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The effect of Stenotrophomonas maltophilia on Gram-negative cystic fibrosis pathogens.

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The effect of *Stenotrophomonas maltophilia* on Gram-negative cystic fibrosis pathogens

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ABSTRACT

Cystic Fibrosis (CF) is the most common lethal genetic disorder in the Caucasian population with an incidence of 1 per 3,000 live births and a median predicted survival of only 47 years. Respiratory failure due to repeated pathological insults to lung tissue by infection is the ultimate cause of mortality in the majority of patients. The lung microenvironment created by CF highly favors colonization by opportunistic pathogens such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cenocepacia*, and *Achromobacter xylosoxidans*. Biofilm formation by multiple bacterial species contributes to the chronic, persistent, and difficult to treat nature of CF infections. This study seeks to further the limited understanding of what polymicrobial biofilm interactions may be occurring in the CF lung. Survival assays of bacterial cells grown under biofilm-forming conditions demonstrated that *P. aeruginosa* survival was inhibited, and no detectable growth occurred for *B. cenocepacia* or *A. xylosoxidans* in co-culture with *S. maltophilia.* Further experimentation including supernatant assays, treatment of biofilms with cell lysate, pH measurements, and laser scanning confocal microscopy have elucidated further hints about the potential mechanisms of this *S. maltophilia*-mediated inhibition. The presence of live *S. maltophilia* cells appears to be necessary for *A. xylosoxidans* inhibition, while *B. cenocepacia* is inhibited by both live cells and filtered *S. maltophilia* supernatant. Characterization of these interspecies relationships may further our understanding of how flora composition influences pathogenesis in the CF lung.

INTRODUCTION

Cystic Fibrosis

Cystic fibrosis (CF) is the most common lethal genetic disorder in the Caucasian population with an incidence of 1 per 3,000 live births and median life expectancy of 46.7 years [1]. Over 30,000 people in the United States alone suffer from the disease with an average of 1,000 newly diagnosed cases every year, primarily through newborn screening [1]. The pathophysiology of the disease is wide-reaching and affects multiple organ systems, but progressive respiratory failure secondary to repeated and sustained pathological insults to lung tissue is the ultimate cause of mortality in the majority of cases [1]. The microenvironment created by the CF lung creates a prime breeding ground for opportunistic pathogens to colonize and establish chronic, persistent, and difficult to treat infections. How these pathogens establish themselves, interact with each other, and deteriorate the clinical course is the primary focus of this thesis.

CF is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). Found on chromosome 7, *CFTR* codes for an ion channel which facilitates the exchange of chloride ions between the intracellular and extracellular environments [2]. There are currently thousands of mutations known to cause and modulate the severity of CF [3]. Mutations are generally categorized into one of five groups depending on the mechanism by which the CFTR protein or its downstream products are disrupted [4]. This categorization guides clinicians in anticipating the phenotype of a particular patient's disease. The most common disease-causing mutation ΔF508 is a deletion of the three nucleotides coding for the phenylalanine amino acid residue at position 508 in the chloride channel protein. Misfolding caused by this deletion primes a large fraction of the mutant CFTR

protein for premature degradation in the endoplasmic reticulum [5]. The remaining defective proteins have difficulty incorporating themselves within the plasma membrane. Their transport efficiency is significantly reduced compared to wild-type CFTR protein and they have half-lives which are much shorter [5, 6]. Roughly 90% of CF patients are heterozygous for at least one ΔF508, while 50% are homozygous [7]. The two next most common mutations are G542X and G551D, neither of which are found in more than 5% of the CF population [1].

Debate continues about the exact genetic, cellular, and molecular mechanisms that contribute to the pathogenesis of CF. Disruption of osmoregulation is a leading theory as to the cause of pathophysiology. Loss of the CFTR protein renders chloride anions unable to cross the lipid bilayer of epithelial cells, creating a hypertonic intracellular environment relative to the extracellular space [8, 9]. In addition, research has also suggested that loss of CFTR increases sodium resorption across the membrane [10]. Both factors lead to an increased osmotic pressure which readily draws water out of the luminal space and into the epithelium. The physiological implications of this change are wide-reaching, affecting the function of nearly every exocrine gland in the body. Complications related to pancreatic, liver, and reproductive system dysfunction are common, but arguably some of the most significant consequences affecting morbidity and mortality are observed in the lungs.

Production of mucus by goblet cells and submucosal glands in the conducting portions of the lungs is an integral process necessary for normal respiratory function and pathogen defense. Normal mucus has an approximate composition of 97% water and 3% protein [11]. In CF, dehydration of the mucus due to changes in osmotic pressure is believed to contribute to impaired clearing of secretions by mucociliary action. Large glycosylated proteins known as mucins form the classic gel-like network characteristic of mucus and act as imitative binding

sites for would-be pathogens. In CF patients, mucin concentrations are much lower than samples from healthy control populations [12]. Loss of the CFTR protein likely contributes directly to the stagnation of secretions and indirectly to infection susceptibility. Retention of thick collections of desiccated organic material creates a reservoir of nutrients ripe for colonization by opportunistic pathogens. CF-related lung infections are characterized by their unusual chronicity and persistence. Formation of biofilms and the diversity of pathogens involved are two factors that severely complicate the management of what would otherwise be routine treatments for typical infections.

Bacterial Biofilms

Bacterial biofilms are thick, adherent mats of cells that form in aqueous or humid environments encased within a matrix of secreted proteins, lipids, and polysaccharides known as the extracellular polymeric substance (EPS) [13]. The unusually persistent and treatment-resistant nature of CF infections is largely attributable to the development of these structures. Cells within biofilms have distinct profiles of gene expression compared to planktonic cells [14]. Biofilms themselves confer survival advantages to the cells within against a variety of biotic and abiotic stressors. Predation by the immune system is impaired as the EPS physically impedes infiltration of the biofilm by antibodies [15]. Previous studies have shown that one biofilm-forming bacterium, *Staphylococcus epidermidis,* is significantly more susceptible to the innate immune responses when there are mutations in key EPS-forming genes [16], suggesting that disruption of biofilm structure may be a potential therapeutic target.

In addition to creating a physical barrier that protects against host defenses, the EPS creates a significant advantage to the cells within due to antibiotic resistance. Compared to their planktonic counterparts, cells existing in a biofilm may be extremely more resistant. *P. aeruginosa* exhibits a 1,000-fold increase in resistance to tobramycin when in biofilms [17]. In addition to the typical resistance mechanisms observed in planktonic cells such as efflux pumps, antibiotic-modifying enzymes, and mutations of antibiotic targets, numerous processes unique to biofilms have been proposed – though none alone are likely to explain the phenomenon in its entirety [reviewed in 18]. The EPS matrix likely inhibits the diffusion of cytotoxic compounds. Confocal imaging has shown that although vancomycin binds to planktonic cells in an average of 5 min, penetration into the matrix of *S. aureus* biofilms may take up to an hour [19]. Gradients of varying antibiotic concentration could theoretically trigger the activation of adaptive resistance pathways well before the minimum inhibitory concentration (MIC) is reached. However, there are also several studies that show antibiotic penetration is not impeded and no reduction in viability is observed despite the presence of concentrations well above the expected MIC [19-21]. The effects of matrix permeability thus vary greatly with the bacterial strain and antibiotic compound studied.

Extracellular DNA (eDNA) released either by biofilms cells themselves or incorporated into the matrix from exogenous sources may play an important role in resistance. *P. aeruginosa* mutants low in eDNA production are more susceptible to aminoglycosides than wild-type cells and the addition of exogenous DNA from lysed polymorphonuclear leukocytes increases biofilm resistance [22]. Some species such as *S. epidermidis* have even been found to produce more eDNA as an adaptive response to subinhibitory concentrations of antibiotics, thus increasing overall biofilm resistance [23]. High concentrations of eDNA create areas of relative acidity in

the biofilm matrix and can facilitate chelation of ions such as Mg^{2+} [24, 25]. Production of eDNA may also facilitate local horizontal gene transfer, increasing the total number of resident resistant cells [26, 27]. Resistant and/or metabolically dormant phenotypic variants deep within the biofilm may act as 'persisters' [28]. Failure to eradicate these cells, which may represent as few as 1% of the biofilm population, helps ensure eventual reestablishment of the colony after all other non-resistant cells are killed [29]. In some *E. coli* strains, it appears that defects in the hydroxyl radical formation pathway allow some subpopulations of cells to exhibit enhanced tolerance to antibiotic exposure [30].

Formation of polymicrobial biofilms can facilitate cooperation and competition between species by bringing multiple organisms in close proximity. Of the biofilms relevant to human health, the oral microbiome is one of the most well studied. Hundreds of articles have explored the web of complex interactions that allow many organisms to survive where mono-cultures ordinarily could not [reviewed in 31-33]. Some of the most common opportunistic pathogens afflicting CF patients include *P. aeruginosa*, *S. maltophilia, B. cenocepacia,* and *A. xylosoxidans* [1]. All are typical biofilm-forming CF species of substantial clinical interest.

Pseudomonas aeruginosa

P. aeruginosa is a leading cause of respiratory infections in CF patients and perhaps the most well studied of the common CF pathogens. It is a Gram-negative facultative aerobe and is the second most common respiratory pathogen in the CF population, found to be infecting 44.6% of all monitored CF registry patients in 2017 [1]. Being the most extensively studied pathogen, *P. aeruginosa* has long served as a model organism for the investigation of biofilm formation,

especially as it relates to human health. The clinical relevance of *P. aeruginosa* is not limited to CF as the pathogen is also a leading cause of burn infections and sepsis [34-38]. Its propensity to form biofilms on both biotic and abiotic surfaces makes it a particularly common cause of healthcare acquired illnesses such as ventilator-associated pneumonia and catheter-based infections [39].

There are typically two variations of *P. aeruginosa* strains: mucoid and non-mucoid phenotypes. Non-mucoid strains generally exist as free-floating planktonic cells and are the main pathogens in acute respiratory disease. Mucoid strains are associated with chronic infections and prolonged biofilm formation [40]. Mucoidy is dependent on the production and secretion of alginate, a polysaccharide made of mannuronic acid and guluronic acid [41] that aids cells by increasing surface volume, thereby reducing predation via immune cells, and increasing resistance to desiccation and osmotic stresses, as it is hygroscopic in nature.

Recent evidence suggests that the transition from free-floating cells to sessile communities is mediated by the increased production of the secondary messenger cyclic-di-GMP upon a bacterial cell's contact with a viable surface [42]. Cells remain attached in a reversible manner while they divide and begin generating precursors required for EPS production. For non-mucoid strains, the exopolysaccharides in EPS are most commonly Psl (containing branched polymers of D-glucose, D-mannose and L-rhamnose) and Pel (comprised of *N*-acetylgalactosamine and *N*-acetylglucosamine polymers) [43, 44]. For mucoid cells, the dominant polysaccharide in the EPS is alginate [45]. After sufficient amounts of the EPS have been manufactured, the biofilm is considered irreversibly attached to a surface. Cells within the biofilm continue to grow within the EPS if sufficient nutrients and water are available through porous channels in the matrix. The mature biofilm will then periodically release cells in a

process known as dispersal. Dispersed cells are free to spread and infect other areas or surfaces, although their transcriptome is still uniquely different from that of their wholly planktonic counterparts [46]. In the CF lung, mucoid biofilms permit chronic infections that are often refractory to therapeutic intervention. In addition to the airway obstruction they cause, prolonged inflammation due to sustained neutrophil activity leads to breakdown of the connective tissues keeping bronchioles patent and elastic. Biofilm formation is thus an important aspect of *P. aeruginosa* that affects disease progression.

P. aeruginosa employs a variety of virulence factors which make it a formidable pathogen. Many bacteria make use of one or more types of secretion systems which allow them to transport proteins across their phospholipid membranes either into the extracellular environment or directly into other cells. *P. aeruginosa* uses a Type III Secretion System (T3SS) which can deliver a suite of exotoxins directly to host cells. Four effectors are currently known: ExoS, ExoT, ExoU, and ExoY. ExoS and ExoT are two closely related toxins whose role includes disruption of the host cell cytoskeleton and induction of apoptosis [47, 48]. ExoU is a potent phospholipase whose activity is associated with rapid epithelial cell necrosis which facilitates dissemination of the pathogen [49]. ExoY is an adenylate cyclase whose activity within host cells interferes with normal pathways of cell signaling, disrupts the cytoskeleton, and inhibits cellular internalization of *P. aeruginosa* [50]. The importance of the T3SS in virulence during acute infections is clear. Patients infected with clinical isolates found to be actively excreting T3SS-linked toxins have worse outcomes than those with clinical isolates not expressing the T3SS [51]. However, this association may not hold for chronic infections. Evidence suggests that the importance of biofilm formation supplants expression of the T3SS in chronic conditions [52]. This may be mediated by cyclic-di-GMP which negatively regulates the

T3SS while positively regulating the genes involved in biofilm formation [53-55]. Clinical isolates from CF patients often express the mucoid phenotype induced by mutations that simultaneously increase the production of alginate and downregulate the production of the T3SS [56]. Although targeting elements of the T3SS for treatment may be useful in acute settings, this evidence suggests that finding ways to disrupt biofilms is a more pressing concern for the CF model. In fact, one study demonstrated that 90% of their CF participants already possessed at least one antibody against a component of the *P. aeruginosa* T3SS [57].

P. aeruginosa is also a model organism for bacterial quorum sensing and its relevance to pathogenesis. Quorum sensing is the process of cell-to-cell communication among bacteria. Cells produce and respond to molecules such as acyl homoserine lactones (AHLs). Bacteria secrete different variations of AHLs which are unique to their specific strain or species. Increasing concentrations of species-specific AHLs in a location indicates increased cell density. Beyond certain thresholds, cells will begin to alter their gene expression in accordance with the AHL gradient. This allows for the coordination of community gene expression, potentially conferring a selective advantage against the host. *P. aeruginosa* employs the LasRI and RhlRI systems as parts of its quorum sensing [58]. Presence of both functional systems is required for the expression of several virulence factors including the elastase LasA, protease LasB, exotoxin A, and alkaline protease [59]. Quorum sensing has also been implicated in biofilm formation. Pel production has been shown to be regulated by quorum sensing processes [60]. Mutant strains lacking the LasRI system either fail to form biofilms or experience significant reduction in accumulated biomass [61, 62]. *Las* and *rhl* mutants produce less extracellular DNA for the EPS and are unable to form the mature biofilms observed in their wild-type counterparts [63]. Production of rhamnolipids is under direct control of the *rhl* system [64]. Their presence is

important both for maintaining the higher-order structure of *P. aeruginosa* biofilms and facilitating detachment of cells in their transition from the sessile to planktonic state [65, 66]. A third quorum sensing system, PQS, operates using quinolone substrates as opposed to AHLs. PQS mutants have been found to produce fewer siderophores, pyocyanins, elastases, and biofilms than wild-type *P.* aeruginosa [67]. These facts make quorum sensing proteins an attractive target for therapeutic interventions, however their importance in chronic infections such as CF is disputed. Although quorum sensing may play an integral role in the establishment and maturation of *P. aeruginosa* biofilms, the accumulation of *las* mutants in the CF lung over time suggests its loss may be an adaptation [68]. The presence of biofilms again complicates the picture.

P. aeruginosa has a notorious reputation for its innate multidrug resistance mechanisms. In the year 2017, 18% of CF patients with a positive culture for *P. aeruginosa* were infected with a multidrug resistant strain [1]. In addition to the elements that may be acquired through horizontal gene transfer, a number of chromosomally encoded resistance mechanisms are present in the *P. aeruginosa* genome. β-lactams are generally poor therapeutic choices due to the species' inducible β-lactamase AmpC [69]. Susceptibility is usually maintained to a select few carbapenems, although mutations in the gene coding for the outer membrane protein OprD and horizontal gene transfer of metallo-carbapenemases are becoming increasingly frequent countermeasures [70, 71]. Development of mutations in the gene *gyrA* encoding for topoisomerase II is a common mechanism by which quinolone resistance is mediated [72]. In non-cystic fibrosis patients, resistance to aminoglycosides may develop due to the presence of aminoglycoside-modifying enzymes and 16S ribosomal subunit mutations [73]. Overexpression of efflux pumps such as MexXY appears to be the primary mechanism of aminoglycoside

resistance in CF patients with chronic *P. aeruginosa* infection [73, 74]. It has also been shown that selective pressure may result in a resistant pathogen less than ten days after initial antimicrobial exposure [75].

P. aeruginosa is an early colonizer of the CF airway. The median age at initial infection in 2017 was 5.2 years [1]. A number of studies have associated early infection by *P. aeruginosa* with unfavorable outcomes including increased rate of pulmonary function decline, increased time of hospitalization, and increased mortality [76-78]. Multiple randomized, high-quality trials have demonstrated that early eradication therapy using inhaled tobramycin is effective for elimination of acute infection by *P. aeruginosa* [79-81]. A follow-up study found that patients who undergo successful eradication therapy have a 74% reduction in risk of contracting *P. aeruginosa* again during 5-year follow up and are 54% less likely to become colonized with the mucoid biofilm-forming phenotype [82]. Failure of early eradication, however, usually results in the progression of the infection to the chronic phenotype. Infections may progress to the mucoid stage in fewer than two years [83]. Novel strategies against *P. aeruginosa* and its biofilms will be required to reduce the burden of morbidity and mortality that the pathogen places on the CF population.

Stenotrophomonas maltophilia

S. maltophilia is the fourth most common CF pathogen, found to be infecting 12.6% of all monitored patients in 2017 [1]. Rates of infection have been steadily climbing over the past several years, and the clinical relevance of *S. maltophilia* as a Gram-negative opportunistic pathogen is under continuous investigation. Like *P. aeruginosa*, it is a frequent cause of

nosocomial infections such as pneumonia, sepsis, and urinary infections secondary to its propensity for biofilm formation on a wide array of abiotic and biotic surfaces [84-86]. *S. maltophilia*'s exact level of clinical importance has been and continues to be a subject of controversy. However, recent research has begun to show some important links between *S. maltophilia* infection and clinical outcomes.

S. maltophilia is host to a wide array of secreted enzymes whose actions facilitate colonization, survival, and infection within hosts. *S maltophilia* has been found to have two gene loci which purportedly code for a Type II Secretion System (T2SS). Of the two loci, the gene encoding the Xps protein has been shown to be essential for the proteolytic activities associated with the presence of a T2SS. *S. maltophilia* K279a mutants lacking the *xps* gene lost the ability to disrupt actin cytoskeleton organization and overall viability of A549 human lung epithelial cell lines [84]. Some of the major effectors delivered by the T2SS are serine proteases. For example, StmPr1 has broad catalytic activity against a number of human connective tissue and serum proteins [87, 88]. Other extracellular enzymes have been consistently found in clinical CF isolates including lipases, DNases, gelatinase, streptokinase, heparinase, and hyaluronidase [89]. Studies into the mechanisms and relative importance of this armamentarium of enzymes are ongoing. Clinical isolates of *S. maltophilia* also exhibit remarkable biofilm-forming capabilities. In one study utilizing the human IB3-1 bronchial cell line, *S. maltophilia* actually outperformed *P. aeruginosa* PAO1 in biofilm adhesiveness and proliferation [85]. Mutations of the *S. maltophilia* genes *spgM*, encoding a phosphoglucomutase, and *rmlA*, encoding a thymidylyltransferase, are believed to be important modulators of biofilm formation in this species [90].

Similar to *P. aeruginosa*, *S. maltophilia* also possess a number of innate antibiotic resistance mechanisms which complicate therapeutic interventions. Two β-lactamases, L1 and L2, are regulated by AmpR, a system similar to that observed in *P. aeruginosa* [91]. This confers resistance against all β-lactams including carbapenems. Over ten different chromosomally-encoded antibiotic efflux pumps of various classes have been found in *S. maltophilia* whose presence and overexpression are key modulators of β-lactam, quinolone, aminoglycoside, trimethoprim/sulfamethoxazole, chloramphenicol, and tetracycline resistance [92]. Unlike other pathogens, *S. maltophilia* has not yet been observed to develop quinolone resistance through genetic mutations of gyrase and topoisomerase [93]. Instead, presence of the gene *qnr* is thought to protect this cellular machinery from the harmful effects of quinolones [94]. Sulfamethoxazole in combination with trimethoprim is the clinician's antibiotic of choice in treating *S. maltophilia* infections because of widespread susceptibility to the agent. However, drug-resistant strains have recently been isolated and have most likely arisen due to acquisition of mobile elements encoding the trimethoprim/sulfamethoxazole resistance gene *sul2* [95]. The combination of all these unique resistance mechanisms can make *S. maltophilia* infections difficult to treat successfully.

Although quite prevalent in the CF population, the consequences of colonization and long-term infection with *S. maltophilia* are debated. A central question to this disagreement is whether *S. maltophilia* is a truly virulent pathogen which worsens clinical outcomes or is simply an innocent bystander whose presence represents advanced stages of disease [96]. Some studies have supported the former claim [97, 98]. Patients infected with *S. maltophilia* generally have poorer lung function, more pulmonary exacerbations, and an increased number of hospitalizations. However, these patients are also more likely to be older and co-infected with

other organisms such as *P. aeruginosa* [98]. Mortality does not seem to be affected by the presence of *S. maltophilia*, and when adjusted for other possible confounding variables such as age, sex, and disease severity markers, patients infected with *S. maltophilia* do not appear to have a significantly higher rate of decline in lung function [97, 98]. More recent models, though, have found that chronic *S. maltophilia* infection is an independent risk factor for acute pulmonary exacerbations requiring hospitalization [99]. *In vitro* studies with airway epithelial and macrophage cell lines have demonstrated the significant immunostimulatory capabilities of *S. maltophilia* [100]. CF patients with chronic infection possess circulating antibodies against the pathogen, suggesting an active immunologic response which could theoretically play a role in worsening the severity of CF lung disease [99]. A recent Cochrane systematic review could identify no randomized control trials specifically testing the efficacy of targeted antibiotic treatment against *S. maltophilia* during acute exacerbations [101]. These findings reflect the need for additional research into the role *S. maltophilia* plays in CF.

Burkholderia cenocepacia

The *Burkholderia cepacia* complex (Bcc) is a group of over twenty-one related, obligately aerobic, Gram-negative bacterial species capable of causing opportunistic infections in humans. *B. cenocepacia* is the most common Bcc member found in the United States CF population [102]. Although not particularly common with a prevalence of only 2.4% in the CF population during 2017 [1], *B. cenocepacia* is an organism of significant clinical importance due to its well-documented effects on pulmonary function.

As Bcc members have also been shown to be plant pathogens, particularly in onions, early investigation into virulence utilized plant models. Genome analysis has shown the presence of two Type IV secretion systems (T4SS), one of which is chromosomally encoded. In one onion model, disruption of the system encoded by a gene cluster on a resident plasmid of *B. cenocepacia* K56-2 demonstrated a loss of pathogenesis. This locus, termed *ptwD4*, showed strong homology to the *VirD4* gene encoding the T4SS in the species *Agrobacterium tumefaciens* [103]. *B. cenocepacia* acts as a facultative intracellular pathogen in humans and is frequently found within the lung epithelium and alveolar macrophages [104, 105]. Further experiments have confirmed that *ptwD4* is a key modulator of intracellular survival. The number of viable mutant cells recovered from IB3 airway epithelial cells and U937 macrophages are significantly smaller than wild-type *B. cenocepacia*. Most mutants were targeted for early lysosomal degradation, whereas normal cells survived and replicated within the endoplasmic reticulum [106]. In addition to secretion systems, the presence of quorum sensing, siderophore production, and lipopolysaccharide (LPS) synthesis have been shown to be essential for pathogenesis in a variety of mammalian models [107].

Like *P. aeruginosa* and *S. maltophilia*, *B. cenocepacia* is also a notoriously pan-resistant organism. In addition to the AmpC system as seen in *P. aeruginosa*, *B. cenocepacia* also expresses a co-regulated penicillinase known as PenB which confers broad-spectrum resistance to most β-lactams when induced [108]. Unique core oligosaccharides that make up the LPS reduce outer membrane permeability to polymyxins and other antimicrobial peptides [109]. Fluoroquinolones are often rendered ineffective by mutations in *gyrA* [110]. Genomic sequencing has revealed that *B. cenocepacia* contains up to 16 different RND-class antibiotic efflux pumps [111, 112]. RND-3 and RND-4 are essential for resistance to tobramycin and

ciprofloxacin in planktonic cells, while RND-3, RND-8, and RND-9 play an integral role in biofilm resistance [113]. All members of the Bcc complex are capable of forming biofilms, and the two producers topping the list (*B. multivorans* and *B. cenocepacia*) are also the most common species found infecting CF patients [114]. Extreme resistance to reactive oxygen species in a small subset of sessile cells enables *B. cenocepacia* biofilms to survive and reestablish themselves after treatment with tobramycin [115]. Neutrophil-like dhl60 cells have been observed to produce less IL-8, exhibit increased rates of necrosis, and stimulate sessile bacterial growth when exposed to mature *B. cenocepacia* biofilms [116]. Thus, biofilm formation is believed to be an important modulator of *B. cenocepacia* virulence.

B. cenocepacia is clinically relevant to CF because of its well-documented association with poor outcomes. CF patients infected with *B. cenocepacia* have been shown to have significantly shortened survival and increased need for medical services when matched with non-*B. cenocepacia* infected individuals [117, 118]. *B. cenocepacia* is capable of establishing chronic infections within the airways just like typical CF pathogens, but its ability to cause sudden and severe acute illness is what sets it apart from other organisms. “Cepacia syndrome” is a complication of Bcc infection that is relatively rare but characterized by a rapid onset of necrotizing pneumonia, sepsis, and frequently results in rapid death of the individual. This dramatic clinical presentation far surpasses the typical consequences of intermittent acute pulmonary exacerbations. Cepacia syndrome is incredibly difficult to treat and considered near universally fatal. Emergence of Cepacia syndrome has been documented as many as 20 years after initial colonization with *B. cenocepacia* [119]. There is ample evidence that *B. cenocepacia* and other Bcc members are easily passed from person to person [120, 121] and have caused numerous outbreaks in CF and other clinics the U.S., Canada, Argentina, India, Israel, and

Europe [122-128]. In order to prevent outbreaks in hospitals and treatment centers, careful separation and isolation protocols are followed to prevent those colonized with *B. cenocepacia* from transmitting the bacteria to other CF patients [129]. Chronic *B. cenocepacia* infection is often a contraindication to lung transplantation due to poor post-surgical outcomes [130]. More research is needed to develop novel preventative and therapeutic strategies against this relatively uncommon but potentially devastating pathogen.

Achromobacter xylosoxidans

A. xylosoxidans is an opportunistic, Gram-negative, aerobic pathogen with a prevalence of 5.8% in the 2017 CF population [1]. Acquisition from the environment is likely the primary method of infection. However, there is evidence to support person-to-person transmissibility [131, 132]. Outbreaks have been observed and studied at a number of health care centers from around the world [133-136]. Evidence points to potential negative impacts resulting from *A. xylosoxidans* colonization. Studies have linked CF colonization by *A. xylosoxidans* with a reduction in forced expiratory volume in one second (FEV_1), greater need for transplantation, and increased incidence of acute pulmonary exacerbations [137-140]. Although results between observational clinical studies are frequently contradictory, a potential chronic inflammatory role of *A. xylosoxidans* in the CF lung at a level approaching *P. aeruginosa* has been documented [141]. Confocal imaging has shown that *A. xylosoxidans* readily forms biofilms both *in vitro* and *in vivo*, conferring an antibiotic resistance ranging from 8- to 1,000-fold higher than planktonic counterparts [142]. Compared to non-CF isolates, CF isolates of the species have higher binding affinities for proteins found in the conducting portion of the lungs such as mucin, collagen, and fibronectin [143]. These findings implicate specific adaptations for colonization, biofilm

formation, and persistence in the CF lung. Investigations into the mechanisms of virulence and clinical implications of long-term infection are ongoing.

Multi-drug resistance profiles are common among *A. xylosoxidans* strains. Innate resistance exists towards aminoglycosides, β-lactams, fluoroquinolones, and chloramphenicol. Genomic analysis of *A. xylosoxidans* strain ATCC 27061 has identified at least 50 antibiotic resistance related genes, many with homology to *P. aeruginosa* [144]. Carbapenem resistance is common despite absence of the well-characterized OprD porin. Only one narrow-spectrum β-lactamase and few RND-type efflux pumps have been characterized in *A. xylosoxidans* [145, 146]. However, putative genes for additional novel β-lactamases and an arsenal of at least 17 unique efflux pump systems have been postulated as other major contributors to resistance [144].

Polymicrobial Biofilms

Much is known about the progression of CF and patient outcomes related to single pathogens, but studies tend to be limited to those species in isolation even though the microenvironment of the CF lung allows for the development of complex microbiological communities. Culture-independent methods reveal a surprising amount of diversity with one study finding that individual sputum specimens obtained from the CF lung contained an average of 36 operational taxonomic units [147]. Clinically, polymicrobial infections are more complex than their mono-species counterparts and are often associated with worse outcomes [148]. Co-infection with *S. aureus* and *P. aeruginosa* in CF patients has been associated with an increased incidence of CF-related diabetes and poor clinical outcomes [149]. Published models have shown that the presence or absence of certain organisms in one year may be associated with an

increase or decrease in the likelihood of harboring a particular pathogen during the next [150]. Relationships may even exist outside of the prokaryotic realm, as *S. maltophilia* has been shown to be an independent risk factor for the development of allergic bronchopulmonary aspergillosis [151] caused by the fungus *Aspergillus fumigatus*. This evidence suggests that the unique and dynamic populations of mixed-species biofilms are likely to modulate the clinical course.

A number of synergistic relationships between potential CF pathogens have already been observed *in vitro* and *in vivo*. *P. aeruginosa* normally competes with *S. aureus* in co-culture planktonic conditions; however, overproduction of alginate by *P. aeruginosa* actually promotes coexistence between the two species [152]. Co-cultured *P. aeruginosa* and *B. cenocepacia* increase biofilm mass *in vitro* and appear to work synergistically to increase host immune response in murine models [153]. Adhesiveness of *S. maltophilia* to IB3-1 airway epithelial cells is significantly increased compared to mono-culture when inoculated in co-culture with *P. aeruginosa* [85]. With the large number of organisms affecting CF patients, a multitude of other interspecies interactions are likely awaiting discovery.

Despite their potential relevance to CF, the knowledge base surrounding polymicrobial biofilms is relatively limited. The purpose of this study is to further explore the relationships observed between the Gram-negative CF pathogens *S. maltophilia*, *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa*. The rationale for studying these specific pathogens comes from data reported by a previous member of the Yoder-Himes lab which showed that *S. maltophilia* appeared to completely inhibit the survival of *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa* in co-culture biofilms [154]. I sought to replicate those findings, investigate the conservation of this effect, and identify possible mechanisms of action. Establishment of *in vitro* interactions between these four species may have implications for patterns of infection observed

in CF patients. Presence or absence of certain microbes can be predictive of subsequent infections, so understanding the potential mechanisms behind these findings carries significance for understanding the progression and treatment of CF lung disease as a whole.

MATERIALS AND METHODS

Bacterial strains used in this study

Bacterial strains were retrieved from freezer stock by single colony isolation streaking on round agar plates containing the proper concentration of antibiotics for selection as indicated in Table 1. All bacterial strains were maintained with Luria Broth (LB, Lennox formulation) unless otherwise noted. Prior to each experiment, single colony isolates taken from freezer stock samples were inoculated into 5 mL LB broth tubes. Liquid overnight cultures were vigorously aerated by placement on a rotary shaker device set at ~250 rpm.

Table 1: Bacterial strains used in this study

Strain	Description	Antibiotic Selection	Source
***S. maltophilia* K279a**	Clinical isolate - blood	N/A	Dr. Nicholas Cianciotto, Northwestern University
***S. maltophilia* K279a + pIN29**	K279a strain transformed with pIN301 encoding eGFP	30 µg/ml chloramphenicol	Present study
***S. maltophilia* K279a + pIN301**	K279a strain transformed with pIN301 encoding dsRedExpress	30 µg/ml chloramphenicol	Present study
***B. cenocepacia* J2315**	Clinical isolate – CF sputum	N/A	Dr. James Tiedje, Michigan State University
***B. cenocepacia* J2315 + pIN62**	J2315 strain transformed with pIN62 encoding dsRedExpress	100 µg/ml chloramphenicol	Rachel Thompson, University of Louisville
***A. xylosoxidans* AU19284**	Clinical isolate – CF sputum	N/A	Dr. John LiPuma, University of Michigan
***P. aeruginosa* C3719**	Clinical isolate – CF sputum; small colony variant	N/A	Dr. Stephen Lory, Harvard Medical School

Mono- and co-culture survival assays

Five mL of liquid LB was inoculated with one of the following bacterial strains: *S. maltophilia* K279a, *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, or *P. aeruginosa* C3719 and grown overnight at 37 ºC. Cultures were diluted 1:50 for fast growing strains (*S. maltophilia* and *P. aeruginosa*) or 1:30 for slow growing strains (*B. cenocepacia* and *A. xylosoxidans*) and grown with aeration at 37 °C until an optical density 600 nm ($O.D._{600}$) of 0.8 – 1.2. Aliquots of each mid-log phase sample were added to autoclavable reagent reservoirs (Excel Scientific #160519) containing tryptic soy broth (TSB) such that the final concentration was 1.0×10^6 cells/mL based on standard curves previously generated in the lab.

Four 96-well PVC plates (Costar #2797) and breathable rayon film covers (VWR #60941-084) were disinfected with 70% ethanol and sterilized under a UV lamp for at least 10 min. Experimental conditions were such that each strain was grown alone in mono-culture, and *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa* were each grown in co-culture with *S. maltophilia*. Mono-cultures were seeded into replicate wells by adding 60 µL of the standardized cultures to 60 µL of TSB for each well. Co-culture conditions were created by adding 60 µL of the standardized sample (*B. cenocepacia*, *A. xylosoxidans*, or *P. aeruginosa*) to 60 µL of the standardized *S. maltophilia* sample in a single well. The control condition was 120 µl of TSB in a single well. Each condition was carried out in triplicate. All samples were added to each of four plates. Empty wells and columns were left between samples to reduce the risk of contamination. After setup was complete, each PVC plate was sealed using a sterilized rayon film and wrapped in aluminum foil. The bottom of a small bin was covered with paper towels and saturated with sterile water to prevent desiccation during incubation. The biofilms were placed in this makeshift

humidifying chamber and the top was sealed with Saran wrap before being placed in the incubator. One plate was removed from the bin at days 1, 3, 5, and 7 and processed.

The harvested 96-well PVC plate was unwrapped from its aluminum foil covering and the rayon film was carefully removed in the biosafety cabinet. Using a multichannel micropipettor, the remaining liquid in all wells was aspirated and discarded into a 10% (v/v) bleach solution. The wells were refilled with 150 µL of sterile water as a rinse and incubated for approximately one min. The water was pipetted off and replaced with 100 µL recovery medium, TSB with 1% Tween-20. An adhesive aluminum foil cover (VWR #75805-268) sterilized under UV light was used to reseal the plate which was placed on the bottom of a small test tube rack. This rack was positioned over an ultrasonic cleaner (Branson 3200) in such a way that the wells of the plate were partially submerged. The plate was sonicated at 60 Hz for 10 min in order to free viable bacterial cells from the biofilms. These conditions were previously identified by a former member of the lab to induce the maximal release of viable bacteria. The plate was removed from the sonicator and its aluminum cover was surface disinfected with 70% ethanol. Each sample was serially diluted 1:10 after using a multichannel micropipettor to pierce the aluminum covering.

Square petri plates containing LB agar (one for each sample) were dried in advance by placing them in the 37 °C walk-in incubator approximately two hours prior to sonication. One at a time, 10 µL was aspirated from all of the wells of a column using a multichannel micropipettor. The aliquot was dripped onto a plate and allowed to run down its length until dry. Once all wells from all conditions had been plated in this manner, the square LB plates were placed in the incubator for one to two days. When colony sizes were large enough to observe without magnification, lanes that had between 10 and 100 colonies were counted based on colony

morphology and their dilution factor was used to calculate the final colony forming units (CFU) per mL recovered from the biofilm for each sample. The averages, standard deviations, and significant differences between conditions were calculated for each comparison in GraphPad Prism v5.0.

Biofilm supernatant survival assays

To generate sterile supernatants, 150 µL of an overnight *S. maltophilia* culture was added to 15 mL of TSB in an autoclavable reservoir. All the wells of a UV sterilized 96-well PVC plate were filled with 120 µL of the inoculated TSB. The plate was covered with breathable rayon film, wrapped in aluminum foil, and placed in a makeshift humidifying chamber where it was incubated for three days. At the conclusion of the incubation period, the liquid in the wells was distributed into 1.5 mL microcentrifuge tubes (VWR #10025-724). The tubes were subsequently centrifuged at 15,000 RPM for 10 min. The supernatant was filtered using a plastic 15 mL Leur-lock syringe with a sterile 0.2 µm polyethersulfone filter (VWR #28145-501) into a sterile 15 mL conical vial.

Overnight cultures of *S. maltophilia*, *B. cenocepacia*, and *A. xylosoxidans* were grown to mid-log phase. Mono-culture wells contained 60 µL of their respectively inoculated TSB combined with 60 µL of PBS. The experimental wells contained 60 µL of inoculated TSB (*B. cenocepacia* or *A. xylosoxidans*) combined with an equal volume of 3-day *S. maltophilia* biofilm supernatant harvested the same day. Wells of 60 µL TSB with 60 µL PBS or 60 µL TSB with 60 µL *S. maltophilia* supernatant were maintained as negative controls. Biofilms were made in triplicate. The plates were again wrapped in aluminum foil, placed in a humidifying chamber,

and incubated for 1, 3, 5, and 7 days. Survival of strains on each day was assessed using the procedures described in the preceding section.

Comparison of pH in single vs. mixed species Biofilms

Overnight cultures of *S. maltophilia, B. cenocepacia, A. xylosoxidans,* and *P. aeruginosa* were inoculated and grown to mid-log phase. Two UV sterilized 96-well PVC plates were divided into four 8×3 sections, and all wells were filled with their respective conditions. Conditions were arranged so that each bacterial species was grown in mono-culture (including *S. maltophilia* and a plain TSB negative control) and pairwise in co-culture with *S. maltophilia*. Mono-culture conditions for each species consisted of wells containing 60 µL of standardized inoculated TSB and 60 µL of plain TSB. Co-culture conditions contained 60 µL of standardized inoculated TSB with 60 µL of standardized *S. maltophilia* inoculated TSB. Plates were covered in a porous adhesive film and incubated at 37 °C. Once per day for the next eight days, plates were briefly removed for pH measurements. For this, small pipette tips were used to puncture the porous adhesive covering both plates, and a narrow-tip pH electrode was placed into each well. Once a stable reading was recorded, the electrode was removed, rinsed with 10% bleach, and used to measure the pH of the next well. Wells were not remeasured.

Effect of medium pH on B. cenocepacia *biofilm survival*

TSB was sterilized and titrated to a pH of 9.0 using 10 M NaOH. Inoculation of overnight cultures and preparation of the biofilms were carried out as described above. Standardized aliquots of both *S. maltophilia* and *B. cenocepacia* were grown in wells containing either 120 µL

of plain TSB at a standard pH (approximately 7.2) or 120 µL of TSB with a pH of 9.0. Three replicates each of either 120 µL plain TSB or 120 µL TSB pH 9.0 were used as negative controls. Survival was assessed on days 1, 3, 5, and 7 as before.

Effect of S. maltophilia *lysate on* B. cenocepacia *and* A. xylosoxidans *biofilm survival*

A 3-day *S. maltophilia* biofilm plate was prepared in the same way described above in the supernatant experiment subsection. On day three, the remaining liquid culture in the wells was removed and biofilm cells were harvested using the method described for standard survival assays. Recovered media was consolidated into a single 15 mL conical vial. The recovery media was then distributed into 1.5 mL microcentrifuge tubes and sonicated at 85% amplitude for 20 min (10 seconds on, 10 seconds off) using an ultrasonic QSonica Q800R sonicator with 6 °C circulating water. Standardized 60 µL inoculates of *B. cenocepacia* and *A. xylosoxidans* were incubated under biofilm formatting conditions either in the presence of 60 µL TSB with 1% Tween-20 (a positive control) or with 60 µL *S. maltophilia* lysate. Each condition was performed in triplicate. Plates were incubated for 1, 3, 5, and 7 days. Retrieval, dilution, and agar plating procedures on each day proceeded in a manner identical to that described in previous survival assays.

Growth Curves for Single Species

To understand the potential for interspecies competition, 24-hour growth rates of *S. maltophilia* K279a, *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, and *P. aeruginosa* C3719 at 37 ºC were measured using spectrophotometry. Using a flat-bottom 96 well polystyrene plate,

three wells were allocated to each condition using a 1:100 dilution of overnight culture in TSB. $O.D._{600}$ measurements were taken every 15 min for 24 hours using a Tecan Infinite F200 plate reader. Four measurements were taken per well and averaged together in the Tecan software.

Generation of fluorescent S. maltophilia *K279a strains*

Two overnight 5 mL LB tubes inoculated with *S. maltophilia* K279a were combined into a 500 mL Erlenmeyer flask containing 200 mL of pre-warmed LB. The flask was incubated until mid-log phase and subsequently chilled in an ice bath for 30 min. The culture was then evenly dispensed into four 50 mL centrifuge tubes (VWR #89039). Cells were centrifuged at 4000 × *g* for 10 min in a Sorvall RC6 floor centrifuge. Pellets were washed in 5 mL ice-cold 10% (v/v) glycerol and resuspended in 2 mL of glycerol/yeast/tryptone (GYT) medium. Aliquots of 200 µL were stored overnight at –80 ºC.

Aliquots of electrocompetent cells were thawed on ice. For each electroporation, 50 µL of electrocompetent cells was combined with 1 µL of purified plasmid (pIN29 or pIN301 - Dr. Anne Vergunst, University of Montpelier, France) in a sterile microcentrifuge tube. Samples were electroporated in a 2 mm electroporation cuvette at 2.5 kV using a Bio-Rad Micropulser Electroporation Apparatus (setting Ec2). Cells were mixed with 1 mL of fresh LB and incubated with shaking for one hour in sterile microcentrifuge tubes. One hundred µL from each sample was spread onto LB + 30 µg/mL chloramphenicol plates. The remaining 900 µL was centrifuged at 15,000 RPM for one min, the supernatant was decanted off, the pellet was resuspended in the small amount of remaining liquid, and this was spread on a second 30 µg/mL chloramphenicol

plate. Transformants were restreaked on fresh selection plates, checked for fluorescence, and stored in the –80 ºC freezer for later use.

Confocal imaging of S. maltophilia *and* B. cenocepacia *biofilms*

Overnight cultures were made in 5 mL LB tubes containing proper antibiotic selection and grown to mid-log phase the following day. Eighteen sterile flat-bottom confocal imaging dishes (Matsunami #D113OH) were seeded with ~1.0×10^6 CFUs in 3 mL of TSB + 30 µg/mL chloramphenicol. Six dishes contained only *S. maltophilia*, six contained only *B. cenocepacia*, and six contained both species. Dishes were placed in humidity chambers and incubated for three or seven days. At each time point, the supernatants were removed, biofilms were washed with 3 mL of PBS, and 3 mL TSB was added to each dish prior to imaging. Five prospectively determined positions on each dish were imaged under 40X oil immersion magnification on a Nikon Eclipse Ti microscope. The bottom of each biofilm was found manually before capturing a *z*-stack of images. *S. maltophilia* mono-culture and *B. cenocepacia* + *S. maltophilia* co-cultures were imaged through 50 µm above the bottom of their biofilms, while *B. cenocepacia* was imaged through 100 µm. All images were captured and displayed using NIS-elements software. GFP (487 nm) and TxRed (561 nm) lasers were used as excitation wavelengths. Mono- and co-culture conditions were scanned using both channels.

RESULTS

Mono- and co-culture survival assays

To confirm the findings of a previous lab member showing complete inhibition of *B. cenocepacia* J2315, *A. xylosoxidans* AU19284, and *P. aeruginosa* C3719 by *S. maltophilia* K279a, all strains were grown under biofilm forming conditions either alone or in co-culture with *S. maltophilia*. Viable cell counts of *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa* on

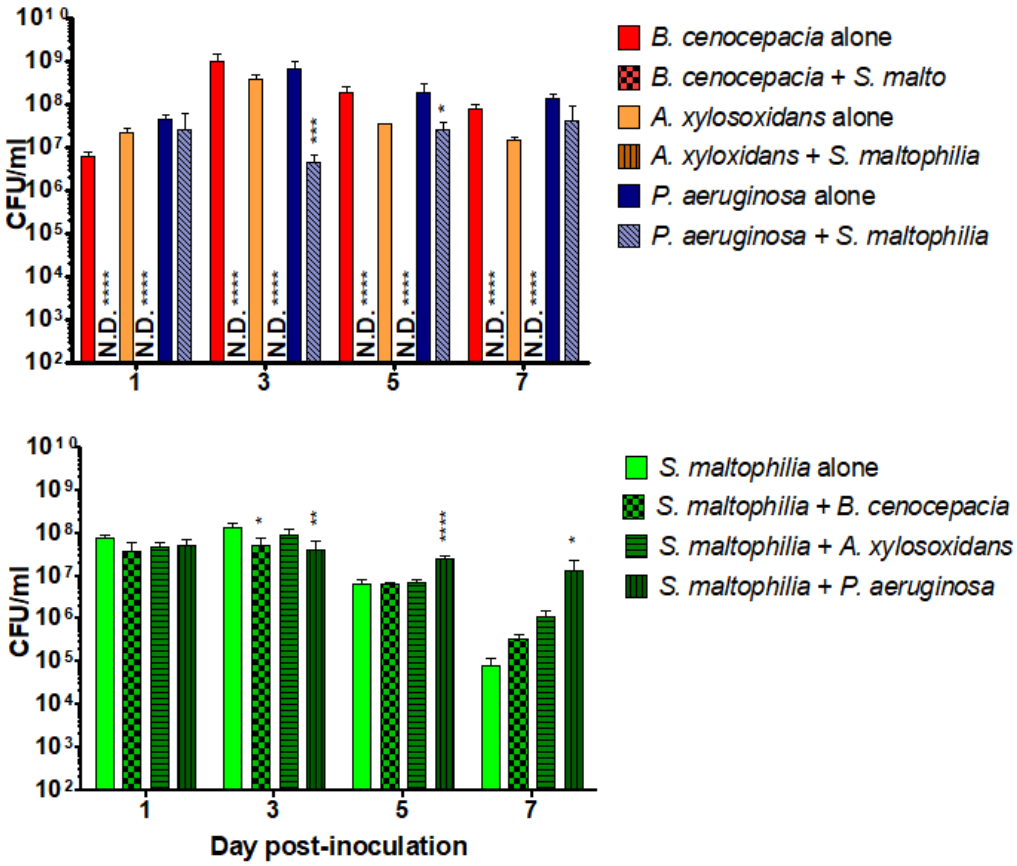

Figure 1: Survival of CF pathogens in co-culture *in vitro.* A) Viable cell counts of *B. cenocepacia*, *A. xylosoxidans*, and *P aeruginosa* in mono-culture conditions or in co-culture with *S. maltophilia*. Asterisks denote statistically significant differences between mono- and co-culture within a given species. Unpaired two-tailed t-tests, *p<0.05, ***p<0.001, ****p<0.0001. CFU = colony forming units. N.D. = not detectable. The limit of detection was 10^2 CFU/ml. **B)** Viable cells counts of *S. maltophilia* in mono-culture and all co-culture conditions. Asterisks denote statistically significant differences from mono-culture. One-way ANOVA w/ Bonferroni's post-test, *p<0.05, **p<0.01, ****p<0.0001. Error bars represent one standard deviation of the data.

days 1, 3, 5, and 7 were analyzed. Neither *B. cenocepacia* nor *A. xylosoxidans* showed any detectable growth in co-culture with *S. maltophilia* on any of the days tested. This was in stark contrast to mono-cultures which showed robust biofilm formation (Fig. 1A). Unpaired t-tests between each day's mono-culture and co-culture conditions showed that there was also significantly less growth of *P. aeruginosa* on day 3 ($p < 0.001$) and day 5 ($p < 0.05$). Viable cell counts of *S. maltophilia* (Fig. 1B) revealed some differences in this species' growth between the mono-culture and co-culture conditions. A one-way ANOVA of each day's conditions using Bonferroni's post-test showed that there was significantly less growth of *S. maltophilia* in co-culture with *B. cenocepacia* on day 3 ($p < 0.05$) and with *P. aeruginosa* on day 3 ($p < 0.01$). In contrast, increased growth was observed in co-culture with *P. aeruginosa* on day 5 ($p < 0.0001$). There was more growth of *S. maltophilia* in all co-culture conditions compared to the monoculture condition on day 7. However, only the increase observed for *S. maltophilia* co-cultured with *P. aeruginosa* was statistically significant ($p < 0.05$). Taken together, these results suggest that live *S. maltophilia* has an inhibitory effect on all the Gram-negative species tested here. There may even be a reciprocal effect, as *S. maltophilia* growth appears to have been enhanced when in co-culture with *P. aeruginosa*.

Biofilm supernatant survival assays

I hypothesized that complete inhibition of *B. cenocepacia* and *A. xylosoxidans* may not require the presence of *S. maltophilia* cells or cell components. A survival assay using filter sterilized supernatant harvested from mature *S. maltophilia* biofilms was used to test this. Mono-culture biofilms were grown in plain TSB for 7 days with either PBS or *S. maltophilia* supernatant. Viable cells were harvested from the biofilms on days 1, 3, 5, and 7 (Fig. 2) A two-

way ANOVA of all *B. cenocepacia* data showed that the presence of *S. maltophilia* was significant ($p < 0.001$), however both time ($p < 0.01$) and the interaction between these two variables ($p < 0.001$) were also significant. Two-way ANOVAs of both all *S. maltophilia* data and all *A. xylosoxidans* showed significant effects for time ($p < 0.01$) but not for supernatant. Unpaired t-tests were used to compare each day's supernatant conditions to their respective controls. *S. maltophilia* had significantly less growth on day 1 only ($p < 0.001$). *B. cenocepacia* had significantly and substantially less growth on day 3 ($p < 0.01$) and 5 ($p < 0.001$), but not on day 7. *A. xylosoxidans* experienced significantly less growth on day 7 only ($p < 0.01$). These results suggest that *B. cenocepacia* was, to a certain degree, susceptible to treatment with *S. maltophilia* supernatant while *A. xylosoxidans* was not.

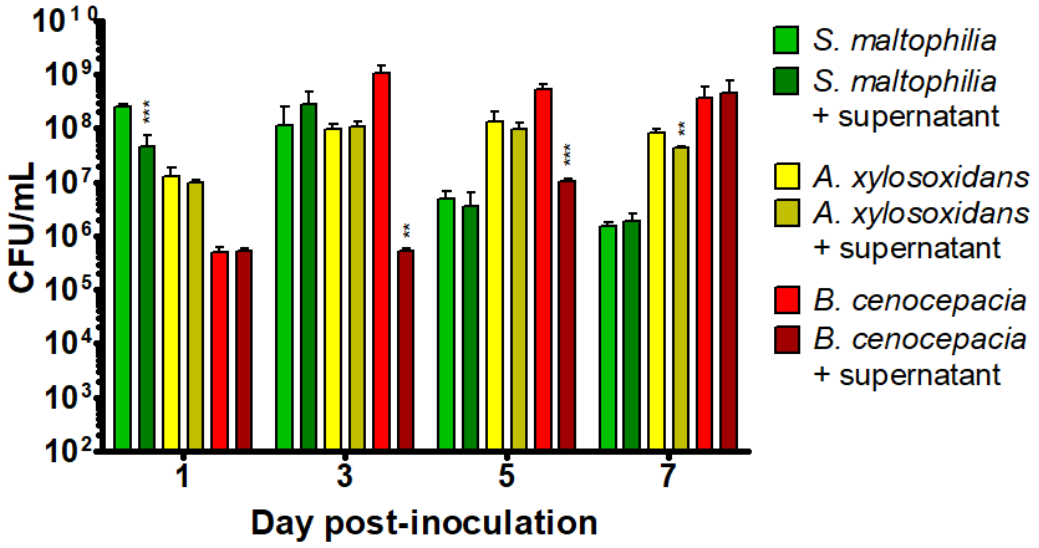

Figure 2: Viable cell counts of Gram-negative pathogens treated with *S. maltophilia* supernatant. Asterisks denote significant differences between each species' control and supernatant condition on each day. Unpaired two-tailed t-tests, *p<0.05, **p<0.01, ***p<0.001. The limit of detection was 10^2 CFU/mL. Error bars represent one standard deviation of the data.

Comparison of pH in single vs. mixed species biofilms

One possible means by which *S. maltophilia* could act to inhibit *B. cenocepacia* and *A. xylosoxidans* would be to alter the pH of the medium. It has been previously shown that pH plays a strong role in biofilm formation by certain species [155-157]. To determine whether pH may play a role in inhibition of *B. cenocepacia* and *A. xylosoxidans* in co-culture biofilms, the pH of both single and mixed species biofilms was assessed by direct measurement. Data for this experiment are shown in Figure 3. Two-way ANOVAs showed a significant effect ($p < 0.0001$) for time, co-culture inoculation with *S. maltophilia*, and interaction between the two variables for all species tested. The average pH of *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa* mono-culture biofilms was significantly less ($p < 0.01$) than the pH of mono-culture *S. maltophilia* biofilms at all measured time points. The pH of the co-culture biofilms only differed significantly from the pH of the *S. maltophilia* monoculture biofilms on days 2 ($p < 0.001$), 4 ($p < 0.05$), and 7 ($p < 0.05$) for *B. cenocepacia* and day 7 ($p < 0.05$) for *P. aeruginosa*. There was no significant difference between *A. xylosoxidans* co-culture biofilms and *S. maltophilia* mono-culture biofilms at any time point. All biofilms and the negative TSB control appeared to become marginally more alkaline as time progressed. This suggests that the pH of the medium in co-cultures with *S. maltophilia* tends to be dictated by *S. maltophilia* rather than its co-culture partner regardless of the species.

Effect of medium pH on B. cenocepacia *biofilm survival*

I hypothesized that pH may be a factor in the inhibition of *B. cenocepacia* as *B. cenocepacia* was at least somewhat dependent on *S. maltophilia* supernatants (Fig. 2). A survival

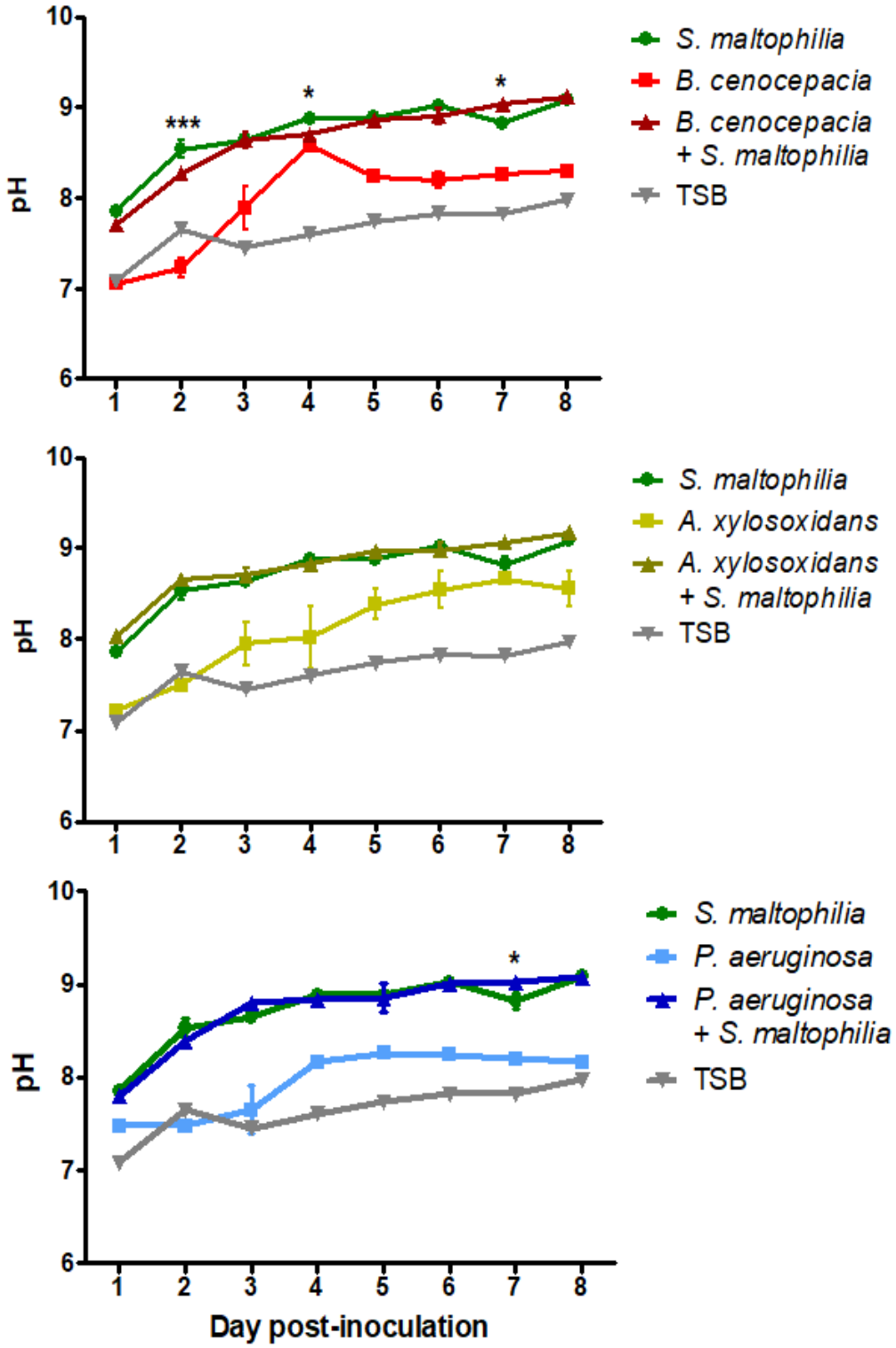

Figure 3: Direct measurements of pH in single and mixed species biofilms between: a) *S. maltophilia* and *B. cenocepacia*. **b)** *S. maltophilia* and *A. xylosoxidans*. **c)** *S. maltophilia* and *P. aeruginosa*. Asterisks indicated significant differences between the *S. maltophilia* mono-culture and co-culture conditions, Two-way ANOVA with Bonferroni post-test, *p<0.05, **p<0.01, ***p<0.001.

assay of mono-culture biofilms grown in TSB titrated to a pH of either 7.0 or 9.0 was conducted to analyze the effect of pH on biofilm formation for mono-cultures of *S. maltophilia* and *B. cenocepacia*. Viable cell counts of both species were determined as in previous experiments (Fig. 4). A two-way ANOVA of all *S. maltophilia* data showed that the effect of pH was not significant, but time ($p < 0.0001$) and interaction ($p < 0.0001$) were significant. Two-way ANOVA of all *B. cenocepacia* data showed the effects of pH ($p < 0.01$), time ($p < 0.05$), and interaction ($p < 0.05$) were significant. Unpaired t-tests comparing each day's controls to the pH 9.0 conditions showed significantly less growth of *S. maltophilia* in the pH 9.0 condition on day 1 ($p < 0.01$) and significantly more growth on day 3 ($p < 0.05$). No detectable colonies appeared on any of the plates for *S. maltophilia* on day 7. This observation is probably due to experimenter error, potential explanations include failure to properly inoculate day 7 *S. maltophilia* pH 9.0 wells or mistakenly drip diluting the negative control instead of the *S. maltophilia* pH 9.0 condition. There was substantially less growth of *B. cenocepacia* in pH 9.0 medium on day 1, though this difference was not statistically significant. Significantly less

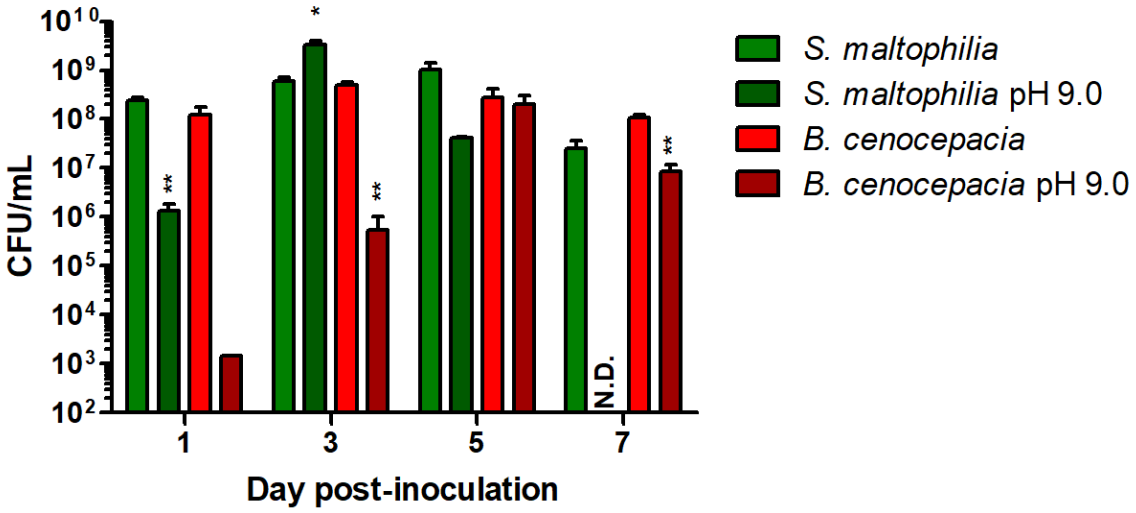

Figure 4: Viable cell counts of *S. maltophilia* and *B. cenocepacia* in plain TSB vs. TSB with a pH of 9.0. Asterisks denote significant differences between each day's control and pH of 9.0 condition, unpaired two-tailed t-test, *$p<0.05$, **$p<0.01$. N.D. = not detectable. The limit of detection was 10^2 CFU/ mL. Error bars represent one standard deviation of the data.

growth was observed on day 3 ($p < 0.01$) and day 7 ($p < 0.01$) but not day 5. The results of this experiment suggest that *B. cenocepacia* may be pH sensitive early in biofilm formation but also fluctuates in susceptibility as the biofilm matures. It is difficult to draw conclusions about the effect of pH on *S. maltophilia* biofilms though as it appears to be quite variable over time.

Effect of S. maltophilia *lysate on* B. cenocepacia *and* A. xylosoxidans *biofilm survival*

Because the data from the supernatant assays showed that inhibition of *A. xylosoxidans* was not due to an effect of the supernatant and that inhibition of *B. cenocepacia* rebounded over time (Fig. 2), I hypothesized that inhibition of *B. cenocepacia* and *A. xylosoxidans* may occur in response to cell-mediated or cell-dependent mechanisms. To test this, I grew *S. maltophilia* in biofilms to day 3, then harvested the biofilm cells and lysed them using sonication. This lysate was applied to *S. maltophilia*, *B. cenocepacia*, or *A. xylosoxidans* and strains were grown under

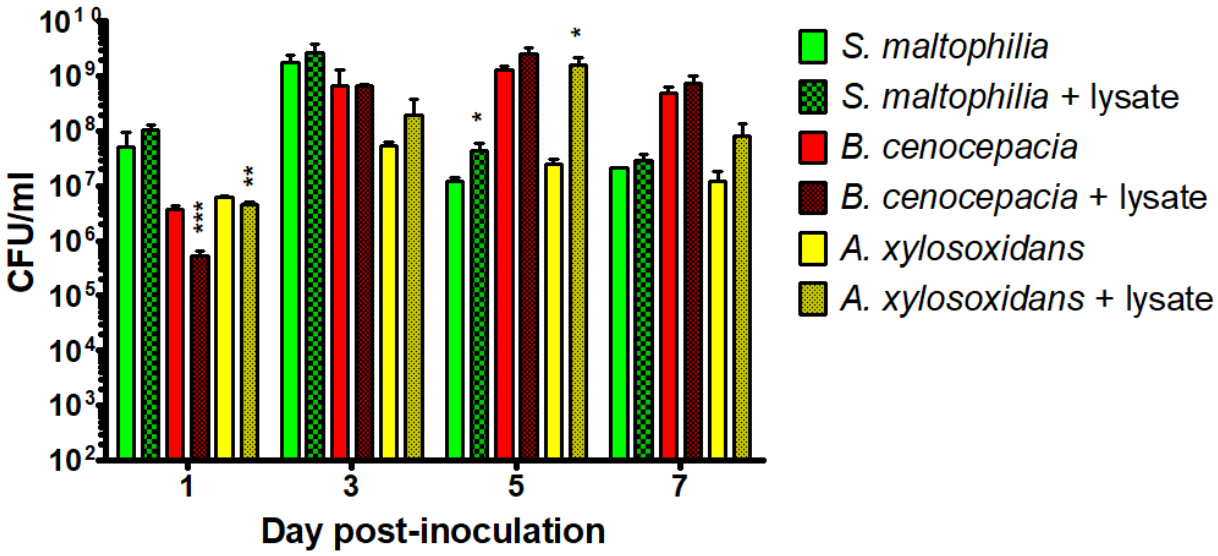

Figure 5: Viable cell counts of Gram-negative pathogens when treated with *S. maltophilia* lysate. Asterisks denote statistically significant differences between each day's control and lysate condition, unpaired two-tailed t-test, *p<0.05, **p<0.01, ***p<0.001. The limit of detection was 10^2 CFU/ mL. Error bars represent one standard deviation of the data.

biofilm-forming conditions over the course of 7 days. On days 1, 3, 5, and 7, the number of viable cells in each biofilm were recorded (Fig. 5). Two-way ANOVAs for each species' data showed a significant effect of time for all three ($p < 0.0001$), a significant effect of lysate for *B. cenocepacia* ($p < 0.05$) and *A. xylosoxidans* ($p < 0.001$), and a significant effect of interaction for *B. cenocepacia* ($p < 0.05$) and *A. xylosoxidans* ($p < 0.0001$). Unpaired t-tests between each day's controls and samples treated with *S. maltophilia* lysate showed significantly less survival of *B. cenocepacia* and *A. xylosoxidans* in the presence of *S. maltophilia* lysate on day 1 ($p < 0.001$ and $p < 0.01$ respectively). There was significantly more growth of *S. maltophilia* in the lysate condition on day 5 ($p < 0.05$) and significantly more growth of *A. xylosoxidans* on day 5 ($p < 0.05$). Taken together, these results suggest that lysed cells alone may be incapable of mediating an antagonistic effect by *S. maltophilia* on either *B. cenocepacia* or *A. xylosoxidans.*

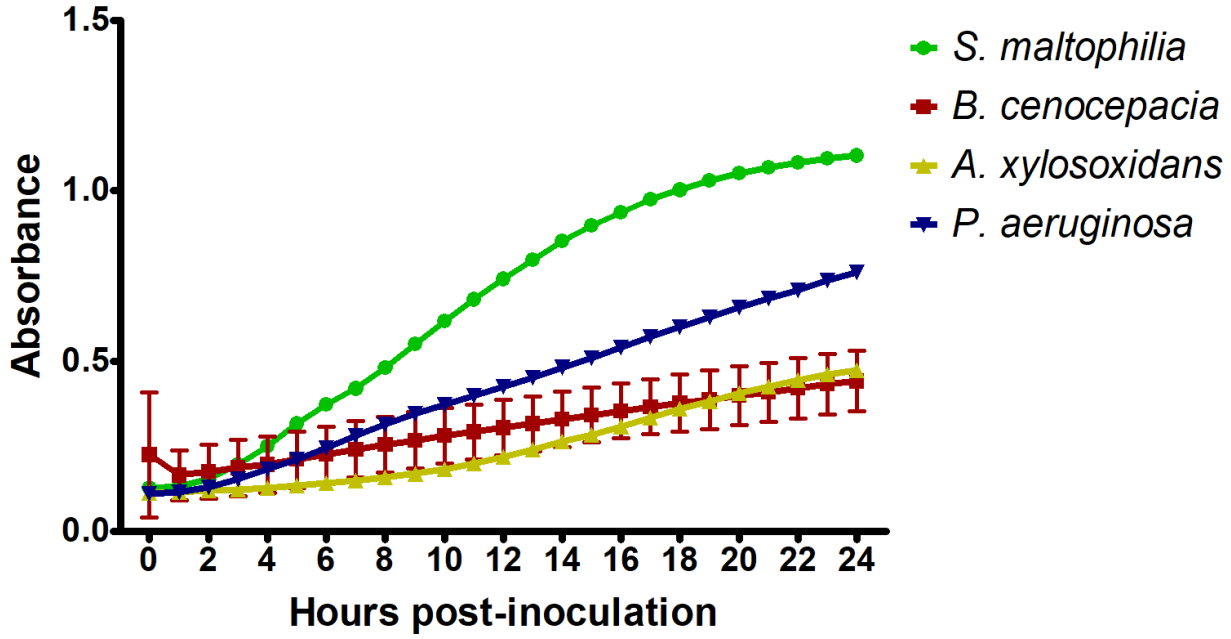

Figure 6: Growth curves of Gram-negative CF pathogens in plain TSB. Absorbances were measured with a wavelength of 600 nm. Data are shown for average absorbance of three biological replicates hourly up to 24 hours. Error bars represent one standard deviation of the data.

Growth curves of single species

To determine how competition for medium resources may influence co-culture biofilms, growth curves for all species of interested were obtained over 24 hours. Optical densities (600 nm) at every hour for each species tested were taken to measure biofilm growth (Fig. 6). Two-way ANOVA with Bonferroni's post-test shows that optical densities for *B. cenocepacia*, *A. xylosoxidans,* and *P. aeruginosa* were significantly less ($p < 0.05$) than that of *S. maltophilia* beginning at six, five, and seven hours, respectively. Readings for *B. cenocepacia* and *A. xylosoxidans* were not significantly different at any time point. *P. aeruginosa* had significantly higher measurements than *B. cenocepacia* and *A. xylosoxidans* at fourteen and eighteen hours, respectively. These results show that *S. maltophilia* is a much faster growing organism than *P. aeruginosa, B. cenocepacia,* and *A. xylosoxidans*. This lends support to the idea that inhibition of *A. xylosoxidans* and *P. aeruginosa* is dependent only upon the presence of live *S. maltophilia* cells which may establish and mature their biofilms in a manner that is prohibitive to the other species' own biofilm formation.

Confocal imaging of S. maltophilia *and* B. cenocepacia *biofilms*

Confocal laser scanning microscopy was used to qualitatively investigate the differences in *S. maltophilia, B. cenocepacia,* and co-culture biofilms over time. Electroporation of *S. maltophilia* K279a was successful in transforming cells with pIN29 (dsRed) and pIN301 (eGFP) plasmids to generate red-fluorescent or green-fluorescent *S. maltophilia* strains. These strains could be imaged in mono-culture or in co-culture biofilms with fluorescent *B. cenocepacia*

strains previously generated in the Yoder-Himes lab to examine the structures produced by each strain alone or in the presence of an interacting partner.

Confocal imaging revealed very different morphologies between *S. maltophilia* and *B. cenocepacia* mono-cultures. On day 3, *S. maltophilia* biofilms appeared as a thin confluent lawn (Fig. 7A). *B. cenocepacia* biofilms on day 3 (Fig. 7C) exhibited a much more organized three-dimensional structure reminiscent of stalks and mushrooms. Volumetric images showed that the concentration of planktonic cells also appeared to be less than that observed in *S. maltophilia* (Fig. 8). At day 7, *S. maltophilia* biofilms appeared thicker than at day 3 but exhibited a similarly confluent lawn (Fig.7B). *B. cenocepacia* biofilms also appeared substantially denser at day 7 (Fig. 7F). Small circular water channels were visible in most images. Co-culture biofilms appeared almost identical in morphology to *S. maltophilia* mono-cultures on both days 3 and 7 (Fig. 7B and E) but with singular pockets of several *B. cenocepacia* cells dotting the lawn. These observations confirm the results seen in the live cell co-culture survival assay (Fig. 1A) and indicate that *S. maltophilia* dominates in co-culture with *B. cenocepacia*.

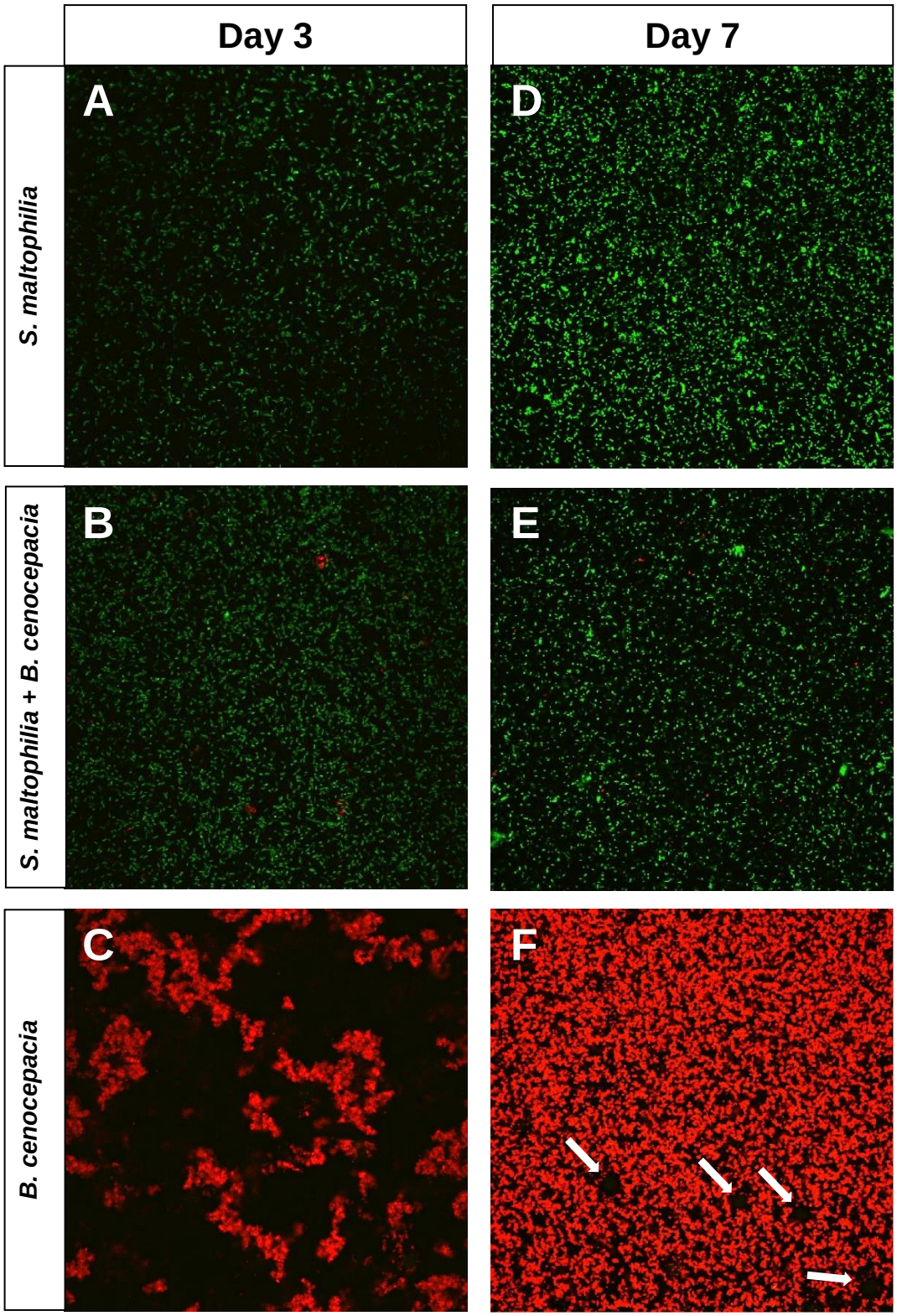

Figure 7: Confocal microscopy of single and mixed species biofilms. Images of *S. maltophilia* K279a (eGFP) and *B. cenocepacia* J2315 (dsRed) biofilms were obtained at 40X magnification in both mono-culture (A, D, C, F) and co-culture (B, E) conditions. Arrows highlight areas of water channel formation. Images shown are representative of over 15 replicates per condition (5 technical replicates of 3 biological replicates).

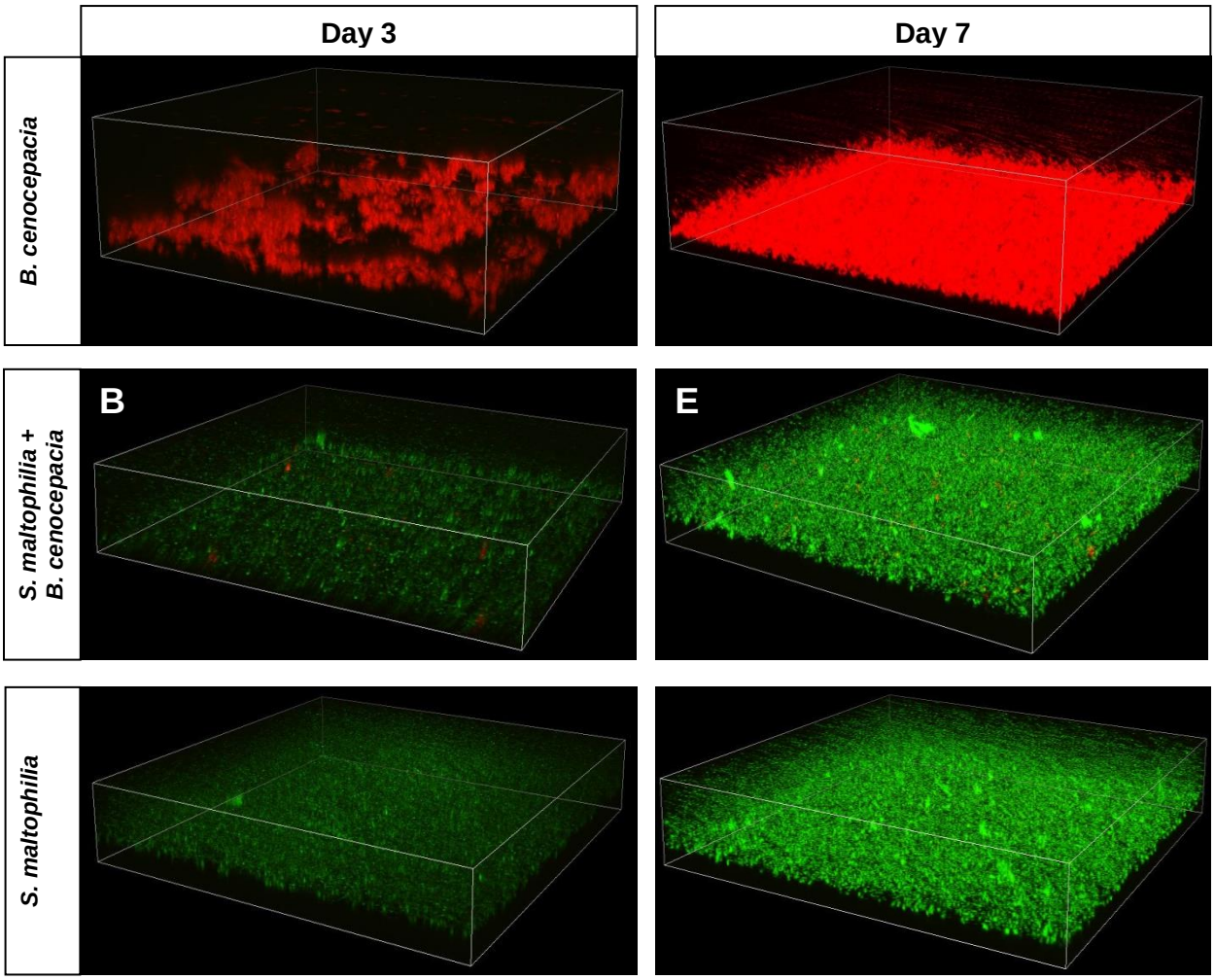

Figure 8: Volumetric confocal images of single and mixed species biofilms. Images of *S. maltophilia* K279a (eGFP) and *B. cenocepacia* J2315 (dsRed) biofilms were obtained at 40X magnification in both mono-culture (A, D, C, F) and co-culture (B, E) conditions. Images shown are representative of over 15 replicates per condition (5 technical replicates of 3 biological replicates).

DISCUSSION

Despite the extensive co-occurrence of pathogens in the CF lung, study of polymicrobial biofilms has been relatively limited. It stands to reason that the positive and negative interactions between various species within the CF lung microenvironment are likely one of the numerous factors influencing disease progression. Synergistic interactions may work to worsen inflammation and lung damage by promoting proliferation of unique mixed species biofilms, while competition may introduce selective pressures that drive one clinical species to dominance over others at the expense of increased tissue damage or treatment resistance. This study sought to investigate the *in vitro* interactions between *B. cenocepacia*, *A. xylosoxidans*, *P. aeruginosa*, and *S. maltophilia*.

Results from co-culture survival assays clearly demonstrated that *S. maltophilia* reduces survival of all the other Gram-negative organisms. Presence of viable *B. cenocepacia* or *A. xylosoxidans* cells was not detectable at or any time after 24 hours in co-culture biofilms. Confocal laser scanning microscopy confirmed these findings for *B. cenocepacia*. Co-culture *S. maltophilia* and *B. cenocepacia* biofilms had a morphology nearly identical to mono-culture *S. maltophilia*. Survival of *P. aeruginosa* in co-culture biofilms was also inhibited albeit to a much smaller degree. Although viable cell counts are a commonly used measure of biofilm quantification, there remains a possibility that all viable cells are not completely released from the EPS after 10 minutes of sonication. Viable cells are generally assumed to be representative of the community as a whole, however biofilms tend to be very heterogenous environments. The retrieval procedure in this experiment was identified by a former lab member to maximize the recovery of biofilm members while minimizing the destruction of what would be otherwise

viable cells. All survival assays in this project were subjected to the same recovery procedure in an effort to ensure that comparisons would be valid.

Although the mechanisms are poorly understood, antagonistic relationships within mixed species biofilms have been observed. *Pseudoalteromonas tunicate* is an aggressive marine biofilm-forming organism that has been shown to directly and indirectly out-compete a number of other prokaryotic organisms [158]. A previous member of the Yoder-Himes also reported evidence of CF pathogen antagonism showing *B. cenocepacia* mediated destruction of *S. aureus* biofilms [154]. Competitive interactions between *P. aeruginosa* and *S. aureus* have even been observed to enhance expression of virulence factors in a way that may cause more harm to the host [reviewed in 159].

Several experiments were undertaken in this study to determine the exact mechanism of complete *B. cenocepacia*/*A. xylosoxidans* inhibition. The supernatant survival assay was employed to test for a cell-independent mechanism of inhibition. Some bacterial species secrete molecules or enzymes that are cytotoxic towards other strains or species. Over 90% of *P. aeruginosa* strains produce antimicrobial pyocins intended to kill competing bacteria [160]. The identification of a novel secreted product by *S. maltophilia* toxic to *A. xylosoxidans* and/or *B. cenocepacia* would be an exciting prospect with potential therapeutic applications. As the biological arms race of antibiotic resistance progresses, novel antimicrobial compounds are in short supply yet desperately needed. However, results showed no susceptibility of *A. xylosoxidans* to the filtered supernatant. This implies that antagonism is cell-mediated. Although unlikely given the choice of a low-binding, hydrophilic polyethersulfone filter, an absence of effect could also be explained if an inhibitory substance was removed from the supernatant during the filtration process. *B. cenocepacia* survival was significantly inhibited by *S.*

maltophilia supernatant, but only on days 3 and 5. The degree of inhibition observed also did not match that seen in co-culture survival assays. *B. cenocepacia* biofilms also appeared to rebound from their disturbance back to a survival level equivalent with mono-culture controls by day 7. This could be perhaps be explained if *B. cenocepacia* was able to degrade *S. maltophilia* cytotoxic compounds over time or if any antagonistic molecules degraded spontaneously under these conditions. *B. cenocepacia* has a large genome that is very adaptable and elastic [111, 161, 162]; thus it is not too surprising that it may be able to sense and overcome inhibition by *S. maltophilia*. However, this makes studying the mechanism underlying this inhibition more difficult and less likely to reveal an effective therapeutic in the future.

Another potential, arguably less interesting cause of cell-independent inhibition that was considered was medium pH. All organisms have an optimal pH range at which they experience the most growth, and any conditions outside of that range could theoretically lead to a reduction in survival. If one species extrudes compounds or waste at much different rate than another species, coexistence may not be possible due to pH differences in the extracellular environment. Direct measurement of single and mixed species biofilms revealed that supernatant pH is dictated by *S. maltophilia* in all conditions tested. The pH of *B. cenocepacia*, *A. xylosoxidans*, and *P. aeruginosa* mono-culture biofilms was significantly less than that of co-culture conditions with *S. maltophilia*. Further, the pH of *S. maltophilia* mono-culture biofilms (approximately 9.0) was nearly identical to that of all co-culture biofilms. However, these data are not sufficient to explain co-culture inhibition in its entirety. Recall that *P. aeruginosa* experienced significantly reduced but still detectable growth in co-culture survival assays despite the differences observed here. Additionally, *A. xylosoxidans* should have been sensitive to the supernatant assay as well, considering that medium pH is a cell-independent process. A few studies have found that biofilm

production by *P. aeruginosa* and *S. maltophilia* is actually enhanced at a higher pH [155, 156]. The media pH survival assay carried out in this experiment showed a varying effect for *S. maltophilia* and a reductive effect for *B. cenocepacia* over time. This is in contrast to a previously published study which showed a marginal increase in viable cell count with increasing pH. However, inoculations were not grown under biofilm forming conditions or for longer than 48 hours in this particular study [157]. Further research on how media pH affects biofilm formation, especially for *B. cenocepacia* and *A. xylosoxidans*, will be required to elucidate whether pH differences might be a significant source of inhibition.

The lysate survival assay was employed to test for cell-dependent mechanisms of inhibition. There is evidence for this type of mechanism in the inhibition of *S. aureus* by *P. aeruginosa* in biofilms which is at least partially mediated by *P. aeruginosa* LPS [162]. If some component of *S. maltophilia*'s membrane or other cellular structure was responsible for inhibition, *B. cenocepacia* and *A. xylosoxidans* survival should be reduced when grown with *S. maltophilia* lysate. Neither species appeared to be sensitive to treatment with 3-day biofilm lysate, meaning that this is likely not the case. Apparently, the presence of dead, sonicated *S. maltophilia* cells is not sufficient to cause inhibition. If a cell-dependent mechanism were to be the cause of inhibition, it would seem that the presence of living cells is required for this to occur. Such cell-mediated, contact-dependent mechanisms of inter- and intra-specific competition have been observed in other Gram-negatives like *Vibrio cholerae* and *Vibrio fischeri*. These two species appear to use their secretion systems to inject toxins directly into other prokaryotic cells [163, 164]. Some Gram-negatives, such as the phytopathogenic *Xanthomonas citri*, possess a type IV secretion system which allows them to directly kill other Gram-negative bacteria in a contact-dependent manner [165]. Should *S. maltophilia* harbor a

similar system, which would not be unexpected given its close phylogenetic relationship with *X. citri*, the presence of live and physiologically active cells would be required for this process to occur.

There also exists the possibility that *S. maltophilia* is not actively destroying other Gram-negative biofilms. Its growth may be rapid enough that it depletes the resources available to other species or establishes biofilms on all available spaces. Growth curve analysis clearly demonstrated that *S. maltophilia* was the fastest growing strain in this experiment. It was followed relatively closely by *P. aeruginosa* while *B. cenocepacia* and *A. xylosoxidans* came in a distant last place. These findings strongly support the possibility that indirect competition is at least a contributing factor to the reduced growth observed in these co-cultures. Perhaps the growth rate of *P. aeruginosa* is rapid enough to allow establishment of itself in co-culture with *S. maltophilia* before it is completely excluded, while *B. cenocepacia* and *A. xylosoxidans* simply lag too far behind. Future work should include time-lapse evaluations of co-culture biofilms utilizing confocal microscopy to better ascertain whether *S. maltophilia* passively outcompetes or actively destroys *P. aeruginosa, B. cenocepacia,* and *A. xylosoxidans* biofilms.

In conclusion, this study demonstrates that *S. maltophilia* inhibits several other Gram-negative CF pathogens in co-culture biofilms. Survival of *P. aeruginosa* is reduced while survival of *B. cenocepacia* and *A. xylosoxidans* is not detectable. No single mechanism was definitively shown to be the cause of this effect. Inhibition of *A. xylosoxidans* appears to require the presence of live *S. maltophilia* cells, while *B. cenocepacia* inhibition is observed with both live *S. maltophilia* cells and filtered *S. maltophilia* supernatant. Inhibition of *A. xylosoxidans* does not appear to be mediated by cell-independent processes and may simply be outcompeted before it has a chance to begin biofilm production. *B. cenocepacia* could be inhibited by a

secreted product, pH differences, competition with *S. maltophilia*, or some combination of the three. Further research that builds on this work may help narrow down the factors contributing to interspecies relationships within clinically relevant polymicrobial biofilms.

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