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Characterization of the Broad-spectrum Inhibitory Capability of Alcaligenes faecalis and A. viscolactis against Potential Pathogenic Microorganisms

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Alcaligenes faecalis and *A. viscolactis* against Potential Pathogenic Microorganisms

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by

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ABSTRACT

Over the past few decades, the rise of multidrug resistant (MDR) microorganisms has grown from an isolated concern to a massive public health crisis. It has become imperative that scientists look for new ways to combat this issue. Due to the selective pressures of competition, bacteria and other microbes possess a host of defenses and weapons designed to exploit vulnerabilities in other microorganisms. Consequently, the study of these systems and microbial interactions has much to reveal in the search for novel antimicrobial treatments. Previous research from our laboratory has discovered that both *Alcaligenes faecalis* and *Alcaligenes viscolactis*, two rarely studied and generally non-virulent bacteria, exert a microbicidal effect on *Candida albicans* and *Staphylococcus aureus*, two pathogenic and frequently drug-resistant organisms. In this study, we confirmed that these effects are via a live-cell, contact-dependent mechanism and showed that both *Alcaligenes* species continue to inhibit *S. aureus* in both planktonic and biofilm forms of growth. Additionally, we found that *A. faecalis* and *A. viscolactis* have the ability to target Gram-positive bacteria outside the genus *Staphylococcus* and certain Gram-negative species as well as *Candida glabrata*. This study also provides evidence for the existence of a Type VI Secretion System in both *A. faecalis* and *A. viscolactis*, which may explain their antimicrobial phenotype. Despite efforts to identify the genetic elements involved via transposon and site-directed mutagenesis, the mechanism of these interactions remain elusive due to the difficulty of gene transfer in these organisms. We hope these results will increase current knowledge of *Alcaligenes'* capabilities and genetic composition as well as establish the groundwork for future efforts to discover its inhibitory system and mechanisms.

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INTRODUCTION

History of Antibiotics & Current Antibiotic Crisis

Sir Alexander Fleming's discovery of Penicillin in 1928 unleashed on the world a seemingly unstoppable power that helped transform medicine into its modern form as we know it today [1]. Bacterial infections once devastating to the human body became immediately vulnerable to these new drugs as an avalanche of new antibiotic classes and derivatives were discovered, developed, and synthetically modified. While antibiotics have undoubtedly saved millions of lives since their discovery, their efficacy has been challenged by their intended targets from the very beginning. In 1940, three years before Penicillin reached mainstream usage as a treatment, Penicillin-resistant strains of bacteria had already been isolated [1]. In 1962, two years after the introduction of Methicillin, deadly Methicillin-resistant strains of *Staphylococcus aureus* were discovered [1]. While decades of new antibiotic discoveries and advancement have helped keep this problem at bay, it has now resurfaced in recent years as one of the most pressing crises of modern medicine for many reasons. A 2013 report by the World Health Organization declared antibiotic resistance to be a worldwide, catastrophic threat with over 2 million cases and 23,000 mortalities occurring from resistant infections in the United States alone [2].

While antibiotic resistance is an unavoidable product of bacterial evolution and the selective pressures of exposure to antibiotics, it has been drastically precipitated by inappropriate prescribing practices, extensive agricultural use, and the collapse of antibiotic research and development in the pharmaceutical industry due to lack of financial profitability and shortage of scientific innovation and new discoveries [3]. Consequently, today's research has begun to shift to a focus on alternatives to traditional antibiotics including bacteriophage therapy, predatory bacteria, and bacteriocins [4]. While promising, none have yet been conclusively shown to equal

the potency of antibiotics [4]. As a result, it is imperative that novel methods of bacterial inhibition continue to be explored, as is the aim of this thesis.

Development of Antifungal Resistance

Unfortunately, the unopposed expansion of antimicrobial-resistance has not been limited to antibiotics, but has also penetrated the sphere of antifungal treatment. Unlike antibiotics, antifungal agents are relatively few in number and even more difficult to develop. As eukaryotes, both fungi and humans employ similar cellular machinery; therefore, antifungals approved for clinical application must exhibit high selectivity and low toxicity, ensuring no disruption to the host's cells during treatment [5]. Only four major classes of antifungal agents currently exist for invasive infections: polyenes, flucytosine, azoles, and echinocandins [5]. Since the introduction of triazoles in the early 1990s, resistance in *Candida* and *Aspergillus* species has become a global threat [6]. Even with treatment, the mortality of patients with fungal infections remains high, with mortality rates of 30% and 50% for aspergillosis and candidemia patients respectively [7,8]. Moreover, since 2006, no new classes of antifungals have been approved as of 2015 [8]. Given the paucity of available drugs and the rapid emergence of resistant strains, advancement and innovation in anti-fungal agents will continue to be an urgent need in the coming years.

Polymicrobial Interactions

One promising area in the search for new mechanisms of microbial inhibition is that of polymicrobial interactions. Polymicrobial interactions between organisms can be studied several ways in vitro via liquid co-culture assays, spot test analyses, and biofilm co-cultures, which can better mimic microbial interactions as they occur in vivo. In recent years, there has been a growing awareness that microbes rarely live in isolated, planktonic colonies but instead coexist as complex, biofilm communities composed of bacteria, fungi, viruses, and even archaea [9]. In

some cases, interactions such as those between *Pseudomonas aeruginosa* and *Staphylococcus aureus* are synergistic, increasing the invasiveness and virulence of infection when present together [10]. In other cases, the interactions are antagonistic such as in *Bdellovibrio* and *Micavibrio*-style predation, parasitism between bacteriophages and bacteria, competition, or amensalism [11,12]. Even outside of the human body, microbes such as bacteria often live in large, heterogenous communities, and competition for resources drives the evolution of many antimicrobial systems and production of compounds such as ribosomally synthesized peptides or proteins called bacteriocins [13,14]. With more than 99% of bacterial species producing at least one bacteriocin, most still unidentified, use of bacteriocins as replacements for antibiotics seems an incredibly promising area of research [14]. There are still many potential barriers yet to be overcome. These include the high cost of commercial production, degradation via proteolytic activity, and narrow spectrum activity for most tested bacteriocins [15]. Fortunately, antagonistic interactions among microbes are not solely limited to bacteriocin production and therefore remain a critical area of focus for the exploration of new inhibitory mechanisms [16]. Other known modes of inhibition include the aforementioned *Bdellovibrio*-style predation, Type III secretion systems, and Type VI secretion systems, which will be discussed more in depth later [17,18,19].

Alcaligenes faecalis

The research involved in this thesis centers on two species of bacteria in the genus *Alcaligenes*, the first being *Alcaligenes faecalis*. *A. faecalis* is a motile, peritrichous, Gram-negative bacillus found in aqueous environments, soil, and human intestinal flora [20]. It is also an aerobic, catalase and oxidase positive nonfermenter, though it can grow in anaerobic conditions in rare cases [21]. These coccoid-shaped rods are 0.5 – 0.6 by 2.0 μm and grow

optimally at temperatures between 25°C and 37°C, usually occurring singly but occasionally in pairs or chains [22]. While generally nonpathogenic, *A. faecalis* can in extremely rare cases induce nosocomial infections in immunocompromised patients and has been implicated in chronic otitis, endocarditis, endophthalmitis, and bacteremia, though with mostly favorable outcomes after treatment [20]. Most recently, a strain of *A. faecalis* has been found to have nematicidal effects on *Caenorhabditis elegans* via an extracellular serine protease virulence factor [23].

Alcaligenes viscolactis

Unlike its relative, few records of *Alcaligenes viscolactis* are found in scientific literature. *A. viscolactis* is an aerobic, nonmotile, Gram-negative, and almost spherical rod with an optimal growth temperature of 10°C to 20 °C [24]. It is also found in aqueous environments, and its size is roughly 0.5-1.0 by 0.8-2.6 μm [24]. Most notably, *A. viscolactis* is known to produce ropiness in milk; however, there is no record of pathogenicity in this organism, opportunistic or otherwise, making it an ideal candidate for potential use as an inhibitor of a variety of pathogenic organisms [24].

Staphylococcus aureus

Despite its commensal presence in approximately 30% of humans, *Staphylococcus aureus* is a common source of nosocomial and community-acquired infections [25]. While it is most commonly implicated in skin infections such as abscesses and boils, this Gram-positive coccus possesses a host of virulence factors such as toxins and immunoglobulin-binding proteins and can cause serious, invasive infections, leading to sepsis and even death [26]. Since the first appearance of Methicillin-resistant strains of *S. aureus* (MRSA) over fifty years ago, MRSA has grown into a worldwide problem, precipitated by its resistance to all available beta-lactam

antibiotics and failure to identify suitable antigens for vaccine development [25]. Despite a substantial decline in MRSA bloodstream infections from 2005 to 2012, the decrease has slowed remarkably since then, with 72,444 cases of invasive MRSA in the U.S. in 2014 [27,28]. Traditionally, severe MRSA infections have been effectively treated with Vancomycin; however, the rise of Vancomycin intermediate-resistant *S. aureus* (VISA) and Vancomycin resistant *S. aureus* (VRSA) poses a severe threat around the globe [27]. Another key feature of *S. aureus* is its ability to form a biofilm composed of polysaccharides, DNA, and protein [29]. These biofilms can attach to bone, heart valves, and implanted materials, leading to chronic infections [29]. Additionally, *S. aureus* biofilms decrease the organism's susceptibility to antibiotics and weakens immune responses, resulting in increased virulence [29].

Candida albicans

Like *S. aureus*, the fungi, *Candida albicans*, is a commensal organism, residing in the gastrointestinal and genitourinary tracts of approximately 70% of humans [30]. In immunocompromised patients, *C. albicans* often becomes an opportunistic pathogen. *Candida* is one of the four most common causes of hospital-acquired bloodstream and cardiovascular infections in the U.S., resulting in mortality rates as high as 50% [30]. *C. albicans* forms protective biofilms and has been developing increasingly high levels of resistance to anti-fungal drugs, a major cause of concern in the medical and scientific communities. It also exhibits morphological switching, primarily between its yeast and hyphal forms, which plays a critical role in its virulence [30].

Type VI Secretion Systems

Due to prior observations that *Alcaligenes* 1) inhibits other organisms via cytolytic effects 2) requires a live-cell, contact-dependent mechanism, and 3) is capable of attacking both

prokaryotic and eukaryotic organisms, we have hypothesized that the presence of a secretion system may be implicated in this phenomenon. Although Type III, IV, and VI secretions systems are able to inject effector molecules directly into other cells via a syringe-like apparatus, many with microbicidal effects, only the Type VI secretion system (T6SS) has been currently shown to kill both bacterial and fungal species [31,32]. Since its discovery in *Vibrio cholerae*, the T6SS has been found in around 25% of all Gram-negative bacteria [33] and confers an incredible advantage on organisms competing for resources. While principally thought of as an antibacterial weapon, the T6SS has many diverse functions and can play an important role in virulence and pathogenesis, as it does with *Pseudomonas aeruginosa* in cystic fibrosis patients [33]. More recently, this system has been shown in *Serratia marcescens* to target fungal cells, including those of pathogenic *Candida* species, in addition to bacterial cells, highlighting its capability as both an antibacterial and antifungal weapon [34]. T6SS machinery is composed primarily of a membrane-spanning unit and a tube-like structure that closely resembles a bacteriophage tail. As the outer sheath contracts, the inner tube and spike propels out of the bacterium and punctures the membrane of the target organism, delivering effector molecules that lead to disruption or lysis of the cell [33]. These effector molecules generally target conserved structures such as the cell wall, membrane compartment, nucleic acids, or cytoskeletal components [33]. Immunity proteins to specific effector molecules protect the organism from self-destruction [33]. In *Vibrio cholerae*, the T6SS is regulated by quorum sensing, catabolite repression, and environmental conditions, among a host of other factors [35]. It is chromosomally encoded within large, variable clusters that contain thirteen essential core genes labeled TssA through TssM, which encode for proteins that form the basic structure of the system [36]. While no T6SS in either *A.*

faecalis or *A. viscolactis* has been previously recorded or characterized in scientific literature, our observations suggest the possibility that this system or one analogous to it may be involved.

Prior and Present Research

Research in our lab has uncovered several previously unknown features regarding *A. faecalis* and *A. viscolactis.* First, both *Alcaligenes* species were shown to have apparent bactericidal effects in a concentration-dependent manner on *Staphylococcus aureus*, *Staphylococcus epidermidis*, and other *Staphylococcal* species in planktonic form [37]. Additionally, heat-killed and cell-free supernatant tests of *A. faecalis* and *A. viscolactis* on *S. aureus* were determined to have no inhibitory effect, proving that this phenomenon is dependent on live *Alcaligenes* cells and on cell-cell contact [37]. Furthermore, our lab has discovered that *A. faecalis* and *A. viscolactis* also inhibit growth of *Candida albicans* in both clinical and laboratory isolates as well as hyphal and yeast-locked strains [38]. This inhibition is dependent on live-cell interaction, suggesting that the mechanism may likely be the same in the killing of both *Staphylococcal* spp. and *C. albicans* [38]. These discoveries show great potential in uncovering therapeutic targets for attacking these pathogenic and often drug-resistant organisms and possibly even for the use of select *Alcaligenes* strains to kill them directly. For these reasons, it is highly desirable to uncover the exact mechanism by which *Alcaligenes* kills its targets.

The objectives of this thesis project were fourfold: 1) to carry out preliminary testing of *A. faecalis* and *A. viscolactis*' ability to inhibit biofilm growth of *S. aureus*, 2) to expand our analysis outside of *C. albicans* and *Staphylococcal* species to determine whether other organisms are susceptible to *Alcaligenes*, 3) to uncover the genetic elements responsible for this microbicidal phenotype via random transposon mutagenesis, and 4) ascertain whether

Alcaligenes contains T6SS machinery and, if present, its potential contribution to our observations.

MATERIALS AND METHODS

Strains	Characteristics	Reference
A. faecalis ATCC® 8750™	used for biofilm & spot assays, screening for inhibition, mutagenesis, & genetic analysis	
A. viscolactis Ward's® # 470179-188	used for biofilm & spot assays, screening for inhibition, mutagenesis, & genetic analysis	
S. aureus ATCC® 25923™	used for biofilm assays, screening for inhibition	
S. aureus Clinical isolate	used for biofilm assays, screening for inhibition, obtained from Quillen COM	
C. albicans SC5314	Used for supernatant spot assays	
E. coli HB101	host for pRK2013 helper plasmid, used for triparental mating	Ditta et al. 1980
E. coli BW20767	host for pRL27-Tn5 plasmid, used for transposon mutagenesis	Nguyen et al. 2016
E. coli SM10λpir	used for biparental conjugation	
Plasmids		
pRL27	Tn5 transposon delivery vector, Kan_R,	Nguyen et al. 2016
pEX18Gm	general cloning vector, Gm_R	Hoang et al. 1998
pEX18GmΔVgrG	pEX18Gm containing SOE product deletion of *VgrG* gene	This work
pRK2013	Helper plasmid for triparental mating, Kan_R	Ditta et al. 1980

Table 1: Primary bacterial strains and plasmids.

Growth Conditions

Unless otherwise noted, all bacterial strains **(Table 1)** except *A. viscolactis* were grown in broth cultures at 37°C on a shaker deck set to 250 RPM. Liquid cultures of *A. viscolactis* were grown at laboratory room temperature, approximately 20-25 °C, on a shaker set to 40 RPM unless co-cultured with other species. Plated cultures were grown under static conditions at the

same temperature range. The primary medium for most strains was Luria-Bertani (LB) broth for liquid cultures and LB agar for plated cultures. Other media and conditions used will be discussed under their specific protocols.

Alcaligenes - *S. aureus* Biofilm Interactions

Liquid cultures of *A. faecalis*, *A. viscolactis,* laboratory *S. aureus*, and clinical *S. aureus* were prepared via inoculation in 5 mL tubes of LB broth and incubated on a rotary shaker for approximately 24 hours.

Using a spectrophotometer set to 600 nm (OD_{600}), the absorbance of the overnight cultures were taken and used to prepare standardized co-cultures with a 10:1 ratio of *Alcaligenes* to *S. aureus* in 5mL LB tubes, along with controls of clinical and laboratory *S. aureus* alone in equivalent amounts. One mL of each preparation was pipetted into separate wells on a six well plate. Additionally, a slide cover slip was placed in one control and one co-culture well. The well plate was placed in a 37°C incubator for approximately 24 hours.

After incubation, the residual liquid in each well was poured off, and the biofilms carefully washed once with 1 mL of phosphate buffer solution (PBS). Another 1 mL of PBS was added to each of the wells, and the biofilm was scraped into the solution with a pipette. Tenfold serial dilutions of the biofilms were performed using PBS. The dilutions were plated on Mannitol Salt Agar (MSA), a selective and differential medium used for *Staphylococcus* species that prevents *Alcaligenes* from growing. The MSA plates were subsequently placed at 37°C overnight, and colony counts were carried out the following day to determine relative biofilm growth of *S. aureus* in the experimental vs control conditions. This procedure was later modified by creating co-cultures with 1x, 2x, and 3x the concentration of the *Alcaligenes* species, allowing

us to ascertain whether increasing the concentration of *Alcaligenes* cells decreases number of viable *S. aureus* colonies in biofilms.

In the wells containing the cover slips, the coverslip containing the intact biofilm was carefully removed, Gram-stained, and visualized under a microscope at 100x magnification.

Co-culture Supernatant Spot Testing

Overnight cultures of *A. faecalis, A. viscolactis, S. aureus*, and *C. albicans* were prepared. As with the biofilm procedure, a spectrophotometer was used to create co-culture samples with a 10:1 ratio of *Alcaligenes* to *S. aureus* or *C. albicans*. Of note, co-cultures with *C. albicans* were prepared in BHI media for better growth. After 24 hours of growth, cell-free supernatant from the co-cultures and from *Alcaligenes, S. aureus,* and *C. albicans* single cultures were obtained by pelleting the cells on a tabletop centrifuge then filter sterilizing the remaining solution with a 0.45 μm filter. Afterwards, 20 μL of the supernatant from the co-cultures were spotted onto lawns of the test organism (*S. aureus* or *C. albicans*) along with controls of live *Alcaligenes* cells, *Alcaligenes* supernatant, and *S. aureus*/*C. albicans* supernatant.

Broad-Spectrum Spot Testing

Spot tests were performed by pipetting 20 μL of an overnight culture of *A. faecalis* and *A. viscolactis* on an LB agar plate streaked with a lawn of the test organism. After allowing the plates to dry, they were placed at 37°C for 24-48 hours. Lawns of *Rhizobium leguminosarum* ATCC #14479 were streaked on Congo Red and incubated at 30°C while *Candida glabrata* was grown on BHI at 20-25°C because of different nutrient and growth requirements. Zones of inhibition, if present, were measured and recorded.

New *A. faecalis* strains C19593 and ATCC #35655 were tested for antibiotic susceptibility by placing antibiotic disks on a lawn of each strain on LB agar. Plates were

incubated overnight. Liquid cultures of the strains were prepared, and 20 μL of each was spotted onto a lawn of *S. aureus* clinical strain to determine if these other strains also possess the inhibitory phenotype observed in *A. faecalis* ATCC #8750.

Transposon Mutagenesis

Overnight liquid cultures of *A. faecalis*, *A. viscolactis*, and *E. coli* BW20767 carrying the pRL27 plasmid were prepared in 5 mL of LB broth. Several methods were then used to induce mating. All overnight incubations were carried out at 37°C except with *A. viscolactis* cultures.

Cross Hatch Technique: Each *Alcaligenes* sp. was crossed with the pRL27 strain on an LB agar plate using sterile cotton swabs. After incubation for 24 hours, 1 mL of PBS was added to the plate, and the colonies were scraped into the solution using a sterilized hockey stick.100 μL of this solution was then plated onto an LB agar plate containing Kanamycin and Ampicillin (LB + KA) at concentrations of 50 μg/ml each and incubated for 24 hours. Isolated colonies were transferred to a fresh LB + KA plate using sterile toothpicks and allowed to grow again before spot testing against *S. aureus*.

Liquid Co-culture Technique: 10 μL of pRL27 and 30 μL of *Alcaligenes* were added to a 5 mL LB tube and placed on a shaker. After 24 hours of growth, 100 μL of the co-culture was plated on LB + KA and again incubated. As before, isolated colonies were transferred to a fresh LB+KA and allowed to grow before phenotype screening.

Spot Co-culture Technique: Using overnight liquid cultures, 500 μL of *Alcaligenes* and pRL27 were mixed in a microcentrifuge tube and pelleted in a tabletop centrifuge. After resuspending in 200 μL of LB, 20 μL of the concentrated co-culture was spotted onto a fresh LB plate and incubated. A loopful of the bacteria was inoculated into a 5 mL LB tube the next day

and plated on LB + KA selective media. Further isolation of suspected mutants were carried out as in the previous techniques.

In Silico Analysis of *A. faecalis* Genome Sequences

Using the National Center for Biotechnology Information (NCBI) GenBank, multiple strains of *A. faecalis* were analyzed for presence of T6SS genes. Three strains, NTCC #10388, BBD4, and ZD02, were primarily analyzed and later used to construct primers. *A. faecalis* ATCC# 8750, our lab's strain, has not been fully sequenced and could not be used for complete genetic analysis.

Primer Design

The primers used in this study (**Table 2)** were designed largely by hand with the help of NCBI Primer Blast and New England Biolab's Tm Calculator.

Name	Sequence (5' – 3')	Use
VgrG-par-F	GCTGACAGAACGCCTTGTAC	Partial *VgrG* amplification
VgrG-par-R	CGATACGTGTAGAAGCGGTC	Partial *VgrG* amplification
VgrG-tot-F	GCTGACAGAACGCCTTGTAC	Total *VgrG* amplification
VgrG-tot-R	GATACACCGCACTGGTCTTG	Total *VgrG* amplification
AF-Hcp-F	TCCAGTCCTTTTCATGGGGC	*Hcp* Amplification
AF-Hcp-R	TGCTTGATATCCCAGGCCAC	*Hcp* Amplification
SOEF1	CGATGGTACCGAGTGGTTCATGCCTTGCAG	Upstream SOE Forward Primer
SOER2	CGAGGACTAAGAATACGCTGGACGGTACAA GGCGTTCTGTCAG	Upstream SOE Reverse Primer
SOEF2	CCGTCCAGCGTATTCTTAGTCCTCGGATCAA GACCAGTGCGGTG	Downstream SOE Forward Primer
SOER1	GCTATCTAGATGGACGTTGCCCAAATACAGC	Downstream SOE Reverse Primer

Table 2: Primers designed and used for this study.

Genomic Extraction

Genomic extraction was completed using 2 mL of an overnight culture of *A. faecalis* and a Promega Wizard gDNA purification kit. Extracted gDNA was tested for purity and concentration using a Thermo Scientific nanodrop and stored at 4°C or -20°C.

Detection of *VgrG* via PCR and Electrophoresis

Aliquots containing primers pairs VgrG-par-F/VgrG-par-R and VgrG-tot-F/VgrG-tot-R along with 1 µg of gDNA, nuclease free H_2O, and NEB 2X Taq Polymerase Master Mix were prepared to amplify partial and total sequences of the *VgrG* gene respectively. PCR parameters were adjusted until optimal conditions were reached **(Table 3)**. PCR products were loaded on a 1% agarose gel containing Ethidium Bromide at a concentration of 0.7 µg/mL, run at 125V for 40-45 minutes, and visualized under UV light. The *Hcp* gene was later amplified under the same PCR conditions (**Table 3)**.

Step	Temp	Time
Initial Denaturation	95°C	2 minutes
34 cycles	95°C 52°C 72°C	30 seconds 30 seconds 1 minute/kb
Final Extension	72°C	5 minutes
Hold	4°C	∞

Table 3: PCR thermocycler protocol for amplification of *VgrG* and *Hcp* genes.

DNA Extraction & Sequencing

Following amplification, 50 µL aliquots of PCR product were extracted from a 1% agarose gel containing no Ethidium Bromide and purified using a Thermofisher gel extraction kit. Purity and concentration of the extracted DNA were analyzed by nanodrop and by running 2-3 µL of the samples on another 1% agarose gel. The extracted DNA was sent to Quillen College

of Medicine's Molecular Biology Core Facility for sequencing along with 15 μL each of the appropriate primers diluted to a working concentration of 3μM.

Analysis of DNA Sequences

Sequencing results were cleaned and analyzed using NCBI Nucleotide Blast, Emboss Merger, EMBOSS Needle, and a reverse complement generator acquired from Bioinformatics.org.

Splicing by Overlap Extension

Splicing by Overlap Extension (SOE) is a method in which regions flanking the gene of interest (approximately 1,000 base pairs (bp) upstream and downstream) are amplified via PCR and fused together, thereby deleting out the gene [42]. This construct can then be ligated into a vector and transferred into the species containing the gene of interest. From there, homologous recombination replaces the original sequence, creating a knockout mutant. This technique was used in an attempt to delete the *VgrG* gene in *A. faecalis*. Primers were designed using the *A. faecalis* genomic sequences noted earlier and previously designed overlap/restriction enzyme sequences [43]. Primer pairs SOEF1 – SOER2 were used to amplify a 944 bp sequence upstream of *VgrG* while primer pairs SOEF2 – SOER1 were used to amplify a 1,041 bp sequence downstream using the PCR parameters outlined previously **(Table 3).** Once the upstream and downstream products were purified via gel extraction, a second, two-step PCR reaction was run, splicing the fragments together and generating an approximately 2,000 bp product with a deletion of *VgrG* **(Tables 4 & 5)**. Following a second purification, the ends of this 2 kb fragment and extracted pEX18Gm plasmid vector were digested using KpnI and XbaI restriction enzymes.

pEX18Gm is a plasmid vector containing a Gentamicin (Gm^R) resistance marker, pUC polylinker, and *SacB* counterselectable marker [41]. After subsequent column purification of

both the vector and insert, ligation using T4 DNA ligase was carried out using a 1:3 picomolar ratio of vector to insert following a protocol from New England Biolabs (NEB). Integration of the SOE product into pEX18Gm formed pEX18Gm*ΔVgrG*. Competent NEBα5 *E. coli* cells were prepared using the Rubidium Chloride ($RbCl_2$) protocol obtained from Mcmanus Lab. The ligation product was used to transform pEX18Gm*ΔVgrG* into competent NEBα5 cells via a heat shock protocol obtained from NEB. Cells successfully transformed with pEX18Gm*ΔVgrG* were isolated using blue-white screening on LB agar plates containing 20 μg/mL of Gentamicin and 20 μg/mL of 5-bromo-4-chloro-3-indolyl-*β*-d-galactopyranoside (X-gal).

Plasmid vector pEX18Gm contains a LacZα gene, which encodes for β-galactosidase, at its multiple cloning site [41]. When treated with X-gal, cells containing the non-recombinant plasmid express a functional β-galactosidase that cleaves X-gal, forming a bright blue pigment. Insertion of DNA into the multiple cloning site disrupts the LacZα gene, and the blue pigment is not produced upon treatment with X-gal and appear white [44]. White colonies were re-plated, and presence of the SOE product was confirmed by colony PCR using primer pair SOEF1-SOER1.

Mixture 1 (25 μL)	**Step**	**Temp**	**Time**
Upstream Template (2 μL)	Initial Denaturation	95°C	3 minutes
Downstream Template (1 μL)	15 cycles	95°C 52°C	45 seconds 45 seconds
Taq 2X Master Mix (12.5 μL)		72°C	2.25 minutes
Sterile H_2O (9.5 μL)	Final Extension	72°C	10 minutes
	Hold	4°C	∞

Table 4: Crossover reaction 1 setup and thermocycler protocol.

Mixture 2 (50 µL)	Step	Temp	Time
Product from Reaction 1 (25 µL)	Initial Denaturation	95°C	1 minutes
SOEF1 Primer (0.5 µL)	30 cycles	95°C	45 seconds
		52°C	45 seconds
SOER1 Primer (0.5 µL)		72°C	2.25 minutes
Taq 2X Master Mix (12.5 µL)	Final Extension	72°C	10 minutes
Sterile H_2O (11.5 µL)	Hold	4°C	∞

Table 5: Crossover reaction 2 setup and thermocycler protocol.

Transfer of pEX18Gm*ΔVgrG* to *A. faecalis*

NEBα5 cells harboring pEX18Gm*ΔVgrG* were then used for triparental conjugation with pRK2013 and *A. faecalis* ATCC #8750 to transfer the deletion product to *A. faecalis*. Overnight cultures of the three strains were grown in antibiotic-containing LB broth, ensuring their purity. NEBα5 was grown in 20 µg/mL of Gentamicin, pRK2013 in 50 µg/mL of Kanamycin, and *A. faecalis* in 35 µg/mL of Ampicillin. One microliter of each culture was pelleted using a tabletop centrifuge and washed twice with sterile 0.85% NaCl to remove any residual antibiotics. After resuspending in 200 µL of saline, 100 µL of each strain was mixed, pelleted, and resuspended in 30 µL of saline or filter sterilized PBS. This mixture was spotted in 10-15 µL aliquots either directly onto LB agar or onto a 0.45 µm nitrocellulose filter disk resting on the agar, and the plates were allowed to incubate for 24 hours at 37 °C. After incubation, the mixture was resuspended in 1 mL of PBS by using an inoculation loop or vortexing the filter disk. Tenfold dilutions were then performed and plated on LB + Gm_{20} agar. Following incubation for 24-48 hours, isolated colonies were re-plated on selective agar and screened via colony PCR. The

pEX18Gm*ΔVgrG* plasmid was also transformed into $RbCl_2$ competent *E. coli* SM10λpir cells by heat shock and biparental mating was carried out following the same procedure as above without use of the pRK2013 strain.

RESULTS & DISCUSSION

Biofilm Interactions

Results from the biofilm co-culture assays reveal that both *A. faecalis* and *A. viscolactis* are able to outcompete *S. aureus* when grown together and cause a significant reduction in the growth of viable *S. aureus* cells during the attachment phase of biofilm formation. Co-culturing clinical *S. aureus* with *A. viscolactis* at a 1:10 ratio resulted in a 97.2% decrease in viable cells while co-culturing *S. aureus* with *A. faecalis* at the same ratio resulted in a 96.1% decrease **(Fig. 1 & 2)**. Doubling the concentration of *A. viscolactis* and *A. faecalis* resulted in *S. aureus* reductions of 98.3% and 97.6% respectively **(Fig. 1 & 2)**. Surprisingly, tripling the concentration of *Alcaligenes* failed to significantly decrease *S. aureus* growth even further and the number of surviving *S. aureus* cells rose slightly in the assays with *A. faecalis*. This demonstrates that while inhibition of *S. aureus* biofilms by *Alcaligenes* follows a concentration-dependent manner, this effect is limited and seems to slow or stop once a certain concentration of cells is reached. A plausible explanation for this observation may be that at high concentrations, rapid depletion of resources occurs, which in turn may effect *Alcaligenes'* ability to prey on other organisms. Gram-stains of the control vs experimental co-cultures visualized with light microscopy at 100x magnification demonstrates the effectiveness of *Alcaligenes* at disrupting *S. aureus* biofilm growth **(Fig. 3)**. Of note, these results show that *A. viscolactis*, whose optimum temperature growth is 20-25°C, does not appear to lose its inhibitory function when incubated at 37°C.

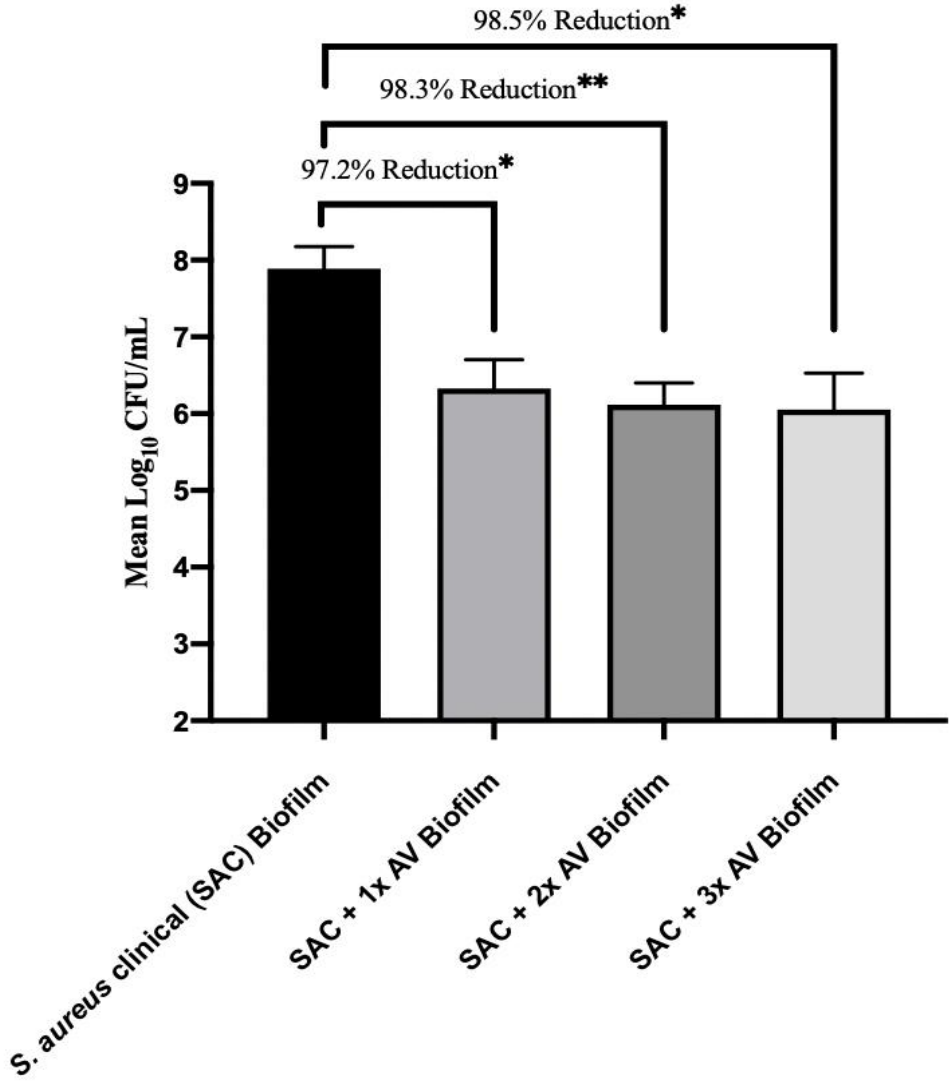

Figure 1: Viable *S. aureus* colonies on Mannitol Salt Agar after 24 hours at 37°C from washed biofilms. Biofilms were grown for 24 hours at 37°C under control conditions with our clinical *S. aureus* strain alone and under experimental conditions with our clinical *S. aureus* strain co-cultured with one, two, and three times concentrations of *A. viscolactis*. Mean $\log_{10}$ CFU/mL data were obtained from three separate trials, and statistical analysis was performed using one-tailed, unpaired Student T-tests comparing the control condition to each of the three experimental conditions. Standard error bars are also included for analytical purposes. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

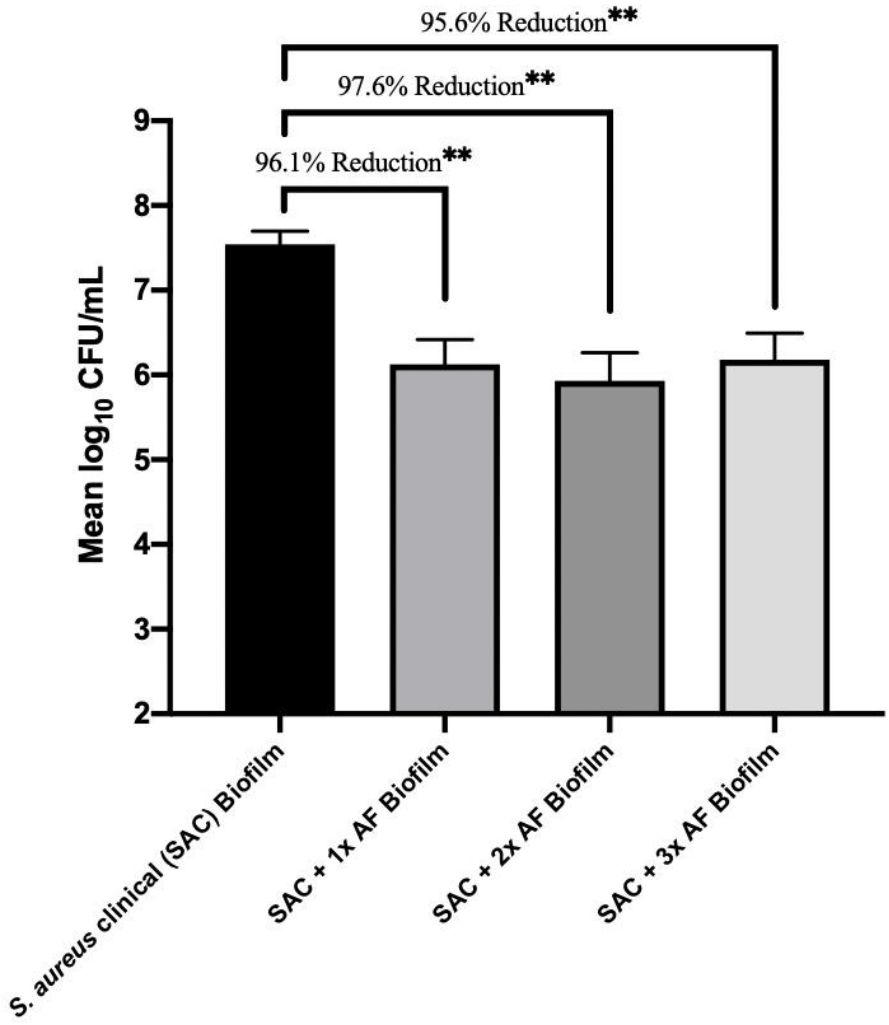

Figure 2: Viable *S. aureus* colonies on Mannitol Salt Agar after 24 hours at 37°C from washed biofilms. Biofilms were grown for 24 hours at 37°C under control conditions with our clinical *S. aureus* strain alone and under experimental conditions with our clinical *S. aureus* strain co-cultured with one, two, and three times concentrations of *A. faecalis*. Mean $\log_{10}$ CFU/mL data were obtained from three separate trials, and statistical analysis was performed using one-tailed, unpaired Student T-tests comparing the control condition to each of the three experimental conditions. Standard error bars are also included for analytical purposes.
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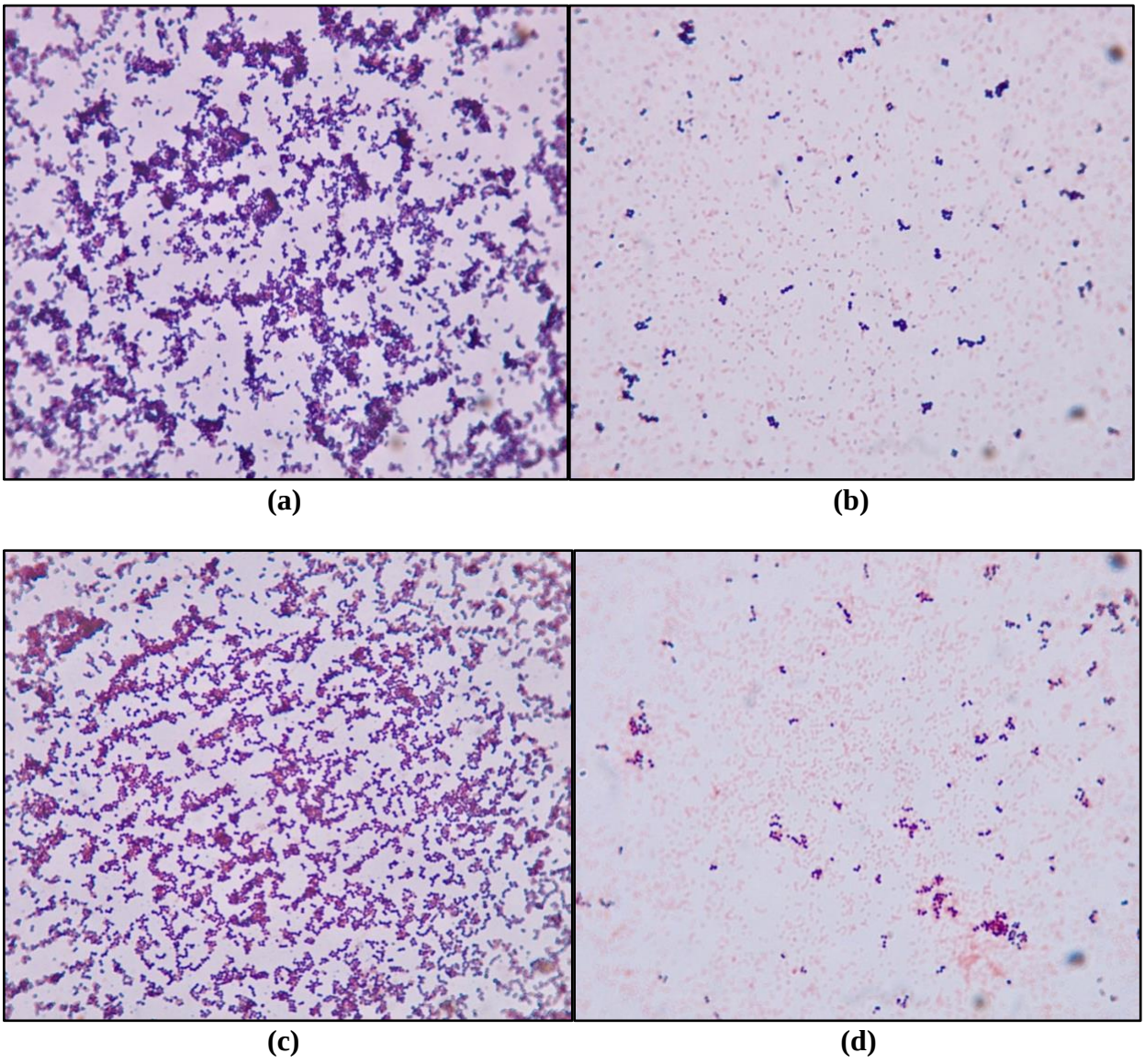


Figure 3: Gram-stains of biofilms grown for 24 hours and visualized at 100x: **(a)** *S. aureus* clinical strain (SAC), **(b)** co-culture of *S. aureus* clinical and 1x concentration of *A. faecalis*, **(c)** *S. aureus* clinical strain, **(d)** co-culture of *S. aureus* clinical and 1x concentration of *A. viscolactis*.

Confirmation of a Contact-dependent Mechanism

To supply further proof that *Alcaligenes'* method of inhibition is contact-dependent and not due to secretion of an antimicrobial compound only in the presence of a competing organism, cell-free supernatant from co-cultures of *A. faecalis* or *A. viscolactis* and *S. aureus or C. albicans* were spotted onto lawns of the test organism along with controls of live *Alcaligenes* cells, *Alcaligenes* supernatant, and *S. aureus*/*C. albicans* supernatant. As hypothesized, only live *Alcaligenes* cells formed zones of inhibition on both *S. aureus* and *C. albicans*, confirming the requirement of live contact of *Alcaligenes* with its microbial prey **(Fig. 4 & 5)**.

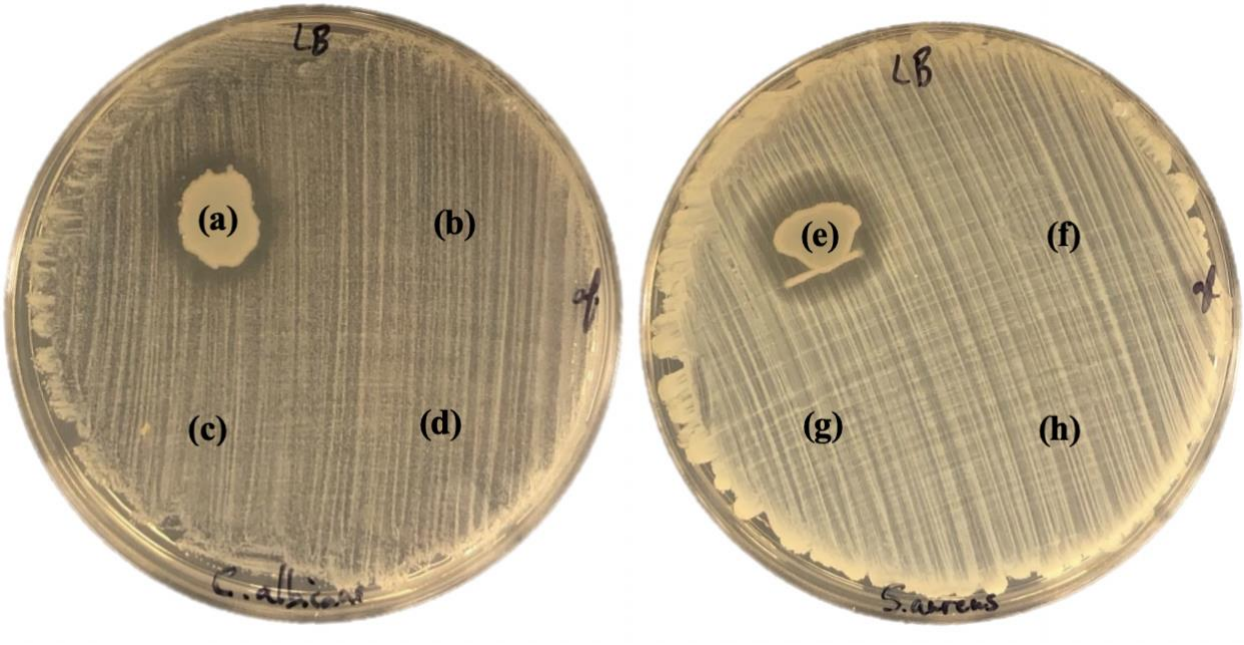


Figure 4: Co-culture-supernatant test: **(a)** *A. faecalis* cells, **(b)** *A. faecalis* supernatant, **(c)** *C. albicans supernatant*, **(d)** *A. faecalis - C. albicans* co-culture supernatant, **(e)** *A. faecalis* cells, **(f)** *A. faecalis* supernatant, **(g)** *S. aureus* supernatant, **(h)** *A. faecalis - S. aureus* co-culture supernatant.

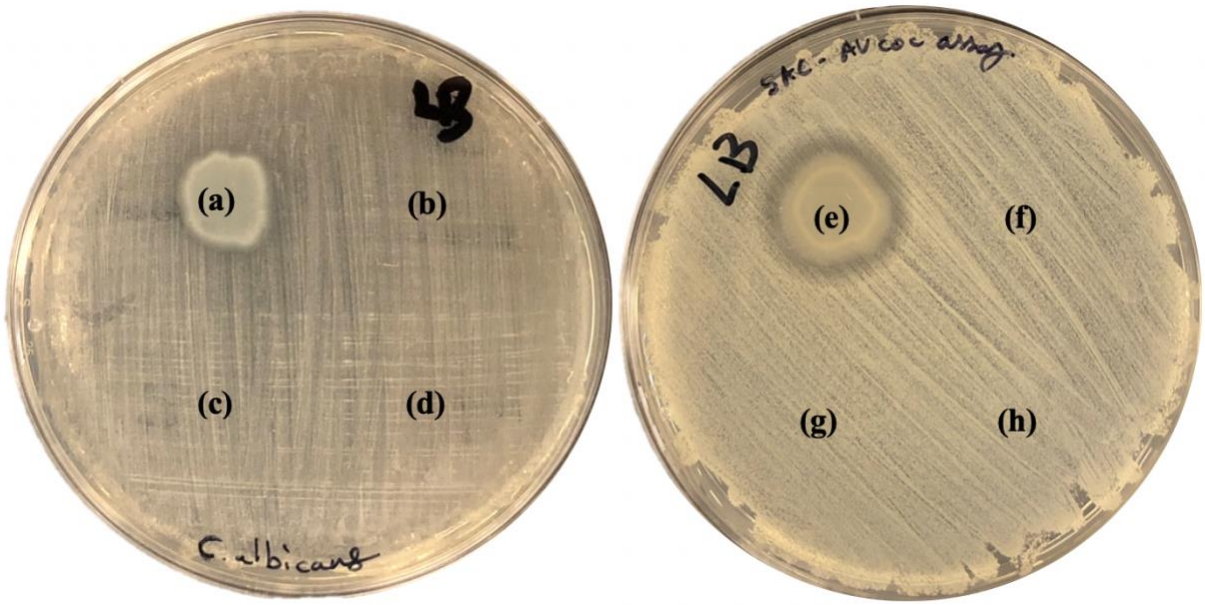


Figure 5: Co-culture-supernatant test: **(a)** *A. viscolactis* cells, **(b)** *A. viscolactis* supernatant, **(c)** *C. albicans* supernatant, **(d)** *A. viscolactis* - *C. albicans* co-culture supernatant, **(e)** *A. viscolactis* cells, **(f)** *A. viscolactis* supernatant, **(g)** *S. aureus* supernatant, **(h)** *A. viscolactis* - *S. aureus* co-culture supernatant.

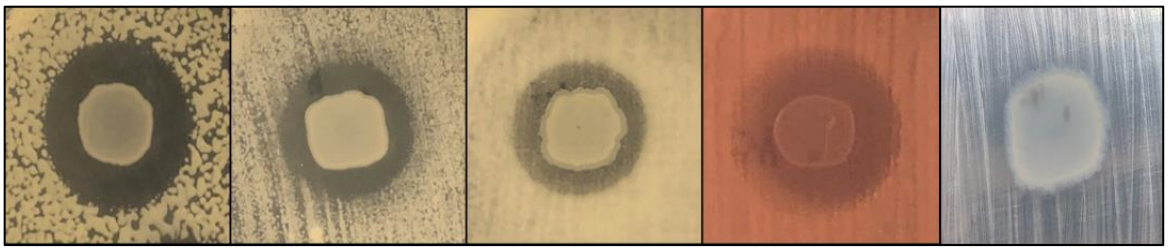
Spectrum of *Alcaligenes* Inhibitory Activity

While previous research had shown that both *A. faecalis* and *A. viscolactis* are significant inhibitors of *C. albicans* and *Staphylococcus* species, it was still unclear whether they were active against other microorganisms. Both *Alcaligenes* strains were spot-tested on a variety of other microbial species (**Table 6**). *A. faecalis* was found to be a relatively strong inhibitor of members of the genus *Bacillus*, including the pathogenic *B. cereus*. **(Fig. 6)**. Interestingly, preliminary testing shows that *A. faecalis* also kills *Candida glabrata*, though not to the degree that it does *C. albicans*, which may be at least explained in part by *C. glabrata*'s yeast morphology and lack of morphological switching into hyphal form as in *C. albicans* [45]. This would be consistent with previous observations in our lab of decreased inhibition of *C. albicans* in yeast-locked strains. Moreover, *Alcaligenes viscolactis* exhibited a lesser effect on these species with only *B. subtilis* being visually inhibited **(Fig. 7)**. In addition, *Klebsiella pneunoniae*,

Shigella dysenteriae, and *Shigella sonnei* showed a small degree of sensitivity to *A. faecalis* but none to *A. viscolactis* **(Fig. 6)**. We also noted that the Gram-negative *Rhizobium leguminosarum* was strongly inhibited by both *Alcaligenes* species, and that it does not appear that *A. faecalis* and *A. viscolactis* inhibit each other's growth.

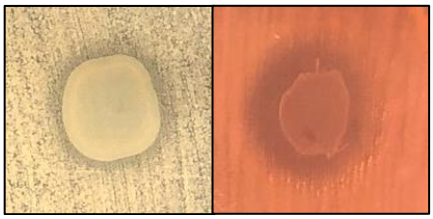
Classification	Species	Inhibition by *A. faecalis*	Inhibition by *A. viscolactis*
Gram-positive	*B. cereus*	++	-
	B. megaterium	+++	-
	B. subtilis	+++	+
	E. faecalis	-	-
	S. aureus	+++	++
	S. pyogenes	-	-
Fungal	*C. albicans*	+++	++
	C. glabrata	+	**nd***
Gram-negative	*C. freundii*	-	-
	E. coli BW20767/prRL27	-	-
	Escherichia coli ATCC #25922	-	-
	Klebsiella aerogenes	-	-
	Klebsiella pneumoniae	+	-
	Proteus vulgaris	-	-
	Pseudomonas aeruginosa	-	-
	Pseudomonas denitrificans	-	-
	Rhizobium leguminosarum	+++	+++
	Salmonella arizonae	-	-
	Salmonella typhi	-	-
	Serattia marcescens	-	-
	Shigella boydii	-	-
	Shigella dystenteriae	+	-
	Shigella sonnei	+	-

Table 6: Spot-Test Assays of *A. faecalis* and *A. viscolactis* on broad range of organisms (* not determined from spot test).



(a) **(b)** **(c)** **(d)** **(e)**

Figure 6: Zones of inhibition of *A. faecalis* on lawns of **(a)** *B. megaterium* **(b)** *B. subtilis* **(c)** *B. cereus* **(d)** *R. leguminosarum* **(e)** *Candida glabrata.*



(a) **(b)**

Figure 7: Zones of inhibition of *A. viscolactis* on lawns of (a) *B. subtilis* (b) *R. leguminosarum*.

These observations raise a pertinent question. While both *A. faecalis* and *A. viscolactis* have been shown to have live-cell dependent, microbicidal effects on a number of organisms, what explains the significant difference in their activity when spot-tested on certain species? One hypothesis is that incubation at 37°C to allow the test organism to grow may stunt the growth of *A. viscolactis* or its expression of intracellular machinery needed to kill more resistant organisms. Results from the biofilm assays appear to contradict this explanation, however. Additionally, its lack of motility and slower growth rate compared to *A. faecalis* may also explain its lesser activity when using a spot-test assay, suggesting that co-culture colony counts may be a better mechanism for determining spectrum of inhibition for *A. viscolactis*.

Mutagenesis with pRL27

Transposon mutagenesis is a technique that uses conjugation to transfer a plasmid containing a transposon element into the desired bacterium. Once inside the cell, the transposon

segment of the plasmid randomly inserts once into the bacterial chromosome with the help of a transposase enzyme, disrupting the gene at the point of insertion. This technique is incredibly valuable for identification of unknown genes integral for a specific function. In this case, *E. coli* strain BW20767 carrying plasmid vector pRL27 was used for conjugation to transport the Tn5 transposon into *A. faecalis* and *A. viscolactis* [40]. The pRL27 plasmid also contains a Km_r gene, which confers Kanamycin resistance to both the donor strain and the mutant upon insertion of the transposon [40]. This enables simple isolation of mutants via growth on antibiotic plates. Kirby-Bauer testing was performed on *E. coli*-pRL27 and on both *Alcaligenes* species to create an antibiotic susceptibility profile (**Table 7)**.

Antibiotic	**Concentration (MCG)**	***E. coli* pRL27 Susceptibility**	***A. faecalis* Susceptibility**	***A. viscolactis* Susceptibility**
Ampicillin	10	+	-	-
Bacitracin	10	-	-	-
Chloramphenicol	30	+	+	-
Erythromycin	15	-	+	-
Kanamycin	30	-	+	+
Novobiocin	5	-	n/a	+
Penicillin	10	-	-	-
Streptomycin	10	+	+	-
Tetracycline	30	+	+	+

Table 7: Susceptibility Profile of *E. coli* pRL27, *A. faecalis*, and *A. viscolactis* to common antibiotics.

Based on the results, Ampicillin and Kanamycin were chosen as the appropriate antibiotics for the selection of mutants. Both *Alcaligenes* species are resistant to Ampicillin while *E. coli* pRL27 is not, which allows only the growth of *Alcaligenes*. Since mutants contain the Km_R gene, treatment with Kanamycin will inhibit growth of all *Alcaligenes* cells except for exconjugants with the transposon inserted into its genome. Despite successful mutagenesis of *Klebsiella pneumoniae* ATCC #13883 using *E. coli* pRL27 by others in our lab, no mutants of

either *A. faecalis* or *A. viscolactis* were obtained by any of the three techniques outlined earlier [46]. While isolated colonies appeared on plates left in the incubator for longer than 48 hours, they either failed to grow when re-plated on selective media or were determined to be *Staphylococcal* contaminants by Gram-staining. The repeated occurrence of contaminants suggest either a breakdown of antibiotics past 48-72 hours or colonization by a strain of *Staphylococcus* resistant to both antibiotics used. Despite numerous screenings, no true *Alcaligenes* mutants were obtained via transposon mutagenesis.

In Silico Analysis of *A. faecalis Genomic DNA*

Using NCBI's genomic database and protein BLAST function, the genomes of several fully sequenced *A. faecalis* strains were analyzed for the presence of T6SS genes. Core genes of conserved T6SS components as well as other proteins of unknown function were found clustered at a single locus in several *A. faecalis* strains (**Table 8)**. Of the essential *TssA*-*TssM* genes, all except for *TssH* were found in every genome containing the T6SS locus. Whether the sheath recycling function of *TssH* in *A. faecalis* is not critical to its T6SS functioning or is sometimes replaced by another protein is currently unknown. Interestingly, an unidentified protein containing a *Duf4150* domain was also found within this locus. In certain proteobacteria such as *Agrobacterium tumefaciens*, this N-terminal domain caps the end of the spike formed by *VgrG,* while the C-terminal contains a toxin_43 domain that functions as a DNase [47]. Despite the conserved *Duf4150* domain, no homology to the toxin_43 domain could be found in *A. faecalis*, suggesting the C-terminal of this protein may possess a different function as a possible effector molecule.

Gene/Component	Conserved/Proposed Function	Source
TssA	Baseplate	Cianfanelli et al.
Contractile Sheath: Small Subunit (*TssB)*	Contractile Sheath	Cianfanelli et al.
Contractile Sheath: Large Subunit (*TssC*)	Contractile Sheath	Cianfanelli et al.
Hcp (TssD)	Expelled Tube	Cianfanelli et al.
TssE	Baseplate	Cianfanelli et al.
TssF	Baseplate	Cianfanelli et al.
TssG	Baseplate	Cianfanelli et al.
*TssH**	Sheath Recycling/ATPase	Cianfanelli et al.
VgrG (TssI)	Expelled Spike	Cianfanelli et al.
TssJ	Membrane Complex	Cianfanelli et al.
TssK	Baseplate	Cianfanelli et al.
DotU (TssL)	Membrane complex	Cianfanelli et al.
TssM	Membrane complex	Cianfanelli et al.
Protein with *Duf4150 domain*	*PAAR* analogue - tip spike/Effector Toxin	Bondage et al.
Protein with *Duf2169 domain*	Links *VgrG* to Effector Protein	Bondage et al.
TagF	Post-translational regulation	Cianfanelli et al.
Protein with *FHA* domain	Post-translational regulation	Cianfanelli et al.

Table 8: T6SS Components Found in *A. faecalis* genome database and their conserved or proposed function in the T6SS of studied bacteria, (* not present in the T6SS loci of all *A. faecalis* strains).

T6SS in *A. faecalis* ATCC 8750 and *A. viscolactis* Wards

Due to lack of a complete genome sequence for *A. faecalis* ATCC #8750, primers were first designed to amplify a partial sequence and then the full sequence of the *VgrG* gene to determine if a putative T6SS might be present in our laboratory's strain. *VgrG* is an approximately 2.3 kb gene that codes for a protein forming the spike of the T6SS. PCR and gel electrophoresis confirmed presence of *VgrG* in *A. faecalis* ATCC #8750 and our strain of *A. viscolactis* **(Fig. 8a & 8b)**. Additionally, we later successfully amplified the *Hcp* (*TssD*) gene in both *A. faecalis* and *A. viscolactis* **(Fig. 8c)**. The *Hcp* gene encodes an essential protein that in T6SS-containing organisms forms a hexameric inner tube structure that is later expelled upon contraction. Because its genome sequence has never been recorded or published, these results are the first to our knowledge that show evidence of a T6SS in *A. viscolactis*, further pointing to the possibility that this system is responsible for the observed microbicidal phenotype of both *Alcaligenes* species.

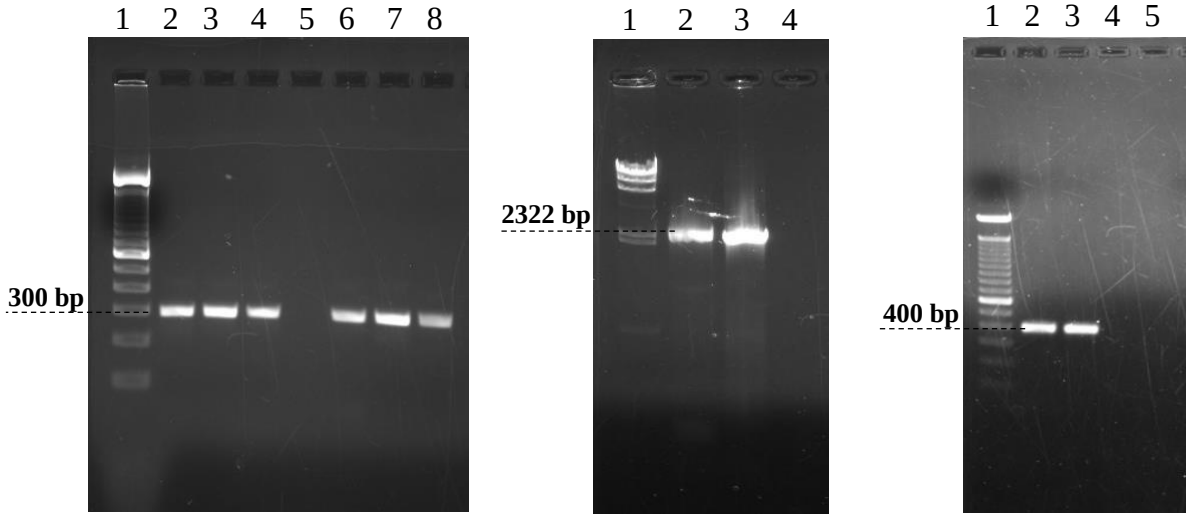


Figure 8: (a): Amplification of small *VgrG* fragment (276 bp): Lane 1: 100 bp ladder, lane 2-4: *A. faecalis* gDNA, lane 6-8: *A. viscolactis* g*DNA,* **(b**) Amplification of large *VgrG* seq. (2.276 kb): Lane 1: λ DNA-HindIII ladder, Lane 2: *A. faecalis* gDNA, Lane 3: *A. viscolactis* gDNA, **(c)** Amplification of *Hcp* (388 bp): Lane 1: 100 bp ladder, Lane 2: *A. faecalis* gDNA, Lane 3: *A. viscolactis* gDNA.

Sequencing of *VgrG*

Forward and reverse nucleotide sequences were merged using Emboss Merger and compared to sequences in the NCBI genome database using BLAST. Th

The 273 bp sequence obtained from *A. faecalis* ATCC 8750 shared a 98.84% similarity with the *VgrG* sequences from *A. faecalis* strains DSM30030 and FDAARGOS_491 and greater than 92% similarity to other strains. The 275 BP sequence obtained from *A. viscolactis* shared 99.27% similarity with the *VgrG* sequences from *A. faecalis* strains DSM30030 and FDAARGOS_491 and greater than 92% similarity to other *A. faecalis* strains. Using EMBOSS Needle, both sequences were aligned and analyzed, and comparison revealed a 97.1% similarity between the obtained *VgrG* sequences of *A faecalis* and *A. viscolactis*. While only a small segment was successfully sequenced, these results confirm that the designed primers amplified the *VgrG* gene and points to a high degree of similarity between the *VgrG* genes of both *A. faecalis* and *A. viscolactis* **(Fig. 9)**.

```
AFVgrGpartial      1 ATG-TGACAGA--GCC-TGTAC-AGTGCATAGCCCGTTGGGGCCGGAAGT     45
                     | |||||||  ||| ||||| ||||||||||||||||||||||||||||
AVVgrGpartial      1 --GCTGACAGAACGCCTTGTACAAGTGCATAGCCCGTTGGGGCCGGAAGT     48

AFVgrGpartial     46 CTTGCTGTTCCAGGCTCTGCAGGGTCGAGAAGCCTTGTCACAGCCCTTTG     95
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
AVVgrGpartial     49 CTTGCTGTTCCAGGCTCTGCAGGGTCGAGAAGCCTTGTCACAGCCCTTTG     98

AFVgrGpartial     96 AATTGCAAGTGGACGTGGTCTGCAATACTCCCTTGCTGGATTTGCGCAGC    145
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
AVVgrGpartial     99 AATTGCAAGTGGACGTGGTCTGCAATACTCCCTTGCTGGATTTGCGCAGC    148

AFVgrGpartial    146 TTGCTGGGCGAGTCCCTGAGCCTGGAAATCAAAACACCTTTTGCGGTGCC    195
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
AVVgrGpartial    149 TTGCTGGGCGAGTCCCTGAGCCTGGAAATCAAAACACCTTTTGCGGTGCC    198

AFVgrGpartial    196 ACGTTTTCTGGATGGCCAGCATCAGTGCGTTTCGCTTGATGGGTAAAACT    245
                     |||||||||||||||| ||||||||||||||||||||||||||||||||
AVVgrGpartial    199 ACGTTTTCTGGATGGCC-GCATCAGTGCGTTTCGCTTGATGGGTAAAACT    247

AFVgrGpartial    246 GGCCGCAGCGACCGCTTCTACACGTATC    273
                     ||||||||||||||||||||||||||||
AVVgrGpartial    248 GGCCGCAGCGACCGCTTCTACACGTATC    275
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Figure 9: Alignment of *A. faecalis* and *A. viscolactis* partial *VgrG* sequences obtained from PCR using constructed primer pair VgrGpF/VgrGpR.

Construction and Transfer of a VgrG Deletion Product via SOE

Because the *VgrG* gene encodes an essential part of the T6SS, construction of a *VgrG* knockout mutant would allow us to determine what role, if any, the T6SS plays in the broad-spectrum, antimicrobial phenotype displayed by *A. faecalis* and, by extension, *A. viscolactis*. Using SOE primer pairs, approximately 1000 bp regions flanking *VgrG* in *A. faecalis* were amplified via PCR **(Fig. 10a)**. A second PCR reaction fused the two segments together, forming a deletion product **(Fig. 10b)**. This product was successfully ligated into pEX18Gm and transformed into *E. coli* NEBα5 by heat shock. Blue/white screening and colony PCR both confirmed presence of the recombinant plasmid carrying the SOE product (**Fig. 10c & 11)**.

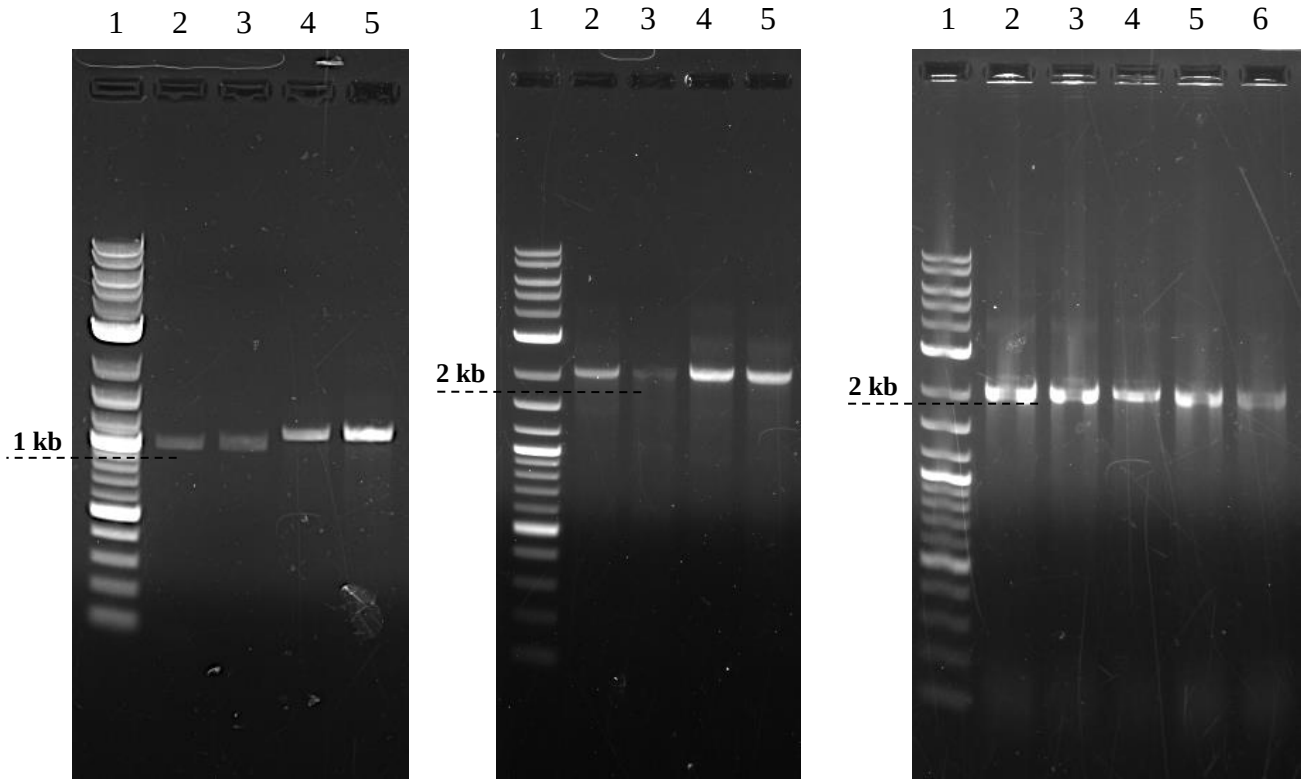


Figure 10: Visualization of PCR amplification on 1% agarose gel with NEB 1 kb plus DNA ladder: **(a)** Lanes 2-3: upstream SOE fragment, Lanes 4-5: downstream SOE fragment, **(b)** Lanes 2-5: SOE crossover product **(c)** Lanes 2-6: SOE product in transformed NEBα5 colonies.

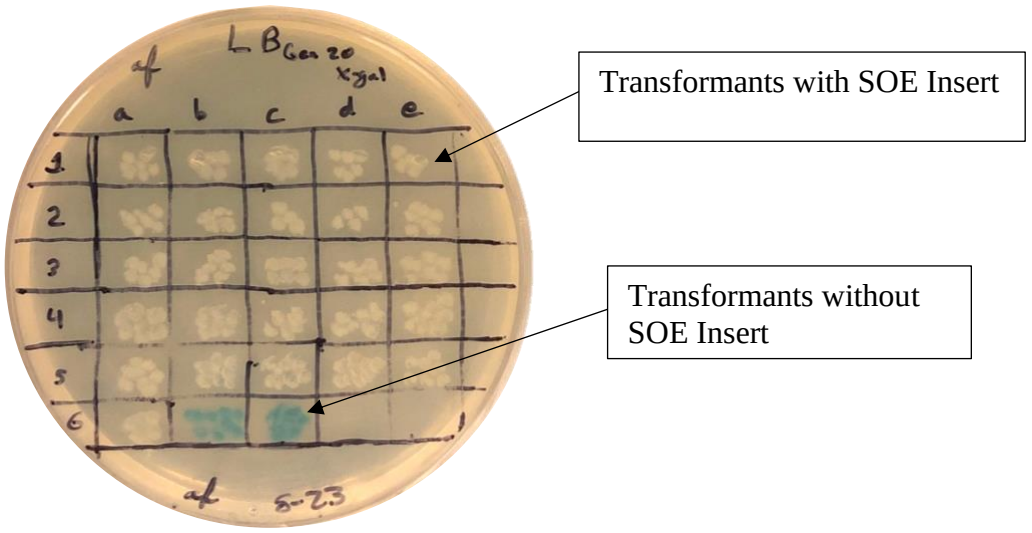


Figure 11: Blue/White Screening for Recombinant NEBα5 Colonies Containing SOE *VgrG* Deletion Product.

Triparental Mating

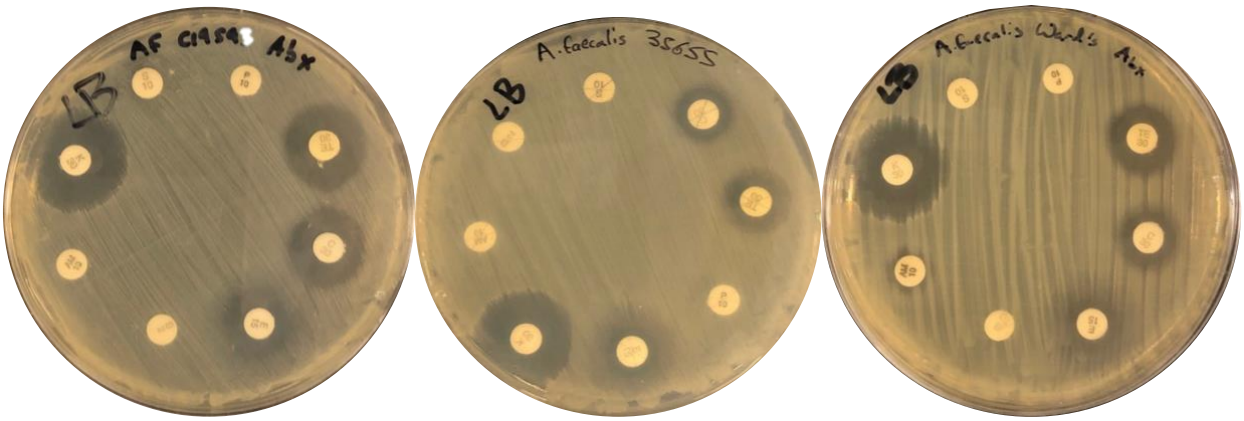
Despite successful construction and insertion of pEX18Gm*ΔVgrG* into NEBα5, triparental mating with pRK2013 repeatedly failed to transfer the plasmid to *A. faecalis*. Increasing incubation time to 48 hours and modifying incubation temperature from 37°C to 42°C had no effect. Biparental mating with SM10λpir and *A. faecalis* was also attempted, and conjugation was unsuccessful as before. Without the ability to insert plasmids into *A. faecalis*, we were unable to create a *VgrG* knockout mutant and ascertain whether the putative T6SS of *A. faecalis* is responsible for our observations. Despite hundreds of attempts by members of our lab and using multiple methods including conjugation, heat shock, and electroporation, both *A. faecalis* and *A. viscolactis* have proved resistant to horizontal gene transfer. Despite other studies' success using conjugation and electroporation with *A. faecalis*, strains other than ATCC #8750 were used [48,49]. This raises the question as to whether this strain along with our lab's strain of *A. viscolactis* possess unknown barriers to the uptake of foreign DNA such as restriction enzyme activity [50].

Antibiotic Susceptibility and Phenotypic Profile of Other *A. faecalis* Strains

An antibiotic susceptibility profile for two new *A. faecalis* strains was carried out so that a suitable selective media for conjugation could be determined (**Table 9**). Unlike strain #8750, strains C19593, #35655, and Wards were resistant to Streptomycin in addition to Ampicillin, Penicillin, and Bacitracin (**Fig. 12)**. While these strains showed the greatest sensitivity to Kanamycin, #35655 demonstrated a large decrease in sensitivity to Chloramphenicol, Tetracycline, and Erythromycin **(Fig 12b)**. All three strains retained the inhibitory phenotype against clinical *S. aureus* **(Fig. 13)**.

Antibiotic	**Concentration (MCG)**	***A. faecalis C19593* Susceptibility**	***A. faecalis* #35655 Susceptibility**	***A. faecalis Wards* Susceptibility**
Ampicillin	10	-	-	-
Bacitracin	2	-	-	-
Chloramphenicol	30	+	+	+
Erythromycin	15	+	+	+
Kanamycin	30	+	+	+
Penicillin	10	-	-	-
Streptomycin	10	-	-	-
Tetracycline	30	+	+	+

Table 9: Susceptibility Profile of *A. faecalis* C19593 and *A. faecalis* ATCC #35655 to common antibiotics.



(a) **(b)** **(c)**

Figure 12: Antibiotic Susceptibility Profile of *A. faecalis* strains **(a)** C19593, **(b)** ATCC #35655, and **(c)** Ward's.

(a) **(b)** **(c)**

Figure 13: Spot-tests of *A. faecalis* strains **(a)** C19593, **(b)** ATCC #35655, and **(c)** Ward's on lawns on clinical *S. aureus*.

CONCLUSION

Due to the rise of drug-resistance in bacterial and fungal species, the search for innovative approaches to combating these often pathogenic organisms is urgent. The realm of polymicrobial interactions is particularly promising as organisms have often developed antimicrobial mechanisms to outcompete other species. One such system can be found in *Alcaligenes faecalis* and *Alcaligenes viscolactis*, which has been found to inhibit *S. aureus* and *C. albicans* via a microbicidal, contact-dependent mechanism. This thesis builds on these previous findings, showing that both *Alcaligenes* species retain this ability when grown in a biofilm with *S. aureus* and kill a broad range of organisms including *C. glabrata* and a variety of Gram-positive and even Gram-negative bacteria. Additionally, it provides preliminary evidence for the potential existence of a putative T6SS in *A. faecalis* and *A. viscolactis*, which may be responsible for our observations. If implicated, this T6SS would be unique due its ability to target not only eukaryotic *Candida* species and certain Gram-negative bacteria but also a range of Gram-positive organisms, an ability not currently considered possible in the studied T6SS of other bacteria [35]. Future studies of the T6SS in *Alcaligenes faecalis* may also lead to discovery of a new class of effector molecules. Because of a consistent inability to induce plasmid transfer via conjugation and other forms of gene transfer, the exact mechanism for *Alcaligenes*' inhibitory effect remains elusive, and an entirely unknown system may be at play. Future directions include RNA isolation and analysis for upregulation of T6SS genes during co-cultures and use of newly acquired *A. faecalis* strains for transposon and site-directed mutagenesis.

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