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### Burkholderia cenocepacia J2315-mediated destruction of Staphylococcus aureus NRS77 biofilms.

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*Burkholderia cenocepacia* J2315-mediated destruction of  
*Staphylococcus aureus* NRS77 biofilms

By

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Submitted in partial fulfillment of the requirements for Graduation *summa cum laude*  
and for Graduation with Honors from the Department of Biology

University of Louisville

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## **Abstract**

Cystic fibrosis (CF) is an inherited disorder that affects over 30,000 people in the US and more than 70,000 people worldwide. Recurring bacterial infections in CF patients result in tissue damage that dramatically lowers respiratory function and are ultimately fatal. The formation of bacterial biofilms in the mucus and on lung epithelial tissue allows pathogens to be protected from antibiotics and the host immune system, making treatment of infection difficult. The interactions between CF pathogens in co-culture biofilms are not well understood and were examined in this study. *Staphylococcus aureus* and *Burkholderia cenocepacia*, two common CF pathogens, were used to examine the effects of co-culture on biofilm formation. We found that *S. aureus* biofilm formation and maintenance is inhibited when grown in co-culture with *B. cenocepacia*. In fact, supernatant from 3-day-old biofilms of *B. cenocepacia* was sufficient to reduce *S. aureus* biofilms, suggesting that a secreted compound may be responsible for this antagonism. The results of this study can be used to better understand the complex microenvironments bacteria experience in CF lungs. Further exploration of this interaction could lead to discovery of a novel therapeutic or a target for treatment of persistent *S. aureus* infection.

## **Introduction**

### **Cystic Fibrosis**

Cystic fibrosis (CF) is a recessive genetic disorder most common to Caucasians, a population in America in which 1 out of every 29 individuals is a carrier for a CF allele [1]. This disease is inherited by obtaining two copies of a mutant *CFTR* gene, which

normally encodes an ion channel in the cell membrane called the cystic fibrosis transmembrane conductance regulator (CFTR). Both chloride and bicarbonate ions have been shown to utilize this protein to exit the cell, and they contribute to the maintenance of salt balance and mucin dissolution for healthy exocrine mucosal secretions [2].

Many different *CFTR* mutations can lead to a mutant phenotype [3], which can consist of either the absence or dysfunction of the CFTR channel. Due to CFTR defects, CF patients have a lowered ability or incapacity to transport chloride and bicarbonate anions outside of the cell [4]. The resulting ion imbalance causes dehydration of the mucus outside of the cell, hindering mucociliary clearance and making clearance through coughing arduous [5]. The altered mucus can affect the antimicrobial function of defensins, lysozymes, and other host pathogen-targeting compounds [5-7]. The mucus inside the lumen is rich in carbon and other nutrients, which can be metabolized by microbes [8]. Thus, the environment in the lumen of the CF lung is conducive to bacterial growth and infection.

Mutations in the *CFTR* gene can also result in compromised cellular and immune function. CF lungs are in a hyper-inflammatory state due to chronic bacterial infections as well as the accumulation of misfolded and degraded proteins inside the cell. The cellular process of autophagy normally assists in removal of unwanted proteins and cytosolic debris through fusion of phagosomes with degradative lysosomes [9]. Defects in autophagy have been observed in CF-derived cell lines and have been shown to lead to increased inflammation in the lung airways [10-12]. Furthermore, airway epithelial cells and neutrophils lacking functional CFTR proteins have altered cytokine responses compared to wild-type cells [13-15], suggesting a possible impairment of immune

responses to pathogens.

Due to the viscosity of CF mucus, epithelial cilia are weighed down and clearance of microbes is severely impeded [16]. This stagnant environment can result in microbial growth in the mucus and allow bacteria to anchor to lung tissue. Bacteria in this environment can form biofilms, which are mats of bacterial cells embedded in an extracellular polymeric substance (EPS) made of polysaccharides, proteins, and extracellular DNA. EPS forms a sticky matrix that protects the enclosed bacteria from antibiotics, desiccation, and predation by immune cells.

The ability of bacteria in biofilms to evade the immune system and resist even the strongest antibiotics contributes to chronic bacterial infections and other symptoms seen in CF patients [17, 18]. Neutrophil and macrophage invasion to the site of infection leads to inflammation, which can chronically damage host cells and lead to tissue damage [19]. There is a decrease in surface area for oxygen exchange in the scar tissue that develops from chronic inflammation [20]. Chronic infection and accumulating tissue damage over the course of their lives cause CF patients to experience a slow suffocation until the severity of lung damage leads to death.

Even though the airway in CF patients is rife with nutrients, only some opportunistic bacterial pathogens succeed in this environment. The most common pathogens in CF patients are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, and members of the *Burkholderia cepacia* complex, including *Burkholderia cenocepacia* [21]. Of these, *S. aureus* and *P. aeruginosa* are the dominant pathogens and can be annually cultured from 80% of CF patients. Infections by these organisms are difficult to treat due to intrinsic

antibiotic resistance. In addition, these pathogens are frequently spread between patients nosocomially [22, 23]. Development of new therapeutics against these bacteria could benefit both CF patients and hospitalized individuals worldwide.

### ***Staphylococcus aureus***

*S. aureus* is a Gram-positive, non-motile bacterial species first discovered in 1880 in a skin abscess by Sir Alexander Ogsten [24]. It is estimated that 30% of the human population carry *S. aureus* as a commensal organism [25], usually in the upper respiratory tract, and it can be found commensally on other mammals [26-34]. The genome of *S. aureus* consists of a single 2.8 megabase pair (Mbp or 1 million base pairs) chromosome and contains pathogenicity islands for a variety of toxins and other virulence factors [34]. Plasmids and several other mobile genetic elements encode antibiotic resistance genes in both methicillin sensitive (MSSA) and methicillin resistant (MRSA) *S. aureus* strains.

As an opportunistic pathogen, *S. aureus* can cause a variety of infections in susceptible hosts. This species is the most frequent infectious agent of infective endocarditis [35] and osteoarticular infection [36]. This species can cause pneumonia [37], infection of prosthetic joints [38-39], and it can form biofilms on catheters [40], prosthetic heart valves [41], pacemakers [42] and other prosthetic devices [43]. Skin and soft tissue infections by *S. aureus* manifest as abscesses, cellulitis, boils, impetigo, and infection of wounds [44].

As a primarily extracellular pathogen, the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) are a critical component of *S.*



*aureus* pathogenicity and contribute to the initial attachment to host cells [53,54]. These proteins are located on the surface of the cell, covalently bound to the peptidoglycan of the cell wall [55]. Components of *S. aureus* MSCRAMM include fibronectin binding proteins, clumping factors, and *S. aureus* protein A (SpA) [56-59]. Fibronectin binding proteins and clumping factors act as adhesins, anchoring the cells to the extracellular matrix of host tissue for colonization [53]. SpA is a protein unique to *S. aureus* and contains several immunoglobulin-binding domains [60]. The function of SpA is to bind the Fc portion of immunoglobulin G. This interaction prevents Fc from being recognized by – and interacting with – phagocytic neutrophils and macrophages, thereby allowing *S. aureus* to avoid detection by the circulating antibodies and evade classical complement formation, opsonization, and phagocytosis [61].

The majority of *S. aureus* strains produce exotoxins that function as superantigens, which are potent mitogens that trigger massive proliferation of T cells and cytokine production [45]. This immune response can overwhelm the human body and lead to septic shock. Enterotoxins secreted by this species and other staphylococci quickly cause food poisoning when consumed from undercooked, unpasteurized, or handler contaminated foods [46, 47]. Another *S. aureus* toxin, called toxic shock syndrome toxin, is a causative agent of toxic shock syndrome [48].

Other toxins produced by *S. aureus* strains do not act as superantigens but directly target host cells for destruction. Leukotoxins produced and secreted by *S. aureus* act to form holes in the membrane of host leukocytes, upsetting the osmotic balance and resulting in cell lysis [49]. The leukotoxin  $\gamma$ -hemolysin targets red blood cells and can cause massive hemolysis [50], while the monomeric  $\alpha$ -toxin (or  $\alpha$ -hemolysin) is highly

cytotoxic and polymerizes to form a water channel in host cell membranes [51]. This leukotoxin leads to apoptosis through activation of a caspase cascade [52]. These leukotoxins extend bacterial infection by eliminating predatory immune cells and enhancing invasion of host tissue.

In addition to producing adhesins for attachment to host tissue during initial colonization, *S. aureus* also produces factors that assist in cell-to-cell binding and biofilm formation. The intercellular adhesin (*ica*) locus is found in *S. aureus* and several other staphylococcal species and encodes enzymes necessary for the production of polysaccharide intercellular adhesin (PIA), a component of staphylococcal EPS [62]. The products of the *ica* locus are necessary for initiation of biofilm formation by assisting in intercellular attachment between cells. The ability of *S. aureus* to form biofilm and attach to biomedical implants contributes to its ability to cause sepsis and recurring infection in host tissue.

Difficulty in treatment of *S. aureus* infection is due to the extensive range of antibiotics to which both MSSA and MRSA strains have evolved resistance. Over 90% of *S. aureus* isolates encode  $\beta$ -lactamase, eliminating certain penicillins as useful antibiotics [63]. While penicillinase-resistant penicillins are available, they are usually limited to treatment of severe MSSA infection. After its introduction in 1961, strains resistant to methicillin – a penicillinase-resistant penicillin – quickly surfaced [64], and MRSA strains have rapidly increased in prevalence of infections over the past 50 years. In the CF community, MRSA infection has increased from 6.1% of patients in 2000 to 26% in 2015 [21].

The *mecA* gene in MRSA isolates is located on the staphylococcal cassette

chromosome *mec* (SCC*mec*) and encodes the enzyme that confers resistance to methicillin, penicillin, and other  $\beta$ -lactams [65]. The SCC*mec* is a mobile genetic element capable of horizontal gene transfer between staphylococcal strains [66]. Some studies suggest vancomycin is inferior to  $\beta$ -lactams as an antimicrobial, but it is still able to effectively eliminate infection of both MSSA and MRSA [67, 68]. However, strains bearing vancomycin resistance (VRSA) have also begun to appear in isolates of *S. aureus* [69], increasing the vital need for discovery of new anti-staphylococcal compounds and vaccine development for *S. aureus*.

*S. aureus* is typically the first organism that colonizes the CF lung and remains the most common CF isolate in individuals under 25, with prevalence of *S. aureus* colonization peaking between ages 11-15 [63,70]. Rates of MRSA infection during childhood are substantially higher in the United States than in Europe [70]. Approximately 25% of annual CF cultures are positive for MRSA, while the prevalence of MRSA in Europe remains about 5%. This indicates that MRSA is a pressing problem in the U.S. and illustrates the need for the development of new treatments for MSSA and MRSA infection.

### ***Burkholderia cenocepacia***

The *Burkholderia cepacia* complex (Bcc) consists of 18 related Gram-negative species that thrive in a diverse array of environmental niches and have a broad array of metabolic capabilities, including nitrogen fixation [71], pollutant metabolism [72, 73], antifungal production [74], and plant pathogenesis [75]. These bacteria are typically found in the soil or water, and this versatility seen in Bcc ecological specialization is

attributable to their uniquely complex genomes. Most Bcc species have genomes over twice the size of the *Escherichia coli* genome [76]. Whereas the majority of bacteria have one circular chromosome, Bcc genomes consist of two or three circular chromosomes and at least one large plasmid [77]. The CF isolate *B. cenocepacia* J2315, for example, has four replicons, with approximate sizes of 3.8 Mbp, 3.2 Mbp, 875 kilobase pairs (kbp) and 92 kbp, which together encode over 7,000 predicted coding regions.

The first isolation of a Bcc member from a CF patient occurred in the 1970s [78], and a CF epidemic in the 1980s involving *B. cenocepacia* increased the significance of the Bcc as opportunistic human pathogens [79]. Analysis of the *B. cenocepacia* J2315 genome has implicated the role of horizontal gene transfer of genomic islands in the development of human virulence factors [77]. Currently, *B. cenocepacia* and *B. multivorans* are the most common Bcc species isolated from CF patients in North America [80] and Europe [81], respectively.

*B. cenocepacia* strains found in either the environment or as clinical isolates can be categorized into four different subgroups [82]. Subgroup IIIA includes *B. cenocepacia* J2315 and the ET12 lineage from which it is derived. The ET12 lineage of *B. cenocepacia* caused a large epidemic in the CF community in the 1980s and 1990s [83]. This strain was transmitted across the United Kingdom, Canada, and Europe due to contact between patients. The ET12 epidemic lineage encodes a cable pilus that can assist in binding to both mucins [84] and host tissue [85], and isolates have been shown to dominate and replace infections originally caused by *B. multivorans* [86].

*B. cenocepacia* J2315 contains genes that confer natural resistance to several

antibiotics, including  $\beta$ -lactams, aminoglycosides, chloramphenicol, trimethoprim-sulfamethoxazole, and gentamicin [77]. This strain also expresses type IV pili that are involved in motility and cellular adhesion to host tissue. Additionally, these pili have DNA binding activity [87] suggesting they may play a role in biofilm formation, in which extracellular DNA is a critical component of the secreted matrix. Like *S. aureus*, the ability of this strain to bind to both host tissue and form biofilms contributes to its virulence as a CF pathogen.

The secretion systems utilized by *B. cenocepacia* J2315 play an important role in the infection of human tissue. When cells of this strain are taken up by macrophages, the bacteria evade degradation and survive inside an endocytic vacuole [88]. The type IV secretion system has been implicated in the ability of *B. cenocepacia* to survive macrophage phagocytosis by evasion of lysosomal fusion [89]. The type VI secretion system has been shown to reduce production of reactive oxygen species inside macrophages [90].

The importance of Bcc as CF pathogens lie in their high transmissibility between patients and the quick progression of CF pathogenesis associated with Bcc infection [91]. “Cepacia syndrome” is characterized by bacteremia and a sudden rapid deterioration of lung function from necrotizing pneumonia [92]. The large majority of incidents of these symptoms occurred when CF patients were infected with a Bcc member. In addition, presence of Bcc is correlated with an increased risk of death in CF patients. Even with the dominance of *S. aureus* and *P. aeruginosa* as the most common CF pathogens, the risks of Bcc infection make exploration of their virulence necessary for the CF community.

## **Bacterial Biofilms**

Bacteria form biofilms as a defensive mechanism against a variety of potentially damaging conditions, such as presence of phagocytic predator cells, abrasive mechanical stimuli, or exposure to antibiotics. Initiation of biofilm formation involves quorum sensing, which is a method of altering gene expression at the population level based on cell density [93]. Initially, cells use adhesins and extracellular structures to reversibly attach to a surface, such as host tissue or synthetic material. During the second stage of biofilm formation, cells begin secreting molecules for formation of the EPS, which results in the irreversible attachment of bacteria to other cells and surfaces.

After permanent attachment to a surface, biofilm cells will begin to replicate, resulting in formation of clusters of bacteria termed microcolonies. Replication and EPS production continue until a mature biofilm with a unique three-dimensional structure is formed. Depending on how bacteria are communicating within or between species, different biofilm morphologies can be observed. *P. aeruginosa*, one of the most well-studied biofilm formers, typically forms globular biofilms that resemble mushrooms [94]. This mushroom model of biofilm morphology is a commonly observed phenotype [95].

After biofilm maturation, the final stage of biofilm formation is the release of cells back to a planktonic, free-swimming lifestyle. This change involves dissolution of a portion of the biofilm EPS to allow cells to leave the biofilm and potentially begin the cycle all over again, initiating biofilm formation on another surface [96]. The biofilm lifestyle can increase the minimum inhibitory concentration of antibiotics 1000-fold [97,98] and make phagocytosis by macrophages difficult [99, 100], thus increasing the virulence and persistence of human bacterial pathogens inside the host.

## Co-Culture Biofilms

Single and polymicrobial biofilms can take on unique structures and spatial organizations that result in complex microenvironments [101, 102]. Different species can lay down in layers like stacks of paper, can arrange in a heterogeneous mixture of bacteria and involve contact with other species, or can remain in localized clusters with little to no cell-to-cell contact between microcolonies. Bacteria at the surface of a biofilm or in the lining of water channels will have different nutrient and oxygen availability than those near the bottom or the center of the biofilm. This arrangement results in unique metabolic states and requirements based on an organism's location within the biofilm.

Co-infection by pathogens in CF lungs is a common occurrence. In one group of CF patients participating in a clinical trial, 43.2% of individuals that were colonized by *P. aeruginosa* were also colonized by *S. aureus* [103]. Of 595 patients sampled, an average of 2.9 strains were cultured per person, including isolates of the biofilm-forming bacteria *P. aeruginosa* [104], *Achromobacter xylosoxidans* [105], *B. cepacia* [106], *S. aureus* [107], and *S. maltophilia* [108]. Given co-infection of CF patients with these bacteria, the CF lung likely acts as an ideal environment for the formation of mixed species biofilms, and the combinations of different strains may result in unique symptoms and severity of disease.

It is clear that a complex polymicrobial community inhabits CF lungs and that the microbial contents of CF airways change throughout a patient's lifetime. Multispecies biofilms and CF pathogen interactions are therefore important areas of research to understand CF pathogenesis. Monospecies biofilms have been studied in depth, such as those of *P. aeruginosa* [93, 109, 110], but only a few studies have explored co-culture

biofilms of CF pathogens. For example, the co-culture biofilm of *P. aeruginosa* and *S. maltophilia* may act to enhance antibiotic resistance compared to single species biofilms [111]. Clinical isolates of *B. cepacia* and *P. aeruginosa* grown in co-culture have been shown to form stable mixed biofilms [112]. In another study, *P. aeruginosa* PAO1 enhanced biofilm formation in *S. aureus* when grown in co-culture [113]. These studies are preliminary, and the interactions between many different CF pathogens remain to be explored. Currently, there is little to no understanding of the interaction between *S. aureus* and *B. cenocepacia* in a multispecies biofilm. The prevalence of the former and the mortality of the later make interactions between the pair an interesting topic of study.

The purpose of this study was to analyze polymicrobial interactions and biofilm formation in co-culture conditions of different CF pathogens. Originally, representative strains of five CF pathogens commonly isolated from the CF lung were tested for co-culture biofilm formation. These pathogens included *P. aeruginosa*, *S. aureus*, *B. cenocepacia*, *A. xylosoxidans*, and *S. maltophilia*. Pairwise combinations of these species were grown in co-culture conditions and analyzed for biofilm formation, and the unique interaction between *S. aureus* and *B. cenocepacia* was chosen for further exploration. The data suggests that *B. cenocepacia* inhibits biofilm formation and maintenance of *S. aureus*. Based on these preliminary data, the main hypotheses for this study were that (1) *S. aureus* biofilm formation would be inhibited when grown in co-culture with *B. cenocepacia* J2315, (2) mature monoculture *S. aureus* biofilm would be diminished when exposed to *B. cenocepacia*, and (3) destruction of *S. aureus* biofilm in the presence of *B. cenocepacia* would occur over time.



# Materials and Methods

## Bacterial Isolates and Materials

Luria Broth Lennox formulation (LB) was used to make both agar plates and liquid culture medium. Unless specified otherwise, all bacterial strains were plated from freezer stocks on LB agar plates and grown at 37°C for 24-48 h. For overnight cultures, single colonies were inoculated into 5 mL LB and incubated at 37°C with aeration until the next day. Plates and broth were supplemented with antibiotics as indicated. All strains can be found in the Yoder-Himes Lab strain collection. A complete list of all bacterial isolates used during the course of this project are listed and described in Table 1.

**Table 1. Strains used in this study.**

Strain	Description	Antibiotic Selection	Yoder-Himes Lab Strain	Source
<i>B. cenocepacia</i> J2315	CF clinical isolate	-	Bcc isolates A1-4	Dr. James Tiedje (Michigan State University)
<i>B. cenocepacia</i> J2315-62	J2315 bearing plasmid pIN62 encoding dsRedExpress	chloramphenicol 100	16-171	This study
<i>B. cenocepacia</i> J2315-301	J2315 bearing plasmid pIN301 encoding GFP	chloramphenicol 100	16-172	This study
<i>S. aureus</i> NRS77	Blood clinical isolate	-	13-012	BEI Resources; also known as NCTC 8325 or PS47
<i>S. aureus</i> NRS72	Bacteremia clinical isolate	-	13-011	BEI Resources; also known as Sanger476
<i>S. aureus</i> AH2183	Derivative of NRS77	-	16-082	Dr. Alexander Horswill (University of Iowa)
<i>S. aureus</i> AH3865	RN4220* containing a plasmid encoding dsRedExpress	chloramphenicol 10	16-081	Dr. Alexander Horswill (University of Iowa)
<i>S. aureus</i> AH1710	RN4220* containing a plasmid encoding GFP	chloramphenicol 10	16-080	Dr. Alexander Horswill (University of Iowa)
<i>A. xylosoxidans</i> AU19284	CF clinical Isolate	-	13-005	Dr. John LiPuma (University of Michigan)
<i>S. maltophilia</i> K279a	Blood clinical isolate	-	HMS Bcc/Pa 3	Dr. Nick Cianciotto (Northwestern University)

<i>P. aeruginosa</i> C3719	CF clinical isolate; small colony variant	-	HMS Bcc/Pa 1 H8-9	Dr. Stephen Lory (Harvard Medical School)
<i>E. coli</i> DH5 $\alpha$ pIN62	DH5 $\alpha$ containing a plasmid encoding dsRedExpress	chloramphenicol 30	16-0103	Dr. Annette Vergunst (University of Montpellier, France)
<i>E. coli</i> DH5 $\alpha$ pIN301	DH5 $\alpha$ containing a plasmid pIN301 encoding GFP	chloramphenicol 30	16-0105	Dr. Annette Vergunst (University of Montpellier, France)
<i>E. coli</i> DH5 $\alpha$ pIN298	DH5 $\alpha$ containing a plasmid pIN298 encoding mCherry	chloramphenicol 30	16-0106	Dr. Annette Vergunst (University of Montpellier, France)

\*Restriction deficient *S. aureus* strain RN4220 is defective in the restriction-modification system that targets foreign DNA for destruction, therefore enhancing genetic manipulation of *S. aureus* in the lab.

## Biofilm Quantitation

### Choosing Material for Optimized Biofilm Formation

Overnight cultures of *S. aureus* NRS77, *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a in LB were diluted 1:100 in TSB, and 100  $\mu$ L of each culture was added to triplicate wells of both a sterilized 96-well PVC plate (Costar, #29442-384) and a 96-well polystyrene plate (Greiner Bio-One, #655-185). The PVC plate was covered with porous sealing film (VWR, #60941-086), and the polystyrene plate was covered with the provided lid. Both plates were incubated at 37°C for 24 h. Quantitation of each monoculture biofilm on both plates was completed using the microtiter dish biofilm formation assay published by O'Toole [114]. Absorbance was measured using a Tecan Sunrise plate reader with optical density at 595 nm.

### Co-culture Biofilm Formation Assays and Optimization

*S. aureus* NRS77, *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a were grown overnight and diluted 1:100 in Tryptic Soy Broth (TSB). Equal volumes of each strain were added to centrifuge tubes in

pairs, then vortexed for 5-6 seconds to thoroughly mix the cultures. Two 96-well PVC plates were sterilized using 70% ethanol and allowed to dry in a biosafety cabinet. To both plates, 100  $\mu$ L of mixed co-culture was added to three replicate wells. Positive controls consisted of triplicate wells with 100  $\mu$ L of diluted single cultures. Plain TSB served as a negative control. The plates were sealed using porous sealing film to allow oxygen to freely cross, then covered with aluminum foil to help prevent desiccation. The plates were placed in an acrylic tub lined with damp paper towels and incubated at 37°C for 24 or 72 h.

#### Sonication and Drip Dilutions

Sonication was used to quantitate the number of cells in a biofilm using a procedure adapted from Harrison, *et al* [115]. After the 96-well plate had been incubated for the desired amount of time, the foil and sealing film were removed and the liquid culture dumped into a 10% (v/v) bleach solution. The plate was gently and briefly placed upside down on a paper towel to allow residual liquid to be removed from the surface of the plate. This step helps prevent cross contamination of the wells in the later steps of the procedure. Aliquots of 115  $\mu$ L of sterile water were added to the wells, and the samples were incubated for one min. The water was dumped out of the plate and 115  $\mu$ L of 1% Tween 20 in LB was added to each well to act as the recovery medium. The plate was covered with sealing foil (USA Scientific, #2923-0110) and placed on the bottom level of a test tube rack. The rack was suspended inside an ultrasonic cleaner (Branson 3200) until the liquid in the wells was submerged below the level of the water in the cleaner. The plate was subjected to sonication at 60 Hz for 10 min to disrupt biofilms, as

suggested in the protocol by Harrison, *et al* [115].

The foil was wiped down with 70% ethanol to ensure it was sterilized. A pipet tip was used to punch through the foil and transfer 20  $\mu$ L from the well to 180  $\mu$ L phosphate buffer solution (PBS) in row A of a separate flat bottom polystyrene 96-well plate. After the entire row had been inoculated, 10-fold serial dilutions were performed. Using an eight-channel pipettor, 10  $\mu$ L of the entire serial dilution were transferred to a square plate containing LB medium, which was set on its side and slanted to allow the drops to run down the agar. The drops were allowed to run three quarters of the way down the plate before replacing the lid and setting the plate down flat.

Plates were incubated at 37°C for 24-48 h, or until colonies became distinguishable. Supplementary Table 1 shows the colony morphology and incubation time for each strain. Typically, *S. aureus* colonies were counted after 24 h, while *B. cenocepacia* colonies were counted after 48 h. Colonies were counted in the drip line that contained between 11-100 colonies for each specific strain, and the number of live bacteria per milliliter of recovery medium (colony forming units per milliliter or CFU/mL) was calculated using both these values and the dilution factor of the most appropriate dilution.

#### Standardizing Viable Cell Count

To eliminate the variability in the viable cell count of overnight cultures between strains, drip dilutions were performed for cultures grown to an optical density at 600 nm (O.D.<sub>600</sub>) of 0.8, which corresponds to mid-log phase growth. Overnight cultures were grown by inoculation of 5 mL LB with the desired strain, followed by incubation at 37°C

with aeration until the next day. Overnight cultures were made for *B. cenocepacia* J2315, *S. aureus* NRS77, *S. aureus* NRS72, *S. aureus* AH3865, *S. aureus* AH2183, *S. aureus* AH1710, and *S. aureus* AH2547. For each strain, 100  $\mu$ L of the overnight culture was added to fresh, pre-warmed LB. These were allowed to grow at 37°C with aeration until O.D.<sub>600</sub> reached 0.8, and the tubes were placed on ice until all strains reached this O.D. The cultures were serially diluted in PBS, and then drip dilutions were performed to quantitate the viable cell count of each strain at O.D.<sub>600</sub> of 0.8.

## **Co-Culture Biofilm Assays**

### Co-culture Biofilms of five CF Pathogens in Pairwise Combinations

The methods described under the sections Co-culture Biofilm Formation Assays and Optimization and Sonication and Drip Dilutions were followed using all ten pairwise combinations of *B. cenocepacia* J2315, *S. aureus* NRS77, *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a. For each combination, equal volumes of cultures were added to microfuge tubes and vortexed for 5-6 seconds. Triplicate wells of two 96-well PVC plates received 100  $\mu$ L of each mixture and plates were incubated for 1 or 3 days. During drip dilutions, only the 10<sup>3</sup>-10<sup>7</sup> dilutions were plated. Plating of 10  $\mu$ L of this serial dilution therefore placed the limit of detection of the viable cell counts at 10<sup>5</sup> CFU/mL.

### The effect of *B. cenocepacia* J2315 on multiple *S. aureus* strains

To examine the ability of *B. cenocepacia* J2315 to affect various *S. aureus* strains, four *S. aureus* isolates were used and paired with *B. cenocepacia* J2315 in co-culture

conditions. Diluted cultures of each *S. aureus* strain, NRS77, NRS72, AH2183, and AH3865 were mixed 1:1 (v/v) with diluted *B. cenocepacia* J2315 culture. In addition to each co-culture, monocultures were also inoculated in triplicate wells of three different 96-well PVC plates, which were incubated for 1, 3, or 7 days. Wells containing TSB acted as the negative control. The procedure for the co-culture biofilm formation assay, with sonication and drip dilutions, was followed for this experiment.

#### Inoculation of Pre-established *S. aureus* NRS77 Biofilms with *B. cenocepacia* J2315

*S. aureus* NRS77 was grown to an O.D.<sub>600</sub> of 0.8, then diluted 1:10 in TSB. In a sterilized 96-well PVC plate, 100  $\mu$ L of the culture was added to 10 sets of triplicate wells. Another set of wells was inoculated with TSB as a negative control. The plate was sealed with porous film, covered with aluminum foil, and placed in an acrylic bin lined with damp paper towels. After covering the bin with plastic wrap, the bin was placed in a 37°C room for 72 h. Water was added to the bin daily to prevent desiccation of the biofilms.

The same day that the *S. aureus* monoculture biofilm plate was to be removed, *B. cenocepacia* J2315 and *S. aureus* NRS77 cultures were grown to an O.D.<sub>600</sub> of 0.8 then diluted 1:100 or 1:10 in TSB, respectively, in a microfuge tube. Based on the results from the Standardizing Viable Cell Count experiment in the section above, the dilutions of both strains had approximately the same cell count. Three different cultures were then made from these dilutions: *B. cenocepacia* J2315 monoculture, *S. aureus* NRS77 monoculture, and co-culture of *B. cenocepacia* J2315 and *S. aureus* NRS77. In addition to equalizing the cell count between strains, the total cell count of each strain must be the

same in the co-culture condition as in the single culture condition. This feat was accomplished by aliquoting 1 mL of diluted *B. cenocepacia* J2315 culture into a microfuge tube, centrifugation of the cells for one min at 21,130 x g, pouring off the supernatant, then resuspending the pellet in 1 mL of the diluted *S. aureus* NRS77 culture. All microfuge tubes were vortexed for 6 seconds to homogenize the mixtures.

The covers from the established *S. aureus* biofilms were removed and the biofilm supernatant was poured into a bleach solution. After dabbing off excess liquid from the surface of the plate, 100  $\mu$ L of diluted *S. aureus* NRS77 was added to three sets of triplicate wells, one set each for day 1, 3 and 7. This step was repeated for the culture of diluted *B. cenocepacia* and the co-culture mixture. In addition, 100  $\mu$ L of diluted *B. cenocepacia* was added to three triplicate non-biofilm containing wells to act as a positive control for biofilm formation at the point of inoculation. TSB was used as a negative control.

At this point, 9 sets of the triplicate *S. aureus* NRS77 biofilm wells were inoculated with either experimental or control treatments. The tenth set of *S. aureus* biofilm wells and the negative control from initial *S. aureus* inoculation were washed with 115  $\mu$ L sterile water then scraped into 115  $\mu$ L of 1% Tween 20 recovery medium using a P200 pipette tip. The medium was pipetted out of the wells, serially diluted, and plated for drip dilutions to determine the starting CFU/mL of the *S. aureus* biofilm at the point of *B. cenocepacia* inoculation.

The biofilms were once again covered with porous sealing film and aluminum foil, and then placed on damp paper towels in an acrylic, plastic-wrap-covered bin. The plate was incubated for 1, 3, and 7 days. On each day, the plate was uncovered, the liquid

culture of the wells of interest were removed with a pipette, and the wells were washed with sterile water, as described above. Because wells containing conditions for 1, 3, and 7 day biofilm formation were all on the same plate, biofilms were disrupted by scraping with a 200  $\mu$ L pipette tip, instead of through sonication. Biofilms were quantitated using drip dilutions. The plate was covered and incubated at 37°C between time points. On the final day, the negative control was also plated.

#### Fractionating *B. cenocepacia* J2315 cultures

*B. cenocepacia* J2315 cultures were separated into the supernatant and cellular components. Supernatant was isolated in two ways. First, *B. cenocepacia* J2315 was grown in a biofilm for three days in 64 wells of a 96-well PVC plate, as described above for the growth of monoculture *S. aureus* biofilm. On day three, the liquid from each well was removed and added to a microfuge tube. The cells were pelleted at 21,130 x *g* in a microfuge for one min, and the supernatant was filter-sterilized through a 0.45  $\mu$ m syringe filter. Aliquots of this sterilized supernatant were plated to ensure no viable *B. cenocepacia* J2315 cells remained. The total volume of collected supernatant was approximately 6.4 mL. To this, 6.4 mL of 20% glycerol in LB was added to result in a 10% glycerol solution that could be stored at -20°C until use.

Overnight culture supernatant was also isolated. *B. cenocepacia* J2315 was grown overnight in LB, centrifuged at 21,130 x *g* for one min, and the supernatant was filter-sterilized. Each supernatant solution was added 1:1 (v/v) with TSB and added to *S. aureus* biofilm to yield a 25% biofilm supernatant treatment and a 50% overnight supernatant treatment, both with 50% TSB. Additionally, the pellet formed after the



centrifugation of the overnight *B. cenocepacia* J2315 culture was resuspended and diluted 1:100 in TSB before addition to *S. aureus* biofilms.

#### Sonication of *B. cenocepacia* J2315 cells and biofilm assay

To determine if live cells were needed for the toxic effect on *S. aureus*, dead *B. cenocepacia* cells were made by placing an overnight culture of the J2315 strain in a 65°C water bath for 30 min then sonicated at 60% amplitude for 10 second pulses over a period of 10 min in a Qsonica cup sonicator (Qsonica, Q800R). This culture was not diluted, but mixed with TSB in a 1:1 (v/v) ratio. Plating of serial dilutions of the resulting culture showed no viable cells remained after sonication.

Live cells, sonicated cells, or either supernatants were added to triplicate wells containing one-day old *S. aureus* biofilms and also to empty wells to act as controls. One set of triplicate wells containing *S. aureus* biofilm received 100 µL plain TSB to act as a control for *S. aureus* biofilm formation. Another set of empty wells received TSB as a negative control. Two PVC plates were made with these conditions, and one was incubated at 37°C for 24 h, while the second was incubated for 72 h. The protocol described above in Sonication and Drip Dilution Biofilm was used to quantitate biofilm formation.

### **Phenotypes of Co-culture Biofilms**

#### Super Optimal Broth Preparation for *B. cenocepacia* Transformation

Super optimal broth (SOB) and super optimal broth with catabolite repression (SOC) are media used to promote higher plasmid transformation efficiency in bacteria.

To make SOB, 20 g tryptone, 5 g yeast extract, and 0.5 g NaCl were added to 950 mL sterile water and shaken until dissolved. To this solution, 10 mL of 250 mM KCl was added and the pH adjusted to 7.0. After autoclaving for 20 min, the medium was placed in a 55°C water bath to cool for about 30 min. Gentamicin (10 µg/mL) was added to the medium to prevent contamination, as suggested by published protocol for transformation of *B. cenocepacia* J2315 [116]. Right before the medium was used, 10 mL of sterile 1 M MgSO<sub>4</sub> and 5 mL 2 M MgCl<sub>2</sub> were added. SOC broth was made by adding 20 mL of a 1 M glucose solution to 1 L SOB.

#### *B. cenocepacia* Electrocompetent Cells

To create electrocompetent cells for transformations, an overnight culture of *B. cenocepacia* J2315 was grown in 5 mL SOB medium. The next day, the culture was diluted into an Erlenmeyer flask containing 50 mL SOB to make a final O.D.<sub>600</sub> of 0.01. The flask was incubated with aeration at 37°C for 2-3 generations, until the O.D.<sub>600</sub> was between 0.04-0.08 based on the published protocol [116]. This equates to about 4-6 h of incubation. Next, the flask was chilled on ice for 5 min to stop bacterial growth, and the culture was aliquoted and centrifuged at 5000 x g for 8 min at 4°C (Kendro Lab Products, Sorvall Legend RT Centrifuge). The supernatant was removed, and the pellet was washed and resuspended in 20 mL ice-cold sterile 0.5 M sucrose, followed by two additional rounds of centrifugation, washing, and resuspension. The final pellet was resuspended in 0.5 mL 0.5 M sucrose. An LB and glycerol solution was added to make the final concentration 10% glycerol. The sample was divided into 500 µL aliquots and stored at -80°C until electroporation.

### Transformation of *B. cenocepacia* J2315

Following the QIAprep Spin Miniprep Kit (Qiagen, #27106) protocol, plasmids encoding fluorescent proteins were purified from the *E. coli* DH5 $\alpha$  strains provided by Dr. Annette Vergunst (University of Montpellier, France). These are listed in Table 2. The purity and DNA concentration for each purified plasmid was determined using a Nanodrop 2000 (Thermo Fisher Scientific). Plasmids were stored at -20°C until use.

The plasmids, thawed electrocompetent *B. cenocepacia* cells, and electroporation cuvettes (VWR, #89047-206) were kept on ice. To 15  $\mu$ L water, 0.1  $\mu$ g of each plasmid was added, followed by the addition of 55  $\mu$ L of electrocompetent *B. cenocepacia*. The negative control consisted of electrocompetent cells without the addition of plasmid. After transferring this volume to a 2 mm electroporation cuvette, cells were electroporated at 2.5 kV (Bio-Rad Micropulser). Samples were removed from the cuvette by adding 950  $\mu$ L SOC medium, and after gentle mixing, the volume was transferred to a microfuge tube. Samples were incubated at 37°C, without aeration, for 4-5 h and plated on LB plates supplemented with 100  $\mu$ g/mL chloramphenicol. Plates were incubated for three days. Single colonies were plated in patches on selective plates, and the resulting growth was observed under a confocal microscope to confirm fluorescence. Colonies that showed the proper fluorescence profile were stored in 20% glycerol at -80°C under the strain list designations shown in Table 1.

### Confocal Laser Scanning Microscopy Preparation and Analysis

To capture images of both single and co-culture biofilms, the fluorescent strains *S. aureus* AH1710 and *B. cenocepacia* J2315-62 were utilized. Each strain was individually

inoculated in test tubes containing 5 mL LB supplemented with 10 µg/ml or 100 µg/ml chloramphenicol, respectively. Cultures were incubated at 37°C overnight then diluted 1:100 in TSB supplemented with 10 µg/mL chloramphenicol. Glass bottom FluoroDishes (World Precision Instruments, FD35-100) were used to grow the biofilms. Three sets of triplicate dishes were made for each monoculture and co-culture conditions. Dishes contained 3 mL of *S. aureus* AH1710, 3 mL of *B. cenocepacia* J2315-62, or 3 mL of a 1:1 by volume mixture of the two. Confocal laser scanning microscopy (CLSM) analysis occurred at 1 day, 3 days, and 7 days post-inoculation of each condition. At each time point, the biofilm supernatant was discarded prior to microscopy. Both 2D and 3D imaging was captured using NIS-Element software with both the 40x and 60x lenses of a Nikon Eclipse Ti microscope. The GFP (487 nm) and TxRed (561 nm) lasers were used for excitation wavelengths. The power and gain of each image was optimized to show maximum fluorescence without saturation of the image.

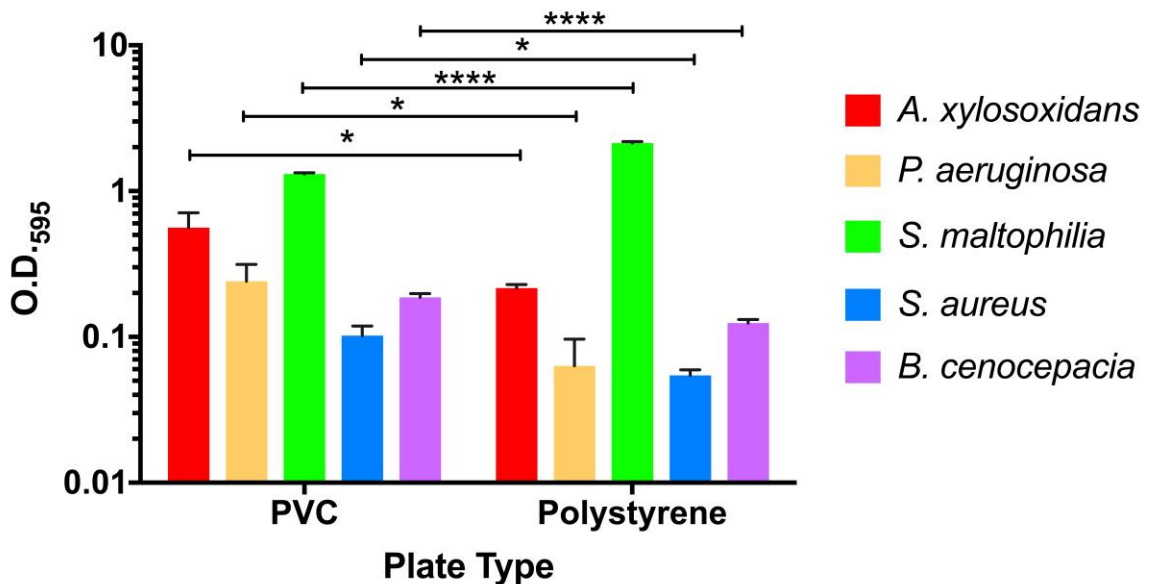
### Statistical analyses

All biofilm assays and viable cell count assays were graphed and analyzed using GraphPad Prism v. 5.1.0. Statistical significance was calculated using either two-way ANOVA analysis, one-way ANOVA analysis in conjunction with Tukey's multiple comparison test, or an unpaired T test, as indicated in each figure legend. Only p-values less than 0.05 were considered statistically significant.

# Results

## Choosing Material for Optimized Biofilm Formation

In order to test for the plate material that optimizes biofilm formation, representative strains of five common CF pathogens were chosen to form monoculture biofilms in 96 well plates made of two different materials. Twenty-four hour monoculture biofilms of *S. aureus* NRS77, *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a were grown in both round-bottom 96-well PVC plates and flat-bottom 96-well polystyrene plates. Biofilm was quantitated using the retention of crystal violet as described in the Materials and Methods. Results are shown in Figure 1.



**Figure 1. Biofilm formation in polystyrene and PVC 96-well plates.** The optical density of biofilm growth was obtained for one-day-old monocultures of *A. xylosoxidans* AU19284 (red), *P. aeruginosa* C3719 (orange), *S. maltophilia* K279a (green), *S. aureus* NRS77 (blue), and *B. cenocepacia* J2315 (purple) grown in both PVC and polystyrene 96-well plates. Two-way ANOVA analysis showed an overall significant effect of the interaction between strain type and plate material ( $p < 0.0001$ ). Unpaired t-tests were used to calculate significance between strains grown on each plate material (\* $p < 0.05$ , \*\*\*\* $p < 0.0001$ ).

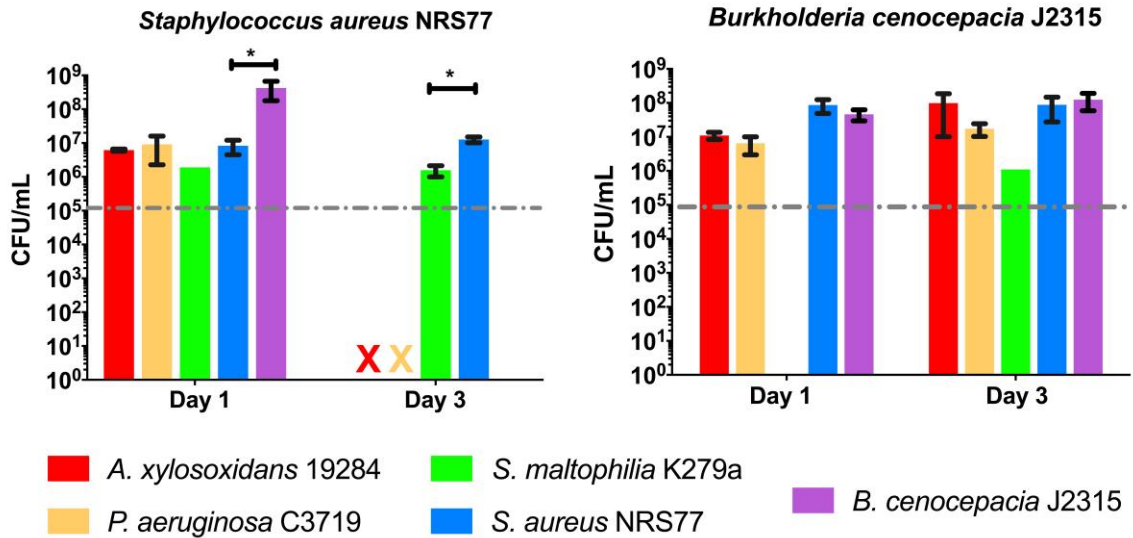
Unpaired t-tests of each strain showed that for all strains except *S. maltophilia* K279a, biofilm formation was significantly greater in 96-well PVC plates compared to polystyrene 96-well plates (Fig. 1). *S. maltophilia* K279a grew optimally in the polystyrene 96-well plate but was still able to form biofilm that was comparable to the biofilm formation of the other four isolates in the 96-well PVC plate. Due to these observations, the 96-well PVC plate was chosen to use in all future biofilm formation assays.

#### Co-culture Biofilms of Five CF Pathogens in Pairwise Combinations

After establishing that round-bottom, 96-well PVC plates were conducive for biofilm formation for a majority of the strains (Fig. 1), the same five isolates were tested for compatibility of biofilm formation in co-culture conditions. All pairwise combinations of *B. cenocepacia* J2315, *S. aureus* NRS77, *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a were inoculated in triplicate wells of a 96-well PVC plate and incubated for 1 or 3 days. Biofilm quantitation was completed using sonication and drip dilutions, and viable cell counts for each strain in co-culture were compared to each strain in monoculture.

Supplementary Figure 1 shows the data and summarizes the results for monoculture and co-culture biofilm formation of *A. xylosoxidans* AU19284, *P. aeruginosa* C3719, and *S. maltophilia* K279a. The data for *B. cenocepacia* J2315 and *S. aureus* NRS77 (Fig. 2) suggested that the presence of *S. aureus* NRS77 has no effect on *B. cenocepacia* J2315 biofilm formation, but the presence of *B. cenocepacia* J2315 results in a reduction in viable cells below the limit of detection for *S. aureus* NRS77

biofilm by day 3. These results suggested that the presence of *B. cenocepacia* J2315 may inhibit *S. aureus* NRS77 biofilm formation over time.

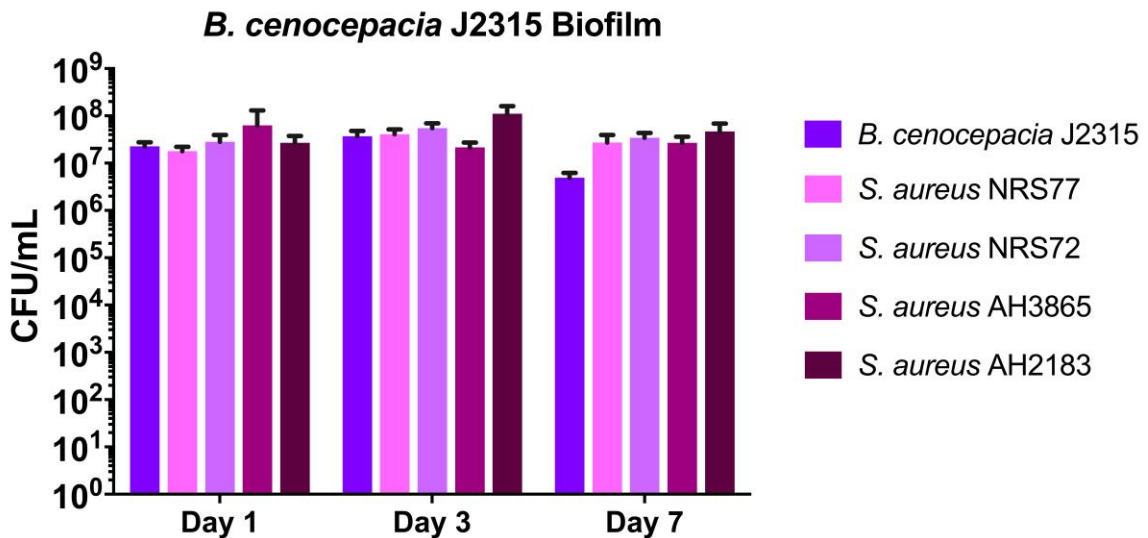


**Figure 2. Biofilm formation of *B. cenocepacia* J2315 and *S. aureus* NRS77 in co-culture conditions.** Viable cell counts of (A) *S. aureus* NRS77 biofilms and (B) *B. cenocepacia* J2315 biofilms when grown in co-culture with *A. xylosoxidans* (red), *P. aeruginosa* C3719 (orange), *S. maltophilia* K279a (green), *S. aureus* NRS77 (blue), or *B. cenocepacia* J2315 (purple). Presence of an “X” indicates exclusion of data due to contamination of samples. Absence of a bar indicates the viable counts are below the limit of detection (gray line), which was  $10^5$  for this experiment. One-way ANOVA analysis showed an overall significant difference among means ( $p < 0.05$ ) in CFU/mL within both *S. aureus* NRS77 and *B. cenocepacia* J2315 data. Tukey’s multiple comparison test was used to compare co-culture and monoculture conditions ( $*p < 0.05$ ).

#### Effect of *B. cenocepacia* J2315 on *S. aureus* Strains

Next, the ability of *B. cenocepacia* J2315 to inhibit biofilm formation in other *S. aureus* strains was tested. It was hypothesized that inhibition of *S. aureus* biofilm by *B. cenocepacia* J2315 would not be limited to the NRS77 strain. Mixes of *B. cenocepacia* J2315 and four *S. aureus* strains – NRS77, NRS72, AH2183, and AH3865 – were made and inoculated in a 96-well PVC plate and allowed to incubate for 1, 3, or 7 days before the co-culture biofilms were quantitated by sonication and drip dilutions. The viable cell count was then compared to viable counts from monoculture biofilms.

There was no statistical significance in biofilm formation of *B. cenocepacia* J2315 between *S. aureus* strains or over time (Fig. 3), while all tested *S. aureus* strains were inhibited over time by the presence of *B. cenocepacia* J2315 (Fig. 4). Viable *S. aureus* NRS77 biofilm was below the limit of detection ( $10^3$  CFU/mL) in the presence of *B. cenocepacia* J2315 (Fig. 4A). Biofilms of *S. aureus* NRS72 were present after growing for 1 day with *B. cenocepacia* J2315, but growth was significantly inhibited in comparison to the monoculture biofilm, and by day 3, viable *S. aureus* NRS72 biofilm was reduced beyond the limit of detection in co-culture with *B. cenocepacia* J2315 (Fig. 4B).

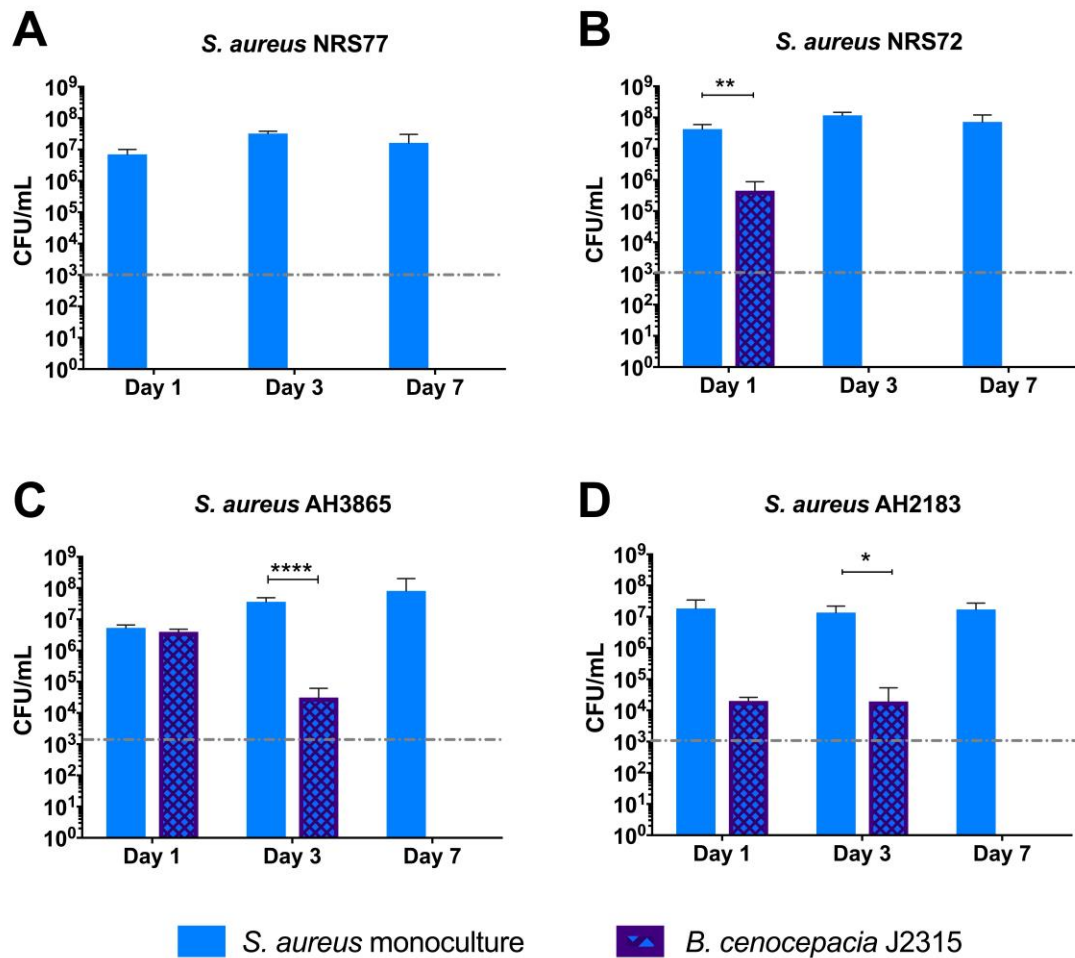


**Figure 3. Effect of *S. aureus* strains on *B. cenocepacia* J2315 survival in co-culture biofilms.** *B. cenocepacia* J2315 was mixed with *S. aureus* strains NRS77, NRS72, AH3862, or AH2183 for 1, 3, or 7 days. Biofilm formation was quantitated using sonication and drip dilutions, and biofilm viable cell counts were compared to monoculture biofilm growth of *B. cenocepacia* J2315. One-way ANOVA with Tukey's multiple comparison test showed no significant difference over time or between co-culture conditions.

*S. aureus* AH3865 was able to form biofilm as effectively in the presence of *B. cenocepacia* J2315 as in monoculture during day 1, but was significantly inhibited by day 3 (Fig. 4C). *S. aureus* AH2183 biofilm formation appeared to be inhibited in the presence



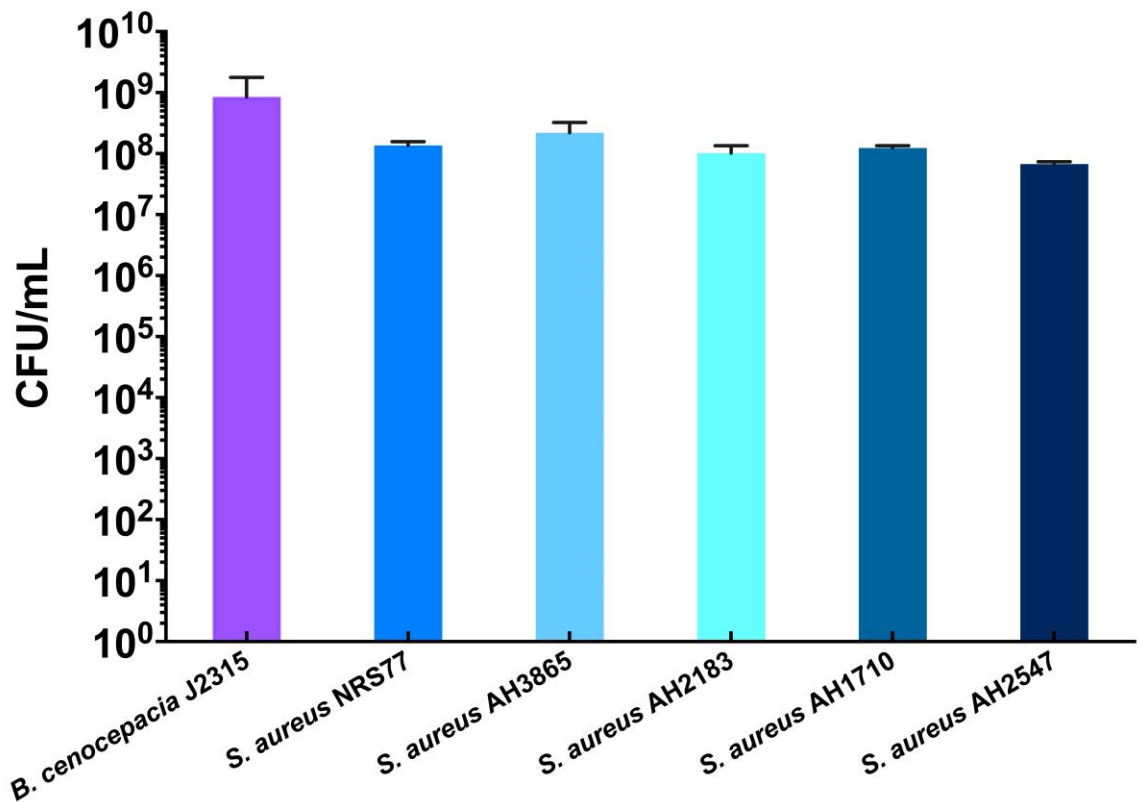
of *B. cenocepacia* J2315 after 1 day, but this effect was not statistically significant (Fig. 4D). On day 3, however, the decrease in *S. aureus* AH2183 co-culture biofilm was statistically significant in comparison to monoculture. All strains of *S. aureus* had no detectable biofilm on day 7 in the presence of *B. cenocepacia* J2315. This data suggests that *B. cenocepacia* J2315 inhibits biofilm formation in multiple *S. aureus* strains.



**Figure 4. Effect of *B. cenocepacia* J2315 on multiple *S. aureus* strains during biofilm formation over time.** *S. aureus* strains NRS77 (A), NRS72 (B), AH3865 (C), and AH2183 (D) were grown in monoculture and in co-culture with *B. cenocepacia* J2315 for 1, 3, or 7 days. Biofilm formation was quantitated using sonication and drip dilutions. Bars representing biofilm formation of *S. aureus* strains grown alone (blue) or grown in co-culture with *B. cenocepacia* J2315 (purple striped) are shown. Limit of detection is indicated by the gray line. Unpaired t-test measured significant differences as indicated by brackets and asterisks (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ).

### Standardizing the Viable Cell Count of Isolates with O.D.<sub>600</sub>

In the previous experiments, an equal volume of each overnight culture was used when inoculating 96-well plates for co-culture biofilm formation. However, because *S. aureus* is a coccus and *B. cenocepacia* is a bacillus, the size difference between species may result in a different concentration of cells per unit volume of medium. Additionally, the viable cell count of overnight cultures within one strain may not be consistent between replicates. We sought to eliminate the difference in cell number between *B. cenocepacia* J2315 and *S. aureus* NRS77 as a possible source of variation.



**Figure 5. Viable cell count of isolates with O.D.<sub>600</sub> of 0.8.** Overnight cultures were diluted in fresh LB and grown to O.D.<sub>600</sub> of 0.8, serial diluted, and plated. Viable cell counts were collected for *B. cenocepacia* J2315, and the indicated *S. aureus*. One-way ANOVA analysis showed no significant differences between strains.

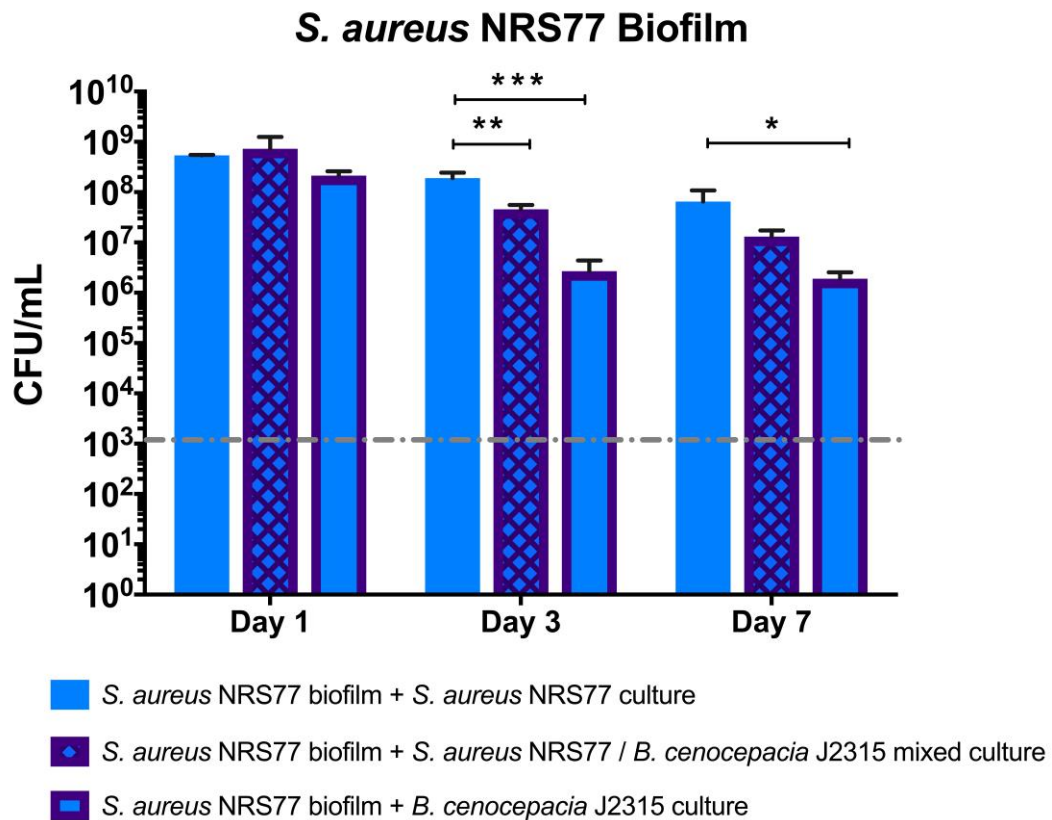
Performing drip dilutions on cultures with an O.D.<sub>600</sub> of 0.8 standardizes the viable cell count of each strain. This process was completed for *B. cenocepacia* J2315 and for *S. aureus* strains NRS77, AH3865, AH2183, AH1710, and AH2547. Results show that all *S. aureus* strains had an average concentration of approximately 10<sup>8</sup> CFU/mL at this O.D.<sub>600</sub> reading, while *B. cenocepacia* J2315 had an average concentration around 10<sup>9</sup> CFU/mL (Fig. 5). Therefore, when creating 50/50 mixes of *B. cenocepacia* J2315 and any of the aforementioned *S. aureus* strains grown to O.D.<sub>600</sub> of 0.08, *B. cenocepacia* J2315 may have 10-fold the number of cells compared to *S. aureus*. This led to a fundamental change in our experimental design. In the following experiments, after strains reached an O.D.<sub>600</sub> of 0.8, *B. cenocepacia* J2315 was diluted 10-fold in comparison to *S. aureus* NRS77 to account for differences in cell size.

#### Effect of *B. cenocepacia* J2315 on Pre-Established *S. aureus* NRS77 Biofilms

The results from the above experiments show that the J2315 strain of *B. cenocepacia* is able to prevent *S. aureus* from forming and maintaining the biofilm lifestyle. We sought to determine if *B. cenocepacia* J2315 is also able to destroy mature *S. aureus* NRS77 biofilms. We hypothesized that mature *S. aureus* biofilms would be able to survive inoculation of *B. cenocepacia* J2315, but biofilm viable cell counts would show a progressive decrease over time, while *B. cenocepacia* J2315 biofilms would be unaffected.

*S. aureus* biofilms were formed for 3 days before the addition of either more *S. aureus* NRS77, a 50/50 mixture of *S. aureus* NRS77 and *B. cenocepacia* J2315, or *B. cenocepacia* J2315. After 1 day, there was no significant difference between *S. aureus*

biofilms of any treatment (Fig. 6). On day 3, there was a significant decrease in *S. aureus* NRS77 biofilm in both the treatment with *B. cenocepacia* J2315 / *S. aureus* NRS77 mixture and the treatment with *B. cenocepacia* J2315. Only the treatment with *B. cenocepacia* J2315 showed a statistically significant decrease in *S. aureus* NRS77 biofilm formation on day 7. In addition, compared to monoculture conditions, there was no statistically significant difference in *B. cenocepacia* J2315 biofilm formation in the presence of *S. aureus* NRS77 biofilms (data not shown).



**Figure 6. Inhibition of mature *S. aureus* NRS77 biofilms by *B. cenocepacia* J2315.** Mature, 3-day old *S. aureus* NRS77 biofilms were inoculated with *S. aureus* NRS77 (blue), a 50:50 mixture of *S. aureus* NRS77 and *B. cenocepacia* J2315 cultures (purple striped), or *B. cenocepacia* J2315 (blue bar, purple outline) and grown for 1, 3, and 7 days. Biofilms were quantitated for each condition using sonication and drip dilutions. Limit of detection is indicated by a gray line. One-way ANOVA with Tukey's multiple comparison tests analyzed significant differences of biofilm formation between treatments (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

Together, these data continue to support the hypothesis that *B. cenocepacia* J2315 is diminishing the ability of *S. aureus* NRS77 to form or maintain a biofilm state. Not only does *B. cenocepacia* J2315 prevent biofilm formation of *S. aureus* NRS77 upon simultaneous inoculation, but it also results in the reduction of *S. aureus* NRS77 biofilm that has already been established. It is not clear if this inhibition is due to the presence of planktonic *B. cenocepacia* J2315, *B. cenocepacia* J2315 biofilm, or a combination of both.

#### Exploring the Mechanism of *S. aureus* Biofilm Inhibition

This experiment was designed to determine if inhibition of *S. aureus* biofilm by *B. cenocepacia* was through a secreted or shed product, and to determine if live *B. cenocepacia* cells are necessary for *S. aureus* biofilm destruction. Five *B. cenocepacia* J2315 treatments for *S. aureus* biofilms were chosen to answer these mechanistic questions and are depicted in Table 2. *B. cenocepacia* J2315 seems to specifically inhibit biofilm formation in *S. aureus* and does not appear to reduce biofilm of other tested species (Supplementary Fig. 1). Due to these observations, it was hypothesized that *B. cenocepacia* J2315 needed to recognize the presence of *S. aureus* NRS77 to mediate biofilm reduction, and would, therefore, require the presence of live *B. cenocepacia* J2315 cells.

For clarification, the results from this experiment have been separated into four sections: the effect of sonicated *B. cenocepacia* J2315 cells, *B. cenocepacia* J2315 biofilm supernatant, *B. cenocepacia* J2315 overnight supernatant, and *B. cenocepacia* J2315 pelleted planktonic cells on *S. aureus* NRS77 biofilms. The effects of all *B.*

*cenoepeacia* J2315 fractions on *S. aureus* NRS77 biofilms were compared to monoculture *S. aureus* NRS77 biofilm formation. In addition, results could be compared to the inhibition of *S. aureus* NRS77 biofilms treated with whole *B. cenoepeacia* J2315 culture. This was the same condition tested in previous experiments and therefore acted as a positive control for *S. aureus* NRS77 biofilm inhibition.

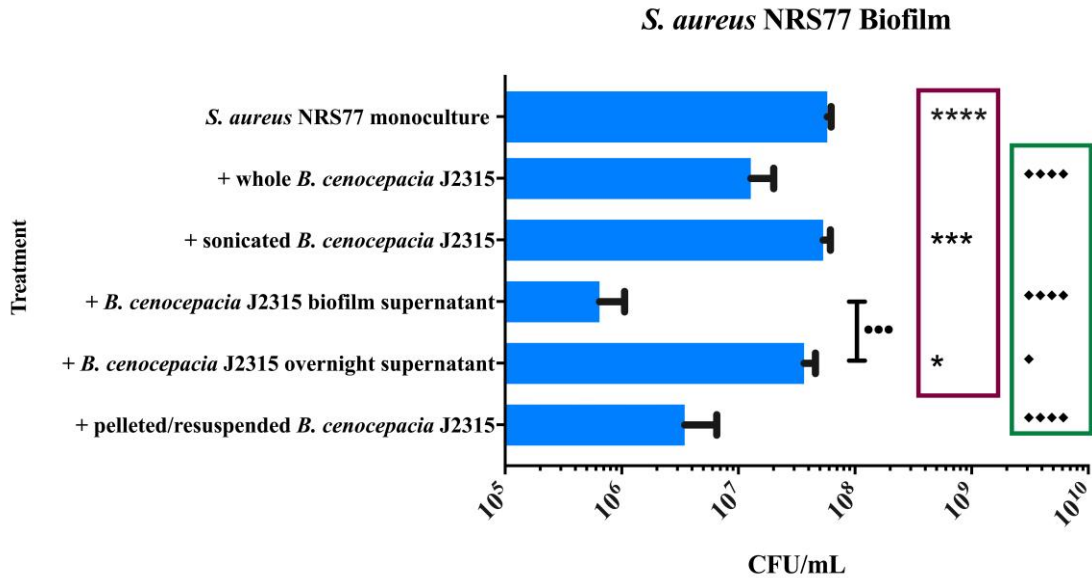
**Table 2. Experimental design for exploring possible mechanisms of *B. cenoepeacia* J2315-mediated destruction of *S. aureus* NRS77 biofilm**

Treatment	Source	Purpose or question addressed
Whole <i>B. cenoepeacia</i> J2315	Overnight culture	Positive control for <i>S. aureus</i> NRS77 biofilm inhibition
Sonicated <i>B. cenoepeacia</i> J2315	Overnight culture, sonicated	(1) Is live <i>B. cenoepeacia</i> J2315 necessary for <i>S. aureus</i> biofilm reduction?
<i>B. cenoepeacia</i> J2315 biofilm supernatant	Filter-sterilized supernatant of a 3-day-old monoculture biofilm	(1) Do biofilm secretions result in <i>S. aureus</i> biofilm reduction? (2) Is cell-to-cell contact necessary for <i>S. aureus</i> biofilm reduction?
<i>B. cenoepeacia</i> J2315 overnight supernatant	Filter-sterilized supernatant of an overnight culture	(1) Do planktonic secretions result in <i>S. aureus</i> biofilm reduction? (2) Is cell-to-cell contact necessary for <i>S. aureus</i> biofilm reduction?
Pelleted/resuspended <i>B. cenoepeacia</i>	Overnight culture, pelleted and resuspended in fresh medium	(1) Are cells alone sufficient to induce <i>S. aureus</i> biofilm reduction? (2) Is cell-to-cell contact necessary for <i>S. aureus</i> biofilm reduction?

*The effect of sonicated B. cenoepeacia J2315 cells on S. aureus NRS77 biofilms*

*B. cenoepeacia* J2315 was subjected to sonication in order to lyse cells. Following the sonication treatment, serial and drip dilutions of lysed cells suggested no detectable levels of viable *B. cenoepeacia* cells remained (data not shown). This sonicated mixture was mixed with TSB 1:1 (v/v) and added to one-day-old *S. aureus* NRS77 biofilm. Biofilms were incubated for 3 days. The resulting data shows that there

was no significant difference in the viable cell count of the *S. aureus* NRS77 biofilm and the *S. aureus* biofilm exposed to sonicated *B. cenocepacia* J2315 (Fig. 7). In comparison, the significant inhibition of the *S. aureus* NRS77 biofilm due to the *B. cenocepacia* J2315 control on day three suggests that the process that leads to *S. aureus* biofilm antagonism requires live *B. cenocepacia* J2315.



**Figure 7. Effect of *B. cenocepacia* J2315 components on *S. aureus* NRS77 biofilm.** One-day-old *S. aureus* NRS77 biofilm was inoculated with either TSB or one of five experimental treatments. *S. aureus* NRS77 biofilm was quantitated after three days using sonication and drip dilutions. One-way ANOVA analysis showed a significant effect of treatment ( $p < 0.0001$ ), and Tukey's multiple comparison test analyzed for significant difference of treatments in comparison to the treatment with whole *B. cenocepacia* J2315 (Red box; \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ) and the *S. aureus* monoculture condition (Green box; ♦ $p < 0.05$ , ♦♦♦♦ $p < 0.0001$ ). Statistical difference of *S. aureus* NRS77 biofilm formation between *B. cenocepacia* J2315 overnight and biofilm supernatant treatments is also shown (••• $p < 0.001$ ).

#### *The effect of B. cenocepacia J2315 biofilm supernatant on S. aureus NRS77 biofilms*

*B. cenocepacia* J2315 biofilm was grown for three days, and the supernatant was collected and filter-sterilized before being mixed with TSB for addition to a one-day-old *S. aureus* NRS77 biofilm. After incubation for 3 days, the *S. aureus* NRS77 biofilm viable cell count was measured following sonication and drip dilutions. Analysis of the

resulting data shows exposure to the biofilm supernatant caused a statistically significant decrease in *S. aureus* NRS77 biofilms compared to the *S. aureus* NRS77 monoculture biofilms. There was no statistically significant difference in *S. aureus* NRS77 biofilm inhibition when comparing the exposure to whole *B. cenocepacia* J2315 to the exposure to *B. cenocepacia* J2315 biofilm supernatant.

Both the presence of *B. cenocepacia* J2315 cells and the exposure to *B. cenocepacia* J2315 biofilm supernatant results in a reduction of *S. aureus* NRS77 biofilm. This suggests that *B. cenocepacia* J2315 is secreting or shedding a substance during biofilm formation that is a potential inhibitor of *S. aureus* NRS77 biofilms. Additionally, *B. cenocepacia* J2315 does not require the presence of *S. aureus* NRS77 for production of the inhibiting substance.

#### *The effect of B. cenocepacia J2315 overnight supernatant on S. aureus NRS77 biofilms*

The overnight culture of *B. cenocepacia* J2315 was centrifuged and the supernatant was collected, filter-sterilized, and stored in a 1:1 mixture of a glycerol solution for storage until use. On the day of inoculation of one-day-old *S. aureus* NRS77 biofilm, the supernatant glycerol solution was mixed 1:1 (v/v) with TSB and added to *S. aureus* NRS77 biofilms. Quantitative analysis through sonication and drip dilutions was completed for *S. aureus* NRS77 biofilms after three days of incubation.

*B. cenocepacia* J2315 overnight supernatant decreased *S. aureus* NRS77 biofilm to a mild but significantly lower level compared to treatment with whole *B. cenocepacia* J2315 (Fig. 7). These results suggest that *B. cenocepacia* J2315 may be able to secrete or shed a product during growth of overnight culture that results in a slight reduction of *S.*



*aureus* NRS77 biofilms. However, because the effect of the overnight supernatant treatment did not result in the same level of inhibition as whole cultures, this suggests that either an inhibitory molecule is not optimally expressed during overnight growth or that there is an additive effect of *B. cenocepacia* J2315 cells and supernatant on *S. aureus* biofilm inhibition.

#### *The effect of pelleted planktonic cells on S. aureus NRS77 biofilms*

An overnight culture of *B. cenocepacia* J2315 was centrifuged, and the supernatant was removed. The resulting pellet was resuspended and diluted in fresh TSB then added to one-day-old *S. aureus* NRS77 biofilm and incubated for one or three days. Biofilm was quantitated through sonication and drip dilutions. Statistical analysis showed that after three days the viable cell count of *S. aureus* NRS77 biofilms was statistically lower than the monoculture *S. aureus* NRS77 biofilms but not statistically different than the *S. aureus* NRS77 biofilms exposed to whole *B. cenocepacia* J2315 (Fig. 7). This result indicates that *B. cenocepacia* J2315 cells, both with and without supernatant from overnight growth, are capable of reducing *S. aureus* NRS77 biofilm to the same extent.

Altogether, the data presented above suggest that our original hypothesis regarding a potential mechanism of *S. aureus* biofilm reduction was likely incorrect. *B. cenocepacia* J2315 does not require the presence of *S. aureus* NRS77 in order to produce an inhibitory effect on *S. aureus* NRS77 biofilm viability. It appears as if there is an antagonistic substance in the supernatant of *B. cenocepacia* J2315 – both from cells in a planktonic and biofilm lifestyle – that interacts with *S. aureus* NRS77 biofilm in a way that leads to a reduction in the viable cell count of cells in the biofilm.

### Transformation of *B. cenocepacia* J2315

To better understand the interactions between *S. aureus* strains and *B. cenocepacia* J2315 in biofilms, strains encoding fluorescent proteins were obtained. *S. aureus* strains bearing GFP- and dsRedExpress-encoding plasmids (Table 1) were obtained from Dr. Alexander Horswill at the University of Iowa. To generate fluorescent *B. cenocepacia* J2315, a protocol established by Dubarry et al. [116] was followed, and transformants of *B. cenocepacia* J2315 expressing GFP (J2315-301), mCherry (not named) and dsRedExpress (J2315-62) were created. The mCherry transformants showed weak fluorescence and were quick to photobleach, while both the GFP strain and dsRedExpress strains showed strong fluorescence that did not fade quickly. The latter two strains were used in conjunction with the provided *S. aureus* strains to observe co-culture biofilms with confocal laser scanning microscopy, which allows 3-dimensional biofilm structures to be observed.

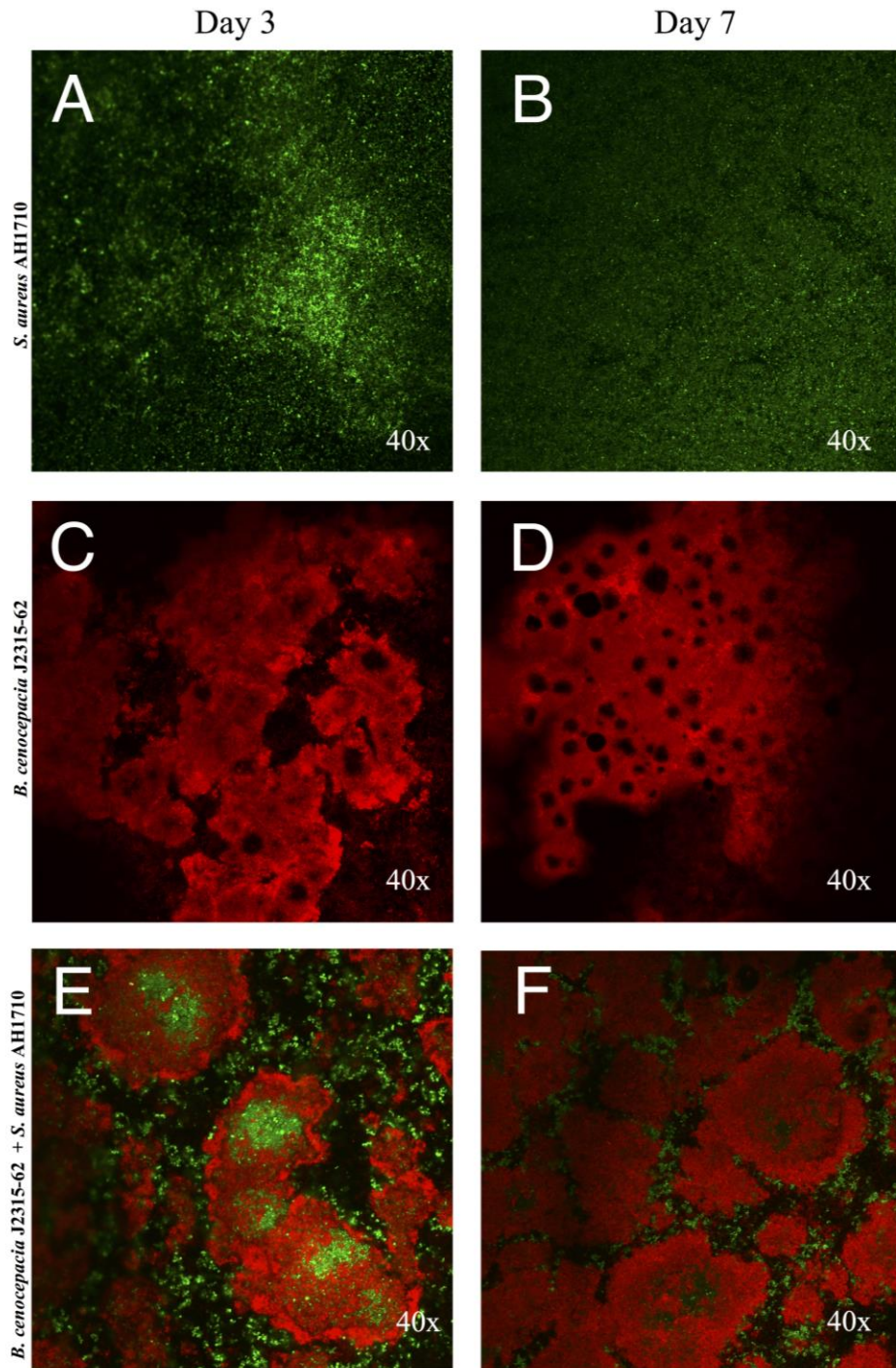
### Phenotypes of Co-culture Biofilms

To observe the phenotypic and spatial interaction between these organisms, strains *B. cenocepacia* J2315-62 (red fluorescence) and *S. aureus* AH1710 (green fluorescence) were grown in triplicate in both co-culture and monoculture conditions for 1, 3, or 7 days in glass-bottomed petri dishes. In addition, another set of dishes were inoculated with *S. aureus* AH1710 monoculture and incubated for 3 days. The *S. aureus* supernatant was then replaced with filter-sterilized supernatant of 3-day-old *B. cenocepacia* J2315 biofilm, and the dishes were incubated with the remaining plates. It was hypothesized that the destruction of *S. aureus* biofilm formed in co-culture with *B.*

*cenoepectacia* J2315-62 would be observable over time. Additionally, because *B. cenoepectacia* J2315 reduces biofilm in several *S. aureus* strains, we also predicted that *S. aureus* AH1710 biofilm exposed to *B. cenoepectacia* J2315 biofilm supernatant would be reduced.

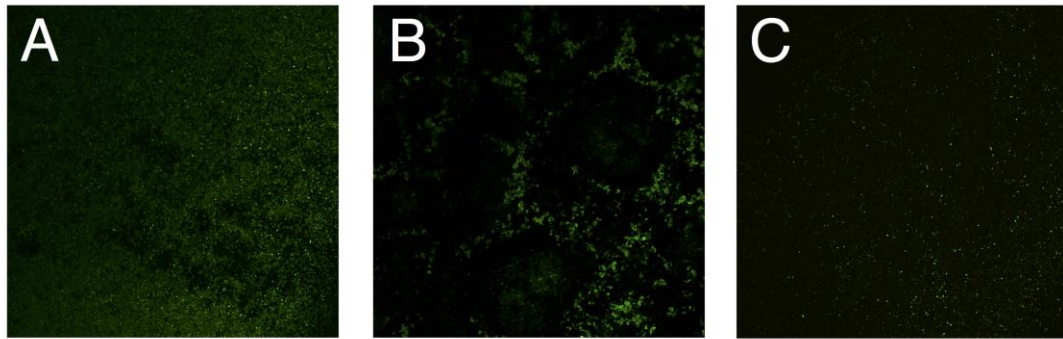
First, *S. aureus* AH1710 and *B. cenoepectacia* J2315-62 form morphologically different biofilms in monoculture compared to co-culture. Single cultures of *S. aureus* AH1710 laid down a biofilm in a flat mat, with little to no clustering morphology (Figs. 8A and B), while *S. aureus* AH1710 cells in co-cultures with *B. cenoepectacia* can regularly be seen in both flat mats on the surface of the dish and in mixed species mushroom structures with *B. cenoepectacia* J2315-62 (Figs. 8E and F). The biofilm formed by *B. cenoepectacia* J2315-62 in monoculture is highly clustered and porous, resembling honeycomb (Figs. 8C and D). These structures are less apparent when grown in co-culture with *S. aureus* AH1710, where as *B. cenoepectacia* biofilm phenotype appears to switch from a porous morphology to a mushroom-like structure that surround clusters of *S. aureus* (Figs. 8E and F).

While co-culture experiments using 96-well PVC plates showed an absence of biofilm of four *S. aureus* strains by day 7 when inoculated simultaneously with *B. cenoepectacia* J2315 (Figs. 1 and 2), *S. aureus* AH1710 is still present in the co-culture biofilm after the same amount of time in co-culture, albeit to a reduced degree (Fig. 9B). However, *S. aureus* AH1710 biofilm exposed to 3-day-old sterile supernatant of *B. cenoepectacia* J2315 appears markedly decreased in comparison to the co-culture condition. This difference in *S. aureus* biofilm reduction can be seen in Figure 9B and C.



**Figure 8. CLSM imaging of single and co-culture biofilms of *B. cenocepacia* J2315-62 and *S. aureus* AH1710 over time.** Triplicate glass bottom dishes were inoculated with either single cultures (A-D) or co-cultures (E and F) of *B. cenocepacia* J2315-62 and *S. aureus* AH1710 and grown for 3 (A, C, and E) and 7 days (B,D, and F). Images were captured using NIS-Elements software, and settings were optimized for each image.

Based on these observations, it seems as if the presence of *B. cenocepacia* cells may be slowing the progression of *S. aureus* biofilm destruction that results from *B. cenocepacia* J2315 secretions when grown in the glass bottom dishes. Alternatively, the timing of biofilm inhibition may be variable between cultures grown in PVC 96-well plates and cultures grown in glass-bottom dishes. A longer time period may be required to see the same levels of inhibition in the glass bottom dishes that is observed in the 96-well PVC plate.



**Figure 9. Effect of *B. cenocepacia* J2315 biofilm supernatant on *S. aureus* AH1710 biofilm.** (A) Monoculture, 7-day-old *S. aureus* AH1710 biofilm. (B) A 7-day-old co-culture biofilm image with the red channel (*B. cenocepacia* J2315-62) removed, and only the green channel (*S. aureus* AH1710) displayed. (C) Three-day-old *S. aureus* AH1710 biofilm inoculated with 25% *B. cenocepacia* J2315 biofilm supernatant in TSB, and incubated for an additional four days.

Although it was shown that both *B. cenocepacia* J2315 cells and biofilm supernatant reduce *S. aureus* biofilm to the same extent (Fig. 7), it is clear that some sort of alternate interaction is occurring under the conditions of this experiment. The presence of *B. cenocepacia* cells still reduces *S. aureus* biofilm, but appears to slow that reduction compared to the presence of biofilm supernatant alone. This effect could be due to the difference in the experimental design between the 96-well plate experiments and the CLSM experiments. The glass-bottom dishes used for biofilm attachment in this experiment held a larger volume of inoculated medium compared to the 96-well PVC

plates used in other experiments. Difference in material or total medium volume may influence the time it takes for reduction in *S. aureus* biofilm to occur.

Another possibility is that the substance in the biofilm secretion that results in *S. aureus* biofilm destruction is regulated and taken back up by *B. cenocepacia*. The presence of *B. cenocepacia* J2315-62 would decrease the concentration of the biofilm-destroying-substance, thereby slowing *S. aureus* biofilm degradation in a dose-dependent manner. This is speculative, and further experimentation needs to be done to identify the source of *S. aureus* biofilm-degradation in the *B. cenocepacia* J2315 biofilm supernatant.

## **Discussion**

Most of the published literature chronicling CF pathogenesis focus on single culture biofilms of pathogens either *in vitro* or *in vivo*; however, co-culture biofilms are common in nature and in human hosts during the course of disease and thus warrant further study. Multiple strains and species are commonly cultured from CF lungs [103], and the unique interactions that may occur in the biofilms of these isolates could provide key insight into the varying pathogenicity of the disease. The analysis of co-culture biofilm formation of *B. cenocepacia* and *S. aureus* explored in this study has shown a novel interaction with important implications.

In this study, we found that *B. cenocepacia* J2315 secretions result in the degradation of *S. aureus* biofilm and prevent *S. aureus* biofilm formation in several strains. Even when given the opportunity to form mature biofilms, *B. cenocepacia* J2315

degrades *S. aureus* biofilm over time. In contrast, the presence of *S. aureus* NRS77 does not seem to alter biofilm formation or maintenance in *B. cenocepacia* J2315.

The exact mechanism of this interaction remains unclear, although there exist several explanative possibilities. First, *B. cenocepacia* secretion may be altering the pH of the medium. However, destruction of *S. aureus* biofilm is not likely occurring due to decreasing the pH. The CF lung, which *S. aureus* frequently infects, is acidic in comparison to a healthy lung due to the improper transport of bicarbonate ions [117]. Additionally, low pH can be an environmental signal to initiate biofilm formation in staphylococcal species [118]. Its ability to survive in low pH conditions suggests a different mechanism is causing degradation of *S. aureus* biofilm. Further, our data suggests this inhibitory effect may be specific to *S. aureus*, as similar inhibition was not observed for *P. aeruginosa* or *S. maltophilia* (Supplementary Fig. 1). A generalized mechanism such as decreased pH would theoretically affect biofilm formation in other species as well. Absence of this effect insinuates that the substance secreted by *B. cenocepacia* J2315 specifically targets *S. aureus* or a component of the *S. aureus* biofilm.

Another possible mechanism leading to *S. aureus* biofilm destruction is through the destruction of the EPS holding the biofilm together, such as through secretions of polysaccharide hydrolases, DNases, and/or proteases. This destruction would result in release of cells from the bacterial matrix and back to planktonic growth. This explanation is easily testable by removing the liquid from a co-culture biofilm, quantitating planktonic bacteria, and comparing the viable cell count to the CFU/mL of the planktonic culture of *S. aureus* monoculture biofilm. The presence of a greater viable cell count of planktonic *S. aureus* in co-culture with *B. cenocepacia* compared to *S. aureus* in single

culture would suggest the biofilm matrix is being dissolved and releasing the cells back into the medium.

Destruction of *S. aureus* biofilm in such a manner would not be a novel finding. A serine protease secreted by *Staphylococcus epidermidis* termed Esp has been shown to degrade both molecules in the matrix of *S. aureus* biofilm and molecules essential for biofilm formation and colonization, such as fibronectin binding proteins [119]. Exposure to Esp results in degradation of established *S. aureus* biofilm over time in a similar manner as exposure to *B. cenocepacia* and its biofilm supernatant.

Other potential mechanisms to explain the antagonism of secreted compounds to *S. aureus* biofilm could be the production of reactive oxygen species (ROS) in the medium or the production of biofilm-inhibiting lipids. ROS secretion by *B. cenocepacia* is less likely, as some Bcc members are killed by certain antibiotics through a resulting increase in ROS production [120].

Production of biofilm-inhibiting lipids, on the other hand, is a possible mechanism of *S. aureus* biofilm reduction. Rhamnolipids produced by *P. aeruginosa* have anti-staphylococcal activity [121] and act to decrease biofilm formation in order to maintain water channels [122]. *B. cenocepacia* J2315 has been shown to possess the operon required for rhamnolipid production [123], although the production of rhamnolipids has not yet been reported in this species. However, the porous biofilm phenotype shown in our study suggests the importance of water channels in *B. cenocepacia* biofilm maturation and maintenance, hinting at a possible role or expression of the rhamnolipid regulating operon. As a typically aerobic species, access to oxygen is critical for *B. cenocepacia* survival. Maintenance of biofilm water channels for access to



fresh nutrients and oxygen implicate the potential importance of rhamnolipids in *B. cenocepacia* virulence, and is therefore a potential mechanism of *S. aureus* biofilm inhibition.

In addition to rhamnolipids, *B. cenocepacia* J2315 produces a lipopeptide capable of lysing human cells and inhibiting growth of both *S. aureus* and *P. aeruginosa* strains [124]. However, this lipopeptide was only produced at effective concentrations with vigorous shaking for three days. Our data suggest that the substance secreted by *B. cenocepacia* J2315 that inhibits *S. aureus* biofilm is produced under static conditions for the same amount of time. It is therefore probable that the inhibition of *S. aureus* observed in our data is not occurring through actions of this lipopeptide.

Further research into the identity of the *B. cenocepacia* J2315 secreted substance could clarify the discrepancy seen in the significant reduction of *S. aureus* AH1710 biofilm exposed to *B. cenocepacia* supernatant during CLSM imaging. Co-culture imaging showed that *S. aureus* AH1710 was still present in 7-day-old co-culture biofilm with *B. cenocepacia* J2315, while *B. cenocepacia* supernatant in the absence of viable *B. cenocepacia* cells resulted in intensified *S. aureus* AH1710 biofilm reduction.

A possible mechanism explaining this *S. aureus* biofilm reduction is the secretion and re-uptake of a toxic or catabolic substance by *B. cenocepacia*. *P. aeruginosa* has been shown to produce siderophores that are toxic to *S. aureus* and lead to cell lysis as a way to scavenge iron [125]. Siderophores are secreted to collect iron from the environment and are then taken back up by the bacteria. A similar mechanism used by *B. cenocepacia* may explain the presence of *S. aureus* biofilm in co-culture with *B. cenocepacia* (Fig. 7E and 7F) in the CLSM experiment, whereas the presence of the *B.*

*cenoepeacia* biofilm supernatant without *B. cenoepeacia* cells resulted in *S. aureus* biofilm destruction (Fig. 9). If *B. cenoepeacia* is secreting a molecule that destroys *S. aureus* biofilm but the molecule can be taken back up into the cell, then the presence of *B. cenoepeacia* could be somewhat ameliorating to *S. aureus* biofilm degradation in such a situation. *B. cepacia* complex isolates have been shown to produce a variety of siderophores including ornibactin, pyochelin, salicylic acid, and cepabactin [126-129].

There are many hypotheses to be explored and tested as a result of the data presented in this thesis. Though the exact mechanism underlying the *B. cenoepeacia* mediated destruction of *S. aureus* biofilm remains to be elucidated, the degradation of *S. aureus* biofilm by a potentially novel substance is an exciting finding. The increasing prevalence of antibiotic resistance in both MSSA and MRSA necessitates the discovery of both new antibacterials and targets for therapeutics.

There are antibacterials on the market that can effectively treat *S. aureus* infection in CF and other individuals [130], but antibiotic resistance by bacteria protected in biofilms prevents treatment of the underlying infection. Should further experimentation reveal that the substance secreted or shed by *B. cenoepeacia* results in dissolution of *S. aureus* biofilm matrix, it could implicate a potential therapeutic solution to elimination of *S. aureus* biofilms.

Future directions for this research include the identification of the substance of interest being secreted by *B. cenoepeacia* J2315, use of tissue cultures as a surface for co-culture biofilm formation, analysis and comparison of gene expression between *B. cenoepeacia* planktonic and biofilm lifestyles, as well as continuous capture of CLSM images to create and observe a time lapse of co-culture biofilm formation and *S. aureus*

biofilm degradation. Results from these proposed experiments, used in conjunction with the findings of this paper, could reveal a novel antimicrobial agent or a new target for *S. aureus* biofilm reduction.

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## **Appendix**

**I. Supplementary Table**

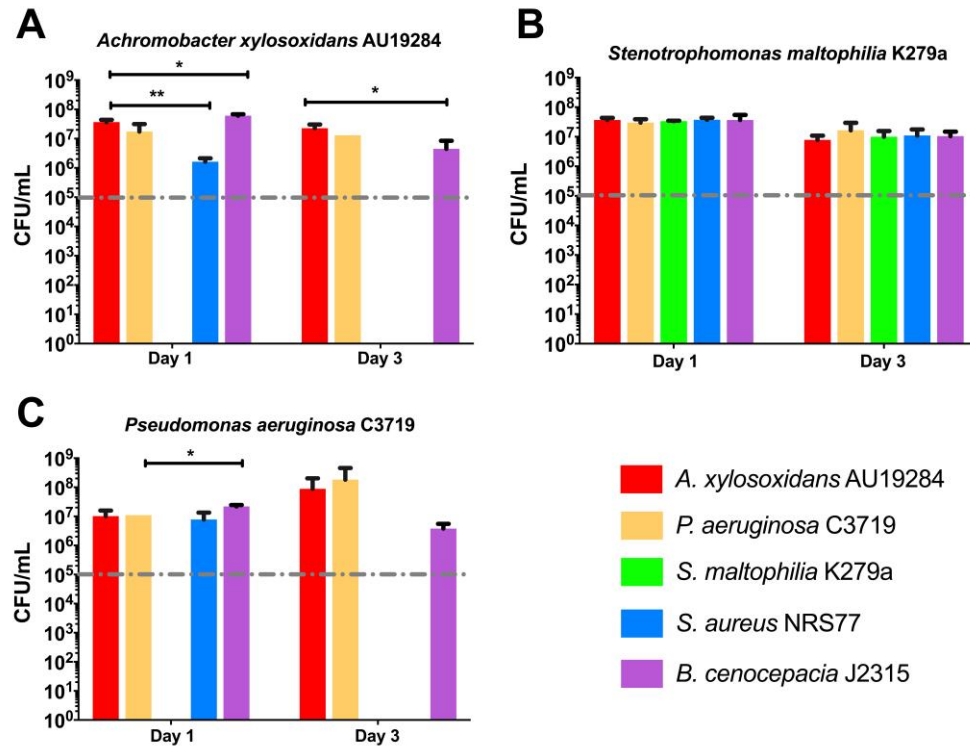
**II. Supplementary Figure**

## **I. Supplementary Table**

**Supplementary Table 1. Description of incubation times and colony morphology by strain.**

<b>Strain</b>	<b>Colony Morphology</b>	<b>Incubation Time</b>
<i>A. xylooxidans</i> AU19284	Small colonies, white	48 h
<i>P. aeruginosa</i> C3719	Large colonies, tinged pink	24-48 h
<i>S. maltophilia</i> K279a	Largest colonies, dark yellow	24h
<i>S. aureus</i> NRS77	Large colonies, white	24 h
<i>B. cenocepacia</i> J2315	Smallest colonies, tan, produce a brown pigment	At least 48 h

## **II. Supplementary Figure**



**Supplementary Figure 1. Co-culture biofilm formation of common CF pathogens.** Viable cell counts of *A. xylosoxidans* AU19284 (A), *S. maltophilia* K279a (B), and *P. aeruginosa* C3719 (C) biofilm grown were compared when grown in with *A. xylosoxidans* (red), *P. aeruginosa* C3719 (orange), *S. maltophilia* K279a (green), *S. aureus* NRS77 (blue), or *B. cenocepacia* J2315 (purple). Presence of “X” indicates contamination, and therefore exclusion of data. Absence of a bar indicates the CFU/mL is below the limit of detection, which was 10<sup>5</sup> for this experiment. One-way ANOVA analysis with Tukey’s multiple comparison test was used to determine significance (\*p < 0.05, \*\*p < 0.01).

The ability of *S. maltophilia* K279a to form biofilm was not affected by the presence of the tested strains. There was no significant difference in *S. maltophilia* K279a biofilm formation in monoculture compared to co-culture conditions on both day one and day three of biofilm formation. On the other hand, the presence of *S. maltophilia* K279a in co-culture reduced biofilm formation below the limit of detection in both *A. xylosoxidans* AU19284 and *P. aeruginosa* C3719. The biofilm formation of *A. xylosoxidans* AU19284 in co-culture with *B. cenocepacia* J2315 was significantly greater than monoculture on day one, but was significantly lower than monoculture on day three. Interestingly, the presence of *S. aureus* NRS77 significantly inhibits *A. xylosoxidans* AU19284 biofilm formation on day one, and reduces biofilm below the limit of detection on day three. *P. aeruginosa* C3719 biofilm was significantly increased due to the presence of *B. cenocepacia* J2315 on day one, but biofilm formation on day three in this co-culture condition was not statistically significant in comparison to monoculture. Repeated experimentation over a longer period of time or with a lower limit of detection would further elucidate the interactions between some of these isolates in co-culture conditions.