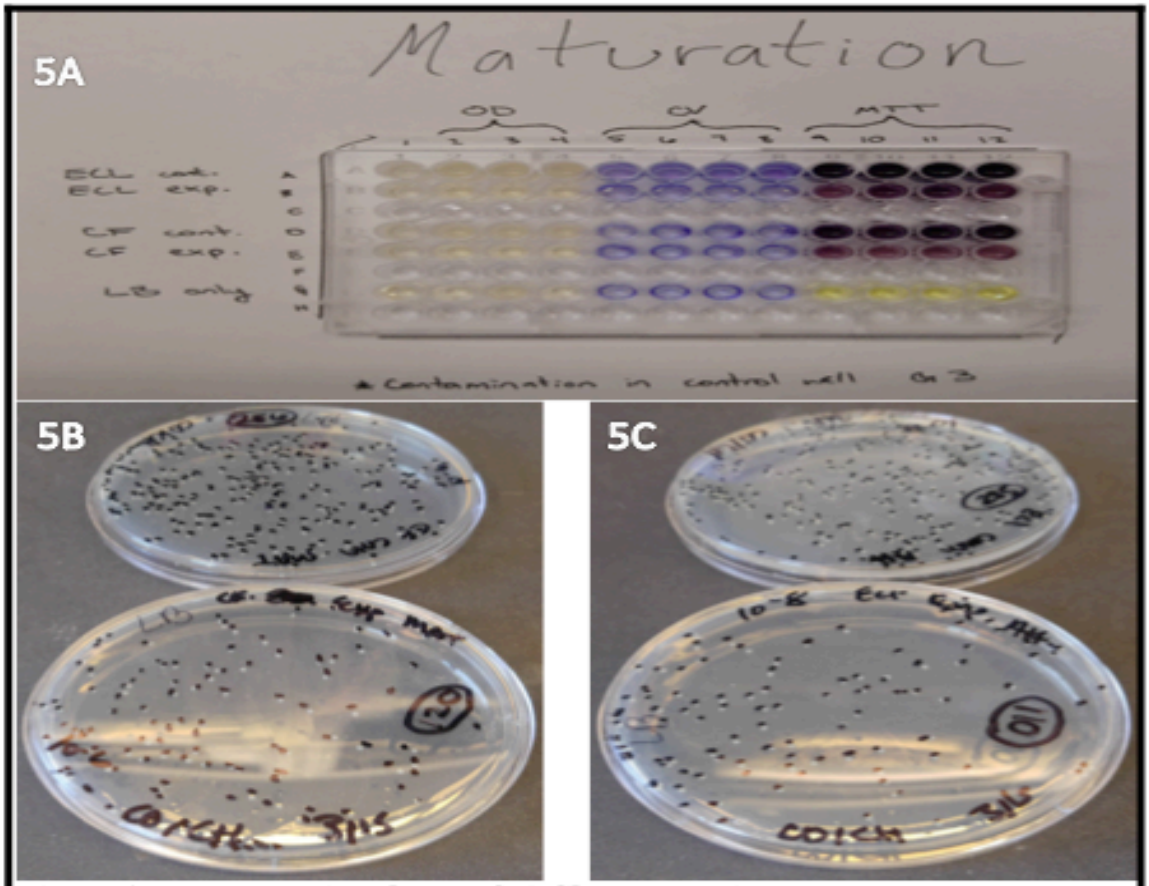

Figure 5: Representative Photos of Biofilm Maturation Assays.
(5A) Representative 96 well microtiter plate with the completed biofilm OD, CV, and MTT assays for the biofilm maturation phase. ***(1B)*** Representative of a reduction of 256 CFUs to 120 CFUs between the CF control and experimental plate at a 10^{-6}dilution. ***(1C)*** Representative of a reduction from 285 CFUs on the ECL only plate down to 110 CFUs on the ECL with KP product plate at a 10^{-8}dilution.

6A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	0.732	0.68	0.744	0.718666667	0.034019602
CF+KP mol	0.374	0.277	0.305	0.318666667	0.049923274
Ecl	0.487	0.683	0.707	0.625666667	0.120686923
Ecl+KP mol	0.293	0.158	0.203	0.218	0.068738635

6B

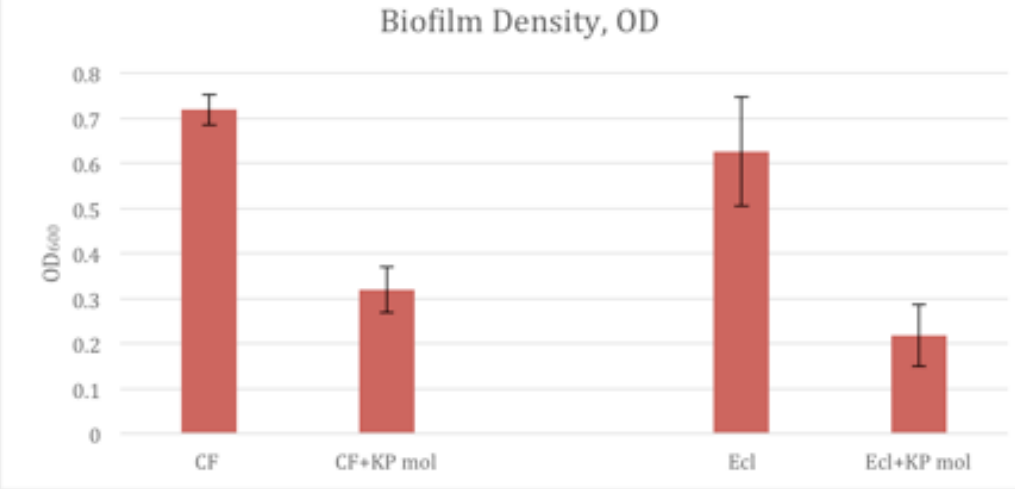

Figure 6: Numerical and Graphical Results of OD Measurements for Maturation Phase Biofilm Density. ***(6A)*** Shows numerical OD values from wells 2-4 in all 3 Attachment trials with calculated overall averages and standard deviations. ***(6B)*** Graphically illustrates the overall OD averages to determine biofilm density for all 3 Attachment trials with confidence interval bars.

The same method of using CV to stain attached cells on the 96 well plates that was used for the attachment phase was used in the maturation trials. The mass can be calculated as a proportion by absorbance at OD_{595}, as before. For this series, when CF was treated with the KP molecule there was a 89% reduction in biofilm mass in comparison to the CF untreated control, demonstrating what appears to be superior action of the KP molecule when compared with the attachment data (Figure 7A/7B). Contrarily, ECL only exhibited an average reduction of 71% in biofilm mass when added 4 hours after incubation began, which is >10% less effective than when the filtrate was introduced immediately (Figure 7A/7B).

7A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	1.664	1.605	1.774	1.681	0.085772956
CF+KP mol	0.144	0.105	0.313	0.187333333	0.110563707
Ecl	1.81	2.49	2.46	2.253333333	0.384230833
Ecl+KP mol	0.619	0.675	0.68	0.658	0.033867388

7B

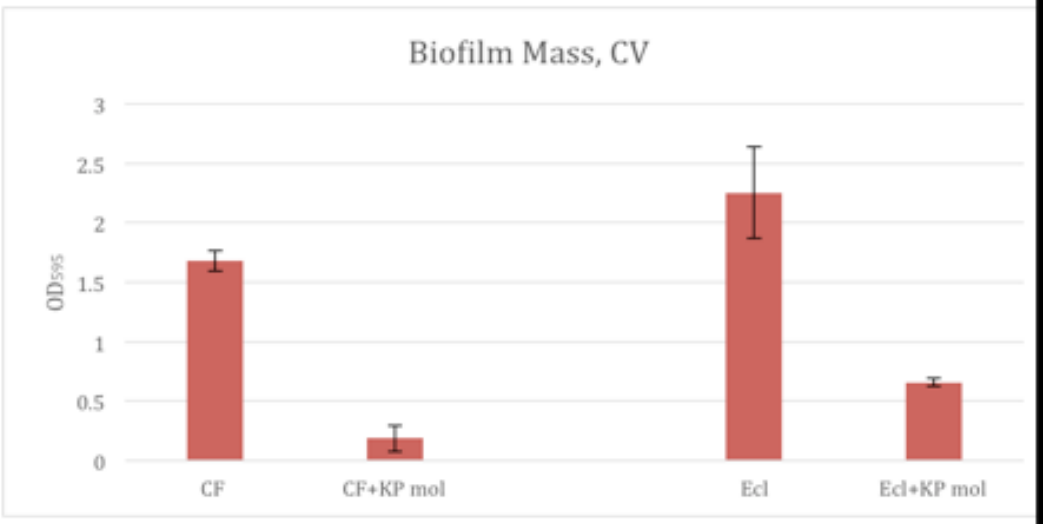

Figure 7: Numerical and Graphical Results of OD Measurements for Maturation Phase Biofilm Mass. ***(7A)*** Shows the OD values of CV wells from all 3 Attachment trials with calculated overall averages and standard deviations included. ***(7B)*** Shows the graphical representations of the overall Optical Density averages for biofilm mass in all 3 Attachment trials with confidence interval bars.

To determine if the cells present were alive or dead, an MTT assay was utilized. The amount of reduced product was determined by spectrophotometer readings (OD_{570}) and the proportion of cells that are viable was then calculated. 61% average viable cell reduction was found between the control and experimental categories for CF, which is ~8% greater efficacy than when the KP molecule was added to inhibit the attachment phase of biofilm formation (Figure 8A/8B). Inversely, the ECL averages indicated a 60% reduction of viable cells due to the filtrate's addition at the maturation stage which is >10% less efficacy than when it was added for attachment (Figure 8A/8B). For both CF and ECL at the maturation

stage of biofilm formation, we can conclude that there is a similar quantifiable decrease in viable cells present in the cultures read by the spectrophotometer (OD_{570}) as compared to the attachment stage trails.

8A

Category	Trial 1 Average	Trial 2 Average	Trial 3 Average	Overall Average	Standard Deviation
CF	1.63	2.56	1.956	2.048666667	0.471874277
CF+KP mol	0.731	1.2	0.474	0.801666667	0.368122715
Ecl	2.19	2.49	2.21	2.296666667	0.167729942
Ecl+KP mol	1.04	1.17	0.536	0.915333333	0.334881073

8B

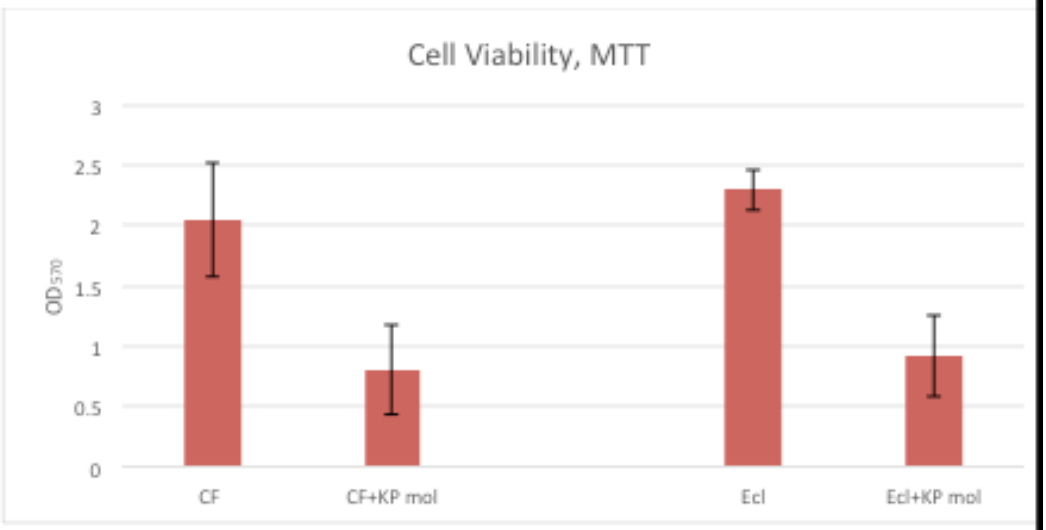

Figure 8: Numerical and Graphical Results of OD Measurements for Maturation Phase Biofilm Cell Viability. ***(8A)*** Shows us the numeric OD values of MTT wells from all 3 Attachment trials with calculated overall averages and standard deviations determining the presence of viable cells in the solutions tested ***(8B)*** Shows, graphically, the overall Optical Density averages of amounts of viable cells for all 3 Attachment trials with confidence interval bars as determined via MTT assay.

Determination of KP molecule Zones of Inhibition (ZOI)

Lastly, it was determined from the data that the unknown molecule secreted from KP has the capability to create a ZOI that closely resembles the same action enacted by lower concentrations of the antibiotic Ampicillin on lawns of CF and ECL. In too low dilutions, the antibiotic won't create any ZOI of CF lawns (Figure 9A/9B).

However, the filtrate is capable of creating a ZOI very similar to what would be produced if the KP cells were placed on the lawn. With ECL, which appears to be more susceptible to Ampicillin than CF, the KP filtrate proved to be slightly less effective than the lowest antibiotic dilution we tested, but more effective than the KP cell trial.

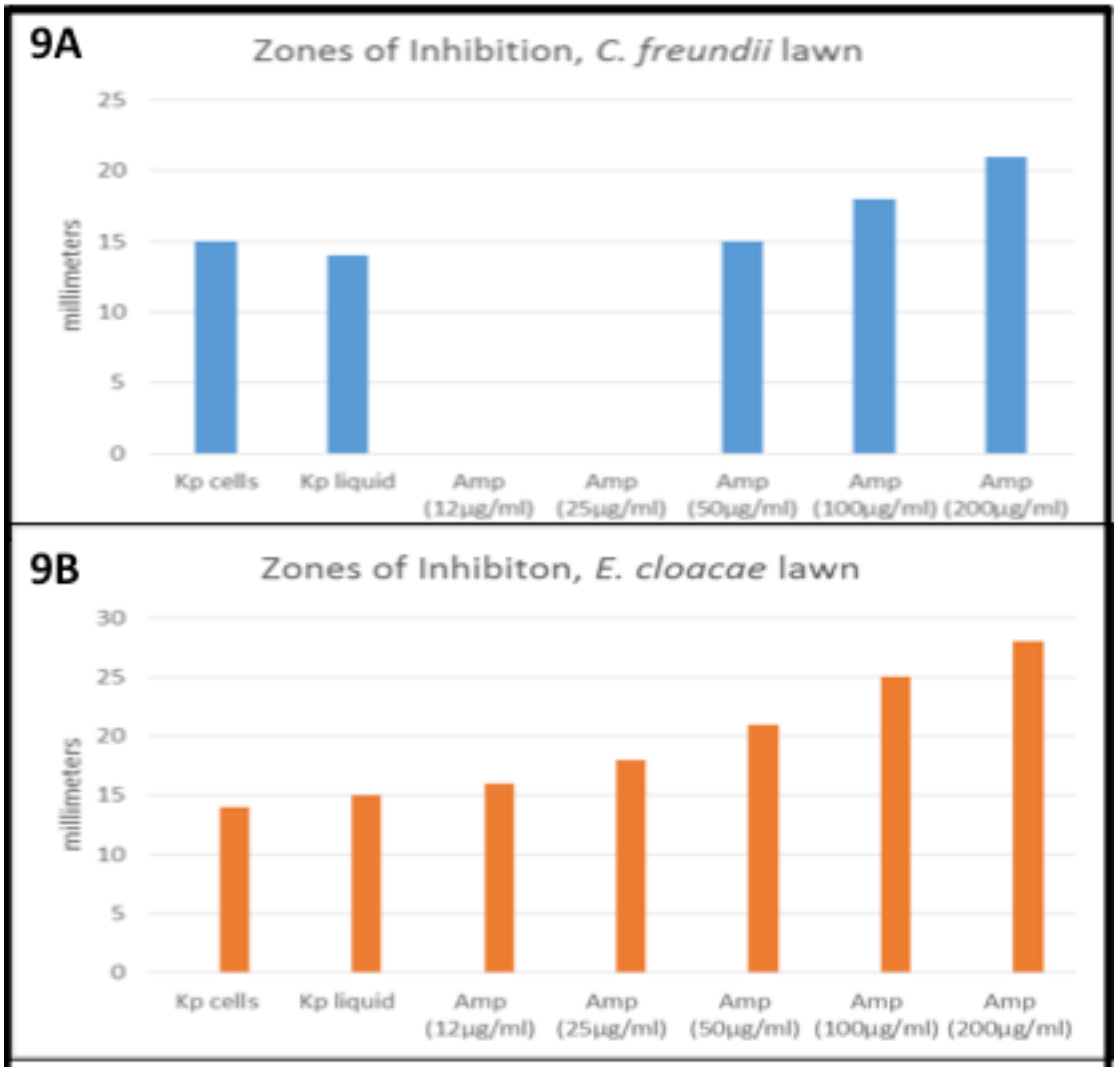

Figure 9: Graphical representation of KP ZOIs when compared with multi-dilution Ampicillin. ***(9A)*** Illustrates the ZOI left in millimeters by each individual component when 15 ul was dropped on a CF lawn ***(9B)*** Shows the ZOI left in millimeters by each individual component when 15 ul was dropped on a ECL lawn

Conclusions

The results of this study are indicative of the uncharacterized KP molecule possessing inhibitory effects on CF and ECL biofilm formation at both stages of attachment and maturation at the dilutions used. For each condition tested, there was substantial reduction by more than 50% between control groups and those exposed to the uncharacterized product. This shows that the molecule in question has inhibitory effects on the bacteria's biofilm cell density, mass, and cell viability, ultimately weakening these bacteria's biofilms.

The mechanism of how exactly this molecule does this, as well as what exactly this product from KP is categorized as, is still in question. We are not certain if this product exhibits strictly bacteriostatic effects or if there is possibility for more dramatic, bactericidal action upon purification. Again, only the liquid, dilute form of this product was used for this study, and the behavior of the KP product in higher concentrations with higher concentrations of bacteria remains to be tested.

The future goals of this series of experiments would include the isolation, identification, and successful purification of this unknown product secreted by KP so that the full extent of its antimicrobial effects could then be analyzed. At present, the results of this study are highly optimistic for multiple practical applications of the product in question. There is the possibility of alternative solutions to CF and ECL infections rather than traditional, high-dose antibiotics. Should an infection develop biofilms and run the risk of becoming a chronic illness in a patient, we now have an antimicrobial agent that may be capable of treating MDR CF and ECL, giving medicine a new weapon in microbial warfare.

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