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Deciphering the Mechanisms of Alcaligenes faecalis' Inhibition of Staphylococcus aureus and Synergism with Antibiotics

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Deciphering the Mechanisms of *Alcaligenes faecalis'* Inhibition of *Staphylococcus aureus* and Synergism with Antibiotics

by

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ABSTRACT

Staphylococcus aureus has developed resistance to several antibiotics including vancomycin, which is often used as a "last resort" treatment. There is an ever-increasing need to develop novel antimicrobial treatments to combat *S. aureus* and other drug resistant bacteria. Microorganisms are most often found in polymicrobial communities where they either exhibit synergistic or antagonistic relationships. Competition between microorganisms can lead to the discovery of new antimicrobial targets as the specific mechanisms of resistance are elucidated. In addition, synergistic treatments are being evaluated for their combined effect and potential to decrease the concentration of drugs needed, and thus the side effects also. *Alcaligenes faecalis* is a microorganism that our lab has previously shown to inhibit *S. aureus* and other various bacterial species. In this study, we found that *A. faecalis* reduces the planktonic growth of *S. aureus* by 94.5% and biofilm growth by 76.6%. A*. faecalis* also has a synergistic effect when paired with bacitracin to reduce the planktonic growth by 99.9% and biofilm growth by 99.7%. Transposon mutagenesis was successfully performed on *A. faecalis*, and loss of function mutations were attained. Two mutants were no longer able to inhibit the growth of *Staphylococcus aureus*, *Candida albicans*, or *Bacillus megaterium*. Further analysis and genomic sequencing of these mutants is needed to determine the gene(s) that were interrupted and the mechanism of *A. faecalis*' antimicrobial activity. The findings of this study may aid in the identification of new therapeutic targets for novel *S. aureus* treatments.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Sean Fox for introducing me to research and allowing me to work on this project. His guidance and mentorship have been remarkable and allowed me to learn and grow in many ways. I am also grateful for his endurance through the COVID-19 pandemic and desire to safely allow students to continue research. Our research has greatly benefitted from his persistence, encouragement, and patience. I would also like to Dr. Erik Petersen for his support and microbiological expertise. Finally, I thank Dr. Karen Kornweibel and the Honors College for providing support and resources to complete this research.

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INTRODUCTION

Staphylococcus aureus Infections

Staphylococcus aureus is a gram-positive bacterium that often acts as a commensal organism, inhabiting parts of the human body including the skin and mucous membranes of the nose and gut (Lakhundi and Zhang 2018). The anterior nares of 20-25% of the human population is persistently colonized with *S. aureus*, while 30% is intermittently colonized and 50% has never been colonized (Lister and Horswill 2014; Archer et al. 2011; Lakhundi and Zhang 2018). Persons colonized with *S. aureus* are more likely to become infected with the bacteria (Lakhundi and Zhang 2018). *S. aureus* is a leading cause of both nosocomial and community-acquired infections, causing 119,247 bloodstream infections and 19,832 deaths in 2017 (Kourtis et al. 2019). It is often the causative agent in bacteremia, endocarditis, osteomyelitis, hospital-acquired pneumonia, and infections of surgical sites, skin, medical devices, and implants (Parastan 2020).

Development of Antibiotic Resistance

The fight against *S. aureus* has proven tedious as it developed resistance to penicillin a mere two years after its discovery and resistance to methicillin just a few years after its appearance. Methicillin-resistant *S. aureus* (MRSA) was identified in 1960 and with it came higher mortality and morbidity rates and increases in hospital stays and health care costs. MRSA strains are the result of an acquired gene, *mecA*, which encodes an altered penicillin-binding protein that has a decreased affinity for most penicillins, rendering them ineffective. This resistance is easily transferred to susceptible strains via transfer of *mecA* by a mobile genetic element. MRSA is now accountable for 25-50% of *S. aureus* infections found in a

hospital (Lakhundi and Zhang 2018). However, MRSA is not confined to a hospital setting, and community-acquired infections are on the rise (Lakhundi and Zhang 2018; Kourtis et al. 2019). Hospital-acquired MRSA infections may be caused or promoted by invasive procedures, indwelling devices, skin lesions or wounds, antimicrobial therapy, or preexisting conditions, and the immunocompromised are most at risk. Community-acquired MRSA is most often present in skin or soft tissue infections, and even the young and healthy are susceptible to its invasion (Lakhundi and Zhang 2018). The opioid crisis is thought to be partially responsible for the increase in community-acquired *S. aureus* infections, and persons who inject drugs are at a 16-fold risk for invasive MRSA infection (Kourtis et al. 2019).

While infection control efforts are decreasing the prevalence of nosocomial MRSA infections, antibiotic resistance remains a pandemic that requires action (Kourtis et al. 2019). The natural ability of bacteria to acquire resistance genes is being accelerated by an abundant misuse of antimicrobial agents. Over-prescribing antibiotics, a lack of knowledge about antibiotic resistance, and patient noncompliance to drug use regimens contribute to the increasing level of antibiotic-resistance (Aminov 2010). In addition, antibiotic use in agriculture has led to a prevalence of MRSA among livestock and a greater risk of MRSA colonization or infection for humans that handle livestock. A recent survey found that 39.2% of retail meat samples were contaminated with *S. aureus*, which could be transmitted to humans by environmental contamination, direct contact, or the handling of the products of an infected animal (Lakhundi and Zhang 2018).

Vancomycin is an antibiotic that has often been used as a last resort drug in MRSA infections. However, in 2002, a vancomycin-resistant *S. aureus* strain was isolated in the U.S.

(Parastan et al. 2020). Because vancomycin is slowly bactericidal and *S. aureus* strains are increasingly losing their susceptibility to it, other drugs are being used to treat drug-resistant gram-positive pathogens. These include daptomycin, telavancin, ceftaroline, and linezolid. There are pros and cons to each drug, and they are reserved for the most resistant and dangerous infections because of their toxicity and potential for resistance (Choo and Chambers 2016).

Biofilm Formation and Mechanisms

Another factor that increases the resistance of *S. aureus* is its ability to form biofilms. Biofilms are involved in 80% of microbial infections and can increase a bacterium's resistance to antibiotics by up to 1000-fold more than if it grew planktonically (Mohammad et al. 2015). A biofilm is a sessile community with attachment to a common base and/or each other with an extracellular matrix and an altered phenotype. *S. aureus* biofilms are most often found on orthopedic implants like plates, screws, and prosthetic joints; in addition, they can be found on indwelling medical devices including ventilators, heart valves, pacemakers, stents, catheters, and implants (Archer et al. 2011). The formation of a biofilm is initiated by reversible attachment to a surface. If the bacteria are not challenged by antibiotics or another treatment, they use structures like pili to become irreversibly attached, then multiply and form colonies. Next, an extracellular matrix is developed through secreted polysaccharides, proteins, and lipids (Warraich et al. 2020). The biofilm matures via cell division and matrix formation and is then capable of forming new biofilms by dispersal (Lister and Horswill 2014).

Biofilms have several mechanisms that contribute to their resistance to antibiotics. First, the matrix acts as a barrier and inhibits antibiotic diffusion (Lister and Horswill 2014). Mature

biofilms also exhibit slow growth because of a decreased metabolic rate, which impedes antibiotics that target rapidly diving cells (Warraich et al. 2020). Biofilms can undergo a dispersal phase in which cells or clusters of cells detach and move to new infection sites (Otto 2008). *S. aureus* produces exoenzymes and surfactants that degrade the matrix to allow dispersal (Lister and Horswill 2014). Dispersal is the cause of chronic infections, which may seemingly be cured by antibiotic therapy until the biofilms disperse planktonic cells to maintain a persistent infection (Warraich et al. 2020). In *S. aureus* the biofilm thickness and rate of dispersal are maintained by quorum sensing through its *agr* system (Otto 2008). Antibiotic therapy alone is not effective against biofilms, and often, the infection or indwelling medical device must be surgically removed. Rifampin has been found to be active against biofilms, but it should be paired with vancomycin or another antibiotic that is active against *S. aureus* in order to prevent resistance (Archer et al. 2011). Another possible treatment under investigation is the intentional dispersal of a biofilm paired with antibiotic therapy (Lister and Horswill 2014).

Shift in Antibiotic Research

Although antibiotic resistance is increasingly problematic, the development of new antibiotics has been declining for decades (Moellering 2011). Novel drug discovery takes 10 to 17 years, and only 10% of these drugs are successful (Shang et al. 2019). Pharmaceutical companies are not interested in antimicrobials for several reasons: many antibiotics are still effective against several microorganisms; antibiotic resistance is transferred between microorganisms very rapidly; and the duration of antibiotic treatment is much less than that of treatments for neurological, musculoskeletal, or cardiovascular diseases, so there is less profit to be made. Most of the research on antimicrobials is now conducted by academic centers and

small biotechnology companies. Some current approaches to antimicrobial research include searching ecological niches, using antimicrobial compounds from plants and animals, targeting pathogenic traits or metabolic processes, coupling antibiotics with phage therapy, and targeting resistance mechanisms (Moellering 2011). This thesis investigates the inhibitory behavior of a common soil microbe, *Alcaligenes faecalis*, to determine the mechanism of its inhibitory behavior toward other microbes.

Polymicrobial Interactions

Microorganisms rarely exist in planktonic forms, but rather they are often found in polymicrobial communities (Peters et al. 2012); therefore, it is essential to investigate interspecies relationships. Interactions may be physical, chemical, or genetic and include cell to cell contact, metabolite exchange, and gene transfer. Relationships between microbes may be mutualistic, synergistic, or antagonistic and determine the composition and function of a community (Vinoth et al. 2016). There are two broad categories of competition: passive, also called scramble, and active, or contest (Bauer et al. 2018; Hibbing et al. 2010). In passive competition, a competitor seeks to gain the best access to resources, which may be accomplished by producing siderophores or altering metabolism to uptake resources more efficiently or by producing adhesive molecules in order to colonize in an area of abundant nutrients. Some organisms have been termed “cheaters” because they are able to reduce their expression of costly genes and instead utilize the products of other strains. Active competition involves damaging another cell to eliminate it. This can be the use of a type VI secretion system to inject toxins into other cells or the use of quorum quenching to disrupt signaling (Bauer et al. 2018). Antimicrobials released from various microorganisms are often investigated and utilized

as antibiotic treatments. Microbial interactions are important clinically because co-infecting pathogens are common and more difficult to eliminate. In addition, other microorganisms that make up the human microbiome are essential in preventing and fighting off infection (Vinoth et al. 2016).

Synergistic Treatments

As microbes can be synergistic in their colonization and survival, there are also treatments and antimicrobials that have seen synergistic successes. Combination therapy with antibiotics plus another antimicrobial compound has the potential to improve the efficacy of treatment and prevent microbes from obtaining resistance. In addition, synergism can reduce the dosage of drugs to minimize toxicity and adverse effects. Antimicrobial peptides (AMPs) are known to disrupt the cell membrane, but these have a high toxicity when used alone. However, a study found that AMPs have a synergistic relationship with antibiotics because they allow the antibiotic better access to the cell. When paired with penicillin, ampicillin, erythromycin, or tetracycline, both the AMP and antibiotic had significantly reduced minimum inhibitory concentrations (MIC) against planktonic *Staphylococcus epidermis*. Penicillin combined with the AMP also showed an antibiofilm effect (Shang et al. 2019). In another study, it was found that acidic amino acids paired with ciprofloxacin can disperse and inhibit biofilm formation in *S. aureus* (Warraich et al. 2020). A thiazole compound was also discovered to have a synergistic effect when paired with vancomycin against *S. epidermis* and *S. aureus*. This thiazole compound was also able to resensitize vancomycin-resistant *S. aureus* to vancomycin, while also decreasing the MIC (Mohammad et al. 2015).

Alcaligenes faecalis and Its Antagonistic Activity

Alcaligenes faecalis is an organism that has been found to be both an antibacterial and antifungal agent. It is a gram negative, obligate aerobe that is often found in soil and water (Huang 2020). *A. faecalis* is currently used in sewage treatment as it produces enzymes that degrade organic contaminants. It is also essential to the pharmaceutical industry because it supplies a precursor to several drugs (Ju et al. 2016). Also found in the hospital setting, *A. faecalis* is an opportunistic pathogen that has been involved in bacteremia, endocarditis, pneumonia, and various other infections (Huang 2020). Of special interest, it is being investigated for its antagonistic activity as a potential antimicrobial agent. In previous studies, *A. faecalis* has shown antifungal activity against *Candida albicans*, *Aspergillus niger*, *Fusarium oxysporum*, and *Paecilomyces variotii*. In addition, *A. faecalis* exhibits antibacterial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Mycobacterium avium* and of particular interest to my research, *Staphylococcus aureus* (Zahir et al. 2013). As far as I know, the mechanisms of its antagonistic behavior have not been discovered. The goal of this thesis is to investigate the antagonistic behavior of *A. faecalis* against *S. aureus* as well as its synergy with antibiotics against both planktonic growth and biofilm development.

Polymicrobial Infections of *Candida albicans* and *Staphylococcus aureus*

Candida albicans is an opportunistic fungus that can be found in mucosal and moist surfaces on the skin. It is successful as a pathogen largely because of its ability to transition between its yeast and hyphal forms (Schlecht et al. 2015). Twenty percent of *C. albicans* bloodstream infections are polymicrobial with *S. aureus* being the third most common coinfecting microorganism (Carolus et al. 2019). Polymicrobial infections typically have an

increased resistance to antibiotics and other treatments. While these infections are common, research to elucidate their mechanisms and possible treatments is not (Schlecht et al. 2015). A study found that peritoneal infection with either *S. aureus* or *C. albicans* is nonlethal; however, there was a 40 percent mortality rate when the same doses were used for a coinfection (Peters and Noverr 2013). In addition, *S. aureus* exhibits increased tolerance of vancomycin when *C. albicans* is present (Carolus et al. 2019). One study proposes that the coinfection is facilitated by what they term "microbial hitchhiking." *S. aureus* shows an affinity for the hyphal morphology of *C. albicans* with the main adhesin target being Als3p (Schlecht et al. 2015). It has also been found that *C. albicans* enhances the ability of *S. aureus* to form biofilms. *C. albicans* serves as the scaffolding and *S. aureus* gets coated in its matrix to form a stable and extremely resistant biofilm (Harriott and Noverr 2009). Many treatments only target a specific kingdom, so polymicrobial infections are extremely difficult to combat. *A. faecalis* has shown antagonistic activity towards both *S. aureus* and *C. albicans*, so it can be investigated for its effect on a co-culture of these two organisms.

METHODS

Growth Conditions

All bacterial strains were grown from freshly isolated cultures in Luria-Bertani (LB) broth at 37°C on a shaker at 250 rpm, unless otherwise noted. Plated cultures were also grown at 37°C and the media used will be noted in the specific protocols below. The specific strains used were *Staphylococcus aureus* ATCC 25923, *Alcaligenes faecalis* ATCC 8750, *Candida albicans* SC5314, and *Bacillus megaterium* ATCC 14581. A Kirby Bauer test was performed to find an antibiotic that would inhibit the growth of *S. aureus* but not *A. faecalis*. Bacitracin was chosen,

and the concentration used was 25 µg/ml to allow some growth of *S. aureus*, unless otherwise noted.

S. aureus, *A. faecalis*, and Bacitracin Co-cultures and Synergism

Overnight liquid cultures of *S. aureus* and *A. faecalis* were grown in 5 ml of LB broth. A spectrophotometer set to 600 nm (OD_{600}) gave the absorbance of the overnight cultures to allow preparation of a 10:1 ratio of *A. faecalis* to *S. aureus* co-culture. Four 5 mL LB tubes were inoculated with the same amount of *S. aureus*. Into the second tube, the appropriate amount of *A. faecalis* was added to give the 10:1 ratio. Into the third tube, Bacitracin was added. In the fourth tube, both *A. faecalis* and Bacitracin were added. These cultures were incubated for 24 hours. Serial dilution was performed using phosphate-buffered saline (PBS) for all four tubes and 100 µl of each dilution was pipetted onto mannitol salt agar (MSA) plates and spread around the plate using the spread plate technique. MSA was used to select for the growth of *S. aureus*, since *A. faecalis* cannot grow at the high salt concentration. After 24 hours incubation, plate counts of *S. aureus* were obtained.

Biofilm Interactions: *S. aureus*, *A. faecalis*, and Bacitracin

Overnight broth cultures of *S. aureus* and *A. faecalis* were grown in 5 ml of LB broth. *S. aureus* was inoculated into 4 wells of a 6-well plate with 2 mL of LB. Into the second well, the appropriate amount of *A. faecalis* was added to give the 10:1 ratio. Into the third well, Bacitracin was added. In the fourth well, both *A. faecalis* and Bacitracin were added. The concentration of Bacitracin used was 62.5 µg/ml. The well plate was incubated for 48 hours to allow biofilm growth. After incubation, the remaining liquid was poured out, and 1 mL of PBS was used to rinse each well and was discarded. Upon addition of an additional 1 mL of PBS, the

edges of the wells were scraped and stirred to move the biofilm into the solution. Serial dilution was performed for each well in the same manner as before and the solutions were plated onto MSA plates. After 24-hour incubation, viable plate counts were obtained. This procedure was repeated and cover slips were added to each well before the 48-hour incubation. The cover slips were sterilely transferred to microscope slides and allowed to dry. They were then Gram-stained and viewed under a microscope at 100x magnification, and photos were obtained.

S. aureus and *A. faecalis* Interactions

S. aureus and *A. faecalis* were inoculated into separate 5 mL LB tubes and incubated for 24 hours. 25 µL of the *S. aureus* culture was added into each of 2 fresh 5 mL LB tubes. 50 µL of *A. faecalis* broth was added into one of these tubes and another 50 µL into a third tube. After 24-hour incubation, the specimens were mounted onto microscope slides, Gram-stained, and viewed under 100x magnification. Pictures were taken.

Transposon Mutagenesis

Electrocompetent *A. faecalis* cells (Sharma and Schimke, 2018) were combined with EZ-Tn5 (Epicentre) transposon and electroporated at 2500 kV. 1000 µL of SOC media was added, and the cells were allowed to recover for 60 minutes at 37°C on a shaker set to 250 rpm. Aliquots were plated onto LB + Kanamycin (100 µg/mL) plates. The resulting mutants were patch plated onto LB plates with an *S. aureus* lawn and incubated for 24 hours. The mutants were observed for a loss of function compared to wild-type *A. faecalis*. Two mutants with loss of function were also plated onto lawns of *Candida albicans* and *Bacillus megaterium* to investigate if the mutants were no longer able to inhibit these species.

Genomic Extraction & Identification of Transposon Insertion

Genomic extraction of the *A. faecalis* mutants was accomplished using a Promega Wizard Genomic DNA Purification Kit. The concentration and purity of the extracted DNA was obtained via a Thermo Scientific nanodrop and stored at -20°C. A unique PCR method named "Rapid Amplification of Transposon Ends (RATE)" was utilized to amplify the region flanking the transposon for sequencing (Anriany et al. 2006). This involved three continuous rounds of PCR with the following cycles: *PCR1*: 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, 72^{o}C for 3 minutes (repeated for 30 cycles); *PCR2*: 95^{o}C for 30 seconds, 30^{o}C for 30 seconds, 72^{o}C for 2 minutes (repeated for 30 cycles); *PCR3*: 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, 72^{o}C for 2 minutes (repeated for 30 cycles). PCR fragments were purified and sequenced by Quillen College of Medicine's Molecular Core facility using primers directed towards the transposon.

C. albicans, *S. aureus*, and *A. faecalis* Interactions

C. albicans, *S. aureus*, and *A. faecalis* were inoculated into three separate 5 mL LB tubes and incubated for 24 hours. 20 µL of all three cultures were added into a 5 mL Brain Heart Infusion (BHI) tube. Into a second BHI tube, 20 µL each of *C. albicans* and *A. faecalis* were added. Into a third BHI tube, 20 µL each of *C. albicans* and *S. aureus* were added. After 24-hour incubation, the co-cultures were mounted onto microscope slides, Gram-stained, and viewed under 100x magnification. Pictures were taken. Serial dilution was performed for all three tubes and the solutions were plated onto different agars to allow the growth of only one organism: MSA for the growth of *S. aureus*, LBK plates (LB with 50µg/ml Kanamycin) for *C. albicans*, and LB agar with Bacitracin and Amphotericin B added for *A. faecalis*. 100 µL from the first tube was plated onto all three media. 100 µL from the second tube was plated onto LBK and

Bacitracin/Amphotericin B media, and 100 µL from the third tube was plated onto MSA and LBK media. Plate counts were obtained after 24-hour incubation.

RESULTS AND DISCUSSION

Alcaligenes faecalis' Synergism with Bacitracin

Results from the co-cultures show that both *A. faecalis* and bacitracin inhibit the planktonic growth of *S. aureus*, and when these are paired together, they reduce the growth of *S. aureus* by more than 99%. As seen in **Figure 1**, a 10:1 ratio of *A. faecalis* to *S. aureus* reduced the growth of *S. aureus* by 94.5%, while a 125 µg/ml concentration of bacitracin reduced the growth of *S. aureus* by 91.3%. *A. faecalis* appears to have a similar inhibition on the growth of *S. aureus* as bacitracin at the ratio and concentration used; however, when these two were combined, a synergistic effect was observed. This could potentially decrease the concentration of antibiotics needed to treat an infection when paired with the antagonistic actions of *A. faecalis*.

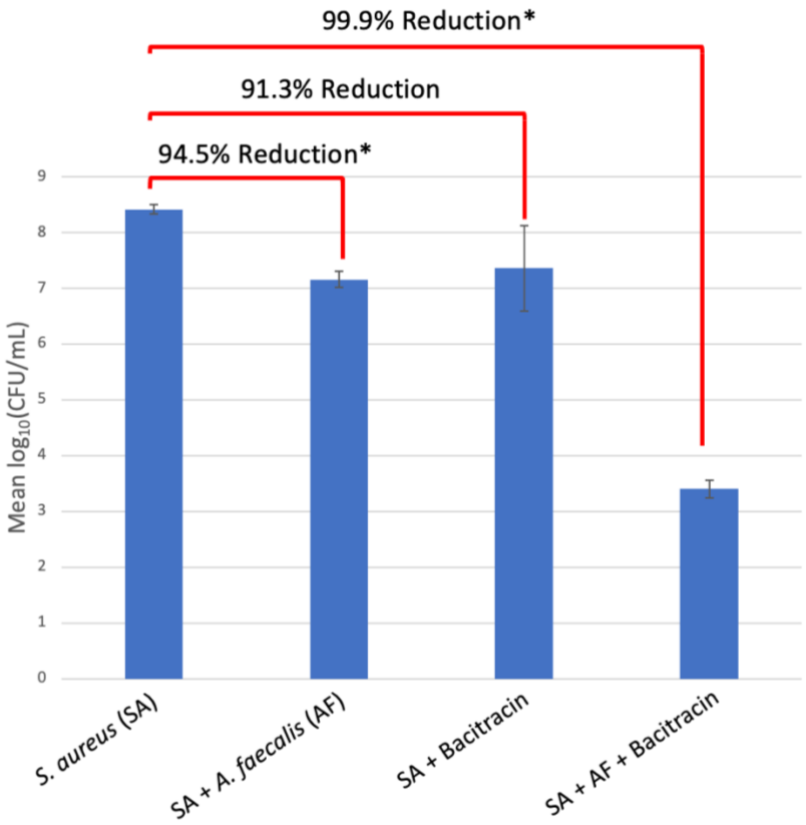

Figure 1: Viable *S. aureus* colonies post 24-hour incubation at 37°C. Co-cultures included *S. aureus* + *A. faecalis*, *S. aureus* + bacitracin, and *S. aureus* + *A. faecalis* and bacitracin, all compared to the control culture of *S. aureus*. Mean $\log_{10}$(CFU/mL) was calculated from three separate trials. Student T-tests were used to determine statistical significance ($p < 0.005$ is denoted by *).

Biofilm Interactions

As *A. faecalis* was a potential inhibitor of *S. aureus* planktonic growth, we turned our attention to *A. faecalis'* inhibition of the attachment phase of *S. aureus* biofilm formation, and similar results were obtained. This time bacitracin had a stronger inhibitory effect than *A. faecalis* when acting alone, but when paired, a synergistic effect was again observed. As shown in **Figure 2**, *A. faecalis* reduced biofilm formation by 76.6%, while bacitracin gave an 81.4% reduction. Together, these two gave a 99.7% reduction. By comparing **Figure 1** and **Figure 2**, it is concluded that while *A. faecalis* and bacitracin have an inhibitory effect on both planktonic

and biofilm growth of *S. aureus*, their effect is greater on planktonic cells. The results from each trial deviated greatly, so further investigation is needed for statistically relevant results. Visualization at 100x magnification under a microscope revealed that *A. faecalis* greatly inhibits the growth of *S. aureus* biofilms and is able to outcompete *S. aureus* even though it has a much slower growth rate. A low dosage of bacitracin was used, and the images obtained do not show a decrease in biofilm growth when grown with bacitracin; however, there appears to be a change in morphology. *A. faecalis* is better than bacitracin at slowing the biofilm growth of *S. aureus*, and it appears to use a contact-dependent mechanism as it is grouped around the *S. aureus* cells. The co-culture of *S. aureus*, *A. faecalis*, and bacitracin still shows a reduction in the biofilm growth of *S. aureus*. (**Figure 3**).

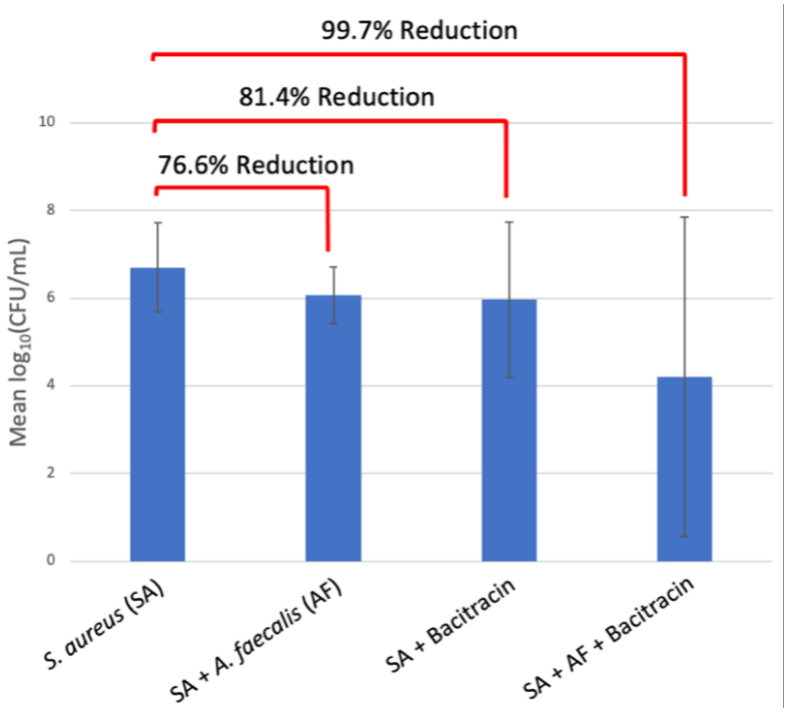

Figure 2: Viable *S. aureus* colonies from biofilms post 24-hour incubation at 37°C. Co-cultures included *S. aureus* + *A. faecalis*, *S. aureus* + bacitracin, and *S. aureus* + *A. faecalis* and bacitracin, all compared to the control culture of *S. aureus*. Biofilms were grown for 48 hours at 37°C. Mean $\log_{10}$(CFU/mL) was calculated from three separate trials. P-values were > 0.05, so the results are not statistically significant.

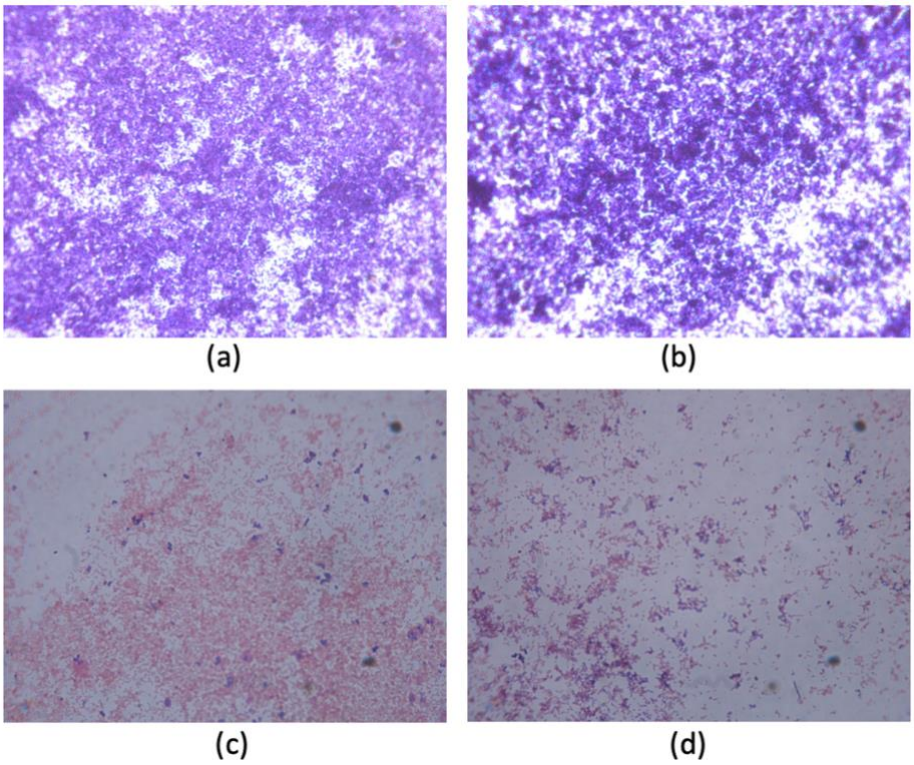

Figure 3: Gram stains of biofilms grown for 48 hours and viewed at 100x: (a) *S. aureus*, (b) *S. aureus* and bacitracin with concentration of 62.5 µg/mL, (c) *S. aureus* and *A. faecalis* (d) *S. aureus*, *A. faecalis*, and bacitracin

S. aureus and *A. faecalis* Interactions

When *S. aureus* and *A. faecalis* are grown together, it is apparent that *A. faecalis* can outcompete *S. aureus* as there is a reduction in growth of *S. aureus* when compared to the control. Under 100x magnification, the presence of longer chains of *A. faecalis* results in less *S. aureus* cells and clusters. In (d) there is a greater amount of *S. aureus* cells and the *A. faecalis* rods appear to be shorter than those in (c). This may suggest that *A. faecalis* experiences a change in morphology when grown with larger amounts of *S. aureus*. Many of the *S. aureus* cells are in contact with the *A. faecalis* rods and are more prevalent near shorter rods (**Figure 4**). This may support the previous findings that *A. faecalis* inhibits other microorganisms via a contact-dependent mechanism (Fuqua n.d.)

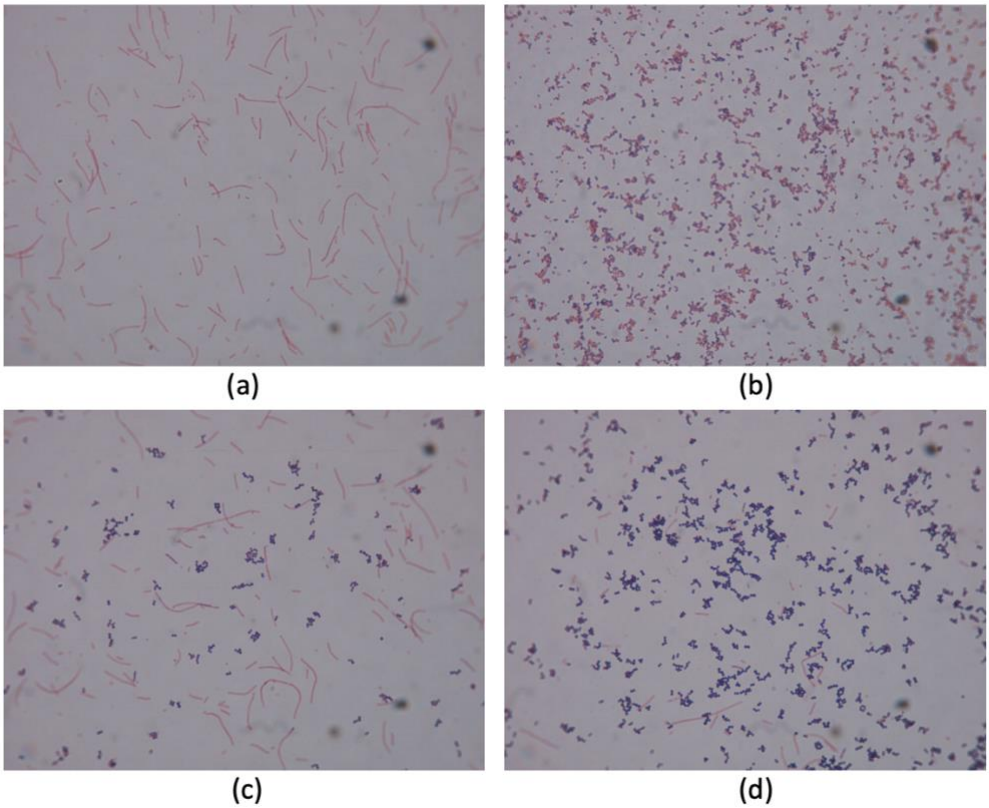

Figure 4: Gram-stains of cultures grown for 24 hours and viewed under 100x: (a) *A. faecalis*, (b) *S. aureus*, (c) *A. faecalis* and *S. aureus* showing longer rods, (d) *A. faecalis* and *S. aureus* showing more *S. aureus* clusters and shorter rods.

A. faecalis Transposon Mutants Obtained via Electroporation

Transposon mutagenesis was successfully achieved through electroporation. From the *A. faecalis* mutants scanned, two were observed to have a loss of function mutation. These no longer showed a zone of inhibition on an *S. aureus* lawn. In addition, these mutants were not able to inhibit the growth of two other species studied in our lab, *Candida albicans* and *Bacillus megaterium* (**Figure 5**).

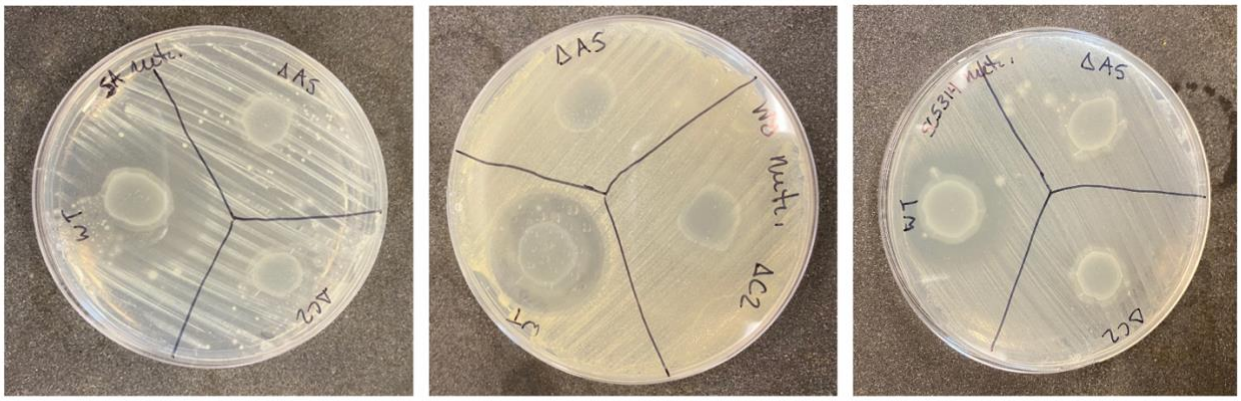

Figure 5: Zones of inhibition or lack thereof for mutant ΔA5, mutant ΔC2, and wild-type (WT) *A. faecalis* on lawns of (a) *Staphylococcus aureus* (b) *Bacillus megaterium* and (c) *Candida albicans*.

The gene(s) that were interrupted by the transposon most likely have a function in *Alcaligenes faecalis'* mechanism of inhibition of the growth of competing microorganisms. The unique RATE method of PCR amplification of the transposon was then used to generate numerous fragments flanking the transposon site. This method uses three rounds of PCR and lower annealing temperature to randomly amplify the transposon ends **(Figure 6).** Attempts were made to sequence the DNA flanking the transposon insertion of these *Alcaligenes* mutants, but were unsuccessful. The DNA sequence that was obtained matched the transposon, so we know that the primers successfully found the transposon (**Figure 7**). However, the sequence did not match the genome of *A. faecalis*, so something caused a misidentification of nucleotides in the sequence. The contents of the final clean-up reagent were not known and may have contributed to sequencing errors.

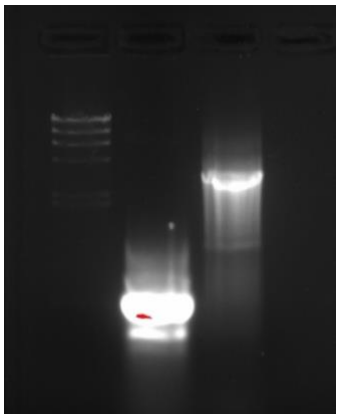

Figure 6: PCR amplification of transposon insertion of mutants for sequencing. Using the RATE method of PCR amplification, transposon sites from the constructed mutant *Alcaligenes* were amplified (Lane 1: Lambda/Hind III DNA ladder, Lane 2: RATE amplification of transposon using INV2 primer, Lane 3: RATE amplification of transposon using INV1 primer).

Sequence ID: Query_9745 Length: 1221 Number of Matches: 1

Range 1: 1 to 103 Graphics ▼ Next Match ▲ P

Score	Expect	Identities	Gaps	Strand
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            ||||| |||| ||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  103  AGTTTTATTGTTCATGATGATATATTTTT-ATCTTGTGCAATGTAACATCAGAGATTTTG  45

Query  60   AGACACAATTCATCGATGATGGTTGAGATGTGTATAAGAGGACAG  104
            |||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  44   AGACACAATTCATCGATGATGGTTGAGATGTGTATAAGAG-ACAG  1
```

Figure 7: Alignment of EZtn5 transposon and *A. faecalis* mutant sequence obtained using INV1 primer.

Polymicrobial Interactions of *C. albicans*, *S. aureus*, and *A. faecalis*

The proposed "microbial hitchhiking" is observed when *C. albicans* and *S. aureus* are grown together. *S. aureus* is clustered around and covering *C. albicans*. *A. faecalis* is still effective against both *S. aureus* and *C. albicans*, as there are fewer cells in the presence of *A. faecalis*. In (b) and (c) *C. albicans* is touching *A. faecalis*, indicating a contact dependent mechanism, and it appears to be in a pseudo-hyphal morphology **(Figure 8**). *A. faecalis* may affect the ability of *C. albicans* to transition to its hyphal form. The plate counts also affirm that

A. faecalis remains antagonistic to *S. aureus* even when *C. albicans* is also present. There is a 99% decrease in the growth of *S. aureus* in a co-culture with *C. albicans* when *A. faecalis* is also grown in the culture. There is little difference between the different cultures showing the growth of *A. faecalis* and *C. albicans* (**Figure 9**). More controls are needed to compare how their growth is affected in the presence of other organisms.

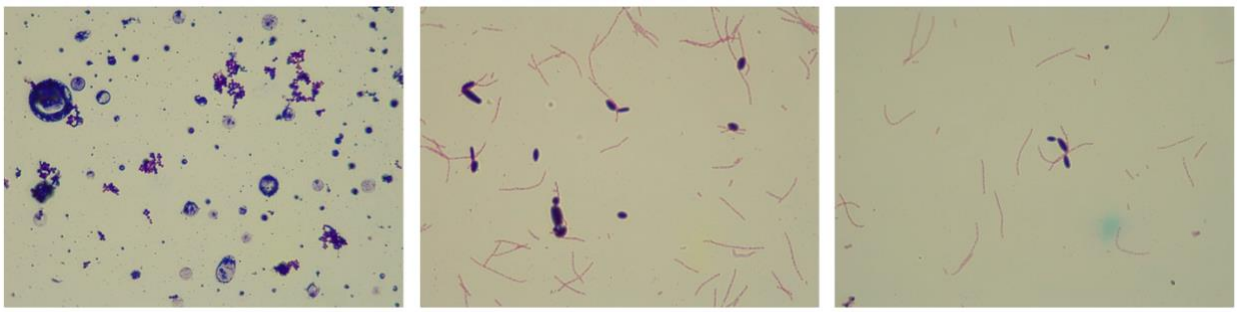

Figure 8: Gram-stains of cultures grown for 24 hours and viewed under 100x: (a) *Candida albicans* and *S. aureus* (b) *C. albicans* and *A. faecalis* (c) *C. albicans*, *S. aureus*, and *A. faecalis.*

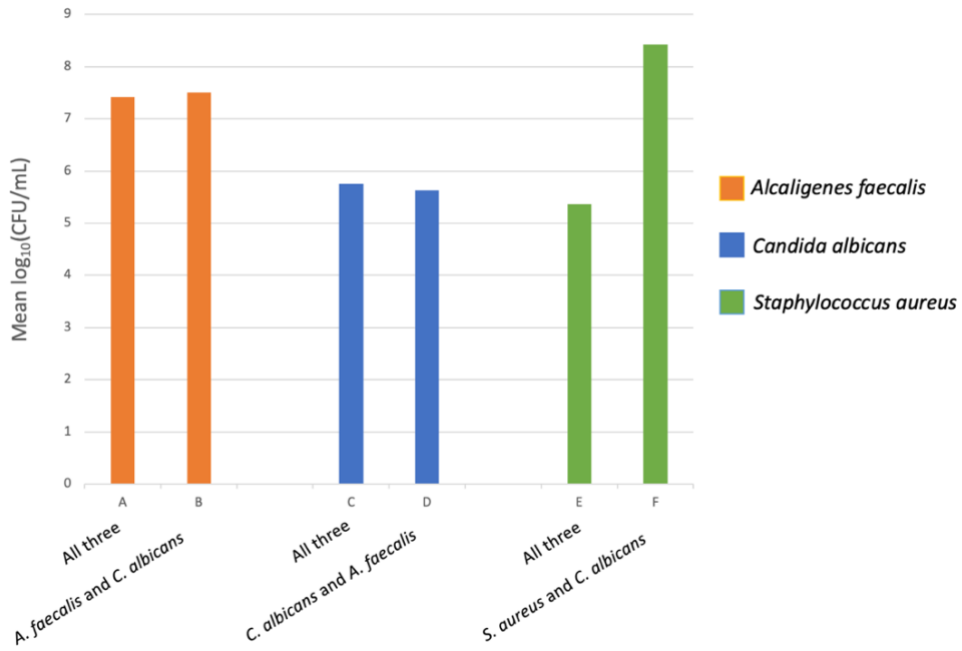

Figure 9: Viable colonies post 24-hour incubation of (A) *A. faecalis* in a co-culture with *S. aureus* and *C. albicans* (B) *A. faecalis* in a co-culture with *C. albicans* (C) *C. albicans* in a co-culture with *S. aureus* and *A. faecalis* (D) *C. albicans* in a co-culture with *A. faecalis* (E) *S. aureus* in a co-culture with *A. faecalis* and *C. albicans* and (F) *S. aureus* in a co-culture with *C. albicans*. Only one trial was completed and further study is needed.

CONCLUSION

The findings of this thesis may lead to a novel antimicrobial treatment for drug-resistant *Staphylococcus aureus*. *Alcaligenes faecalis* was shown to inhibit *S. aureus* in both planktonic and biofilm growth. In addition, a synergistic relationship was observed between *A. faecalis* and bacitracin, which indicates that the bactericidal factor of *A. faecalis* can be paired with a low dose of antibiotics to fight a Staphylococcal infection. Loss of function *A. faecalis* mutants were obtained, amplified, and sequenced. Different purification methods should be used to yield more accurate sequencing. A mutant library is being built for future sequencing and analysis. Determining the genes that were interrupted by the transposon may elucidate the mechanism of *A. faecalis'* inhibition of other microorganisms.

To initiate a future direction, the interactions between *A. faecalis*, *S. aureus*, and *C. albicans* were evaluated. Many infections are polymicrobial, like that of *S. aureus* and *C. albicans*, but multiple microorganisms are rarely studied at the same time. In a preliminary trial, *A. faecalis* was still effective against these other two synergistic organisms and was able to greatly decrease the growth of *S. aureus*. More trials should be completed to determine the significance of the results, and additional controls should be added to evaluate change in growth between co-inoculations and single species inoculations. Further study will determine *A. faecalis'* ability to counteract synergistic relationships and combat polymicrobial infections.

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