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Investigation of the in vitro interactions between two common Cystic Fibrosis pathogens, Staphylococcus aureus and Stenotrophomonas maltophilia.

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Investigation of the *in vitro* Interactions Between Two Common Cystic Fibrosis Pathogens, *Staphylococcus aureus* and *Stenotrophomonas maltophilia*

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Abstract

Cystic Fibrosis (CF) is one of the most common autosomal disorders in Caucasian populations. This disorder creates a very opportune environment for many pathogens within the patient's lung. Two common pathogens that infect CF patient's lungs are *Staphylococcus aureus* and *Stenotrophomonas maltophilia*. These two species of bacteria can colonize host environments and establish mats of cells known as biofilms that become very difficult to eradicate with antibiotics. Once inside a CF lung, these pathogens must not only evade the host immune response but they also interact and compete with each other; however, how bacterial pathogens interact inside the host lung has not been well studied. This study will look specifically at the interactions between these two pathogens *in vivo*. *S. maltophilia* inhibits *S. aureus* biofilm formation over time in a dose-dependent manner though the mechanism is still unclear. The findings of this study could provide insight into these interactions between both *Staphylococcus aureus* and *Stenotrophomonas maltophilia.*

Introduction

Cystic Fibrosis is a prevalent, life-limiting disorder among Caucasian populations. CF is an autosomal recessive disease that results in the production of a defective cystic fibrosis transmembrane conductance regulator (CFTR) protein [1]. The gene that encodes this protein was identified in 1989 by Francis Collins, Lap-Chee Tsui and John R. Riordan [2]. The CFTR gene is found on the long arm of the 7^{th} chromosome and it spans over 190kb [1]. CF is a result of one or more mutations within the gene that encodes the CFTR protein. This protein acts as an ion channel located on the cell membrane. The main role of the CFTR protein is to transport

chlorine and bicarbonate ions in and out of the cell. These mutations can be classified in four different categories [3].

The first class is a mutation that results in defective protein production. This occurs usually due to an incorrect splice site or a frameshift that results in a premature stop codon. This creates a CFTR protein that once inserted into the membrane cannot function correctly. This mutation also often results in degradation of the protein in the endoplasmic reticulum.

The second class is a mutation that results in defective protein processing. This is usually due to the protein failing to be properly modified, usually improper glycosylation of the protein. Glycosylation of the CFTR protein is used to direct the cell to transport it to its final destination within the cell membrane. When the protein is not fully glycosylated, it is typically degraded within the cytoplasm and is never able to reach the cell membrane and is unable to carry out its function as an ion transport channel.

The third class is a mutation that results in incorrect regulation of the CFTR protein. The CFTR protein is activated through binding of various second messengers including cAMP [3,4]. These second messengers require a functional binding domain where they can interact with the CFTR protein in order to regulate the action of the protein. A class III mutation disrupts these binding domains and does not allow these second messengers to regulate the CFTR protein. These mutations have varying ranges of potency but in many cases results in a decrease in CFTR function [3].

The fourth class is a mutation that results in defective conduction of ions across the cell membrane. These mutations usually affect small chains of arginine residues on the membrane spanning domain of the protein. Mutations along this domain cause a reduction in flow of

chloride ions across the protein's channel and significantly decrease the CFTR's functionality [5].

Each of the previously stated classes of mutations within the CFTR gene produce either non-functional or minimally functional CFTR proteins within the cell. This results in a decreased flow of chloride ions out of the cells, which has many effects on multiple organ systems throughout the body, including reduced volume and production of pancreatic secretions [6], and decreased reproductive function due to a defective vas deferens in males [7]. However, the effect that results in around 95% of all morbidity and mortality within human CF patients is due to reduced pulmonary function [8].

Within the lung, mucus clearance through ciliary action of pseudostratified epithelial cells is one of the primary defense mechanisms to prevent infection by microorganisms. Inhaled particles from the air are typically trapped within mucus secreted by goblet cells. The mucus is then transported out of the lungs through ciliary action, which efficiently clears mucus that has been diluted due to a constant flow of water from the epithelial cells. When the CFTR protein is non-functional, there is a disruption of relative ion concentrations inside and outside of the cell. This change in ion concentration disrupts the regular flow of water into and out of the cell via osmosis. In the CF lung there is an observed loss of water efflux which creates a dehydrated state within the lumen. [9] When the lung is dehydrated, the mucus that is created is unable to be constantly removed because the cilia is unable to generate enough force to remove the concentrated mucus. The goblet cells within the lung are unable to detect the accumulation of mucus and continue to produce mucus creating an environment where the microorganisms and other particles that would typically be trapped and removed by ciliary action remain in the lung [9]. Within the thick mucus mats formed in the lung, microbes experience a large excess of

sugars from the many polysaccharides in mucus which allow for colonization and reproduction. These populations of microbe lead to both chronic and acute pulmonary infection throughout the life a CF patient. This increased microbial load within the lungs presents many challenges and ultimately shortens the lifespan of most patients to an average of just 45 years [10].

Several pathogenic microorganisms are found more commonly within the CF lung, including *Staphylococcus aureus* and *Stenotrophomonas maltophilia* [11]. *S. aureus* is the more prevalent of the two bacterial species and, according to the 2015 CF Foundation Annual Patient Registry, was cultured from 56,792 CF sputum samples (from ~30,000 patients) whereas *S. maltophilia* was only cultured from 7,167 sputum samples during the same calendar year [12]. Both have very different physiological characteristics; however it has been found that these two microorganisms are very often found within the same CF patients [11,12]. Using the CF Foundation's Annual Patient Registry, the diagnostic prevalence of 29 common CF pathogens was compared by members of the Yoder-Himes lab to determine if there were any trends in pathogen co-occurence. We found that both *S. aureus* and *S. maltophilia* were cultured from the same CF patient at a higher rate than what would be expected by random chance. This positive trendbetween *S. aureus* and *S. maltophilia* seems to indicate that there is some kind of interaction between the two pathogens when they invade a CF patient's lung. In order to begin to understand these interactions, one must first look at the morphology and various properties that defines each of these species.

Staphylococcus aureus

S. aureus has received a great deal attention within the scientific community due to its prevalence in human populations. *S. aureus* was first clearly identified in 1880 and was found to be the causative agent in both sepsis and abscess formation [13]. Today, *S. aureus* has been

found to be the causative agent in over 40% of all nosocomial (hospital acquired) infections. In addition to being an opportunistic pathogen, *S. aureus* is known to colonize several body sites including the nasal, oral, and vaginal cavities and the skin, in healthy humans. Because of *S. aureus'* ability to survive in multiple body sites, *S. aureus* has been found to colonize 30%-50% of healthy adults [14]. There is a much higher risk of colonization in adults who have Type I diabetes, are intravenous drug users, are surgical patients, or who are immunocompromised [15]. *S. aureus* also acts as a first colonizer in immunocompromised patients and can lead to various subsequent colonization by other species [16].

S. aureus is a Gram-positive coccus that most often appears in clusters when observed under light microscopy [17]. As a species *S. aureus* can be differentiated from the rest of its genus by the gold pigment of its colonies, the presence of coagulase (an enzyme that agglutinates proteins), as well as its ability to ferment mannitol [17]. Being a Gram-positive bacterium, the cell wall of *S. aureus* has a cell wall that is between 20-35 nm thick and is found to have around 50% peptidoglycan by weight [18]. The chains of the peptidoglycan structures are held together by a 6-12 residue long, pentaglycine bridge that is specific to *S. aureus* [19]. This thick cell wall acts as a defense mechanism for *S. aureus* against the many environments. The cell wall allows the bacteria to remain in a steady state of water balance even when placed in various osmotic pressures. It can remain turgid when placed in a very hypotonic environment or it can protect itself from dessication in an extremely hypertonic environment. This feature of *S. aureus* is key to understanding how it is able to survive on both the desolate and dry environment of the skin and in the very moist environment of the nasal cavity.

The *S. aureus* genome consists of one circular chromosome of approximately 2.8Mb of DNA in addition to multiple plasmids, prophages, and transposons that contribute heavily to the

overall virulence of the pathogen [20]. Current proteomic data estimates that this genome encodes between 2,600-2,700 different proteins [21]. These proteins have many functions that range from metabolism to reproduction and include the proteins that promote the virulence of this pathogen.

S. aureus virulence factors have been well studied and can be broken down into four different categories. These groups include: proteins involved in adhesion to host cells, proteins that mediate the degradation of host cells, proteins that help the pathogen evade host immunity, and proteins that assist the pathogen in utilizing nutrients found within the host [22]. These four classes of protein virulence factors all work together to create a pathogen that can invade, infect and persist within a host. A majority of the proteins that contribute to *S. aureus'* virulence are conserved throughout strains within the species and are located on the circular chromosome. However, around 25% of these factors are found on the other genetic elements and are highly variable between strains [20]. Most strains of *S. aureus* have the same basic method of infection however, due to the few variable virulence factors that can be passed among various Staphylococci bacterial strains, there some differences in the potency and methods that each *S. aureus* colony invades and infects.

S. aureus is unusually good at adhering to various tissues and host cells. The exact method that *S. aureus* achieves this adherence is still mostly unknown. However, there is some data that suggests that there are two surface factors, Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) and Wall Teichoic Acids (WTA), on *S. aureus* that assist in its ability to adhere to the host [23]. These two factors seem to play some role in attachment of *S. aureus* to various kinds of epithelial tissue.

Once attached, *S. aureus* can begin to secrete exoproteins, secreted proteins that have toxic effects on hosts [24], that allow for the invasion of the pathogen through the epithelial tissue and into the host. The majority of *S. aureus* virulence factors that are produced are either secreted directly into the medium or are bound to the *S. aureus'* membrane.. The most common way *S. aureus* secretes these proteins is through the Sec pathway, a highly conserved translocation pathway that allows for transport and release of proteins through a hydrophobic channel, though five other pathways have been identified. [20] The absence of the Sec system reduces the virulence *S. aureus* significantly [22]. The exoproteins are used by *S. aureus* to invade and transform the host system into an environment that is suitable for its own survival and reproduction and include the enzymes hemolysins, nucleases, lipases, hyaluronidases, and collagenases [25].

Along with these enzymes, *S. aureus* is also able to produce a variety of enterotoxins, known as pyrogenic toxin superantigens (PTSAgs). These superantigens are extremely variable and have been found to produce a variety of effects within the host. One of the most lethal effects of the PTSAgs is the ability to cause Toxic Shock Syndrome [26]. The exact mechanism of how PTSAgs cause Toxic Shock Syndrome is still being studied, however, one potential contributing mechanism that has been observed is the ability of PTSAg to bind to a highly conserved portion T-cell receptors [25]. This binding results in an over-activation of the host immune system and an over-production of cytokines. An overabundance of cytokine release leads to capillary leak around the site of infection. A leaky capillary is useful to initiate a very quick immune response. However, when PTSAgs are present, the capillary begins to leak too much, leading to hypotension, and ultimately multiorgan failure [25]. PTSAgs along with the

many other exoproteins produced by *S. aureus*, create an extremely effective method of invasion and infection.

Once past the epithelial barrier, *S. aureus* must evade the many other immune response elements produced by the host. One common way *S. aureus* accomplishes this is through the production of anti-opsonizing proteins that block neutrophils within the body from phagocytosing the pathogen [27]. *S. aureus* also kills leukocytes through the production of leukotoxins [28]. There are four known classes of these leukotoxins produced by *S. aureus* and each work to puncture pores in the membranes of various kinds of leukocytes resulting in cell lysis [29].

Once established within a host, *S. aureus* can create large collections or communities of cells known as biofilms. These biofilms form both inside and outside of host tissues and provide many protective properties that a single isolated cell does not possess. In order to establish a biofilm a bacterial colony must first adhere to a surface and produces large extracellular matrices consisting of polysaccharides, lipids, proteins, and genetic material. The microorganisms within these biofilms are able to closely associate with each other and can exist in two forms, a free swimming planktonic form or an adhered form [30]. Once established, biofilms are highly resistant to antimicrobial compounds, such as antibiotics or human defensins, and can allow cells within the biofilm to survive in very harsh environments [31]. This method of resistance and survival is due to: (1) the difficulty of antimicrobial substances to penetrate the biofilm, (2) the close proximity of cells that facilitates the exchange of genetic material including resistance genes, and (3) the slower metabolic rates of the bacteria within the biofilm which decrease the efficiency of growth-dependent antimicrobial agents[31].

Biofilms readily form within the lungs of Cystic Fibrosis patients [32]. The CF lungs create an environment well-suited for adherence and biofilm formation by *S. aureus*. These biofilms allow *S. aureus* to remain present in the lung and can create chronic bacterial infections which persists for months or years, despite the consistent use of antimicrobial agents.

S. aureus is found most commonly to infect CF patients between the ages of 6-10 years of age. It has been found that 65% of all CF patients in this age range were found to be colonized by *S. aureus*. [33] Once *S. aureus* has invaded, through the use of biofilm structures it is able to persist for many years within this population. The overall nature and physiology of *S. aureus* accounts for the large prevalence of this organism in people with CF.

Stenotrophomonas maltophilia

Stenotrophomonas maltophilia virulence and pathogenicity has only recently begun to be studied. The bacterium currently called *Stenotrophomonas maltophilia* was believed to be first cultured in the year 1943; however, it was not given its current name until 1993 [34]. For many years, *S. maltophilia* was classified within the *Pseudomonas* genus. After more intensive study, differences between the Pseudomonas genus and *S. maltophilia* isolates, including differences the 16S rRNA gene sequence, showed that these two genera were distantly related within the *Gammaproteobacteria* class. This species was briefly reclassified as *Xanthomonas maltophilia* before being placed into its own genus of *Stenotrophomonas,* which currently consists of four different species [35].

S. maltophilia has not been a widely analyzed because it is an opportunistic pathogen that mainly infects immunocompromised hosts, especially within hospital environments, and is not very common among human isolates. [36] Recently, the number of nosocomial infections by *S. maltophilia* has been increasing, which has created a greater need for understanding the virulence

mechanism employed by this bacterial species [37]. Other than nosocomial infections, *S. maltophilia* can be found in many humid and aquatic environments [38]. The most common of these include bodies of water, moist soil, and animal feces.

S. maltophilia is a Gram-negative aerobic bacterium. This species is motile through the use of polar, multitrichous flagella [39]. These flagella allow *S. maltophilia* to disperse throughout the various aquatic environments that this bacterial species inhabits, as well as allowing it to better invade susceptible hosts. This species of bacteria has also been found to produce adhesive proteins called fimbriae that allow it to attach to a variety of surfaces [40]. These various fimbriae, known as SMF-1 fimbriae, [41] exist on the outer membrane of the bacterium and are positively charged which allows these mostly negatively charged bacteria to adhere to various negatively charged surfaces. These charges interact mostly though electrostatic attraction and give the bacteria an ability to stick to many surfaces [41]. This ability to adhere to multiple surfaces provides *S. maltophilia* with the first step in the formation of biofilms [41]. Once these biofilms form, *S. maltophilia* is then able to become a very persistent pathogen that becomes extremely difficult to treat and eliminate.

The entire genus of *Stenotrophomonas* has been found to be multi-drug resistant (MDR) [42]. This high level of antibiotic resistance has been associated with an anti-bacterial efflux pumps that allows *S. maltophilia* to secrete these antimicrobial agents [43]. These efflux systems are a very conserved method of antimicrobial resistance and have been found in other Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Burkholderia cepacia* [44]. Other enzymes like β-lactamases and amino-glycoside modifying enzymes along with the efflux system that *S. maltophilia* has evolved, provides this species with resistance to many common clinical drug families such as β-lactams, quinolones, aminoglycosides, tetracycline [45]. In

addition to the intrinsic antibiotic resistance mechanisms that are already encoded in this species' 4.85 Mbp genome, *S. maltophilia* can readily acquire other genetic material, such as plasmids and transposons, through horizontal gene transfer. This ability allows *S. maltophilia* to continue to build on its intrinsic MDR and presents a potential future danger that this organism could develop into an even more potent pathogen [46].

The method *S. maltophilia* uses to induce host virulence is poorly understood, but it has been observed that *S. maltophilia* does have a high level of immunostimulatory properties. *S. maltophilia* has been observed to activate production of both interleukin-8 by epithelial cells and Tissue Necrosis Factor-alpha (TNF-α) by host macrophages [47]. This activation of the immune system within the lungs leads to a large amount of airway inflammation, which results in neutrophil and macrophage recruitment. In the short term, this inflammation significantly decreases lung function and is an indicator of pneumonia. Other than the inflammation caused by *S. maltophilia*, very little is known about the pathogenicity of this bacterial species.

Comparison of *S. aureus* and *S. maltophilia*

S. aureus and *S. maltophilia* have very different morphologies and physical characteristics. They have very different genomic sizes, cell walls, and metabolism. However, these two pathogens both commonly infect CF lungs. Despite their many differences, they are both able to invade and persist in the same environment. However, the lung is not a uniform environment. There are many different microenvironments within the lung that can create unique niches for bacteria to colonize. Examples of microenvironments of the lung include various epithelial cell types, the lumen of alveoli, the lumen of the bronchioles, and the dust cells (alveolar macrophage). Each of these various cell and tissue types are contain different ion

concentrations, sugar availability, osmotic pressure, and predation levels. These biotic and abiotic fluctuations in the lung have an effect on bacterial growth. So when a bacterium invades the lung, there are certain microenvironments that the bacterial species would grow better in and would occupy a specific niche that would amplify the bacteria's ability to persist. However, when multiple bacterial species invade the same niche, a competition for resources takes place and makes it difficult for both species to persist.

In this study, we sought to examine the interactions between *S. aureus* and *S. maltophilia*. The goal of this study is to determine whether negative, independent, or positive interactions exist between these two bacterial pathogens and to provide insight into how these two species might function together within the CF lung. I hypothesized that, *S. aureus* and *S. maltophilia* will interact *in vitro* in an either predatory or inhibitory manner. These predatory or inhibitory interactions would cause a separation of the two bacteria *in vivo*. If true, these bacteria would then have very little interactions within the lung and would, therefore, lack competition that would limit each other's growth. This hypothesis might explain why these two are found very often cultured from the same CF patients.

Materials and Methods

Growth and maintenance of bacterial strains and tissue culture cells

The strains used in this study are found in Table1. Strains were maintained routinely on Luria Broth agar (LB), Lennox formulation, and grown at 37°C. Where indicated, 10 μg/ml or 30 μg/mL chloramphenicol was added to the medium for *S. aureus* or *S. maltophilia* respectively. Murine RAW 264.7 4 macrophage were maintained in Dulbecco's Modified Eagle Medium

(DMEM) containing 10% Fetal Bovine Serum (FBS). For routine growth, 100 µL of penicillin-streptomycin was added to the DMEM to prevent contamination by bacteria.

Table 1. Strains used in this study

Strain Name	Source	Description
S. maltophilia Strains		
M29668	Dr. David Greenburg University of Texas Southwestern Hospital	Clinical strain of *S. maltophilia*; obtained from a CF patient In May 2013
TJB004	Yoder-Himes lab collection University of Louisville	Chloramphenicol 30, *S. maltophilia* M29668 conjugated with pIN301 from Annette Vergunst [57], eGFP-expression vector, fluorescence confirmed by microscopy
K279a	Nicholas P Cianciotto Northwestern University Medical School	Sequenced strain of *S. maltophilia*, originally from a cancer patient
108489	Alan Junkins Norton Hospital, Louisville, KY	Clinical CF isolate
CHB06092	Dr. Greg Priebe Boston Children's Hospital	*Stenotrophomonas maltophilia* clinical isolate from a female CF patient (09-176-00970) co-infected with methicillin resistant *S. aureus.*
SM1511	Dr. Susanna Remold University of Louisville	Environmental *Stenotrophomonas maltophilia* from a household drain in Louisville, KY
100662	Alan Junkins Norton Hospital, Louisville, KY	Clinical CF isolate
S. aureus strains		
AH3865	Alexander Horswill University of Colorado	Chloramphenicol 10, RN4220 containing pCM48 (pCM29_dsRedExpress, camR) [58]
T28260	Dr. David Greenburg University of Texas Southwestern Hospital	Clinical strain of methicillin-sensitive *S. aureus*; obtained from a CF patient in May 2013
NRS 72	Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA)	Clinical isolate of unknown origin isolated in 1960. Also known as NCTC 8325.
NRS 253	Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA)	Clinical isolate from 4 year old male in 2002 Bacteremia isolate
100619	Alan Junkins Norton Hospital, Louisville, KY	Clinical CF isolate

Mono- and Co-culture Biofilm Formation Assays

A single colony isolate was used to inoculate 5mL tubes of LB broth and incubated overnight at 37°C. Antibiotic selection was continued throughout this part of the experiment for the necessary strains. After 24 hours, cultures were diluted 1:50 in 5mL of LB with antibiotics. These tubes were incubated at 37°C until an optical density at 600 nm ($O.D._{600}$) of 0.8-1.2 was achieved which corresponds to mid-log phase growth.

Three 96-well PVC plates (Costar #2797) and rayon film covers (VWR #60941-084) were obtained and each was sterilized under UV light in a biosafety cabinet for 20 minutes and disinfected using 70% ethanol. Once all tubes of culture reached the desired $O.D._{600}$, each sample was aliquoted into autoclaved, sterile test tubes containing Tryptic Soy Broth (TSB) to give a final concentration of $1x10^6$ colony forming units (CFU)/mL based known ratios of CFU/mL concentrations to $O.D._{600}$ absorbance values previously established in the Yoder-Himes lab. Each tube was vortexed for five seconds and added to the wells of the 96-well PVC plates as indicated. For mono-culture biofilms, 60 µl each strain was added individually to at least three replicates wells and 60 µl of TSB was added to each. For co-culture biofilms, 60 µl of *S. maltophilia* culture was added to 60 µl of the *S. aureus* strain culture in at least three replicate well. Control wells containing 120 µl un-inoculated TSB were added to each 96-well plate to account for cross well contamination. Mono- , co-culture, and control wells were separated from each other by at least one row of empty wells to prevent cross-well contamination also. The 96-well plates were sealed with rayon film which allow for gas exchange. Each plate was wrapped in aluminum foil, placed in small humidifying chamber, and incubated at 37°C.

Sonication and Drip Dilution

At indicated time points, the 96-well plates were removed and the rayon film was carefully removed inside a biosafety cabinet. The conditioned media was removed using a micropipetter and each well was washed carefully with 120 µl of sterile water to avoid splashing. One hundred twenty µL of 1% Tween20 was then added to each well and covered with disinfected aluminum sealing foil. Plates were sonicated at 50Hz for 10 minutes in a Branson 3600 water bath sonicator to disrupt the biofilm structures and release the cells. Each sample was transferred to a flat-bottom 96-well disinfected polystyrene plate and serially diluted 10-fold in 180 µl of phosphate buffered saline. A multichannel pipet was then used to extract 10 µl from each well left to drip down square LB agar plates until drips reached 2/3 the way down the plates. The plates were then covered, labelled, and placed in the 37°C incubation room for 24 hours. Viable cell counts were determined for each sample. The row on each plate that had between 11-100 colonies was counted and recorded. The bacterial concentration in CFU/mL was then calculated based on multiplying the number of colonies and all dilution factors. Statistical analyses were performed in GraphPad Prism v 5.0.

Conditioned Medium Harvesting

Two 96-well PVC plates were inoculated solely with *S. maltophilia* M29668 mono-cultures or *S. aureus* AH3865 were prepared as described above and incubated at 37°C room for 7 days. The same protocol was followed in order to create conditioned media for the other *S. maltophilia* strains used [K279a, 108489, CHB06092, SM1511, 100662] except that each strain was added to only half of a 96-well plate in order to generate enough conditioned medium. The rayon films were removed and the conditioned media was transferred into a sterile centrifuge

tube using a micropipette and centrifuged for one hour at 5000 revolutions per minute x gravity (RPMxg). The conditioned media was carefully removed from the tubes and filtered through a 0.2 µm syringe filter to remove any remaining bacteria. The filtered conditioned media were stored for 2-3 days at 4°C.

Conditioned Medium Biofilm Assays

S. aureus AH3865 was grown to mid-log phase and prepared for biofilm experiments as described above. *S. aureus* culture was diluted into five different *S. maltophilia* M29668 conditioned media concentrations. The ratios of conditioned medium to TSB (v/v) used were 0:1, 1:2, 1:1, 2:1, and 1:0. 120 µl of these mixtures were added replicate wells. Each condition was separated from each other condition by at least one well. Control wells containing only TSB or only *S. maltophilia* conditioned medium were added to each plate. Plates were incubated, sonicated at the indicated time points, serially diluted, and plated as described above.

In separate experiments, *S. maltophilia* conditioned medium was combined with *S. aureus* conditioned medium instead of TSB and analyzed as described above. Controls wells containing only *S. aureus* or *S. maltophilia* conditioned medium were inoculated with *S. aureus*. Sonication and drip dilution protocol was performed as described above.

Confocal Imaging of Biofilms

Overnight cultures of *S. maltophilia* TJB004 and *S. aureus* AH3865 were diluted into 5 mL of LB broth with chloramphenicol and grown to mid-log phase. One million cells of each strain were added to flat-bottom confocal imaging dishes (Matsunami #D113OH) either in mono-culture (1 x 10^6 CFU) or co-culture (total of 2 x 10^6 CFU) with 3 mL of TSB +

chloramphenicol. These plates were placed in a humidity chamber and incubated at 37°C for three or seven days. At the appropriate time, these plates were removed from the incubation room and washed with 3 mL of sterile PBS. Three mL of fresh TSB was then added to each dish prior to imaging. Each dish was imaged under 40X oil immersion lens and two excitation wavelengths were used, 480 nm and 580 nm, to visualize both GFP and mCherry fluorescent proteins.

Macrophage Internalization Assays

RAW 264.7 macrophage were grown in T75 flasks to ~80% confluence and diluted approximately 50% confluence. Sterile Corning tissue culture treated 96-well plates were inoculated with 100 µL diluted macrophage culture. The plate was incubated overnight at 37°C with 5% CO_2. On the day of the experiment, the concentration of macrophage was established using trypan blue staining and a hemocytometer using standard protocols. *S. maltophilia* TJB004 and *S. aureus* AH3865 were grown to mid-log phase and diluted to achieve the desired multiplicity of infection (M.O.I. expressed as the number of bacteria per eukaryotic cell) of 50 and 100 (depending on the experiment). Once this value was calculated, the medium from each well was aspirated off and replaced with bacterial mono- or co-cultures suspended in 100 µL fresh DMEM + FBS. These plates were centrifuged at 800 RPMxg for minutes to maximize contact between bacteria and macrophage. These plates were then incubated at 37°C with 50% CO_2. After two hours, the old medium from every well was aspirated off, 100 µL of DMEM containing 5 mg/mL of gentamicin and penicillin-streptomycin mix was added to each well, and the plates were incubated at 37°C and 5% CO_2. At each time point, the medium from three replicate wells containing *S. aureus* or *S. maltophilia* was aspirated off and each well was

washed with 100 µL of fresh DMEM to remove unattached bacteria from the macrophage. This medium was again aspirated off and 100 µL of DMEM containing 0.5% Triton X-100 was added to wells in order to lyse the macrophage. The media was removed from each well, serially diluted 10-fold, and plated via drip dilutions, and analyzed for bacterial survival as described above.

RESULTS

Biofilm analysis of single *S. aureus* strain with panel of *S. maltophilia* strains

To determine if *S. maltophilia* affected the ability of *S. aureus* to survive and reproduce in biofilms, viable cell counts from biofilms were examined over time. To do this, we generated mono- or co-culture biofilms containing 1 x 10^6 or 2 x 10^6 cells respectively and incubated these biofilms for 1, 3, or 7 days. At each time point, *S. aureus* showed a significant decrease in viable cell counts in the presence of each *S. maltophilia* strain. After 3 days, viable cell counts of *S. aureus* significantly decreased in the presence of each *S. maltophilia* strain (Fig. 1A).

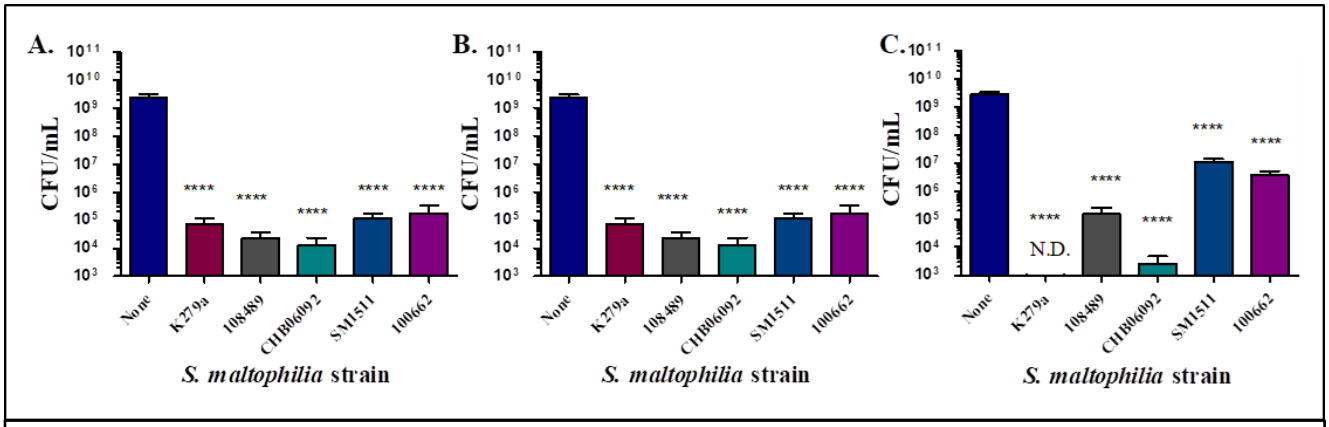


Figure 1. Survival of *S. aureus* in biofilms in the presence of *S. maltophilia*. *S. aureus* viable cell counts from (A) 3 day, (B) 5 day, or (C) 7 day biofilms are shown. Error bars represent one standard deviation from at least three replicate cultures. Co-culture survival was compared to mono-culture survival using unpaired t-tests. **** indicates p-values of <0.0001. N.D. represents not detectable levels of data. Limit of detection was 1.1x10^2 CFU/mL.

Each co-culture condition was statistically compared to the mono-culture condition containing only *S. aureus* AH3865 using an unpaired t-test. Each condition showed a highly significant difference compared to the mono-culture (p-values <0.0001). The same observations are true of both the 5 and 7 day biofilms (Fig. 1B and C respectively). Each condition produced highly significant differences compared to the mono-culture viable cell count (p-values<0.0001), showing that the presence of each *S. maltophilia* strain produced a decrease in the *S. aureus* viable cell counts. One *S. maltophilia* strain, K279a, suppressed *S. aureus* growth in biofilms below the level of detection after 7 days (Fig. 1C). In contrast, it was observed that there was no significant change between the various strains of *S. maltophilia* when grown in mono-culture compared to *S. maltophilia* grown in the presence

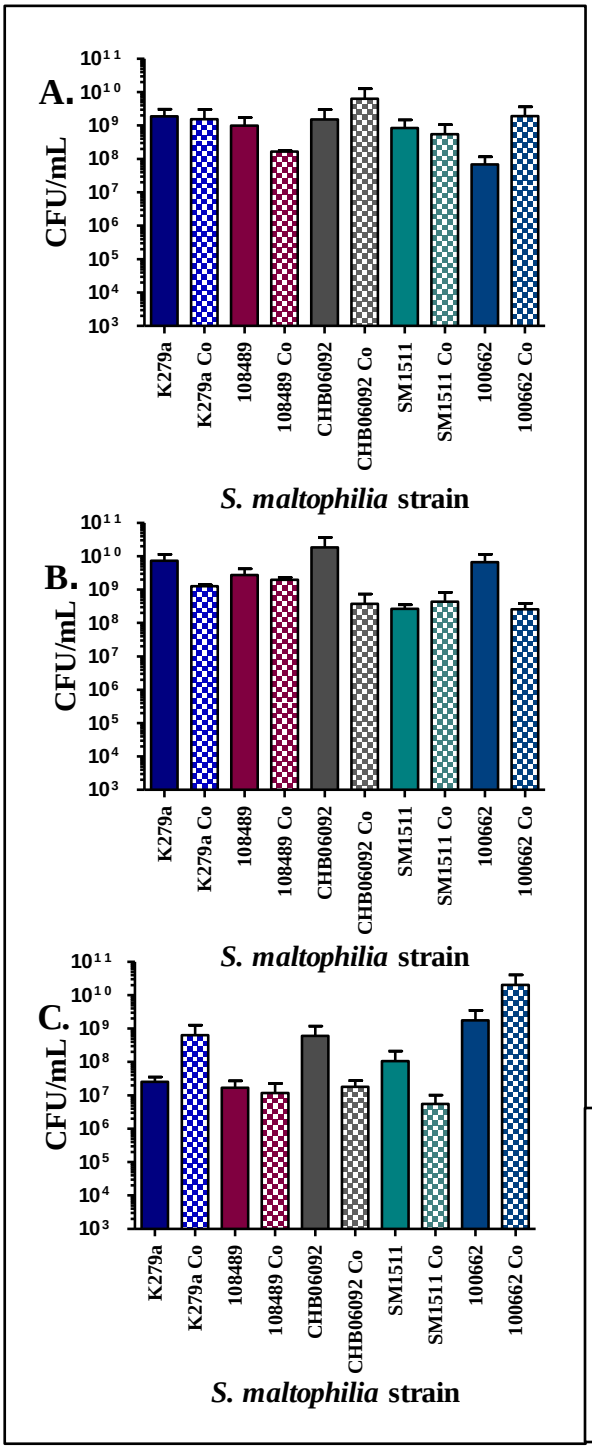


Figure 2. Survival of *S. maltophilia* in biofilms in the presence of *S. aureus*. *S. maltophilia* viable cell counts from (A) 3 day, (B) 5 day, or (C) 7 day biofilms are shown. Error bars represent one standard deviation from at least three replicate cultures. Co-culture survival was compared to mono-culture survival using unpaired t-tests. N.D. represents not detectable levels of data. Limit of detection was 1.1×10^2 CFU/mL.

of *S. aureus* (Fig. 2).

Biofilm analysis of single *S. maltophilia* strain with panel of *S. aureus* strains

To determine if *S. maltophilia* could suppress other *S. aureus* strains in biofilms, the same protocol was used from the previous experiment and viable cell counts for both *S. aureus* and *S. maltophilia* were obtained from mono- and co-cultures at 5 and 7 days post-inoculation. The co-culture condition for each strain was then compared to the mono-culture condition of the same strain using an unpaired t-test. After 5 days (Fig. 3A) every *S. aureus* strain showed a significant decrease in the presence of *S. maltophilia*. The most significant drops were that of strains AH3865, NRS72, and 100619. Compared to the mono-culture, each strain showed a

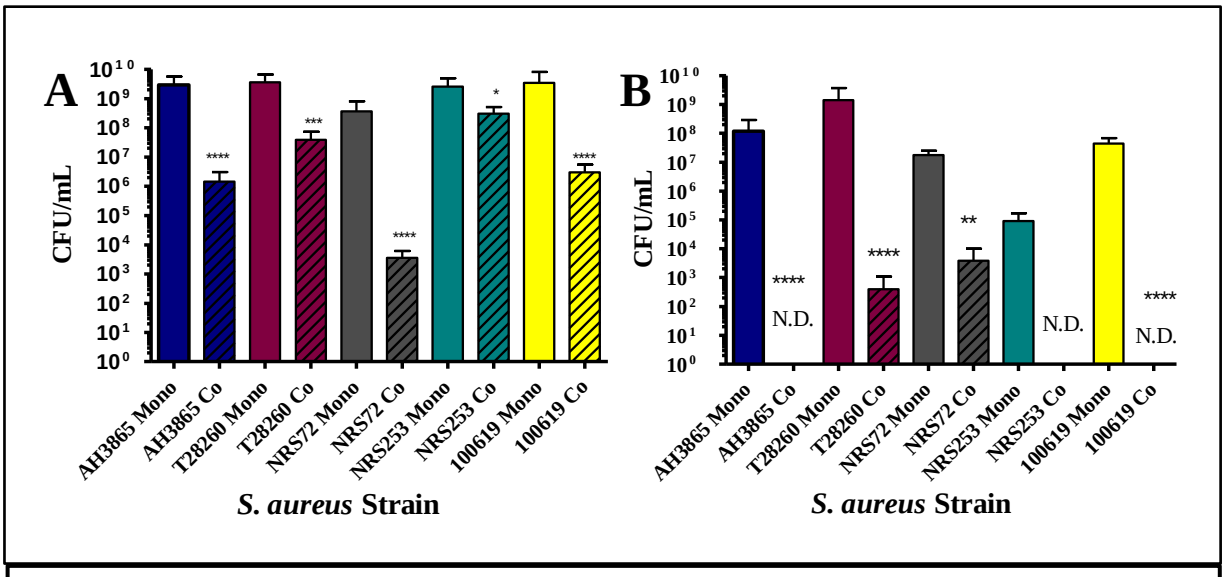


Figure 3. Viable cell counts of various *S. aureus* strains grown in co-culture with *S. maltophilia* M29668. *S. aureus* viable cell counts from (A) 5 day and (B) 7 day biofilms, were measured when grown in mono-culture or in co-culture with *S. maltophilia* M29668. Co-culture survival was compared to mono-culture survival using unpaired t-tests. Error bars indicate one standard deviation from the average from at least three replicate cultures . **** indicates *p*-values of <0.0001, *** indicates *p*-value of <0.001, ** indicates *p*-value of <0.01, *indicates p-value<0.05. N.D. represents levels of survival beneath the limit of detection ($1.1x10^2$ CFU/mL).

highly significant difference (p-value<0.0001). The two other strains, T28260 and NRS253, still showed a significant difference, just not to the extent of AH3865, NRS72, and 100619, with NRS253 showing the least significant drop (p-value<0.05) and T28260 showing slightly more significance (p-value<0.001). After 7 days, every *S. aureus* strain showed a significant decrease when grown in the presence of *S. maltophilia* M29668 except for *S. aureus* NRS253 (Fig. 3B), the latter possibly due to a reduced growth in mono-culture conditions compared to the other strains. After 7 days, *S. aureus* strains AH3865, NRS253, and 100619 were not detected suggesting that *S. maltophilia* strongly suppressed the presence of these strains in biofilms. There was again no significant difference in the viable cell counts of *S. maltophilia* within the co-culture compared to the viable cell counts of *S. maltophilia* alone (Fig. 4).

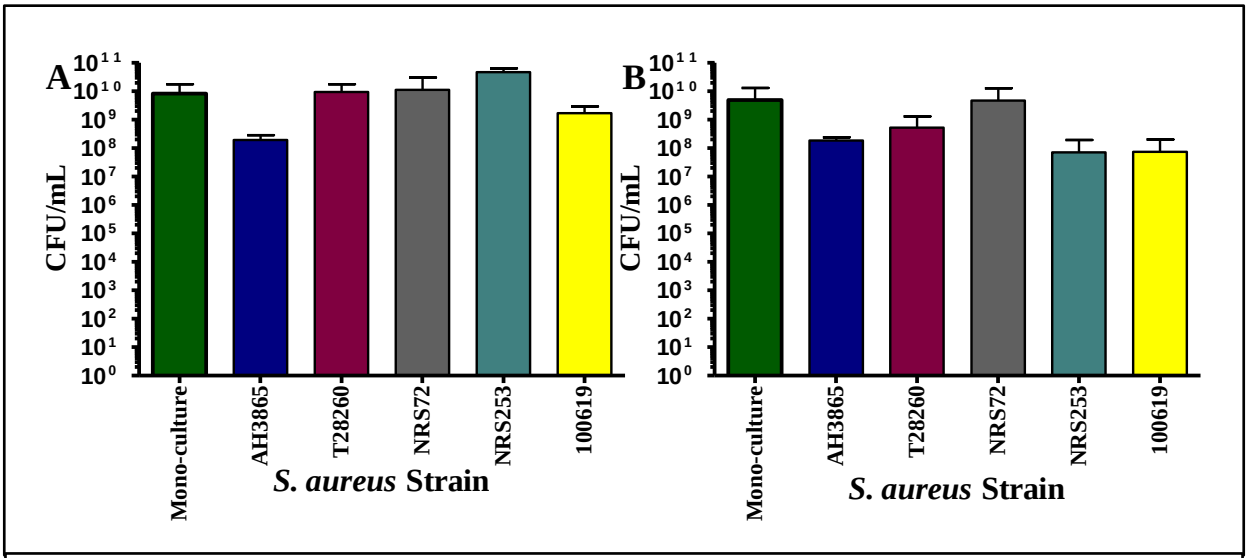


Figure 4. Viable cell counts of *S. maltophilia* M29668 strains grown in co-culture with various *S. aureus* strains. *S. aureus* viable cell counts from (A) 5 day and (B) 7 day biofilms, were measured when grown in mono-culture or in co-culture with *S. aureus* strains listed. Co-culture survival was compared to mono-culture survival using unpaired t-tests. Error bars indicate one standard deviation above the average of at least three replicates. The limit of detection was 1.1×10^2 CFU/mL.

Biofilm analysis using conditioned medium from a single strain of *S. maltophilia*

To assess whether the effect of *S. maltophilia* on *S. aureus* in biofilms is the result of a secreted product, conditioned media was harvested was from 7 day old mono-culture *S. maltophilia* M29668 biofilms. This conditioned media was filter sterilized to remove any live *S. maltophilia* cells and the conditioned media was added in varying concentrations to live *S. aureus* cells under biofilm forming conditions. Each condition was mixed with TSB medium such that it had either 0% conditioned media, 25% conditioned media, 50 % conditioned media, 75% conditioned media, or 100% conditioned media.

Viable cell counts were taken at both 5 days (Fig. 5A) and 7 days (Fig. 5B). At day 5 (Fig. 5A), the viable cell counts showed no significant changes in *S. aureus* concentration in 25% conditioned media and 50% conditioned media compared to the 0% conditioned media condition. However, there was a highly significant decrease with both 75% conditioned media and 100% conditioned media (*p*-values <0.0001) compared to the 0% conditioned media. The control wells at day 5 and day 7, containing only TSB and only conditioned media with no live culture, did not produce any growth when plated.

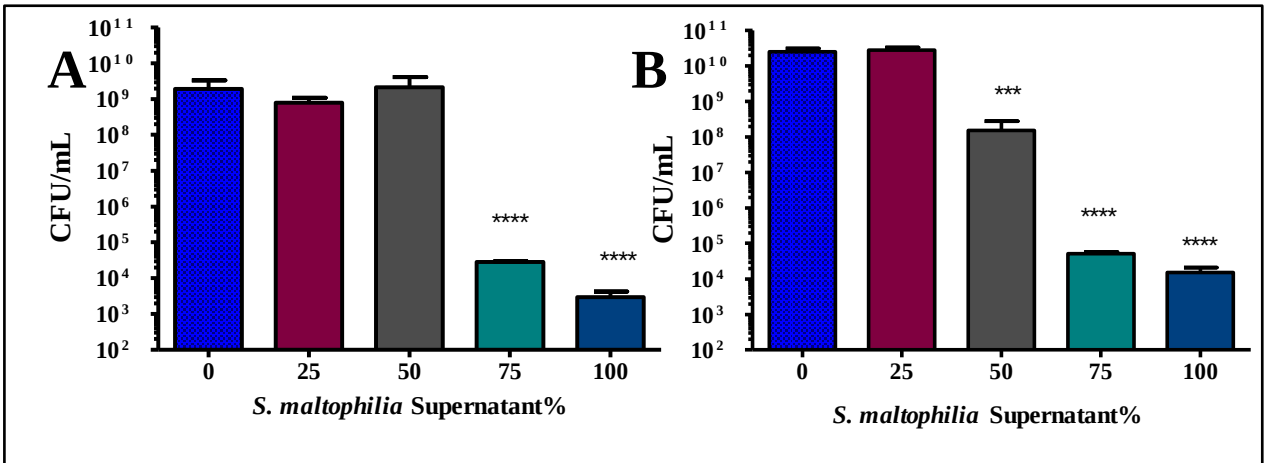
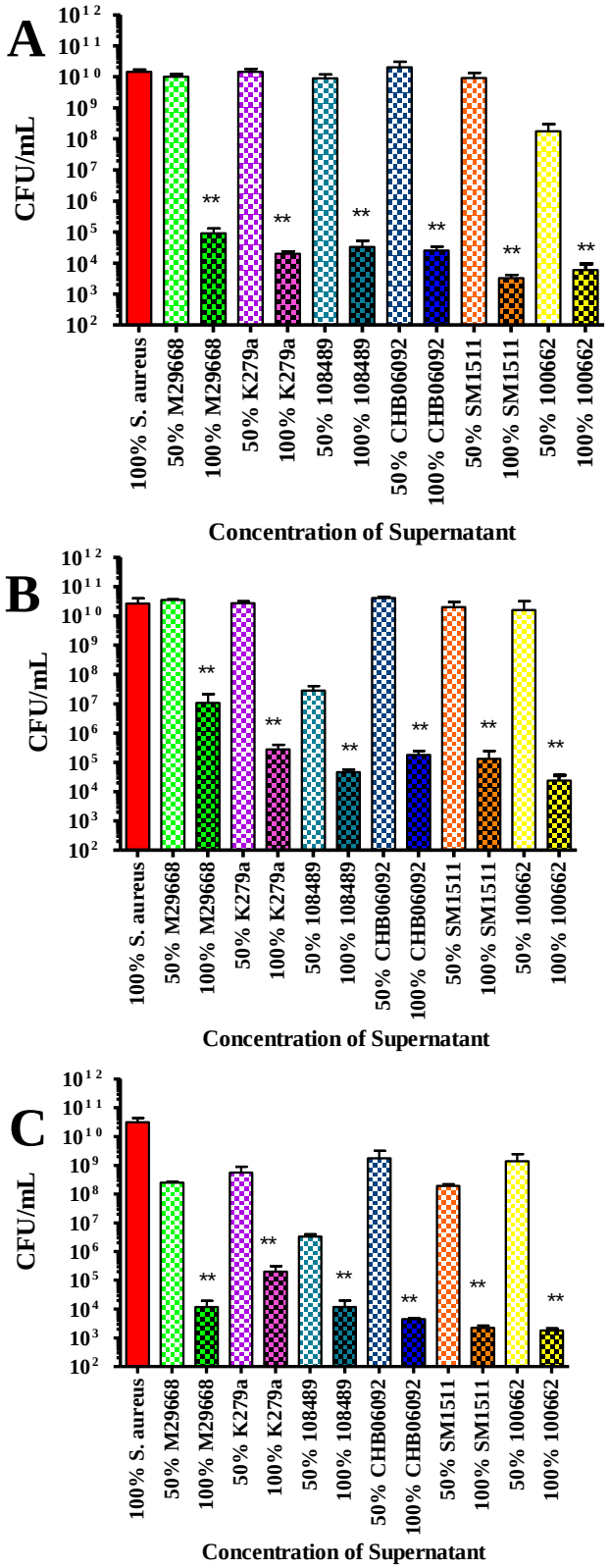


Figure 5. *S. aureus* viable cell counts grown in varying concentrations of *S. maltophilia* M29668 conditioned media. Viable cell counts from (A) 5 day or (B) 7 day biofilms were obtained. Viable cell counts under varying levels of conditioned media concentration were compared to 0% *S. maltophilia* conditioned media concentration using unpaired t-tests. **** indicates *p*-values of <0.0001 ***indicates p values of <0.001 **indicates p values of <0.01. The limit of detection in this assay was $1.1x10^2$ CFU/mL. Error bars indicate one standard deviation above the average.

At day 7 (Fig. 5B), the viable cell counts again showed no significant difference at 25% conditioned media compared to 0% conditioned media. However, at 50% conditioned media there was a 2- log decrease recorded in *S. aureus* viable cell counts (*p*-values <0.001). There was again a 5-log decrease at 75% and 100% conditioned media compared to 0% conditioned media. The control wells at day 7, containing only TSB and only conditioned media with no live cultures, did not produce any growth when plated. Taken together, these results suggest that *S. maltophilia* secretes something that is capable of reducing *S. aureus* growth and persistence in biofilm in a dose-dependent and time-dependent manner.

Biofilm Analysis with conditioned media from multiple strains of *S. maltophilia*

To determine if the conditioned media harvested from various strains of *S. maltophilia* had a similar effect on *S. aureus* AH3865 compared to those conditioned media harvested from *S. maltophilia* M29668, conditioned media from five additional strains of *S. maltophilia* was used in two concentrations, either 50% conditioned media or 100% conditioned media doses. As a control for general conditioned media effects, *S. aureus* conditioned media was harvested and used in parallel. Plates were incubated for 3 days (Fig. 6A), 5 days (Fig. 6B), or 7 days (Fig. 6C). Because this experiment had more than two treatments, a one-way ANOVA statistical test with a Dunnett's multiple comparison test was used to determine if any of the conditioned media conditions produced statistically significant differences when compared to the 100% *S. aureus* conditioned media. We found that every *S. maltophilia* strain at 100% *S. maltophilia* conditioned media concentration resulted in a statistically significant decrease in *S. aureus* viable counts. This is true for the 3 day (Fig. 6A), 5 day (Fig. 6B), and 7 day (Fig. 6C) time points. Conditioned



media from some strains at day 7 also showed strong decreases in *S. aureus* survival, sometimes up to a 10^3-10^4 CFU/mL fold reduction in viability (Fig. 6C).

Assessing mono- and co-culture *S. aureus* and *S. maltophilia* biofilm structures

Bacterial survival gives a gross estimate as to changes in populations in biofilms over time. However, it yields no information into the structures being formed in mono- and co-culture biofilms, which may have implications for the likely

Figure 6. *S. aureus* viable cell counts grown in varying concentrations of *S. maltophilia* M29668 conditioned media. Viable cell counts from (A) 3 day, (B) 5 day, (C) 7 day biofilms were obtained. *S. aureus* was grown in two concentrations of *S. maltophilia* conditioned media. Viable cell counts under varying levels of conditioned media concentration were compared to 100% *S. aureus* conditioned media concentration using a one-way ANOVA with a Dunnett Multiple Comparison post-test. **** indicates *p*-values of <0.0001 ***indicates p values of <0.001 **indicates p values of <0.01. Limit of detection was $1.1x10^2$. Error bars indicate one standard deviation above the average of three replicates.

efficacy of therapeutic agents such as antibiotics. To address whether co-cultures biofilms have different structures or phenotypic properties, we conducted pilot experiments using confocal laser scanning microscopy (CLSM). This type of microscopy allows us to image 3-dimensional structures such as biofilms (Fig. 7). Because these were pilot studies, we have yet to generate a

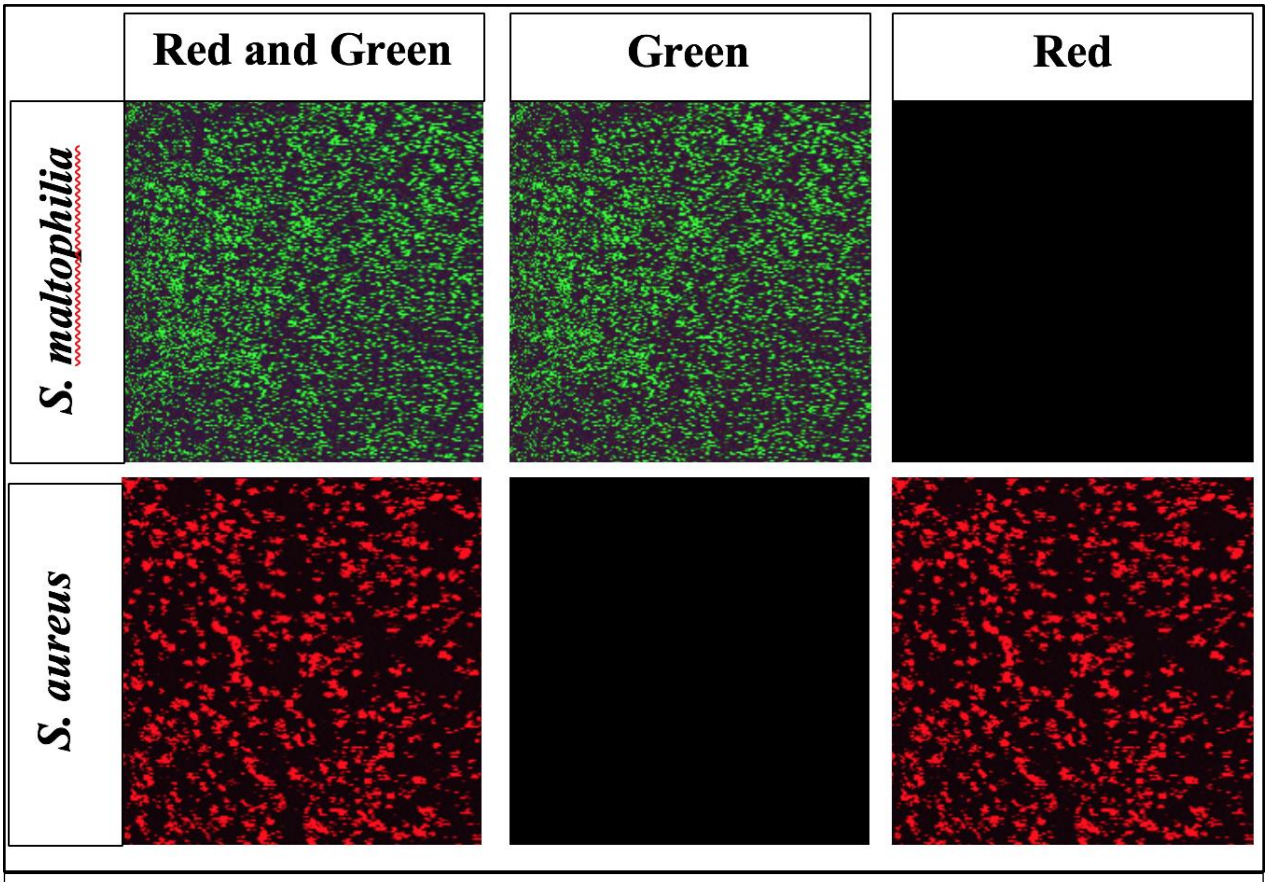


Figure 7. CLSM of mono-culture biofilms. *S. maltophilia* TJB004 (fluoresces green) and *S. aureus* AH3865 (fluoresces red) were imagined under 40X magnification. A-C represent *S. maltophilia* mono-culture biofilms, D-F represent *S. aureus* mono-culture biofilms. Images shown are representative of 9 images taken from biological triplicate cultures. Images represent 3 day biofilm growth.

sufficient of images to allow us to quantitate the physiological properties and establish statistical differences so all conclusions in this section drawn are based solely on qualitative observations made. The mono-culture conditions show standard growth when the bacterial cultures are left alone to grow. When the *S. aureus* mono-culture images (Fig. 7D) are compared to the *S. aureus* assay co-culture images (Fig. 8C), there appears to be a very noticeable decrease in both size of

the structures and number of the structures. When looking at solely the co-culture images, it appears as though there is a greater amount of green fluorescent *S. maltophilia* compared to the red *S. aureus* at both time points (Fig. 8). There also appears to be a slight decrease in both *S. maltophilia* and *S. aureus* from day 3 (Fig. 8A-8C) to day 7 (Fig. 8D-8F).

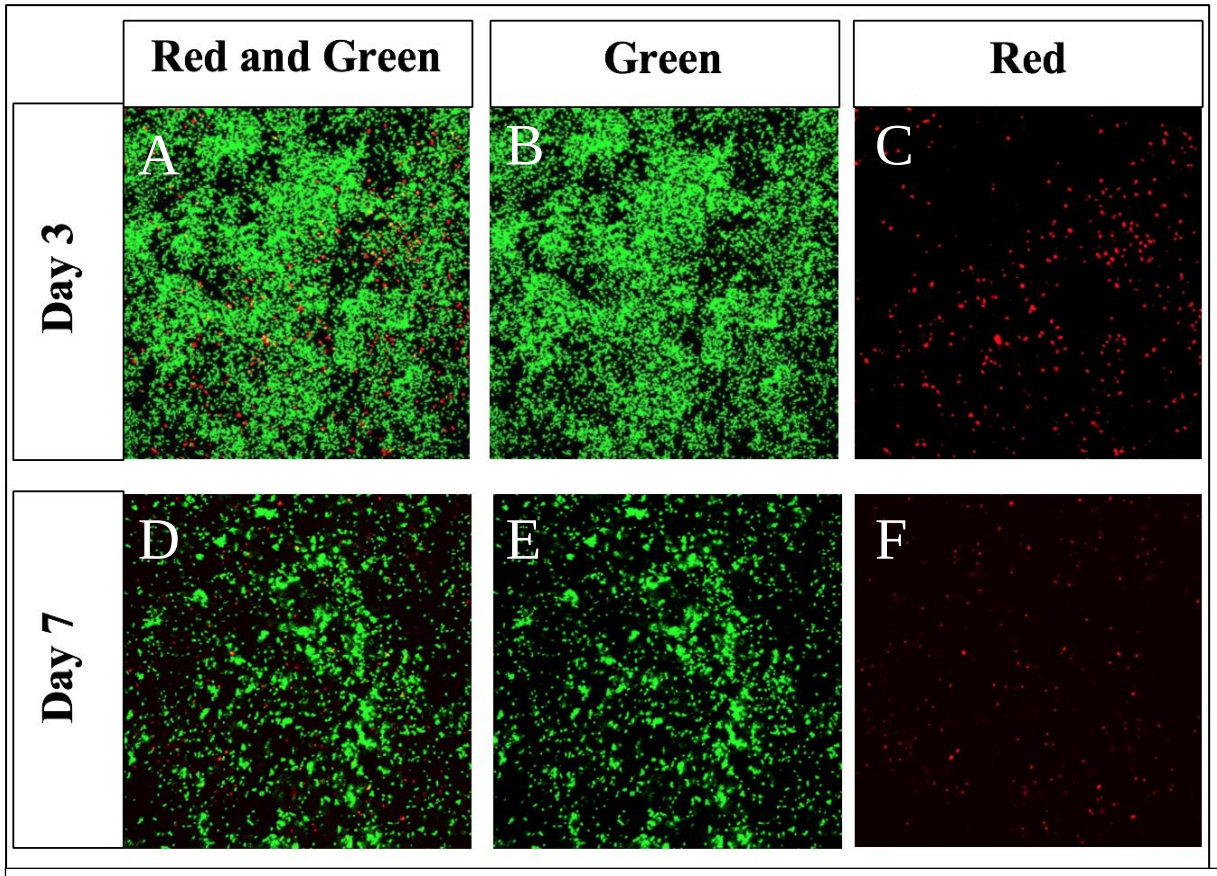


Figure 8. CLSM of co-culture biofilms. *S. maltophilia* TJB004 (fluoresces green) and *S. aureus* AH3865 (fluoresces red) were imagined under 40X magnification. A-C represent 3 day co-culture biofilms, D-F represent 7 day co-culture biofilms. Images shown are representative of 9 images taken from biological triplicate cultures.

Internalization *S. aureus* and *S. maltophilia* by mammalian macrophage

While we observed that *S. maltophilia* can suppress *S. aureus* in biofilms *in vitro*, data from the CF Foundation suggests these two species are often found in the same patient. This data would make sense if these species occupy different niches. Based on other studies, *S. aureus* is

primarily thought to be an extracellular pathogen while *S. maltophilia* may be able to penetrate into host cells and survive. Therefore, these two species may only rarely encounter each other *in vivo*. I conducted internalization assays using immortalized murine macrophages, immune cells known to phagocytose bacteria. This particular macrophage cell line was chosen because it mimics the alveolar macrophages commonly found in the lungs. RAW 264.7 murine macrophage were exposed to either *S. aureus* or *S. maltophilia* in mono-culture or both species in co-culture. After a short period of time, antibiotics were added to the medium to kill extracellular bacteria so we could track only those inside the host cells. We assayed each condition over the course of 24-48 hours to examine the growth dynamic of each mono- and co-culture sample. Bacterial samples from each time point were harvested from the cultured macrophages and assessed for species survival.

Figure 9A shows the viable cell counts for both *S. aureus* (red) and *S. maltophilia* (green) at various hours post-inoculation. The curves as a whole did not show statistical significance using a two-way ANOVA algorithm. However, unpaired t-tests were conducted to compare each individual time point. It was found that at 8 hours, 12 hours, and 24 hours past inoculation there was a highly significant difference (p-value<0.0001) between the viable cell counts of *S. aureus* and *S. maltophilia. S. maltophilia* survived at a 3-log higher rate than *S. aureus* at those hours past inoculation than *S. aureus* had. At an M.O.I. of 100, *S. maltophilia* survived 1,000 fold better compared to *S. aureus* (p-value<0.0001) based on two-way ANOVAs. *S. aureus* seemed to have an overall negative trend, decreasing at each time point, while *S. maltophilia* seemed to fluctuate up and down throughout the duration of the experiment. *S. aureus* was also not detected at 144 hours post inoculation (h.p.i.) suggesting that the macrophage may have effectively

eliminated the *S. aureus* by this time. In contrast, internalized *S. maltophilia* was still detected at 144 h.p.i. at levels of ~10^4 CFU/mL.

Figure 9C shows another set of viable cell counts of *S. aureus* (red) and *S. maltophilia* (green). The bacteria were inoculated at an M.O.I. of 100 and the bacterial cultures were mixed. The invasions occurred with bacterial co-cultures, so each well contained both bacterial species. The viable cell counts represent the number of internalized bacteria from each species. Every time point produced viable cell counts above the limit of detection.

When statistical analysis was carried out using a two-way ANOVAs, these curves were not found to be statistically different. However, when each time point was compared individually using an unpaired t-test, it was found that at 12, 24, and 48 hours

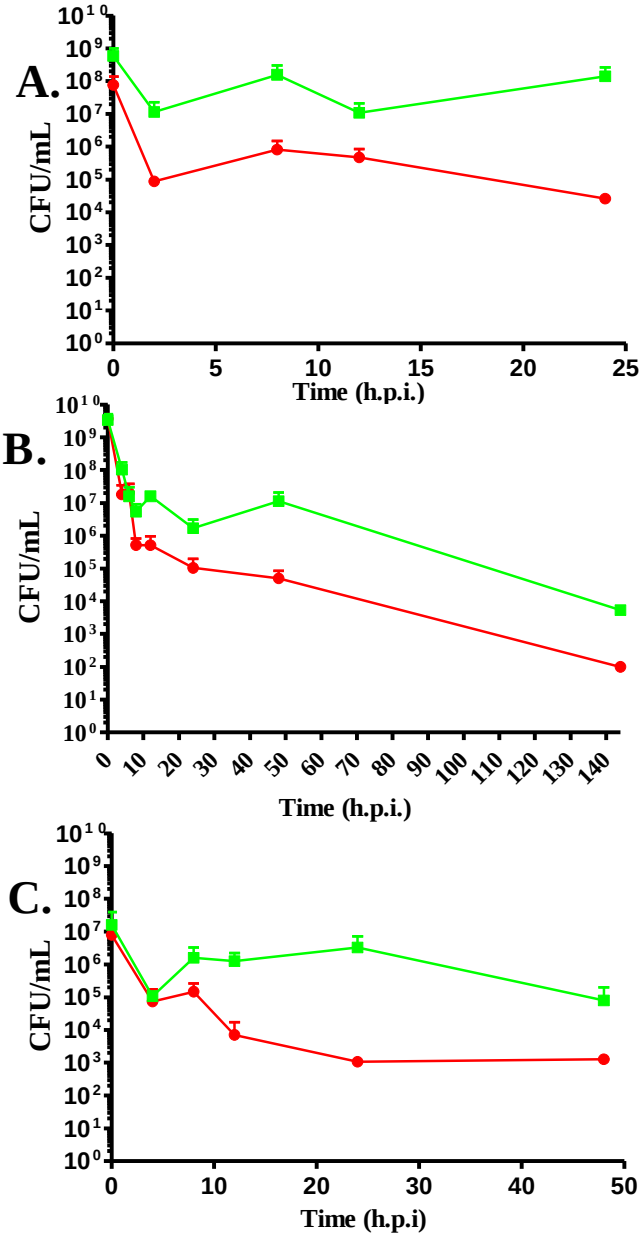


Figure 9. Viable cell counts of *S. aureus* and *S. maltophilia* after internalization by murine macrophage. Viable cell counts (CFU/mL) at various time points represented as hours post- inoculation (h.p.i.) are shown. The red line represents *S. aureus* and the green line represents *S. maltophilia.* Multiplicities of infection of 50 (A) or 100 (B and C) were used. Panels A and B represent mono-culture conditions. Panel C represents co-culture conditions. The limit of detection was 1.1x10^2 CFU/mL in all experiments. Error bars represent one standard deviation above the average of three replicates.

showed statistical significant differences (*p*-values<0.001). This data suggests that after 12 hours, *S. maltophilia* is found internalized at a much higher rate than *S. aureus* is found internalized. In order to determine if the co-culture condition used for inoculation of the macrophages had an effect, the co-culture data from was plotted on the same graph as the monoculture data (Fig. 10). Each time point was then compared for both *S. aureus* and *S. maltophilia* to identify if the co-culture condition produced any statistical significance. For *S. aureus*, (Fig. 10A) every time point except 8 h.p.i. showed a statistically significant difference when compared using an unpaired t-test (*p*-value<0.001). *S. maltophilia* (Fig. 10B) only showed statistically significant difference at 4 h.p.i. and and 48 h.p.i. (p-values<0.001) when using the unpaired t-test. The internalization rates of both bacteria seem to have

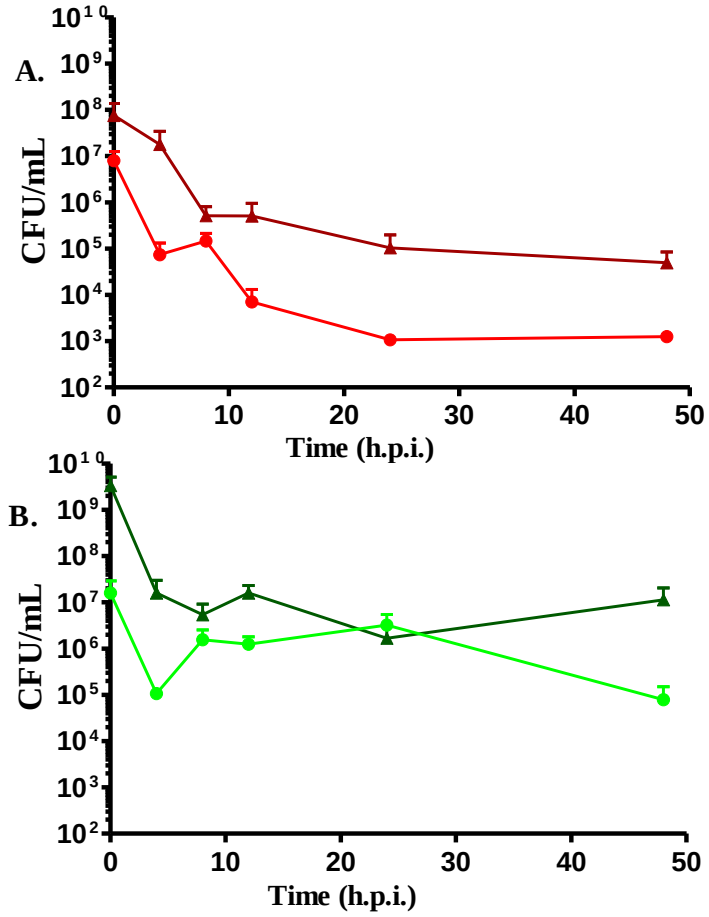


Figure 10. Viable cell counts of *S. aureus* and *S. maltophilia* after internalization by murine macrophage co-culture vs mono-culture. Viable cell counts (CFU/mL) at various time points represented as hours post- inoculation (h.p.i.) are shown. Panel A shows data for *S. aureus* and Panel B shows the data for *S. maltophilia.* M.O.I.s of 100 was used. Dark triangles represent mono-culture, light circles represent co-culture. The limit of detection was 1.1×10^2 in all experiments. Error bars represent one standard deviation above the average of three replicates.

been slightly negatively affected by the co-culture condition. However, it appears as though the condition had a more significant negative effect on the internalization of *S. aureus* than it did on the internalization of *S. maltophilia.*

Discussion

In this study, we present evidence that suggests that *S. maltophilia* can inhibit *S. aureus* biofilm formation *in vitro*. This inhibition is at least partially mediated by one or more secreted substances made by multiple *S. maltophilia* isolates during biofilm formation. Whether the effect of the secreted substance actually leads to lysis or killing of *S. aureus* or whether it simply prevents *S. aureus* from building a biofilm though the inhibition of extracellular polymeric substance remains to be determined. It does appear that this inhibition is time- and concentration dependent as would be expected for a protein-based toxin or other similar inhibitory mechanism.

It does not appear that this inhibition is mediated through nutrient depletion (Fig. 6) because it was observed that this effect is observed in both nutrient rich media (TSB) and nutrient depleted media (*S. aureus* conditioned medium). Further we observed that this inhibition is not strain dependent (Figs. 3, 6), either on the part of *S. maltophilia* or on the *S. aureus* side which suggests that this mechanism of inhibition is fundamental to many strains of *S. maltophilia* rather than a recent acquisition by specific strains of *S. maltophilia*. This data also seems to indicate that live *S. maltophilia* is not required for the disruption of *S. aureus* viability (Fig. 6). It has been previously shown that *S. maltophilia* has the ability to sense the environment around itself and react to that environment through the use of quorum sensing [48]. *S. maltophilia* possesses a very unique system of quorum sensing that works through the use of a diffusible signal factor (DSF). This system has been found to play a role in disrupting growth of

certain organisms, such as the fungus *Candida albicans.* [49]. However, since the *S. maltophilia* generated the conditioned media without the presence of *S. aureus*, this observed inhibitory effect is not a direct result of *S. maltophilia* detecting the presence of *S. aureus* and actively generating something specific to prevent *S. aureus* from growing. Instead, it is more likely that the conditioned medium itself intrinsically contains a substance that makes it harder for *S. aureus* to grow and/or form biofilms.

There are many potential explanations for how *S. maltophilia* conditioned medium could lead to *S. aureus* biofilm inhibition. One potential factor could be changing the pH of the medium. It has been found that pH has a very strong effect on biofilm production by *S. aureus* [48]. In an experiment carried out in 2018 by a member of the Yoder-Himes lab, it was shown that at very high (above 8.5) and very low pH (below 4.0) there is very weak biofilm formation by *S. aureus* [50]. *S. aureus* does not form very thick biofilms at pH values below 4.0 and above 8.5. According to this data, there seems to be an optimal pH value slightly over pH of 6 and in both directions there is a slow decline in biofilm density. That particular study does not reflect viable cell counts, but, based on absorbance data of

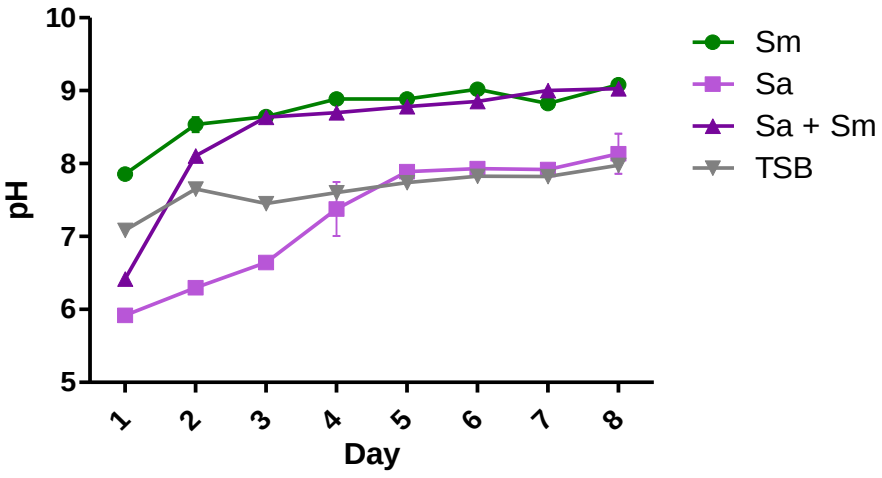


Figure 11. pH values over time of mono-culture and co-culture biofilms. pH values of *S. aureus* (Sa) mono-culture (light purple square), *S. maltophilia* (Sm) mono-culture (green circles), and co-culture with both species (dark purple triangles). Data kindly provided by Josh Stewart, undergraduate in the Yoder-Himes lab, yet unpublished.

the biofilms, there seems to be a definitive decrease in the ability of *S. aureus* to thrive in very high and very low pH levels. Therefore, the negative effect that *S. maltophilia* conditioned media has on *S. aureus* could be attributed to pH if *S. maltophilia* is able to manipulate the pH of its conditioned medium and create a slightly higher or slightly lower pH than *S. aureus*' optimal pH.

A collaborator within the Dr. Yoder-Himes lab carried out an experiment that looked at pH changes biofilms of various microbes. In this experiment (Fig. 11), the collaborator continuously measured the pH values over time of *S. aureus* in mono-culture, *S. maltophilia* in mono-culture, and in both species in co-culture. It was found that when *S. maltophilia* grows and forms a biofilm in mono-culture, its conditioned medium maintains a pH above 8. When *S. aureus* grows and forms a biofilm, its conditioned medium mostly maintains a pH between 6 and 8. When *S. aureus* and *S. maltophilia* are grown together the pH values closely resemble those of *S. maltophilia,* around a pH of 8.5. Since, *S. aureus'* ability to form biofilms significantly decreases when pH increases past a value of 8 [50], there is reason to believe that the elevated pH when grown in co-culture with *S. maltophilia* may be the reason that *S. aureus* is unable to grow and form biofilms at the same rate that this species does when left to grow without *S. maltophilia*.

The elevated pH that *S. maltophilia* generates one possible reason for the observed inhibition, however other potential mechanisms exist in closely related gram-negative species that could give more insight into this inhibitory effect. *S. maltophilia* shares many similarities with other gram-negative bacteria such as *Pseudomonas aeruginosa*. They possess similar methods of antibiotic resistance and cell wall maintenance [51]. This could lead one to propose that *S. maltophilia* and *P. aeruginosa* might also possess similar methods of *S. aureus* inhibition.

An observed method that *P. aeruginosa* uses to limit the biofilm production of *S. aureus* is by forcing *S. aureus* into a fermentation pathway [52]. When *S. aureus* is grown in the presence of *P. aeruginosa* there is an observed shift in *S. aureus* from an aerobic pathway producing acetate, to an anaerobic pathway producing lactate that provides less energy to the organism. The exact mechanism causing this shift is unknown; however, there is evidence that this shift plays an important role in the inhibition of *S. aureus* by *P. aeruginosa* [52]. This shift in metabolism from respiration to fermentation puts *S. aureus* at a competitive disadvantage compared to *P. aeruginosa*. This possibly leads to *S. aureus* being unable to sustain substantial growth in the presence of *P. aeruginosa* and ultimately a lack of survival. This shift from respiration to fermentation in *S. aureus* could potentially also be seen with *S. maltophilia* due to the similarities with it shares with *P. aeruginosa*, and could be a contributing factor to the inhibition seen when *S. aureus* is grown with *S. maltophilia.* However, this is more than likely not the reason for the observed inhibition because the observed shift in metabolism was not seen when *S. aureus* was grown in *P. aeruginosa* conditioned medium [52]. If this same method was solely responsible for the inhibition of *S. aureus* observed with *S. maltophilia* then there would not have been an observed inhibition in fig. 6. Further study is necessary to determine if this shift in metabolism does indeed occur when *S. aureus* is grown in co-culture with *S. maltophilia.* However, there is evidence (Fig. 6) to suggest this method of inhibition could not be solely responsible for the inhibition observed in this paper.

Another potential method of *S. aureus* inhibition by *P. aeruginosa* is through the use of long chain N-acylhomoserine lactones (AHL) [53]. It has been found that multiple types of these AHLs, produced by *P. aeruginosa*, reduce growth and virulence in *S. aureus* [54]. These molecules consist of a homoserine lactone ring that is N-acylated with a fatty acyl group.

Various lengths of acyl groups are produced by *P. aeruginosa* as well as may other gram-negative bacteria. However, the long acyl chains have been found to produce an inhibitory effect in *S. aureus* [55]. This would be an ideal explanation for the potential mechanism of inhibition by *S. maltophilia* since AHLs are produced by many gram-negative species. However, it has been found that no environmental strains of *S. maltophilia* have shown to produce these molecules [56]. Based on the data from this paper, (Fig. 1) suggesting that the inhibitory effect of *S. maltophilia* is not strain specific and is observed in both environmental and clinical strains, it is highly unlikely that AHLs play any role in the inhibition of *S. aureus* by *S. maltophilia*.

pH appears to be the most plausible explanation for this observed inhibitory effect of *S. maltophilia* on *S. aureus*. However, pH may not be solely responsible for this observed effect. There may be other factors that all contribute to this produce this inhibition. More study of the molecular interactions between the two pathogens is needed to determine the exact method of inhibition and if there are more inhibitory factors beyond that of pH.

This inhibitory relationship that exists between *S. maltophilia* and *S. aureus* seems to contradict the data that was found in the CF patient registry that *S. aureus* and *S. maltophilia* are often found within the same CF sputum. However, with my current hypothesis that the two bacteria occupy different niches, this inhibitory effect is plausible. These two bacteria are extremely different and it would make sense that one would not survive in an environment that the other thrives in. However, when the two occupy different niches, there would be very little interactions between the two and there would not be the observed inhibitory effect. If true, there would also be a much smaller amount of nutrient competition which would allow the two to coexist very nicely even within the same lung. However, the question remains, do the two occupy different niches and if so what niches do each occupy?

In order to further investigate this hypothesis, an internalization assay was carried out to determine if there are differences in how each bacterial species invades eukaryotic cells. If it is observed that one has a higher rate of invasion and replication within eukaryotic cells, that might provide evidence that these bacteria localize within different parts of the CF lung. During this invasion a murine model was used to act as the eukaryotic cells. RAW 264.7 macrophages were invaded with both *S. maltophilia* and *S. aureus.* Antibiotics were then used to eliminate any extracellular pathogens, the eukaryotic cells were lysed, and the viable cell counts of each bacterium were recorded. This was first done solely with mono-cultures of each bacterial species. When the data was recorded (Fig. 6A) it was found that there was no statistically significant difference between the two bacteria invasion amounts at 4 h.p.i.. However, after that time point there was a highly significant difference between the two bacterial amounts. *S. maltophilia* showed a much higher amount of invasion and replication within the macrophage. There was a steep decrease in both cell counts during the first two timepoints. *S. maltophilia* seemed to rebound after this decrease at a very high rate and create viable bacterial cells within the macrophage. However, *S. aureus* was found in much lower concentrations and a much smaller rebound effect was observed.

When the invasion was carried out a second time, more time points were recorded but the same trend was still observed. (Fig. 6B) There was the same dip at the very beginning of the experiment but *S. maltophilia* was able to rebound to a very high level. *S. aureus* was unable to achieve any rebound effect and over time decreased in viable to amounts until it was unable to be detected at 144 hours. *S. maltophilia* was still observed even at 144 h.p.i.. This same observation was made when invasion of macrophages was carried out using cocultures containing both *S.*

aureus and *S. maltophilia*. *S. maltophilia;* however, had significantly higher viable cell amounts at 12, 24, and 48 h.p.i. compared to those of *S. aureus.*

This trend seems to indicate that *S. maltophilia* preferentially invades eukaryotic cells to a greater extent than *S. aureus*. Some recent studies corroborate this finding that *S. maltophilia* does have an ability to internalize within host macrophage and replicate efficiently once inside [57]. However, research is still ongoing and far from definitive. The murine tissue invasion does seem to suggest that *S. maltophilia* is able to survive within a specific microenvironment to much greater extent than *S. aureus.* This provides supports the idea that the two species are localized to different niches in the CF lung.

At this point in time, the conclusion that *S. aureus* and *S. maltophilia* occupy different niches within the CF lung is mostly conjecture and requires further study. However, this theoretical conclusion makes sense based on the recorded data that *S. maltophilia* creates a less than optimal environment for *S. aureus* and the statistics from the CF foundation showing that both *S. maltophilia* and *S. aureus* are observed very often within the same CF sputum. Further research and study is necessary to generate more definitive answers. However, this study lays the foundation for understanding the various interactions and relationship between two very common CF pathogens, *S. aureus* and *S. maltophilia*.

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References

1. Fanen, P., Wohlhuter-Haddad, A., & Hinzpeter, A. (2014). Genetics of cystic fibrosis: Cftr mutation classifications toward genotype-based CF therapies. *International Journal of Biochemistry and Cell Biology, 52,* 94-102. doi:10.1016/j.biocel.2014.02.023

2. Riordan, J., Rommens, J., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z. Et, A. (1989). Identification of the cystic fibrosis gene: Cloning and characterization of complementary DNA. *Science (new York, N.y.), 245*(4922), 1066-73.

3. Welsh, M., & Smith, A. (1993). Molecular mechanisms of cftr chloride channel dysfunction in cystic fibrosis. *Cell, 73*(7), 1251-4.

4. Marcorelles, P., Montier, T., Gillet, D., Lagarde, N., & Ferec, C. (2007). Evolution of CFTRprotein distribution in lung tissue from normal and CF human fetuses. *Pediatric Pulmonology, 42*(11), 1032-1040. doi:10.1002/ppul.20690

5. Wilschanski, M., Zielenski, J., Markiewicz, D., Tsui, L., Corey, M., Levison, H., & Durie, P. (1995). Correlation of sweat chloride concentration with classes of the cystic fibrosis transmembrane conductance regulator gene mutations. *The Journal of Pediatrics, 127*(5), 705-10.

6. Durie, P., & Forstner, G. (1989). Pathophysiology of the exocrine pancreas in cystic fibrosis. *Journal of the Royal Society of Medicine, 82,* 2-10.

7. Noone, P., & Knowles, M. (2001). 'cftr-opathies': Disease phenotypes associated with cystic fibrosis transmembrane regulator gene mutations. *Respiratory Research, 2*(6), 328-32.

8. Grubb, B., & Boucher, R. (1999). Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiological Reviews, 79*(1), 193-214

9. Kurbatova, P., Bessonov, N., Volpert, V., Tiddens, H., Cornu, C., Nony, P., & Caudri, D. (2015). Model of Mucociliary Clearance in Cystic Fibrosis Lungs. *Journal of Theoretical Biology, 372,* 81-88.

10. Antoniou, S., & Elston, C. (2016). Cystic fibrosis. *Medicine, 44*(5), 321-325. doi:10.1016/j.mpmed.2016.02.016

11. Mahenthiralingam, E. (2014). Emerging Cystic Fibrosis Pathogens and the Microbiome. *Paediatric Respiratory Reviews: Supplement 1, 15,* 13-15. doi:10.1016/j.prrv.2014.04.006

12. Marshall, B., Elbert, A., Petren, K., Rizvi, S., Fink, A., Ostrenga, J. Loeffler, D. (2015). *Patient Registry Annual Data Report 2015*. Retrieved September 6, 2018

13. Baird-Parker, A. (1965). Staphylococci and their Classification. *Annals of the New York Academy of Sciences, 128*(1), 4-25. doi:10.1111/j.1749-6632.1965.tb11626.x

14. Noble,W., Valkenburg, H., &Wolters, C. (1967) Carriage *Staphylococcus aureus* in random samples of a normal population. *Epidemiology and Infection,* 65(4), 567-573. doi: 10.1017/S002217240004609X

15. Weinstein, H. (1959). The relation between the nasal-staphylococcal-carrier state and the incidence of postoperative complications. *The New England Journal of Medicine, 260*(26), 1303-8

16. Harik, N., MD, Com, G., MD, Tang, X., PhD, Melguizo Castro, M., MS, Stemper, M., MS, & Carroll, J., MD. (2016). Clinical characteristics and epidemiology of methicillin-resistant staphylococcus aureus (mrsa) in children with cystic fibrosis from a center with a high mrsa prevalence. *Ajic: American Journal of Infection Control, 44*(4), 409-415. doi:10.1016/j.ajic.2015.10.015

17. Lowy, F. (1998). *Staphylococcus aureus* infections. *The New England Journal of Medicine, 339*(8), 520-32.

18. Matias, V. R., & Beveridge, T. J. (2006). Native cell wall organization shown by cryo-electron microscopy confirms the existence of a periplasmic space in *Staphylococcus aureus*. *Journal of bacteriology, 188*(3), 1011-1021.

19. Bailey, R., Turner, R., Mullin, N., Clarke, N., Foster, S., & Hobbs, J. (2014). The interplay between cell wall mechanical properties and the cell cycle *in Staphylococcus aureus*. *Biophysical Journal, 107*(11), 2538-45. doi:10.1016/j.bpj.2014.10.036

20. Alibayov, B., Baba-Moussa, L., Sina, H., Zdeňková, K., & Demnerová, K. (2014). *Staphylococcus aureus* mobile genetic elements. *Molecular Biology Reports : An International Journal on Molecular and Cellular Biology,41*(8), 5005-5018. doi:10.1007/s11033-014-3367-3

21. Otto, A., Van Dijl, J., Hecker, M., & Becher, D. (2014). The *Staphylococcus aureus* proteome. *International Journal of Medical Microbiology, 304*(2), 110-120

22. Christoph, K. (2009). *Staphylococcus aureus* bacteremia: Epidemiology, pathophysiology, and management strategies. *Clinical Infectious Diseases, 48*(4), 237. doi:10.1086/598189

23. Weidenmaier, C., Kokai-Kun, J., Kulauzovic, E., Kohler, T., Thumm, G., Stoll, H., . . . Peschel, A. (2008). Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *International Journal of Medical Microbiology : Ijmm, 298*(5-6), 505-13. doi:10.1016/j.ijmm.2007.11.006

24. Amagai, M., Matsuyoshi, N., Wang, Z., Andl, C., & Stanley, J. (2000). Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. *Nature Medicine, 6*, 1275-1277.

25. Dinges, M., Orwin, P., & Schlievert, P. (2000). Exotoxins of *Staphylococcus aureus*. *Clinical Microbiology Reviews, 13*(1), 16-34.

26. Ulrich, R. (2000). Evolving superantigens of *staphylococcus aureus*. *Fems Immunology & Medical Microbiology, 27*(1), 1-7. doi:10.1111/j.1574-695X.2000.tb01404.x

27. Haas, P., De, H., Kleibeuker, W., Poppelier, M., Van, K., Kruijtzer, J., . . . Van, S. (2004). N-terminal Residues of the Chemotaxis Inhibitory Protein of *Staphylococcus aureus* are Essential for Blocking Formylated Peptide Receptor but not c5a Receptor. *Journal of Immunology (baltimore, Md. : 1950), 173*(9), 5704-11.

28. Christoph K. Naber; *Staphylococcus aureus* Bacteremia: Epidemiology, Pathophysiology, and Management Strategies, *Clinical Infectious Diseases*, Volume 48, Issue Supplement_4, 15 May 2009, Pages S231–S237, https://doi-org.echo.louisville.edu/10.1086/598189

29. Yoong, P., & Torres, V. (2013). The effects of *Staphylococcus aureus* leukotoxins on the host: Cell lysis and beyond. *Current Opinion in Microbiology, 16*(1), 63-9. doi:10.1016/j.mib.2013.01.012

30. Allison, D., & Gilbert, P. (1992). Bacterial biofilms. *Science Progress, 76*(3), 305-305.

31. Naicker, P., Karayem, K., Hoek, K., Harvey, J., & Wasserman, E. (2016). Biofilm formation in invasive *Staphylococcus aureus* isolates is associated with the clonal lineage. *Microbial Pathogenesis, 90*, 41-49. doi:10.1016/j.micpath.2015.10.023

32. Wong, J., Ranganathan, S., & Hart, E. (2013). *Staphylococcus aureus* in early cystic fibrosis lung disease. *Pediatric Pulmonology, 48*(12), 1151-1159. doi:10.1002/ppul.22863

33. Goerke, C., & Wolz, C. (2010). Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung. *International Journal of Medical Microbiology : Ijmm, 300*(8), 520-5. doi:10.1016/j.ijmm.2010.08.003

34. Denton, M., & Kerr, K. G. (1998). Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clinical microbiology reviews, 11*(1), 57-80.

35. Coenye, T., Vanlaere, E., Falsen, E., & Vandamme, P. (2004). *Stenotrophomonas africana* Drancourt et al. 1997 is a later synonym of *Stenotrophomonas maltophilia* (Hugh 1981) Palleroni and Bradbury 1993. International Journal of Systematic and Evolutionary Microbiology, *54*(4), 1235-1237.

36. Safdar, A., & Rolston, K. V. (2007). *Stenotrophomonas maltophilia*: Changing Spectrum of a Serious Bacterial Pathogen in Patients with Cancer. *Clinical Infectious Diseases*, *45*(12), 1602-1609.

37. Hoefel, D., Monis, P. T., Grooby, W. L., Andrews, S., & Saint, C. P. (2005). Profiling Bacterial Survival through a Water Treatment Process and Subsequent Distribution System. *Journal of applied microbiology*, *99*(1), 175-186.

38. Hauben, L., Vauterin, L., Moore, E. R. B., Hoste, B., & Swings, J. (1999). Genomic Diversity of the Genus *Stenotrophomonas*. *International Journal of Systematic and Evolutionary Microbiology*, *49*(4), 1749-1760.

39. de Oliveira-Garcia, D., Dall'Agnol, M., Rosales, M., Azzuz, A. C., Martinez, M. B., & Girón, J. A. (2002). Characterization of Flagella Produced by Clinical Strains of *Stenotrophomonas maltophilia*. *Emerging infectious diseases*, *8*(9), 918.

40. Jucker, B. A., Harms, H., & Zehnder, A. J. (1996). Adhesion of the Positively Charged Bacterium *Stenotrophomonas (Xanthomonas) maltophilia* 70401 to glass and Teflon. *Journal of bacteriology*, *178*(18), 5472-5479.

41. de Oliveira-Garcia, Doroti, et al. "Fimbriae and adherence of *Stenotrophomonas maltophilia* to Epithelial Cells and to Abiotic Aurfaces." *Cellular microbiology* 5.9 (2003): 625-636.

42. Martínez, J. L., & Baquero, F. (2002). Interactions Among Strategies Associated with Bacterial Infection: Pathogenicity, Epidemicity, and Antibiotic Resistance. *Clinical microbiology reviews*, *15*(4), 647-679.

43. Zhang, L., Li, X. Z., & Poole, K. (2000). Multiple Antibiotic Resistance in *Stenotrophomonas maltophilia*: Involvement of a Multidrug Efflux System. *Antimicrobial Agents and Chemotherapy*, *44*(2), 287-293.

44. Nikaido, H. (1996). Multidrug Efflux Pumps of Gram-Negative Bacteria. *Journal of Bacteriology*, *178*(20), 5853-5859. doi:10.1128/jb.178.20.5853-5859.1996

45. Sader, H. S., & Jones, R. N. (2005). Antimicrobial Susceptibility of Uncommonly Isolated non-enteric Gram-negative Bacilli. *International journal of antimicrobial agents*, *25*(2), 95-109.

46. Looney, W., Narita, M., & Mühlemann, K. (2009). *Stenotrophomonas maltophilia*: An Emerging Opportunist Human Pathogen. *The Lancet. Infectious Diseases*, *9*(5), 312-23. doi:10.1016/S1473-3099(09)70083-0

47. Waters, V. J., Gómez, M. I., Soong, G., Amin, S., Ernst, R. K., & Prince, A. (2007). Immunostimulatory Properties of the Emerging Pathogen *Stenotrophomonas maltophilia*. *Infection and immunity*, *75*(4), 1698-1703.

48. Peyman, A., Henry, M., Massimiliano, C., Christin, Z., María, B., José, L., & Gabriele, B. (2013). The dsf Quorum Sensing System Controls the Positive Influence of *Stenotrophomonas maltophilia on plants*. Plos One, 8(7). doi:10.1371/journal.pone.0067103

49. De, R., García, C., Alcaraz, E., & Franco, M. (2014). *Stenotrophomonas maltophilia* Interferes via the dsf-mediated Quorum Sensing System with *Candida albicans* Filamentation and its Planktonic and Biofilm Modes of Growth. *Revista Argentina De Microbiologia*, *46*(4), 288-97. doi:10.1016/S0325-7541(14)70084-7

50. Tango, C., Akkermans, S., Hussain, M., Khan, I., Van, I., Jin, Y., & Oh, D. (2018). Modeling the effect of ph, Water Activity, and Ethanol Concentration on Biofilm Formation of *Staphylococcus aureus*. *Food Microbiology, 76*, 287-295. doi:10.1016/j.fm.2018.06.006

51. Calvopiña, K., & Avison, M. (2018). Disruption of MPL Activates β-lactamase Production in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* clinical isolates. *Antimicrobial Agents and Chemotherapy, 62*(8). doi:10.1128/AAC.00638-18

52. Filkins, L., Graber, J., Olson, D., Dolben, E., Lynd, L., Bhuju, S., & O'Toole, G. (2015). Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *Journal of Bacteriology, 197*(14), 2252-2264. doi:10.1128/JB.00059-15

53. Hotterbeekx, A., Kumar-Singh, S., Goossens, H., & Malhotra-Kumar, S. (2017). *In vivo* and *In vitro* Interactions Between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Frontiers in cellular and infection microbiology, 7*, 106. doi:10.3389/fcimb.2017.00106

54. Qazi, S., Middleton, B., Muharram, S., Cockayne, A., Hill, P., O'Shea, P., Williams, P. (2006). N-acylhomoserine Lactones Antagonize Virulence Gene Expression and Quorum Sensing in *Staphylococcus aureus*. *Infection and Immunity, 74*(2), 910-919. doi:10.1128/IAI.74.2.910-919.2006

55. Vfselova, M., Kholmeckaya, M., Klein, S., Voronina, E., Lipasova, V., Metlitskaya, A., . . . Chernin, L. (2003). Production of *n*-acylhomoserine Lactone Signal Molecules by Gram-Negative Soil-Borne and Plant-associated Bacteria. *Folia Microbiologica : Official Journal of the Institute of Microbiology, Academy of Sciences of the Czech Republic,48*(6), 794-798. doi:10.1007/BF02931516

56. Roscetto, E., Vitiello, L., Muoio, R., Soriano, A. A., Iula, V. D., Vollaro, A., De Gregorio, E. Catania, M. R. (2015). In vitro Interaction of *Stenotrophomonas maltophilia* with Human Monocyte-Derived Dendritic Cells. *Frontiers in microbiology, 6*, 723. doi:10.3389/fmicb.2015.00723

57. Mesureur, J., Feliciano, J., Wagner, N., Gomes, M., Zhang, L., Blanco-Gonzalez, M., . . . Vergunst, A. (2017). Macrophages, but not neutrophils, are critical for proliferation of *Burkholderia cenocepacia* and ensuing host-damaging inflammation. *Plos Pathogens, 13*(6), 1006437. doi:10.1371/journal.ppat.1006437

58. Mootz, J., Malone, C., Shaw, L., & Horswill, A. (2013). Staphopains modulate *Staphylococcus aureus* biofilm integrity. *Infection and Immunity, 81*(9), 3227-38. doi:10.1128/IAI.00377-1