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Anti-Staphylococcal Activity of Variovorax paradoxus EPS

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Anti-Staphylococcal Activity of *Variovorax paradoxus* EPS

A Thesis

Presented to the

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In Partial Fulfillment

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by

Patricia S. Holt-Torres

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Introduction

Evolution of Antibiotics and Antibiotic Resistance in environmental and soil bacteria

Antibiotic resistance genotypes evolved over two billion years ago (1). Antibiotic resistance genes were acquired by gram-positive bacteria through horizontal gene transfer approximately 800 million years ago (1). Human use of antibiotics, including clinical use and agricultural production, has exacerbated the development of antibiotic resistance since the 1940's when industrial production of antibiotics began. Examination of plasmids isolated from pre-1940 bacterial collections contain few resistance genes suggesting that the presence of these genes may significantly contribute to the alteration of microbiota. This also demonstrates that human use of antibiotics is linked to the spread of antibiotic resistance (2).

Multiple theories have been suggested to explain the evolution of antibiotics and antibiotic resistance genes in bacteria. One theory is that these genes evolved both as a form of protection against competing microorganisms in the environment and for protection against self-made antibiotics. An alternative hypothesis is that low levels of antibiotic production will support functions such as communication between bacteria as well as gene expression in some cases (1,3).

Antibiotic Classes and Mechanisms

Antibiotics can be bacteriostatic or bactericidal in action with different chemical structures, and target a variety of cellular mechanisms based on antibiotic class (4). The quinolone class of antibiotics target both DNA gyrase and topoisomerase IV(5) during DNA replication through the formation of a reversible quinolone-topoisomerase-DNA complex that

prevents DNA replication. This prevents of the formation of phosphodiester bonds (4) and incites an SOS stress response and cell filamentation (5).

Macrolides are a class of antibiotics synthesized by non-ribosomal peptide synthetases during secondary metabolism and are typically bacteriostatic in action. Macrolides bind to ribosomal RNA machinery at the nascent peptide exit tunnel (NPET) during translation. This results in inhibition of bacterial growth by interrupting the elongation of new polypeptides (6,7,8). Erythromycin, isolated from *Saccharopolyspora erythraea* is an example of the macrolide class of antibiotics (6,7).

β-lactam antibiotics such as penicillin and methicillin are also synthesized by non-ribosomal peptide synthetases (1). The chemical name for β-lactam is 2-azetidinone and is known as "the enchanted rings" for its safety in use. The β-lactam ring is the minimum structural component needed for antibiotic activity. (**Figure 1a**). The chemical structure of a β-lactam is a four-membered, cyclic amide ring with a carbonyl group consisting of three carbons, one nitrogen, and five hydrogen atoms (C_3NH_5). Methicillin ($C_{17}H_{20}N_2O_6S$) is a semi-synthetic, β-lactamase resistant, and bactericidal derivative of penicillin that has been in use since 1960 (**Figure 1b**).

β-lactam rings are structurally similar to the D-ala-D-ala linkages between N-acetylmuramic acid subunits in peptidoglycan (**Figure 1c**). β-lactam rings target and covalently bond to transpeptidase active sites of Penicillin-binding proteins (PBPs), causing inhibition of cell wall synthesis (8,9). There are two classes of high molecular weight PBPs, aPBPs (class A) and bPBPs (class B) as well as low molecular weight PBPs. Class A PBPs contain glycosyltransferase and transpeptidase activity. The glycosyltransferase domain polymerizes glycan elements and

transpeptidase activity cross-links them to create D-Ala-D-Ala linkages between N-acetylmuramic acid subunits. Currently, the only known function of bPBPs is transpeptidase activity.

Peptidoglycan bonds are cleaved by low molecular weight PBPs and are also targeted by β-lactams (8). Inhibition of peptidoglycan synthesis by β-lactamases leads to weakening of the cell wall followed by cell lysis (8).

Antimicrobial Resistance

Bacteria resist antibiotics through modification of the target, modification or hydrolysis of the antibiotic, or through efflux pumps. Efflux pumps may be single substrate specific or have the ability to export multiple substrates of unrelated classes (10, 11). The latter of these efflux systems can confer resistance to multiple antibiotics with the gain of a single gene, affording multidrug resistance to bacteria that possess them (10). Single point mutations within antibiotic resistance genes may generate modification of antibiotic targets. Alterations in the target confer antibiotic-resistance while allowing cellular function to continue (11). In addition, the creation of "mosaic" genes may occur as a result of uptake of environmental DNA that provide antibiotic resistance through alteration of antibiotic target proteins (11).

The gain of homologous genes that produce an altered antibiotic target provides an additional mechanism of resistance. Staphylococcal Cassette Chromosome *mecA* (SCC*mecA*) encodes for Penicillin-binding-protein 2a (PBP2a), which allows *Staphylococcus* to continue peptidoglycan synthesis in the presence of β-lactam antibiotics targeting Penicillin-binding-proteins (PBPs) (11).

Enzymatic activity of phosphotransferases, acetyltransferases, and nucleotidyl transferases alter aminoglycoside antibiotics such as Gentamycin. This activity produces steric hindrance and prevents antibiotic molecules from complexing with target proteins. This results in antibiotic

resistance in host bacteria such as *Campylobacter coli.* A strain of *C. coli* identified in China contains a novel genomic island encoding for phosphotransferases, acetyltransferases, and nucleotidyl transferases. Aminoglycoside antibiotics are used to treat *Campylobacter* infections (11).

Bacteria are ubiquitous to most environments and prokaryotic genes that encode for antibiotic synthesis and antibiotic resistance pathways are ancient and may have evolved over a time span of billions of years. Bacteria isolated within Lechuguilla Cave, New Mexico for over four million years were found to be reservoirs for antibiotic resistance genes. Surprisingly, resistance to modern semi-synthetic antibiotics such as daptomycin was identified within the genomes of some of the culturable isolates (2). The development of resistance to antibiotics has become a worldwide challenge affecting over two million individuals and causing over twenty-three thousand deaths annually (12). In 2013, the World Economic Forum (WEF) stated that, "arguably the greatest risk…to human health comes in the form of antibiotic-resistant bacteria. We live in a bacterial world where we will never be able to stay ahead of the mutation curve. A test of our resilience is how far behind the curve we allow ourselves to fall." (13)

Exposure of pathogens to antimicrobials has not been limited to the "antibiotic era" of the past 100 years, which began with the development of Salvarsan as a treatment for Syphilis during the early 1900's. *Bald's Leechbook*, a 1000-year-old English medical text, contains a plant-based recipe to treat a "wen" (thought to be a sty). This recipe was recently tested by Harrison, F., et al. (2015) and was shown to have bactericidal activity against pathogens such as *Staphylococcus aureus* (14). Also, traditional Chinese medicine has used qinghaosu, extracted from *Artemisia* species, to treat illnesses for thousands of years (14,16). Finally, honey has been used as an

antimicrobial by Egyptians and was used in armed combat during the 20th century for antimicrobial treatment of wounds (15).

Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus are Gram-positive cocci that commonly inhabit the skin of 20% of individuals and nasal passages of 33% of the human population, as well as being carried by some animals (18,19). Resistance to methicillin was acquired by *Staphylococcus aureus* in 1961, just one year after this drug was introduced as the first semi-synthetic antibiotic developed to treat Staphylococcal infections (18). Now, 80% of all *Staphylococcus aureus* isolates are resistant to penicillin and many have developed resistance to methicillin and vancomycin (20). Vancomycin is seen as a "last resort" antibiotic for the treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) and its use has led to several MRSA strains that now carry vancomycin resistance genes. These strains are usually multi-drug resistant as well, and pose a major threat in the treatment of staphylococcal infections (21,22).

Resistance to methicillin was originally acquired in a hospital setting and MRSA is now the most commonly recognized antibiotic-resistant microorganism in the USA and is known as Healthcare-Associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) (17). Some clones of HA-MRSA have spread globally, including the archaic clone ST250/SCC*mecI* and strain N315 discovered in Japan, where they have evolved and adapted in different regions of the world (3,20). Approximately 70% of HA-MRSA infections worldwide are caused by 6 pandemic clones. These MRSA strains carry SCC*mec* types I-III (3,17).

Community-Acquired Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) emerged after HA-MRSA and was identified in 1981 with the detection of SCC*mec* type IV (17). The Centers for

Disease Control defines CA-MRSA as, “MRSA from outpatients (patients in the community) with no history of hospitalization, surgery, dialysis, or indwelling percutaneous medical devices and catheters in the past one year or inpatients isolated within 48 hours after hospital admission.” CA-MRSA infections typically occur in family and athletic groups, and in disaster relief evacuees. Infections occur in healthy individuals through skin-to-skin contact as skin and soft tissue infections (SSTIs), and from deep tissue infections such as carbuncles and cellulitis. The most common strain causing CA-MRSA found in the United States is USA300 (3).

Before acquiring resistance to methicillin, *Staphylococcus aureus* may be methicillin-susceptible and is designated Methicillin-Susceptible *Staphylococcus aureus* (MSSA) (17,23,24). MSSA gains resistance to methicillin through acquisition of a plasmid containing the mobile genetic element SCC*mecA* (23). Acquisition of β-lactam resistance occurs when *SCCmecA* is inserted into the chromosome of MSSA (23). *MecA* encodes for Penicillin- Binding Protein 2a (PBP2a or PBP2’ in the United Kingdom) (19). *SCCmec* also contains site-specific cassette chromosome recombinases A, B, or C (*ccrA*, *ccrB*, or *ccrC*) and their surrounding open reading frames (ORF’s) (23).

J regions are non-essential, sometimes called “junkyard” or “joining” regions, and carry supplementary antimicrobial resistance elements. There are three identified J regions for SCC*mec*: J1, which is located between the right junction of the chromosome to the *ccr* genes; J2, located between the *ccr* genes and the *mec* complex; and J3, located between the *mec* complex and the left portion of SCCmec (19).

Currently there are eight types of Scc*mec* elements that have been described. Classification is based upon a combination of one of five *ccr* gene complexes (1 through 5) and one of four *mec* gene complexes (A, B, C1, and C2) (23).

Novel antibiotics

The necessity for research and development of novel antibiotics has increased as antibiotic-resistant bacteria have spread during the “antibiotic era”. Conversely, pharmaceutical development of novel antibiotics has decreased significantly in the past 30 years (25,26). Insufficient economic incentives are the most common reason given for decreasing research and development (25), which can span ten to twelve years before release to market occurs (27). Historically, pharmaceutical companies base their income on a price-per-volume model with an expectation of higher pricing and/or higher volumes sold to increase sales profits and a return on investment for research funds expended. Generic antibiotics, which typically have lower consumer costs and are still effective in some circumstances, compete with novel antibiotics in clinical settings. This competition typically maintains lower costs overall for both generic and novel antibiotics and creates a situation where the return on investment for research and development of new drugs is difficult to achieve, further reducing incentives for future development (28). Low profits related to antibiotic sales combined with limited markets and limited government research funding has caused pharmaceutical companies to reallocate funding of antibiotic research and development to areas that will provide greater revenue. By 2013, several major pharmaceutical companies had reduced or discontinued antibiotic research and development altogether (25).

Non-ribosomal peptide synthetases

Non-ribosomal peptide synthetases (NRPSs) are large, multimodular enzymes produced by bacteria and fungi. Through condensation reactions of amino acids in secondary metabolism, NRPSs produce nonribosomal peptides (NRPs) (42,43,44) that consist of approximately five

hundred different monomers, including fatty acids, alpha-hydroxy acids, and non-proteinogenic amino acids (42,45). There are three catalytic domains required for synthesis of NRP's and are crucial to peptide formation. The first is an adenylation (A) domain that activates amino acids, the second is a thiolation or peptidyl carrier protein (PCP) which elongates the peptide chain, and the third are condensation (C) domains that act on amino acids. Termination and release of peptide chains occurs catalytically at a thioesterase domain (TE) (4,46), which is located at a termination module. Some examples of NRP's include the antibiotic daptomycin and the immunosuppressant drug cyclosporin (42).

***Variovorax paradoxus* EPS**

Variovorax paradoxus EPS is a ubiquitous Gram-negative beta-proteobacterium of the family *Comamonadaceae*. Many members of genus *Variovorax* have been identified as constituents of various soils and pond waters through rRNA gene-based detection (29,30,31,32). Catabolic pathways of genus *Variovorax* allow it to breakdown toxins and/or complex carbon compounds in polluted environs and this activity has been the subject of bioremediation studies. Known anabolic activities of genus *Variovorax* include synthesis of the enzymes *N*-acyl-D-amino acid amidohydrolase (function unknown), D-aminase (hydrolyses amino acid amides), and *α*-Methyl serine *aldolase* (catalyzes *α*-Methyl-serine to D-alanine and *α*-hydroxymethylserine to D-serine) (35,37). Currently there are no strains of *V. paradoxus* that have been identified as pathogenic (32).

Variovorax paradoxus EPS strain has previously been identified as producing an exopolysaccharide (30). Exopolysaccharide production combined with type IV pilus production

results in the conversion of *V. paradoxus* EPS from a sessile to a mobile growth phenotype when the correct physical and nutrient conditions are present (30).

The genome of *V. paradoxus* EPS consists of a circular chromosome that is 6,550,056 bases in length with a G+C content of 66.48% (38). Previous genetic analysis has identified three loci suspected to encode for a NRPS. These loci are Varpa_4324--27, Varpa_4519, and Varpa_2887--88. The function of Varpa_4519 has been characterized for surfactant production (34). Varpa_2887--88 is an amino acid adenylation domain (41).

In a study completed by C.W. Johnston, et al. (39), a novel non-ribosomal peptide synthetase-polyketide synthase (NRPS-PKS) gene cluster was identified in *Variovorax paradoxus* P4b (gene cluster identified as Var3 through Var7). The products of this gene cluster are novel metabolites, Variobactin A and Variobactin B. Variobactin A has a molecular formula of $C_{47}H_{84}N_{11}O_7$ and a mass-to-charge ratio (*m/z*) of 1074.60. In a separate study, C. Kurth, et al. (40) studied the plant associated bacterium *Variovorax boronicumulans* BAM-48 to identify a NRPS-PKS gene cluster whose product, Variochelin A, is an acyl-peptide and functions as a photoreactive lipopeptide siderophore. The molecular formula and mass-to-charge values of Variochelin A are equivalent to Variobactin A. In their same study, C. Kurth et al. (40) identified a *Variovorax paradoxus* EPS gene cluster whose function is also a siderophore. This gene cluster consists of genes that correspond to Varpa_4324-27 but are different in both size and organization in comparison to gene clusters identified in both *V. paradoxus* P4b and *V. boronicumulans* BAM-48.

Hypothesis

Preliminary research has shown that *Variovorax paradoxus* EPS has anti-staphylococcal activity in liquid and solid co-culture. Solid plating of co-culture of *V. paradoxus* EPS and *Staphylococcus aureus* has determined that this activity is replicable. Based on activity in broth culture, we predict that the activity is mediated by a small, soluble molecule. Anti-staphylococcal activity of Wild type and *V. paradoxus* EPS **Δ**4519 on 0.5% YE agar with embedded *S. aureus* AH1710 supports the concept of a soluble molecule as the agar acted as a physical barrier between *V. paradoxus* EPS and *S. aureus* colonies. The small molecule is suspected to be the product of a non-ribosomal peptide synthetase (NRPS) (34). Genetic analysis of *V. paradoxus* EPS has identified three loci that are suspected to express the molecule of interest. Preliminary data failed to detect expression at two of the three identified loci and a mutation at this third locus continues to produce anti-staphylococcal activity.

I hypothesize that the microbial agent is expressed at a different locus or loci that have yet been identified. These products are in-part or wholly responsible for the production of the microbial agent and will be controlled by exposure to *Staphylococcus aureus.* The use of a *V. paradoxus* EPS Δ 4519 transposon library to identify phenotypic expression and the genes responsible for this expression, will allow us to identify the genetic component responsible for expression of the small molecule.

We used direct phenotypic and genetic methods to identify the active molecule and the genes involved in its production.

Methods and materials

Media and Culture Conditions

Bacterial strains, relevant genotypes, and culture conditions that included antibiotics required for standard growth conditions used in this study can be found in **Table 1** below.

Overnight cultures of Wild type *Variovorax paradoxus* EPS and *V. paradoxus* EPS **Δ**4519 were grown in selective media (**Table 1**). Overnight cultures of *Staphylococcus aureus* strain AH1710=*S. aureus* RN4220 + pCM29 Cm^R (PsarA_RBSsod_SGFP) or *S. aureus* strain AH3849=*S. aureus* LAC(AH1263) +pHC48 Cm^R (pCM29_dsRed) were grown in Tryptic Soy broth (TSB) + Chloramphenicol 25 mg/L. Overnight cultures of *E. coli* S17-1 λpir (pOT182:Tn5) were grown in LB broth + Tetracycline 25 mg/L or Gentamycin 10 mg/L.

All cultures were incubated at 30℃ with shaking (New Brunswick Scientific Classic Series C24 Incubator Shaker) for 24-48 hours. For quantitative assays, cultures were incubated at 30℃ with shaking (New Brunswick Scientific Classic Series C24 Incubator Shaker) for 24- 72 hours.

Cultures grown with antibiotics were washed 2 times in selective media (**Table 1**), centrifuged at 4000 x *g* for 12 minutes, and re-suspended in selective media (**Table 1**).

T-streak method to determine anti-staphylococcal activity

Wild type *V. paradoxus* EPS and *S. aureus* AH1710 or *S. aureus* AH3849 were inoculated as perpendicular streaks onto 0.5% YE agar (5 g/L Yeast Extract + 0.5% agar). Plates were incubated 480 hours and examined every 24 hours for determination of anti-staphylococcal activity.

RNA extraction of Wild Type *V. paradoxus* EPS

Wild type *V. paradoxus* EPS in FWS media and co-culture Wild type *V. paradoxus* EPS/*S. aureus* AH1710 in FWS media were diluted to density OD_{600} of 0.2 to 0.3 (Thermo Spectronic, Genesys 20, Model number 4001/4) to meet density requirements of the Qiagen protocol. Both culture and co-culture were stored in Qiagen RNAlater RNA Stabilization Reagent (Catalog No./ID: 76104) and if necessary, frozen at -20℃ for future use. For an approximately equivalent number of Wild type *V. paradoxus* EPS cells, 500 µl of culture and 1.0 ml of co-culture were used. RNA extraction was completed using Qiagen RNeasy Mini kit (Catalog No.74104) (48) with 15 mg/ml Lysozyme in TE buffer (10 mM Tris, 1 mM EDTA) increased to 5 mg/ml. The volume of reagents used were: 200 µl of 5 mg/ml Lysozyme in TE buffer, 700 µl Buffer RLT with β-mercaptoethanol, and 500 µl 100% ethanol. Promega DNAse treatment (5 µl RQ1 RNase-Free DNase, 2 µl RQ1 RNase-Free DNase 10X Reaction buffer, 63 µl molecular-grade water) was completed on column. Extracted RNA was re-suspended in 50 µl RNase-free water (48).

Phenol/Chloroform/Isoamyl alcohol 25:24:1 pH 8.0 (Fisher Scientific, Catalog No. BP17521-100) extraction was completed with 50 µl extracted RNA (modified from N.E. Biolabs protocol and Short Protocols in Molecular Biology, 5th Edition pg 2-3). Extracted RNA was mixed with 20 µl of 5 M ammonium acetate, and diluted with RNase-free water to a volume of 500 µl. An equal volume of Phenol/Chloroform/Isoamyl alcohol (PCI) was added and briefly vortexed to mix, followed by centrifugation at maximum speed for 15s. The aqueous layer was transferred to a clean tube. This process was repeated twice followed by chloroform extraction using the same protocol twice. The extracted RNA was precipitated by addition of two volumes of absolute ethanol and incubation at -20°C for a minimum of 30 minutes. The precipitation was centrifuged

at maximum speed for 10 minutes and the supernatant was removed. The pellet was rinsed with 70% ethanol followed by centrifugation at maximum speed for 5 minutes. Ethanol was carefully removed to prevent disturbing the pellet, followed by air drying. RNA was re-suspended in 40 µl RNase-free H_2O. Samples were stored at -20°C or below if not used immediately.

Reverse Transcription of Wild type *V. paradoxus* EPS

Reverse transcription was completed using Promega GoScript Reverse Transcription System (Catalog No. #A5000). The protocol was followed as written using the following component volumes, 2.0 µl experimental RNA, 2.0 µl random primers (500 µg/ml, 1.0 µl luciferase (100 pg/µl), 5.0 µl Nuclease-Free Water, 4.0 µl GoScript 5X Reaction Buffer, 3.2 µl $MgCl_2$, 1.0 µl PCR Nucleotide Mix, 0.5µl Recombinant RNasin Ribonuclease Inhibitor, and 1.0 µl GoScript Reverse Transcriptase.

Expression analysis of anti-staphylococcal activity of *Variovorax paradoxus* EPS

Real-time qPCR using was completed using Wild type *V. paradoxus* EPS cDNA and Wild type *V. paradoxus* EPS/*S. aureus* AH1710 co-culture cDNA at 0 and 46 hours to examine expression of Varpa_2887-88, Varpa_4524-27, and Varpa_4519 (Applied Biosystems StepOnePlus Real-time PCR system v2.2.2). A 40 cycle, three-step run method was used with hold temperature at 95.0℃ for 10 minutes. Temperature cycles for Cycle and Melt curve stages were 95℃ for 15 seconds followed by 59.0℃ for one minute. Primers for qPCR were tested for efficiency using Wild type *V. paradoxus* EPS genomic DNA for Varpa_2887-88, Varpa_4324-27. This determined the most compatible forward and reverse primer sets for each RNA sequence (Refer to **Table 2** for final primer sets used).

Quantitative analysis of anti-staphylococcal activity

Overnight cultures of Wild type or *V. paradoxus* EPS **Δ**4519 in Freshwater Succinate (FWS) media (54) were washed one time in fresh media and re-suspended to a density at OD_{600} of 0.2 to 0.3 (Thermo Spectronic, Genesys 20, Model number 4001/4). The *S. aureus* AH1710 was washed and re-suspended in FWS media at an OD_{600} to create a 2:1 density ratio with *V. paradoxus* EPS. Each culture type and a 1:2 co-culture was incubated at 30℃ for 72 hours. At approximately 24-hour intervals, the CFU's in each culture were determined by dilution plating. *S. aureus* AH1710 culture and co-culture were spot plated onto Tryptic Soy Agar (TSA) and *V. paradoxus* EPS was spot plated onto YE agar. Culture CFU was determined in this manner at 0, 24, 48, and 72 hours. All plates were incubated at 30℃ for 24 to 48 hours. For CFU quantification, six spots of 5 µl culture or co-culture for each dilution value were plated in three replicate series. CFU's were counted after 24 hours incubation for *S. aureus* AH1710 and 48 hours incubation for Wild type or *V. paradoxus* EPS **Δ**4519.

Embedded *S. aureus* AH1710 with Wild type or *V. paradoxus* EPS Δ4519

Experimentation with *S. aureus* AH1710 at density OD_{600} 0.3 (Thermo Spectronic, Genesys 20, Model number 4001/4) was embedded in 0.3% YE agar and spot or spread plated with Wild type *V. paradoxus* EPS at density OD_{600} 0.5 allowed for examination of bactericidal activity over 240 hour post inoculation .

To determine if viable *S. aureus* AH1710 colonies in areas of minimal growth and fluorescence were resistant to *V. paradoxus* EPS, quantitative analysis of a randomly chosen viable *S. aureus* AH1710 colony was completed. Replicated assays determined these *S. aureus*

AH1710 were vulnerable to the anti-staphylococcal molecule at similar rates of previous quantitative analysis and that Green Fluorescent Protein expression was maintained.

Analysis of *V. paradoxus* EPS activity on embedded *S. aureus* AH1710 with pour-over plating

Overnight cultures of Wild type *V. paradoxus* EPS in YE broth or *V. paradoxus* EPS **Δ**4519 in YE broth + Kanamycin 50 mg/L were washed one time and re-suspended to a density at OD_{600} of approximately 0.3 (Thermo Spectronic, Genesys 20, Model number 4001/4). An overnight *S. aureus* AH1710 culture was washed twice in YE broth and re-suspended to a density at OD_{600} of approximately 0.5 (Thermo Spectronic, Genesys 20, Model number 4001/4). Embedded *S. aureus* AH1710 plates were created using cell culture diluted to 10^{-3}. This was combined with YE broth at a 1:5 ratio. This *S. aureus* AH1710/YE broth mixture was combined 1:50 with 0.5% YE agar and plated at 15 ml per plate.

Wild type or *V. paradoxus* EPS **Δ**4519 was diluted to 10^{-4}. This dilution was added at a 1:5 ratio with YE broth. This mixture was spot plated centrally at 5 µl onto embedded *S. aureus* AH1710 plates and allowed to dry at room temperature. Once dry, 5 ml of 0.5% YE agar was poured over inoculated plates and incubated at 30℃ for 24 hours and at room temperature thereafter. Plates were examined every 24 hours to identify *V. paradoxus* EPS colonies displaying anti-staphylococcal activity.

Creation of *Variovorax paradoxus* EPS Δ4519 transposon library

The creation of a *V. paradoxus* EPS **Δ**4519 mutant transposon library used bi-parental mating of *E. coli* S17-1 λpir (pOT182:Tn5, **Figure 2** below) as the donor strain and *V. paradoxus* EPS **Δ**4519 as the recipient strain. The donor strain was grown in LB agar + Gentamicin 10 mg/L broth and incubated at 30℃ for 24 hours. The recipient was grown in YE + Kanamycin 50 mg/L

broth and incubated at 30℃ for 24 hours. Each culture was washed 2 times, centrifuged at 4000 x *g* for 12 minutes, and re-suspended in YE broth. Each culture density was normalized at OD_{600} to 1.0 (Thermo Spectronic, Genesys 20, Model number 4001/4), followed by spot plating of a mixture containing 100 µl of each culture onto YE agar + 10 mM $MgSO_4$ and incubated for 24 hours at 30℃. The mixture was scraped from plates in 1X PBS and stored as 1 ml aliquots containing 15% glycerol in microcentrifuge tubes frozen at −80°C for future use.

Transconjugated *V. paradoxus* EPS **Δ**4519 contain Kanamycin resistance (from **Δ**4519 construction), and Tetracycline resistance (from parental strain *E. coli* S17-1 λpir (pOT182:Tn5). This allowed for selection screening of transconjugated *V. paradoxus* EPS **Δ**4519 on YE agar + Kanamycin 50 mg/L + Tetracycline 25 mg/L. The *V. paradoxus* EPS **Δ**4519 and *E. coli* mixture was spread plated and incubated at 30℃ for 48 hours. Only colonies of transconjugated *V. paradoxus* EPS **Δ**4519 would survive plating with both antibiotics.

Transconjugated *V. paradoxus* EPS **Δ**4519 colonies were scraped from plates in 1X PBS and stored as 1 ml aliquots containing 15% glycerol in microcentrifuge tubes frozen at −80°C for future use. Each 1 ml of culture was determined to have approximately 10,200 CFU/ml of transconjugants.

Confirmation of transconjugation of *V. paradoxus* EPS Δ4519 by Blue-White Screening

Plasmid pOT182 contains a promoterless lacZ gene, allowing for blue-white screening to confirm successful transconjugation of **Δ** Varpa_4519 *V. paradoxus* EPS. Colonies grown on YE 1.5% agar + Kanamycin 50 mg/L + Tetracycline 25 mg/L were picked onto YE agar + 40 µl X-gal and incubated at 30℃ for 48 hours.

***V. paradoxus* EPS Δ4519 transposon screening**

Co-cultures of *V. paradoxus* EPS Δ4519 transposon library or Wild type *V. paradoxus* EPS transposon library, and *S. aureus* AH1710 were grown on YE agar to isolate colonies that over and under expressed the molecule of interest. The *V. paradoxus* EPS Δ4519 transposon library was brought to a density of OD_{600} of 0.000006 (Thermo Spectronic, Genesys 20, Model number 4001/). *S. aureus* AH1710 was brought to a density OD_{600} of 0.0005 (Thermo Spectronic, Genesys 20, Model number01/4). A 1:1 co-culture was spread plated at 100 µl onto approximately one-hundred YE agar plates and incubated at 30℃ for 24-48 hours. Colonies that were isolated included the presence of or lack of a mucoid phenotype, a clear perimeter or no perimeter surrounding a transposon mutant colony, and unusual pigmentation.

Spot plate screening of transposon mutants

S. aureus AH1710 was grown overnight in TSB + chloramphenicol 25 (25 mg/L). This culture was washed two times in YE broth, resuspended to OD_{600} 0.3 (Thermo Spectronic, Genesys 20, Model number 4001/4) and 100 µL was spread plated onto YE agar and TSA. Plates were air dried before spot plating occurred. *V. paradoxus* EPS Δ4519 transposon or Wild Type *V. paradoxus* EPS transposon cultures at a density of OD_{600} of 1.0 (Thermo Spectronic, Genesys 20, Model number 4001/4), were spot plated onto the *S. aureus* AH1710 lawns. Plates were be incubated at room temperature or 30℃ for 24 hours.

Optimal density of *S. aureus* AH1710 for phenotypic expression and zone of inhibition formation by *V. paradoxus* EPS Δ4519

Optimal density was determined using 1:2 and 1:10 dilutions of *S. aureus* AH1710 OD_{600} 0.3 plated as a lawn onto YE agar and TSA and allowed to air dry. *V. paradoxus* EPS Δ4519 was spotted at 5µl onto *S. aureus* AH1710 and incubated at 30°C or room temperature overnight.

Plates were examined for zone of inhibition formation to determine optimal *S. aureus* AH1710 density.

Identification of phenotypic expression and zone of inhibition formation in *V. paradoxus* EPS Δ4519 transposon mutants

To reexamine the effect of incubation temperature and density of *S. aureus* AH1710, the 76 selected transposon mutants of interest were spot plated onto a lawn of *S. aureus* AH1710 to determine phenotypic expression and zone of inhibition. The optimal density of *S. aureus* AH1710 at OD_{600} 0.003 was spread at 100 µl onto YE agar and TSA and allowed to air dry. Plates were incubated at room temperature and examined 24-hours post inoculation for identification of zone of inhibition formation, mucoid phenotype production, and additional phenotypes of interest (Refer to **Tables 5** and **6** below**).**

Production of chemically competent cells

A colony of *E. coli* Top 10 F' cells was inoculated into 1 ml SOB-Mg growth media (Bacto Tryptone 20 g/L, Bacto Yeast Extract 5 g/L, 10 mM 1M NaCl, 2.5 mM 1M KCl) and incubated at 37°C with shaking (New Brunswick Scientific Classic Series C24 Incubator Shaker) overnight. At mid-log phase of growth (OD_{600} density of ~0.3), 500 µl of overnight culture was inoculated into 50 mls of SOB-Mg growth media and incubated at 37°C with shaking (New Brunswick Scientific Classic Series C24 Incubator Shaker) until the culture grew to OD_{600} density ~0.3. This 50-ml culture was divided equally into two 50 ml sterile polypropylene tubes and placed in ice for 10 minutes. Cultures were centrifuged at 2500 rpm for 14 minutes at 4°C to pellet cells and the supernatant discarded. Pelleted cells were gently re-suspended in 8.3 ml of CCMB (10 mM Potassium acetate 1M pH 7, glycerol 100 g/L, $CaCl_2 \cdot 2H_20$ 11.8 g/L, 2.5 mM $MgCl_2 \cdot 6H_20$) followed by incubation on ice for 20 minutes. Cultures were centrifuged at 2500 rpm for 10 minutes at 4°C

and the supernatant discarded. Pellets were re-suspended in 2 ml of CCMB and aliquoted on ice followed by storage at -80°C (49).

Genomic DNA extraction

Genomic DNA was extracted from *V. paradoxus* EPS Δ4519 transposon mutants using Wizard Genomic DNA Purification Kit with protocol as written (50). Genomic DNA was stored at 4°C.

Rescue Cloning of *V. paradoxus* EPS Δ4519 transposon mutants

Rescue cloning of transposon mutants that displayed phenotypes of interest was completed using protocols described by Pehl M, et al. (36) with the following alterations: 10 U *Hin*dIII (Promega) restriction enzyme was used for digestion of genomic DNA. After one-hour incubation at 37°C, the product was spiked with an additional 1 U of *Hin*dIII restriction enzyme and incubated for an additional one hour at 37°C to ensure complete digestion.

Ligation of the digestion product was completed using T4 ligase (Promega). The length of time for heat-shock was increased to one minute and SOC recovery media was increased to 450 µl per 50 µl cells. Cells were plated onto LB agar + Tetracycline 25 mg/L and incubated at 37°C overnight. Cultures of successfully ligated *E. coli* were grown overnight in LB broth + Tetracycline 25 mg/L. Plasmids were extracted using Promega Wizard SV Minipreps DNA Purification System (51) and Sanger sequenced at Retrogen Inc., San Diego, CA (Refer to **Table 5** below). Sequencing results were compared to published *V. paradoxus* EPS genome data (38).

Results

T-streak evaluation of Anti-staphylococcal activity

Current studies of anti-staphylococcal activity began with T-streak plating of Wild type *V. paradoxus* EPS and *S. aureus* AH1710 or *S. aureus* AH3849 onto YE agar and incubated at 30℃ for 480 hours. T-streak analysis demonstrated Wild type *Variovorax paradoxus* EPS spread rapidly through *S. aureus* AH1710 streaks which displayed a corresponding decrease in fluorescence. This provided qualitative analysis of a phenotypic response of Wild type *V. paradoxus* EPS to the presence of *S. aureus* (**Figures 3 and** 4.

Real time qPCR

Real-time qPCR (RT-qPCR) using Wild type *V. paradoxus* EPS RNA extracted from culture and co-culture with *S. aureus* AH1710 at zero and 46 hours was performed to examine expression of Varpa_2887-88, Varpa_4524-27, and Varpa_4519 to determine the locus or loci responsible for expression of the anti-staphylococcal molecule.

Preliminary results of RT-qPCR indicate that Varpa_4519 is expressed by Wild type *V. paradoxus* EPS when exposed to *S. aureus* in liquid co-culture from zero to 46 hours, but Wild type *V. paradoxus* EPS liquid culture minimally expressed Varpa_4519 for the same time period (**Figure 5**)**.** Expression of Varpa_2887-88 and 4324-27 could not be detected.

Quantitative analysis of anti-staphylococcal activity in liquid co-culture

Quantitative analysis of anti-staphylococcal activity was completed using Wild type or *V. paradoxus* EPS **Δ**4519, and *S. aureus* AH1710 in both culture and co-culture. All cultures were incubated in FWS media and sampled at approximately 24-hour periods to for viable plate counts (**Figures 6-9**). FWS is a minimal media broth that provides succinate as a carbon source and NH_4Cl

as a nitrogen source. Exponential growth of *V. paradoxus* EPS is possible with these limited nutrient resources. In a preliminary time course assay, it was found that *S. aureus* populations subsist in FWS, but did not grow in density. This variation in growth rates of *V. paradoxus* EPS and *S. aureus* allow for measurement of anti-staphylococcal activity without concern for separation of growth and death rates during analysis. These assays allowed for comparison of Wild type and *V. paradoxus* EPS **Δ**4519 to determine if anti-staphylococcal activity occurred in the mutant strain and if the rate of anti-staphylococcal activity differed in comparison of wild type and mutant strains. **Figure 6** demonstrates CFU formation of Wild type *V. paradoxus* EPS and *S. aureus* AH1710 in co-culture and *S. aureus* AH1710 in culture. Growth of Wild type *V. paradoxus* EPS in culture and co-culture indicate that over 70 hours, CFU's remain consistent between groups. In comparison, *S. aureus* AH1710 CFU's decrease approximately 99% at 70 hours in co-culture, but remains consistent between groups in mono-culture (**Figure 7)**. **Figures 8** and **9** demonstrate similar CFU formation of *V. paradoxus* EPS **Δ**4519 and *S. aureus* AH1710 in co-culture and *S. aureus* AH1710 in culture. Comparison of anti-staphylococcal activity between Wild type *V. paradoxus* EPS and *V. paradoxus* EPS **Δ**4519 demonstrates that *S. aureus* AH1710 in co-culture with *V. paradoxus* EPS **Δ**4519 decreased more significantly at 48 hours than in co-culture with Wild type *V. paradoxus* EPS.

Preliminary results of RT-qPCR for Wild type *V. paradoxus* EPS indicate Varpa_4519 is expressed in the presence of *Staphylococcus aureus* **(Figure 5).** In co-culture, *V. paradoxus* EPS **Δ**4519 exhibits anti-staphylococcal activity, indicating Varpa_4519 is not required for anti-staphylococcal activity. When examined together, results for RT-qPCR of Wild type *V. paradoxus* EPS and quantitative analysis of Varpa_4519 suggest there is either overexpression of precursors

to Varpa_4519 that are responsible in whole or part for the anti-staphylococcal activity, or exposure to *S. aureus* initiates a global recognition system that expresses the anti-staphylococcal molecule of interest.

Embedded *S. aureus* AH1710 with Wild type or *V. paradoxus* EPS Δ4519

Experimentation with *S. aureus* AH1710 embedded in 0.3% YE agar and spot or spread plated with Wild type *V. paradoxus* EPS allowed for examination of bactericidal activity over 240 hour post inoculation (**Figure 10**). A variation to this protocol used embedded *S. aureus* AH1710 with spot plated Wild type or **Δ**4519 with the addition of a 5 ml 0.5% YE agar overlay.

To determine if viable *S. aureus* AH1710 colonies in these areas of minimal growth and fluorescence were resistant to *V. paradoxus* EPS, quantitative analysis of a randomly chosen viable *S. aureus* AH1710 colony was completed. Replicated assays determined that these *S. aureus* AH1710 were vulnerable to the anti-staphylococcal molecule at similar rates of previous quantitative analysis and that Green Fluorescent Protein expression was maintained (**Figures 11 and 12**).

Isolation of TN5 Transconjugated *V. paradoxus* EPS Δ4519

Transconjugated *V. paradoxus* EPS **Δ**4519 will contain Kanamycin, Tetracycline, and Gentamicin resistance. This allowed for selection screening of transconjugated *V. paradoxus* EPS **Δ**4519 on YE agar + Kanamycin 50 mg/L + Tetracycline 25 mg/L. Because *E. coli* does not grow on YE agar, we expected viable colonies of transconjugated *V. paradoxus* EPS **Δ**4519 only to grow on this agar with selected antibiotics.

Confirmation of transconjugation of *V. paradoxus* EPS Δ4519

Plasmid pOT182 contains a *lacZ* gene, allowing for blue-white screening to confirm successful transconjugation of *V. paradoxus* EPS Δ4519. Successfully transconjugated colonies express β-galactosidase for this screening (**Figure 13**).

Screening of *V. paradoxus* EPS Δ4519 transposon library

Co-cultures of *V. paradoxus* EPS Δ4519 transposon library or Wild type *V. paradoxus* EPS transposon library, and *S. aureus* AH1710 were grown on approximately 100 YE agar plates to screen transposon mutants displaying phenotypes of interest, such as mucoid expression a clear perimeter surrounding a transposon mutant colony, or a change in pigmentation. A total of 206 colonies were chosen for isolation and further study in combination with *S. aureus* AH1710.

Spot plate screening of *Variovorax paradoxus* EPS Δ4519 transposon mutants

Two-hundred six *Variovorax paradoxus* EPS Δ4519 transposon mutants were chosen for examination of anti-staphylococcal activity. These mutants were randomly assigned numbers from one to 97 and 100 to 208 for identification only. Phenotypic expression and zone of inhibition formation were identified for each transposon on TSA agar. Examples of phenotypes examined included *V. paradoxus* EPS Δ4519 transposon mutant colonies that leave remnants of *S. aureus* AH1710 as a hollow shell of green fluorescent protein ("ghosts of staph", **Figure 14** below), with or without a dark ring at the perimeter (**Figure 14** below), colonies with a hazy green or bright green ring at the perimeter (**Figure 15** below), a change in pigment from yellow to creamy/white and mucoid colonies **(Figure 16)**. Of the 206 transposon mutants, 76 were identified for further screening. Twenty-eight of these were selected for rescue cloning and Sanger sequencing in preparation for complementation (Refer to **Table 4** below).

Optimal density of *S. aureus* AH1710 for phenotypic expression and zone of inhibition formation by *V. paradoxus* EPS Δ4519

Plates spot plated to determine optimal density of *S. aureus* AH1710 were examined 24 hours post incubation. It was determined that OD_{600} 0.003 *S. aureus* AH1710 provided sufficient density for zone of inhibition formation by *V. paradoxus* EPS Δ4519 transposon mutants when plated onto YE agar and TSA (**Figures 17** and **18** below).

Identification of phenotypic expression and zone of inhibition formation in *V. paradoxus* EPS Δ4519 transposon mutants

The 76 *V. paradoxus* EPS Δ4519 transposon mutants plated onto TSA at optimal density and room temperature or 30°C incubation displayed varied phenotypic expression and zone of inhibition production amongst the transposon mutants. A zone of inhibition greater than the Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 controls occurred in 18 *V. paradoxus* EPS Δ4519 transposon mutants (ten of these were chosen for Sanger sequencing). Zones of inhibition at both incubation temperatures were produced by 5 transposon mutants (5 sent for Sanger sequencing). Zones of inhibition less than or equal to the control strains were produced by 5 transposon mutants (not sequenced). Please refer to **Tables 3** through **7** below. Mucoid colonies at room temperature occurred in five transposon mutants and in six transposon mutants at 30°C incubation. Mucoid colonies in both room temperature and 30°C incubation occurred in one transposon only (Refer to **Tables 3** through **7** below).

Transformation Efficiency of Chemically Competent Cells

Transformation efficiency of chemically competent cells is 6.0 X 10^6 cfu/µg.

Sanger sequencing

Sequencing results for the 28 *V. paradoxus* EPS Δ4519 transposon mutants selected for over or under expression of anti-staphylococcal activity. Published functions (58) indicated a widespread selection of genotypes that produced zones of inhibition and phenotypes of interest. Of interest is the identification of Varpa_1031, a transposon mutant without zone of inhibition, mucoid colony, and pigmented creamy/white. It is thought to be partially responsible for the production of Exopolysaccharide in *V. paradoxus* EPS. Also, transposon mutant 139 was identified as Varpa_4679 and Transposon mutant 96 as Varpa_4680. Together, these loci are known to express for exopolysaccharide production in Variovorax paradoxus EPS. Transposon mutants 164 and 178 were identified as the same gene, Varpa_4665. A mutation in this gene produces a denser biofilm in culture, is not involved in swarming motility, and is currently under investigation as a regulator of global patterns of gene expression.

Hypothetical proteins were identified for seven of the gene sequences (Refer to **Tables 8** through **11** below).

Discussion

Hypothesis and Non-ribosomal peptide synthetases

In our study of *Variovorax paradoxus* EPS, we have demonstrated that *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 have anti-staphylococcal activity in liquid and solid co-culture under various conditions. When plated with *S. aureus* AH1710, zones of inhibition are present after room temperature incubation. These zones are a classic indicator of antibiotic activity and their presence suggests the antimicrobial produced by *V. paradoxus* EPS is a small soluble molecule similar to known antibiotics. We predicted that a small molecule, the product of a non-ribosomal peptide synthetase, was responsible for anti-staphylococcal activity. Preliminary data suggested that three loci, Varpa_2887-88, Varpa_4524-27, and Varpa_4519 were putative candidates for expression of the small molecule. Expression analysis of these loci indicated a lack of expression of Varpa_4519 in mono-culture at each time point, but significant expression in co-culture at 46 hours. This suggests that Varpa_4619 is controlled at least in part by exposure to *Staphylococcus aureus*. Lack of expression by Varpa_2887-88 and Varpa-4524-27 suggests they are not involved in phenotype expression with exposure to *S. aureus*.

Non-ribosomal peptide synthetases

From our preliminary data, we expected a non-ribosomal peptide synthetase to be responsible for the production of the anti-microbial molecule, however expression analysis did not support this outcome. By screening the large number of colonies produced by our transposon library, it is conceivable, based upon the large size of the *V. paradoxus* EPS genome and the relative size of NRPS genomes, that if an NRPS was responsible for expression of phenotypes of interest, it would have been identified. It could be however, that our screen was insufficient in size or format, and

rescreening of this transposon library may yield results that identify expression by one or more NRPS.

Establishment of Anti-Staphylococcal Activity

To identify *Variovorax paradoxus* EPS and *S. aureus* colonies in co-culture, a gift of Green and Red Fluorescent Protein *S. aureus* was made from Alex Horswill, University of Iowa. This gift allowed for comparison of colony phenotypes and *S. aureus* viability in culture and co-culture as fluorescence diminished over time.

Initial qualitative analysis was completed using T-streak plating of Wild type *V. paradoxus* EPS against *S. aureus* AH1710 or *S. aureus* AH3849 to determine if anti-staphylococcal activity was present. It was determined that fluorescence, and therefore cell viability, decreased almost 100% over the assay period. With these results, it was determined that screening assays for optimal growth conditions to induce anti-staphylococcal activity would need to be developed.

Real-Time qPCR data led us to ask if Varpa_4519 was solely responsible for the anti-staphylococcal activity. However, deletion of this locus did not result in a loss of the anti-staphylococcal activity, indicating that Varpa_4519 was not solely responsible. To avoid the influence of *S. aureus* on Varpa_4519, it was decided to use a Wild Type Transposon mutant, *Variovorax paradoxus* EPS Δ4519, for further examination of anti-staphylococcal activity. In this study.

We have shown on that anti-staphylococcal activity occurs on solid media, but its effectiveness as an anti-staphylococcal drug is unknown at this time. Once the molecule or molecules are identified, determination of toxicity levels may indicate a specified clinical use,

such as topical ointment. This may be an effective delivery system since many Staphylococcal infection occur on or through the skin.

Screening for Optimal Growth Conditions

At the inception of this study, growth conditions to promote expression of anti-staphylococcal activity were unknown. To determine these conditions, assays were developed using various media in liquid and solid forms, and incubation temperatures in different ranges. Optimal results occurred when *S. aureus* was at density OD_{600} 0.003, incubation occurred at room temperature and plating was completed on Tryptic Soy Agar. These results are in part contradictory to previous studies of which indicated that anti-staphylococcal activity does not occur on TSA at 30°C incubation but does occur on YE agar under the same conditions. It is currently not known why there is a significant change in expression when incubation occurs at different temperatures.

During this study, we identified zone of inhibition formation using a dense inoculum of *V. paradoxus* EPS transposon mutants plated onto *S. aureus* AH1710 at a lower density than previously used in assays. This, combined with lower incubation temperature, provided growth conditions that allowed *V. paradoxus* EPS transposon mutants to grow at approximately the same rate as *S. aureus* AH1710, allowing us to observe the expressed inhibitory function. This result supports our hypothesis that *V. paradoxus* EPS produces a small molecule that inhibits or halts the growth of *S. aureus* AH1710. Alternatively, our results indicate that it is not clear if zone of inhibition formation is the result of relative growth rates, a change in anti-microbial expression, a change in staphylococcal susceptibility, or some combination of these effects.

Creation of a *V. paradoxus* EPS Δ4519 Transposon library

Real-time qPCR results indicated that Varpa_4519 was expressed by *V. paradoxus* EPS in co-culture. To determine if Varpa_4519 was responsible in whole or in conjunction with additional gene sequences, a *V. paradoxus* EPS Δ4519 transposon library was created. This library provided the ability to screen for phenotypes that over or under-expressed for anti-staphylococcal activity. Twenty-eight transposon mutants that exhibited phenotypes of interest or a loss of these phenotypes were rescue cloned, Sanger sequenced, and compared to the *V. paradoxus* EPS genome for identification of both the locus and its function (53). Of these Transposon mutants, eleven did not produce mucoid colonies or zones of inhibition, and were pigmented creamy/white. Sequencing results indicate functions that range from hypothetical proteins to regulatory enzymes and transcriptional regulators. Of interest is the identification of Varpa_1031, thought to be partially responsible for the production of exopolysaccharide in *V. paradoxus* EPS.

The remaining sixteen transposon mutants each produced a zone of inhibition at room temperature incubation. Zones of inhibition were not produced by Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 at 30°C incubation, but five of these eleven did so. The implications for this change in expression are currently unknown. Sequencing results indicate functions that also range from hypothetical proteins to regulatory enzymes and transcriptional regulators. Of interest are transposon mutants identified as Varpa_4679-80, members of a putative operon that expresses for Exopolysaccharide in *V. paradoxus* EPS. As identified was Varpa_4665 whose function is biofilm formation and is currently under investigation in the Orwin laboratory as a global regulator of phenotype. With disruption of either Varpa_4680 or Varpa_4665, we see an

increase level of biofilm formation and an increased level of anti-microbial activity. However, it is currently unknown if one or multiple compounds are responsible for observed anti-staphylococcal activity on plates and in liquid culture. Our transposon library analysis has identified genes related to exopolysaccharide production and biofilm formation, but our findings do not conclusive show that antibiotic production is regulated by a system that also controls exopolysaccharide and biofilm formation. Our findings do suggest that their expression may overlap in some way.

Future Plans

Continued work for this study will use the following approaches: The *V. paradoxus* EPS Δ4519 transposon library will be re-screened under optimal conditions for *S. aureus* AH1710 (density OD_{600} 0.003 and room temperature incubation on TSA). This will allow for continued screening of transposon mutants that overexpress for anti-staphylococcal activity as well as the potential identification of a null transposon, a necessity for comparison of phenotypic expression and identification of locus or loci responsible for anti-staphylococcal activity. Rescue cloning will be completed by complementation of genes of interest identified previously. This will confirm the expression of desired phenotypes by *V. paradoxus* EPS Δ4519 transposon mutants. Finally, a comparison of Wild type *V. paradoxus* EPS transposon and *V. paradoxus* EPS Δ4519 transposon genotypes and comparable phenotypes may provide additional information that may lead to the identification of a global regulatory system putatively thought to be controlled in part by Varpa_4665.

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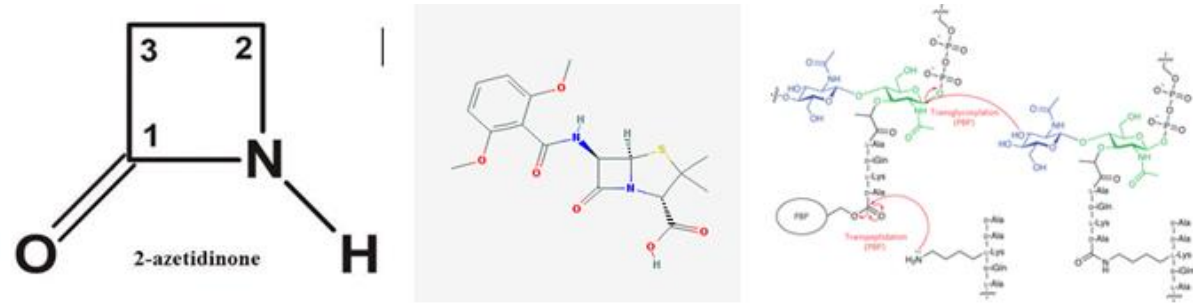
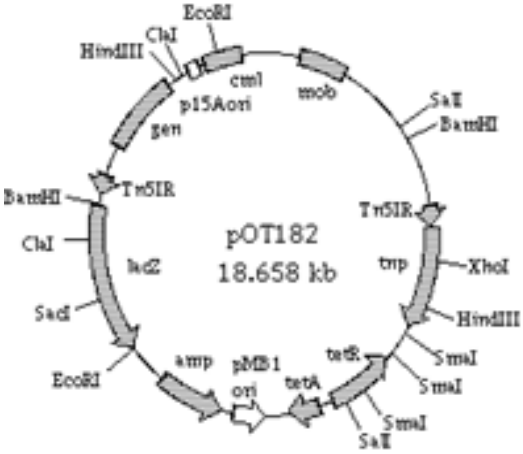
Appendix 1: Figures



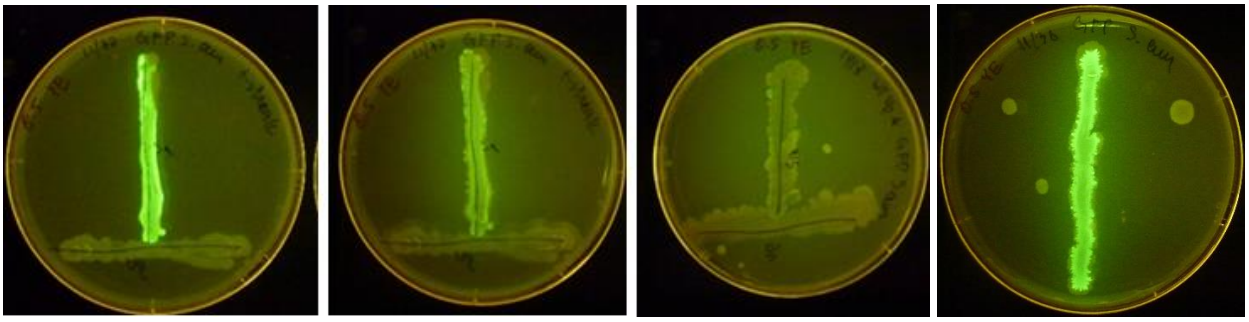
A B C

Figure 1: Class β-lactam structures
Left panel: **B-lactam ring structure** (Chatterjee, S., Ahmed, M., & Wang, F., 2016)
Middle panel: Methicillin (National Center for Biotechnology Information)
Right panel: Peptidoglycan
(http://www.nature.com/nchembio/journal/v12/n7/full/nchembio.2109.html?WT.feed_ name=subjects_chemical-tools)



(https://shigen.nig.ac.jp/ecoli/strain/file/registryFile/pOT182.gif)

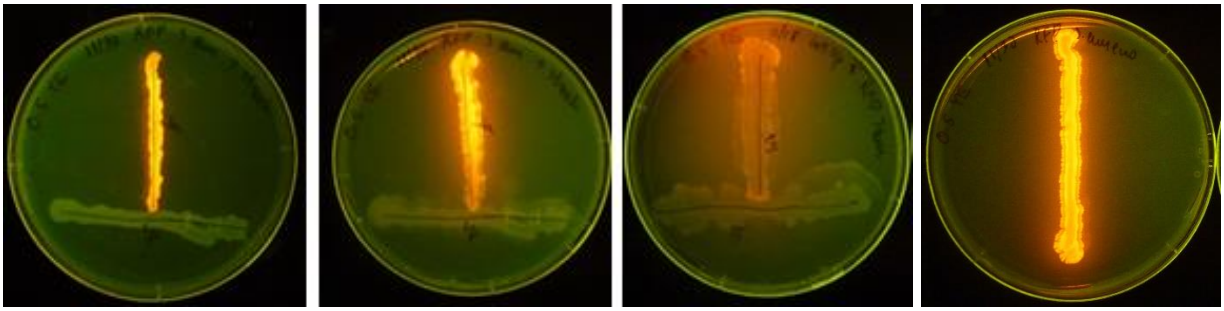
Figure 2: Map of pOT182



a) 48 hours p.i. b) 192 hours p.i. c) 480 hours p.i. d) 220 hours p.i.

Figure 3: T-streak evaluation anti-staphylococcal activity of Wild type *V. paradoxus* EPS and *S. aureus* AH1710 on 0.5% YE agar

Wild type *V. paradoxus* EPS on 0.5% YE agar expresses a mucoid phenotype that can be viewed over time as it grows into and spreads throughout *S. aureus* AH1710. Green fluorescent protein expression diminishes over time as *S. aureus* AH1710 cells loose viability through the activity of *V. paradoxus* EPS. *c*. At 48 hours p.i. *V. paradoxus* EPS has spread through the *S. aureus* streak but florescence has not diminished significantly (Figure 3a). At 192 hours p.i., *V. paradoxus* EPS has continued to spread throughout *S. aureus* but fluorescence has become diminished (Figure 3b). By 480 hours p.i., *V. paradoxus* EPS has spread beyond the border of S. aureus and there is minimal fluorescence at the outer edges only of *S. aureus* only Figure 3c). A control plate of *S. aureus* AH1710 at 220 hours demonstrates the fluorescence is maintained at a higher rate of expression compared to 192 hours plated with *V. paradoxus* EPS (Figure 3d). Images taken using UV lighting (Entela UVP UL3101-1) with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.



a) 48 hours p.i. b) 192 hours p.i. c) 480 hours p.i. d) 220 hours p.i.

Figure 4: T-streak evaluation anti-staphylococcal activity of Wild type *V. paradoxus* EPS and *S. aureus* AH3849 on 0.5% YE agar

Wild type *V. paradoxus* EPS with *S. aureus* AH3849 in a replicate T-streak experiment to Figure 5. The same mucoid phenotype can be seen spreading throughout *S. aureus* AH3849 with the same reduction in fluorescence of *S. aureus* AH3849 over 480 hours p.i. A control plate of *S. aureus* AH1710 at 220 hours demonstrates the fluorescence is maintained at a higher rate of expression compared to 192 hours plated with *V. paradoxus* EPS (Figure 4b). Images taken using UV lighting (Entela UVP UL3101-1) with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.

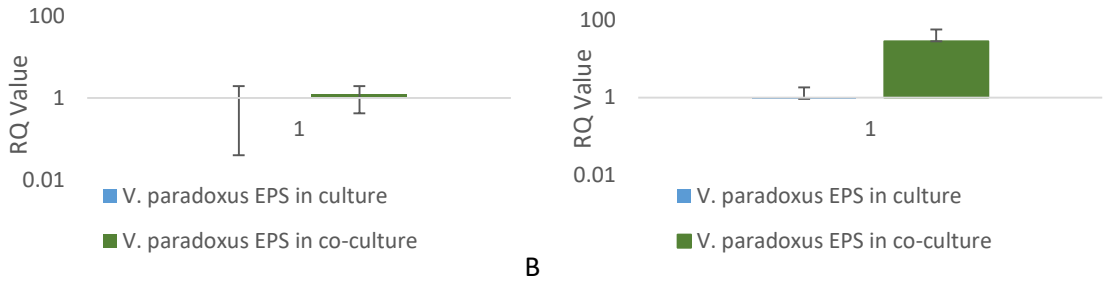


Figure 5: RT-qPCR indicates expression of Varpa_4519 in co-culture only

Zero-hour results indicate approximately equal expression of Varpa_4519 in mono- and co-culture B) 46-hour results indicate approximately equal expression of Varpa_4519 in mono-culture to 0 hour. 46-hour expression of Varpa_4519 in co-culture is significantly increased in comparison to mono-culture at 0 and 46 hours. Expression of Varpa_2887 and Varpa_4324 was not detected. A student's unpaired t-test was performed to compare expression of Varpa_4519 in culture and co-culture at 0 hour (p = 0.87) and 46 hours (p = 0.039).

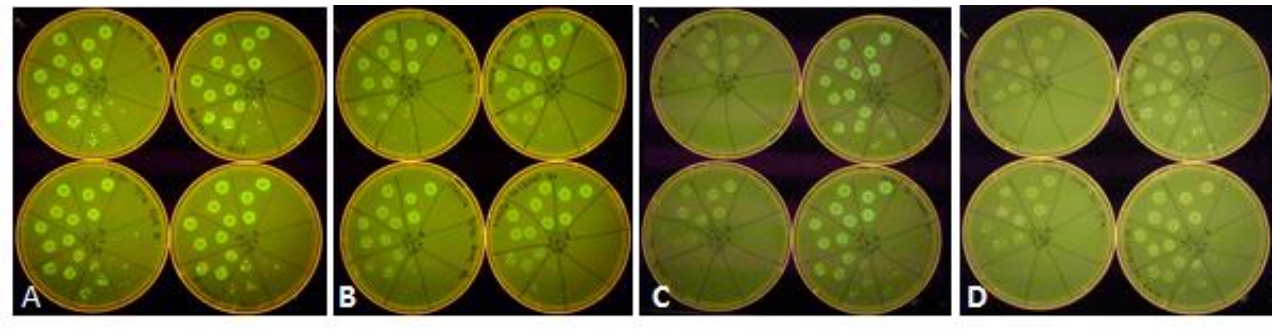


Figure 6: Wild type *V. paradoxus* EPS and *S. aureus* AH1710 co-culture and *S. aureus* AH1710 culture

V. paradoxus EPS/*S. aureus* AH1710 (left side plates) and *S. aureus* AH1710 culture (right side plates). Anti-staphylococcal activity is evident over 70 hours on co-culture plates when compared to 70 hour *S. aureus* AH1710 plates. Images taken using UV lighting (Entela UVP UL3101-1) with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.

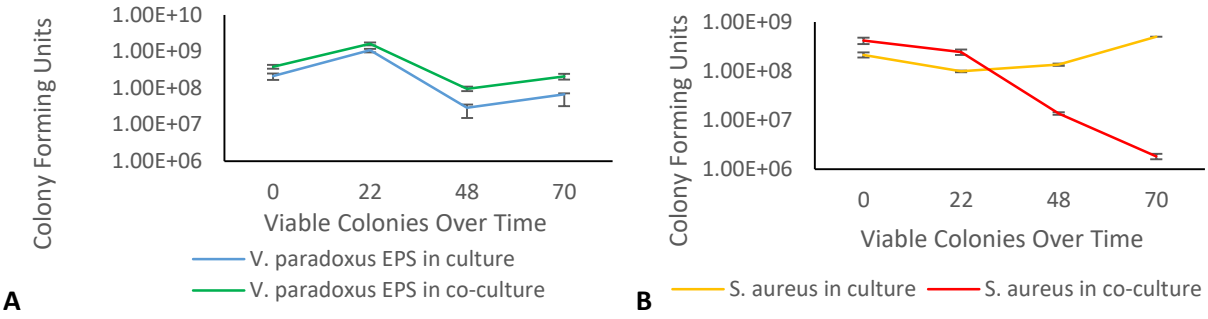


Figure 7: Wild type *V. paradoxus* EPS and *S. aureus* AH1710 Co-culture and *S. aureus* AH1710 Quantitative Analysis

A) Growth of Wild type *V. paradoxus* EPS culture and in co-culture over 70 hours B) Growth of *S. aureus* AH1710 culture and in co-culture over 70 hours. Results indicate anti-staphylococcal activity of *V. paradoxus EPS* occurs when in co-culture with *S. aureus* AH1710.

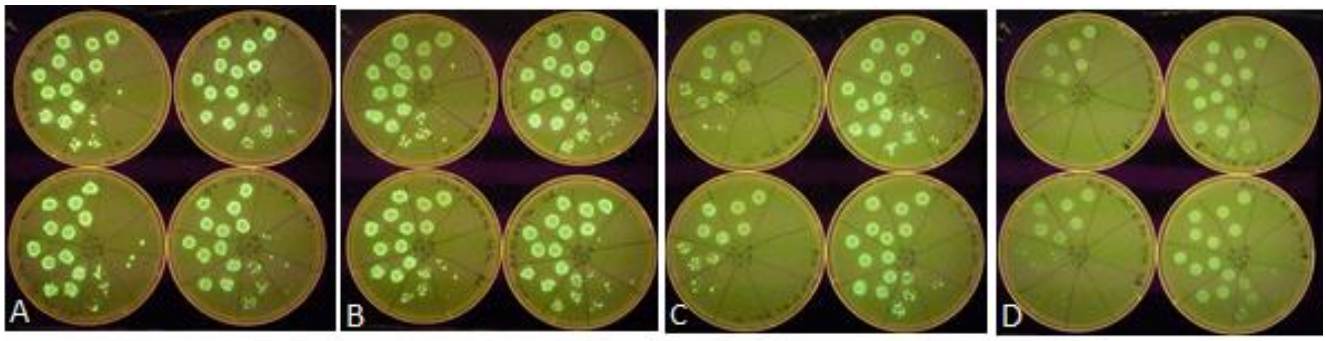


Figure 8: *V. paradoxus* EPS Δ4519 and *S. aureus* AH1710 co-culture and *S. aureus* AH1710

A) 0 hour B) 22 hours C) 48 hours D) 71 hours dilution series comparison of co-cultured

V. paradoxus EPS **Δ**4519/*S. aureus* AH1710 (left side plates) and *S. aureus* AH1710 culture (right side plates). Results suggest that expression of Varpa_4519 is not required for anti-staphylococcal activity to occur. Images taken using UV lighting (Entela UVP UL3101-1) with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.

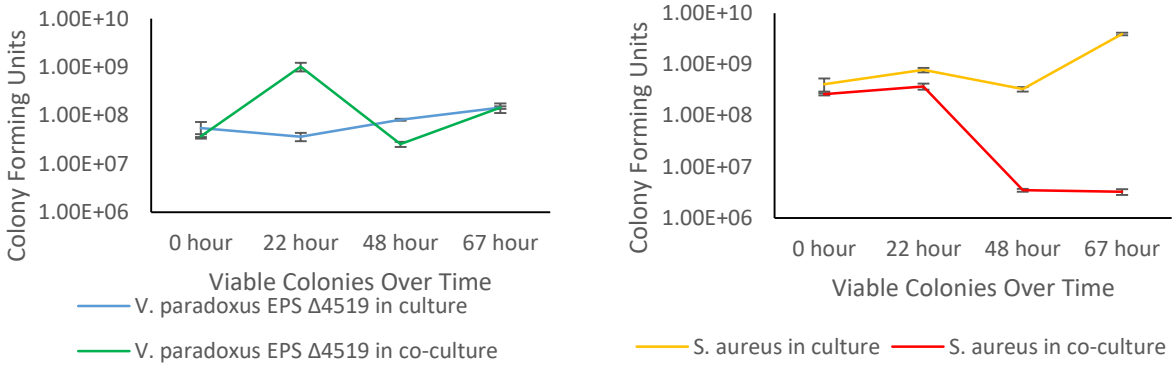


Figure 9: *V. paradoxus* EPS Δ4519 and *S. aureus* AH1710 co-culture and *S. aureus* AH1710 quantitative analysis

A) Growth of *V. paradoxus* EPS **Δ**4519 in culture and co-culture over 67 hours B) Growth of *S. aureus* AH1710 culture and in co-culture over 70 hours. Results suggest that anti-staphylococcal activity occurs without expression of **Δ**4519 but does not exclude possible expression of related genes within this locus or interactions with currently unidentified loci.

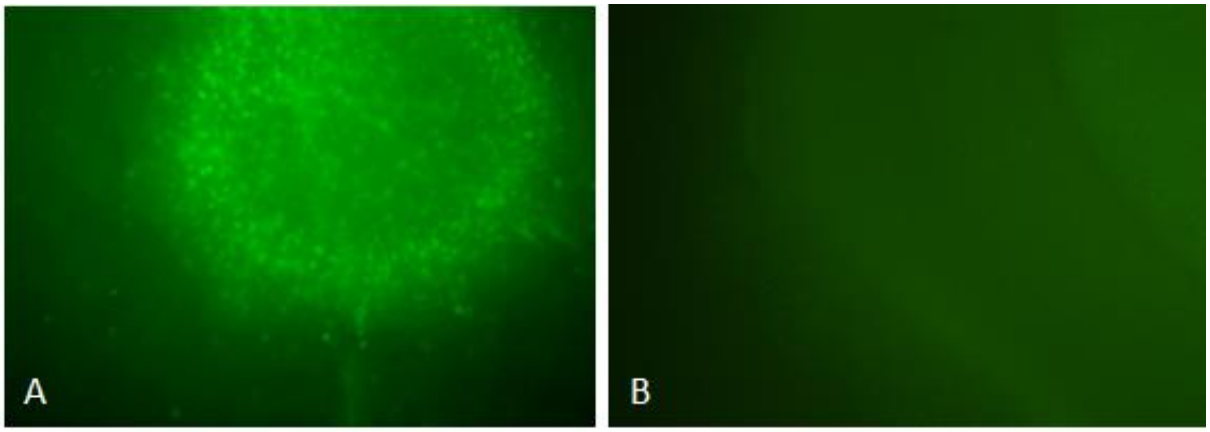


Figure 10: Wild type *Variovorax paradoxus* EPS grown on embedded *Staphylococcus aureus* AH1710

A) Wild type *V. paradoxus* EPS on 0.3% YE with *S. aureus* AH1710 embedded in agar 64h post inoculation. Fluorescence indicates viable *S. aureus* AH1710 under 5 µl *V. paradoxus* EPS spotted onto agar B) Wild type *V. paradoxus* EPS on 0.3% YE with *S. aureus* AH1710 embedded in agar 290h p.i.. A lack of fluorescence suggests that *S. aureus* AH1710 is no longer viable in the presence of *V. paradoxus* EPS. A Nikon Model CD-S microscope and an Andor Technology camera (model no. DR328G-CO2-SIL) was used for this image.

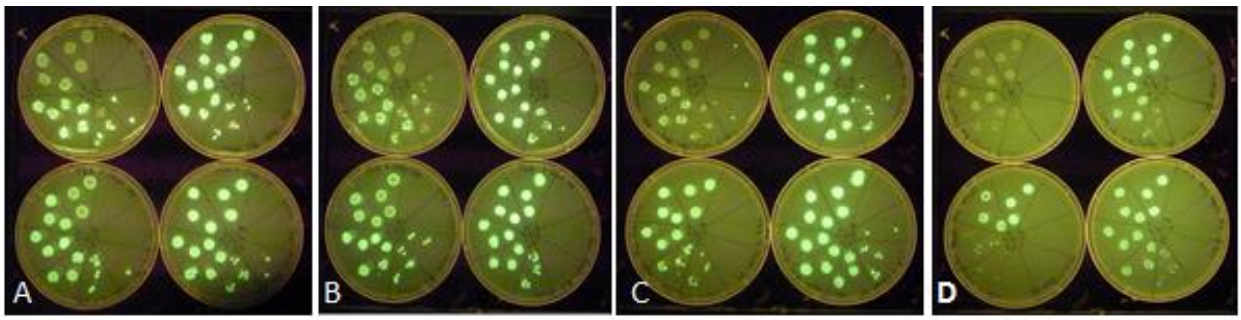
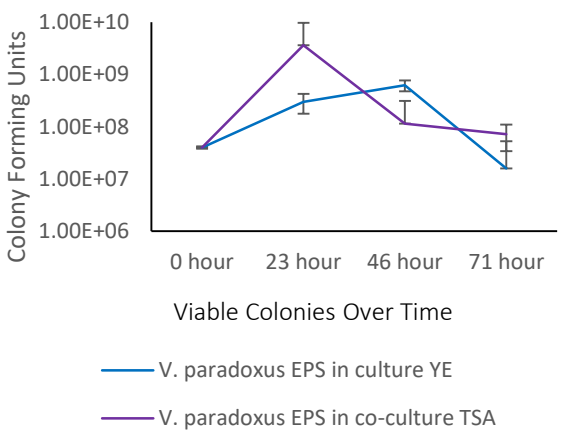
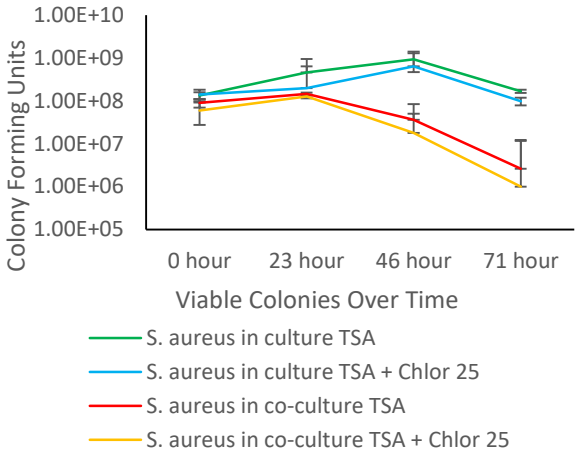


Figure 11: Quantitative analysis of Wild type *V. paradoxus* EPS and *S. aureus* AH1710 isolated from minimal fluorescence

A) 0 hour B) 23 hours C) 46 hours D) 71 hours p.i. for dilution series comparison of co-cultured Wild type *V. paradoxus* EPS and *S. aureus* AH1710 (left side plates) and *S. aureus* AH1710 culture (right side plates). *S. aureus* AH1710 was isolated from an area of minimal fluorescence. These staphylococci are vulnerable to anti-staphylococcal activity in co-culture with similar results to previous Wild type *V. paradoxus* EPS/*S. aureus* AH1710 quantitative analysis. Images taken using UV lighting (Entela UVP UL3101-1) with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.



A



B

Figure 12: Quantitative Analysis of potentially resistant *S. aureus* AH1710

A) Growth of Wild type *V. paradoxus* EPS over 71 hours B) Growth of *S. aureus* AH1710 culture and in co-culture over 71 hours. These results demonstrate that *S. aureus* AH1710 isolated from areas of minimal colony formation and fluorescence have a similar level of susceptibility to *S. aureus* AH1710 grown from maintained culture. A control of Chloramphenicol 25 mg/L (Chlor 25) determined if *S. aureus* AH1710 resistance to this antibiotic provided additional resistance to *V. paradoxus* EPS in co-culture.

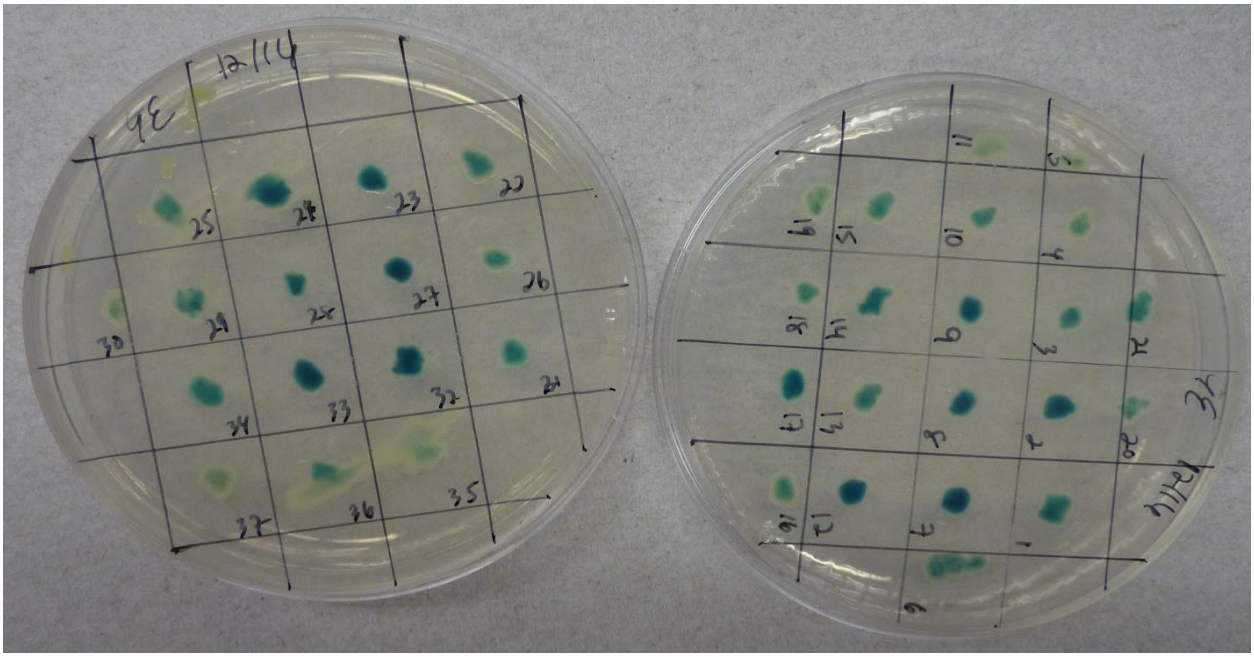


Figure 13: Confirmation of transconjugation of *V. paradoxus* EPS Δ4519 using blue white screening

Thirty-seven colonies were picked at random from transconjugated *V. paradoxus* EPS **Δ**4519 onto YE 1.5% agar + X-gal. All colonies picked have β-galactosidase expression confirming successful transconjugation of pOT182 plasmid into *V. paradoxus* EPS **Δ**4519. Images taken with a Panasonic DM-ZS1, 12X optical zoom, 25mm wide angle camera.

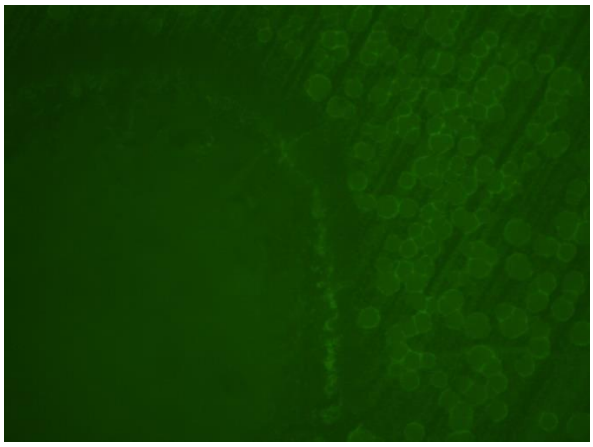
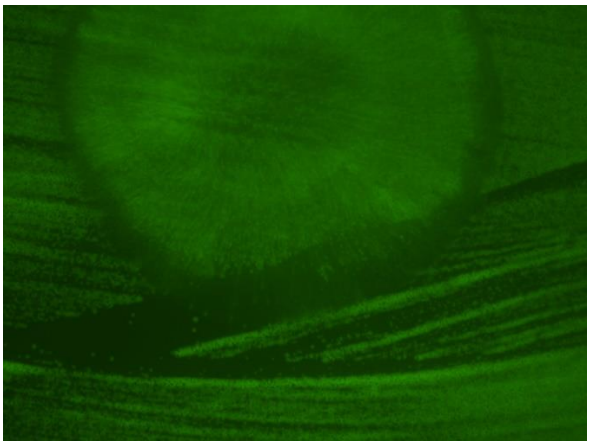
A   B

Figure 14: Examples of phenotypic variances of *V. paradoxus* EPS Δ4519 transposon mutants

Image A) Remnants of Green Florescent Proteins in the coccoid shape of *Staphylococcus aureus* ("Ghosts of staph") when plated with *V. paradoxus* EPS Δ4519 transposon 160 on YE agar 7 days p.i. (Transposon mutant 160 YE agar) Image B) A dark ring surrounding V. paradoxus EPS Δ4519 transposon 167 on YE agar 1 day p.i. (Transposon mutant 167 YE agar)

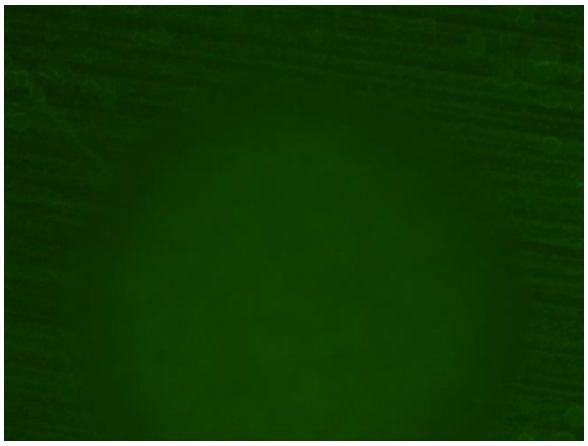
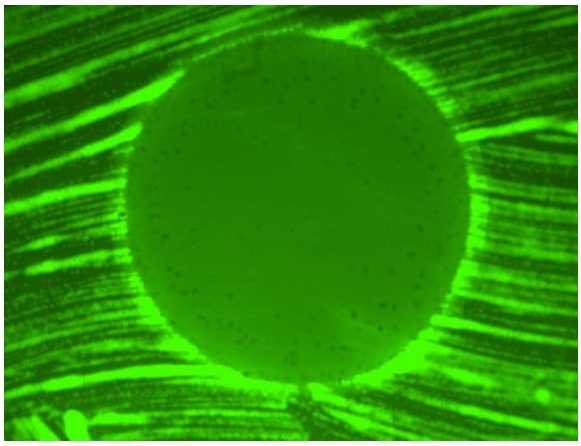
A   B

Figure 15: Examples of phenotypic variances of *V. paradoxus* EPS Δ4519 transposon mutants

Image A) A hazy green ring surrounding *V. paradoxus* EPS Δ4519 transposon 160 on YE agar 3 days p.i. (Transposon mutant 160 YE agar). Image B) A bright green ring surrounding *V. paradoxus* EPS Δ4519 transposon 87 on TSA seven days p.i. (Transposon mutant 87 TSA)

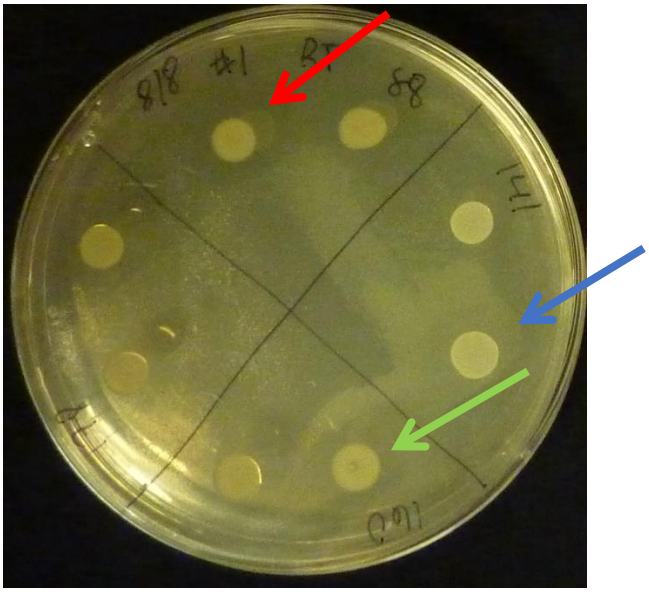


Figure 16: Examples of variation in pigmentation and mucoid colonies of *V. paradoxus* EPS Δ4519 transposon mutants

V. paradoxus EPS Δ4519 transposon mutant 88 expresses normal pigmentation (red arrow). Transposon mutant 141 expresses a cream/white pigmentation (blue arrow). Transposon mutant 160 expresses for mucoid colonies (green arrow).

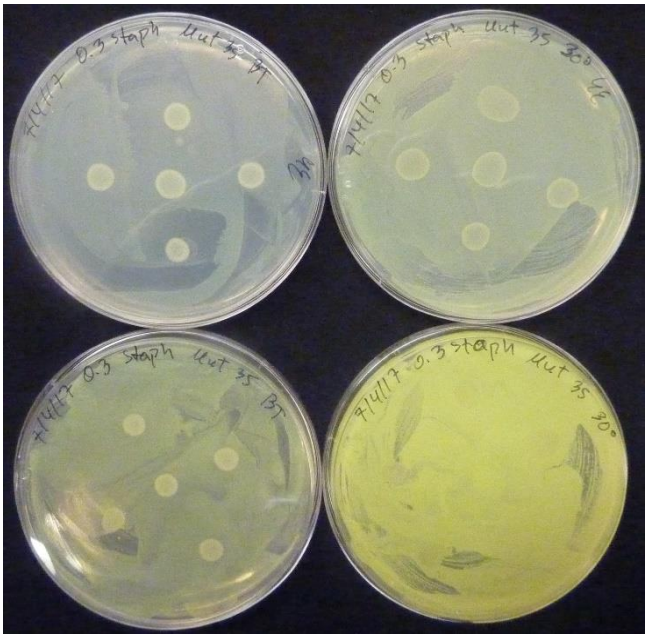
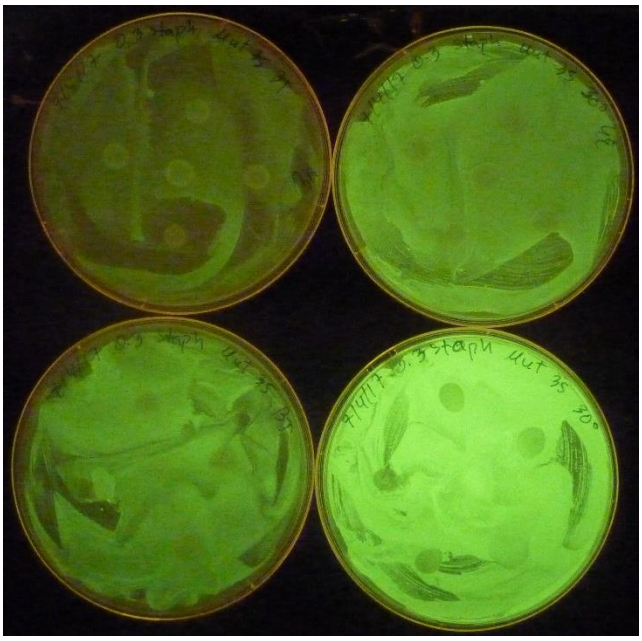
A  B 

Figure 17: Zone of Inhibition formation at Room temperature and 30°C Incubation with OD_{600} 0.3 *S. aureus* AH170

V. paradoxus EPS Δ4519 spot plated onto OD_{600} 0.3 density *S. aureus* AH170. Images taken under a) white light and b) fluorescent lighting. Zones of inhibition do not form on TSA at room temperature or 30°C incubation temperatures at 24 hours post inoculation (images: 10-2 staph (0.3 control) Mut 35 day 1 temp test 070517 and 10-2 staph (0.3 control) Mut 35 day 1 temp test 070517 UV)

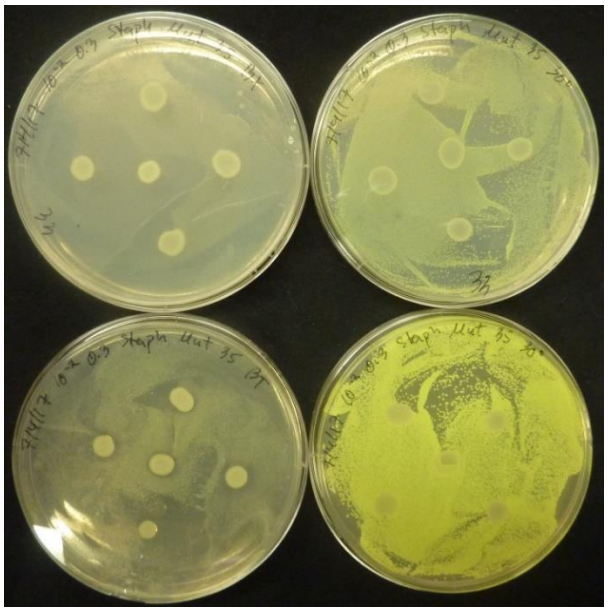
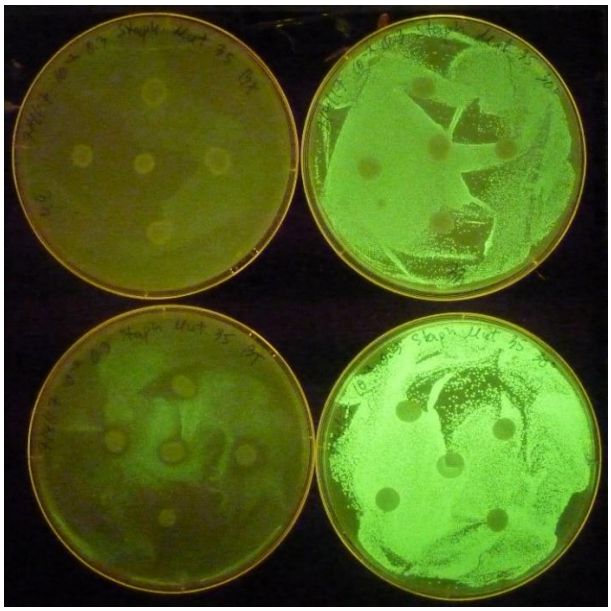
A  B 

Figure 18: Zone of Inhibition formation at Room temperature and 30°C Incubation with OD_{600} 0.003 *S. aureus*

V. paradoxus EPS Δ4519 spot plated onto OD_{600} 0.003 density *S. aureus* AH170. Images taken under a) white light and b) fluorescent lighting. Zones of inhibition form on TSA at room temperature but not 30°C incubation at 24 hours post inoculation (10-2 staph *V. paradoxus* EPS Δ4519 control and 10-2 staph *V. paradoxus* EPS Δ4519 control UV)

Appendix 2: Tables

Table 1: Cell Types Used and their Maintenance

Cell type	Strain	Source	Growth media
Variovorax paradoxus EPS	Wild type	Orwin laboratory	YE agar (5 g/L + 1.5%) or YE broth (5 g/L) Freshwater Succinate broth (47)
Variovorax paradoxus EPS	**Δ**4519 (Km^R)	Orwin laboratory	YE agar (5 g/L + 1.5%) or YE broth (5 g/L) + Kanamycin 50 mg/L Freshwater Succinate broth (47)
Staphylococcus aureus	AH3849=*S. aureus* LAC(AH1263) +pHC48 Cm^R (pCM29_dsRed)	Alexander Horswill, University of Iowa	Tryptic Soy Agar (40 g/L) or Tryptic Soy broth (30 g/L) + Chloramphenicol 25 mg/L
staphStaphylococcus aureus	AH1710=*S. aureus* RN4220 + pCM29 Cm^R (PsarA_RBSsod_SGFP)	Alexander Horswill, University of Iowa	Tryptic Soy Agar (40 g/L) or Tryptic Soy broth (30 g/L) + Chloramphenicol 25 mg/L Freshwater Succinate broth (47)
Escherichia coli	S17-1 λpir (pOT182:Tn5)	DeShazer D., (1997)	LB agar (25 g/L + 1.5%) or LB broth (25 g/L) + Tetracycline 25 mg/L or Gentamycin 10 mg/L YE broth (5g/L)
Escherichia coli	Top 10 F’	Daniel Nickerson, CSU San Bernardino	LB agar (25 g/L + 1.5%) or LB broth (25 g/L)

Incubation of inoculated plates was completed using a Fisher Scientific Isotemp Incubator. Incubation of Biosafety level 2 bacteria was completed using a New Brunswick Scientific Classic Series C24 Incubator Shake

Table 2: Primers for RT-qPCR

Sequence name	**Forward Primer**	**Reverse Primer**
2887	GCGTCTTCAACAACGTGCT	ATCCTGGAACGAGAACATCG
2888	CGCTGTGCGGGTGGTCGT	GCGTTGCCTTCGTGGCG
4324	CTGGATGCAGGAGACCTACC	AGTTCAGGGTGGTGATCTGG
4327	AGAGCGGGTGAAGGATGCG	GCGGGGGCGAAGCGTT
4519	GATTCCGTACCTCGACCTCA	GTCGATGTTCCGGGTGTAGT

Table 3: Phenotype and Zone of Inhibition Formation for transposon mutants of interest not sequenced

	Zone of Inhibition formation		Mucoid phenotype		Additional notes		Zone of Inhibition formation		Mucoid phenotype		Additional notes
	Tryptic Soy Agar		Tryptic Soy Agar				Tryptic Soy Agar		Tryptic Soy Agar		
Δ4519 transposon	**RT**	**30°C**	**RT**	**30°C**		**Δ4519 transposon**	**RT**	**30° C**	**RT**	**30°C**	
4	9-10 mm	–	+	–		**78**	–	–	–	–	RT and 30°C: Cream/white pigment
7	7 mm	–	+	+		**93**	–	–	–	–	RT and 30°C: Cream/white pigment
17	9-10 mm	–	+	–		**94**	–	–	–	–	RT and 30°C: Cream/white pigment
18	12 mm	–	+	–		**95**	–	–	–	–	RT and 30°C: Cream/white pigment
21	–	–	–	–	RT and 30°C: Cream/white pigment	**97**	–	–	–	–	RT and 30°C: Cream/white pigment
24	–	–	–	–	RT and 30°C: Cream/white pigment	**107**	–	–	–	–	RT and 30°C: Cream/white pigment
29	–	–	–	–	RT and 30°C: Cream/white pigment	**122**	–	–	–	–	RT and 30°C: Cream/white pigment
36	–	–	–	–	RT and 30°C: Cream/white pigment	**133**	–	–	–	–	RT and 30°C: Cream/white pigment
41	–	–	–	–	RT and 30°C: Cream/white pigment	**138**	–	–	–	–	RT and 30°C: Cream/white pigment
42	–	–	–	–	RT and 30°C: Cream/white pigment	**140**	–	–	–	–	RT and 30°C: Cream/white pigment
49	–	–	–	–	RT and 30°C: Cream/white pigment	**147**	–	–	–	–	RT & 30°C: pigmented creamy/white
56	–	–	–	+		**149**	–	–	–	–	RT & 30°C: pigmented creamy/white
57	–	–	–	–	RT and 30°C: Cream/white pigment	**153**	9-10 mm	–	–	–	30°C: pigmented green
62	N/A	–	N/A	–	30°C: Cream/white pigment	**154**	–	–	–	–	30°C: Creamy/white

63	N/A	–	N/A	–	30°C: Cream/white pigment	**159**	–	–	–	–	RT & 30°C: pigmented creamy/white
65	N/A	–	N/A	–	30°C: Cream/white pigment	**166**	12 mm	–	–	+	RT: pigmented green
67	–	–	–	–	RT & 30°C: pigmented creamy/white	**183**	11-12 mm	–	–	–	30°C: pigmented green
68	–	–	–	–	RT & 30°C: pigmented creamy/white	**185**	13-14 mm	–	–	+	30°C: pigmented green
69	–	–	–	–	RT & 30°C: pigmented creamy/white	**189**	6-7 mm	7-8 mm	–	+	30°C: pigmented green
72	–	–	–	–	RT & 30°C: pigmented creamy/white	**191**	13 mm	–	–	+	30°C: pigmented green
74	15-16 mm	–	–	–	30°C: pigmented green	**192**	15 mm	8 mm	–	–	30°C: pigmented green
75	–	–	–	–		**193**	7-8 mm	7 mm	–	+	30°C: pigmented green
76	–	–	–	–	RT and 30°C: Cream/white pigment	**194**	11-12 mm	–	–	+	
77	–	–	–	–	RT and 30°C: Cream/white pigment	**196**	12 mm	–	+	–	

Determination of anti-staphylococcal activity included production of mucoid phenotype and zone of inhibition formation. Phenotype production was most prominent on TSA plated with *S. aureus* AH1710 at density OD_{600} 0.003.

Table 4: Transposon mutants without Zone of Inhibition or Mucoid phenotypes (RT & 30°C) selected for Sanger Sequencing

	Zone of Inhibition formation		Mucoid phenotype		Notes for *V. paradoxus* EPS Δ4519 transposon phenotypes
	Tryptic Soy Agar		Tryptic Soy Agar		
	RT	30°C	RT	30°C	
***V. paradoxus* EPS Wild type (WT)**	10 mm	–	–	–	TSA at 30°C: Pigmented green and difficult to visualize. *S. aureus* viable under and outside spotted WT.
***V. paradoxus* EPS Δ4519**	7-10 mm	–	+	–	TSA at 30°C: Colonies are pigmented green
Δ4519 transposon:					
26	–	–	–	–	TSA at 30°C: Creamy/white pigment
27	–	–	–	–	RT and 30°C: Cream/white pigment
37	–	–	–	–	RT and 30°C: Creamy/white pigment
39	–	–	–	–	Hazy green ring at perimeter of spots on TSA (**Figure 19**) TSA at 30°C: Cream/white pigment
47	–	–	–	–	RT and 30°C: Cream/white pigment
50	–	–	–	–	Dark ring at perimeter of spots on TSA (**Figure 18**) TSA at 30°C: Creamy/white pigment
54	–	–	–	–	TSA at 30°C: Creamy/white pigment
60	–	–	–	–	TSA at 30°C: Creamy/white pigment
86	–	–	–	–	TSA at 30°C: Creamy/white pigment
87	–	–	–	–	TSA at 30°C: Creamy/white pigment
90	–	–	–	–	TSA at 30°C: Creamy/white pigment

Determination of anti-staphylococcal activity included production of mucoid phenotype and zone of inhibition formation. Phenotype production was most prominent on TSA plated with *S. aureus* AH1710 at density OD_{600} 0.003.

Table 5: Transposon mutants with Zone of Inhibition greater than Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 and with or without Mucoid phenotypes (RT & 30°C) selected for Sanger Sequencing

	Zone of Inhibition formation		**Mucoid phenotype**		**Notes for *V. paradoxus* EPS Δ4519 transposon phenotypes**
	Tryptic Soy Agar		**Tryptic Soy Agar**		
	RT	**30°C**	**RT**	**30°C**	
***V. paradoxus* EPS Wild type (WT)**	10 mm	**–**	**–**	**–**	TSA at 30°C: Pigmented green and difficult to visualize. *S. aureus* viable under and outside spotted WT
***V. paradoxus* EPS Δ4519**	7-10 mm	**–**	**+**	**–**	TSA at 30°C: Colonies are pigmented green
139	25 mm	**–**	**+**	**–**	TSA at 30°C Green pigment
151	15 mm	**–**	**+**	**–**	
100	15 mm	**–**	**+**	**–**	TSA at 30°C*: S. aureus* shriveled only under spotted transposon TSA at 30°C: Green pigment
162	15 mm	**–**	**–**	**–**	TSA at 30°C: Green pigment
96	15 mm	**–**	**–**	**–**	TSA at 30°C: Green pigment
84	14-15 mm	**–**	**+**	**+**	TSA at 30°C: Green pigment
169	14 mm	**–**	**+**	**+**	
91	12-13 mm	**–**	**+**	**–**	
164	12-13 mm	**–**	**+**	**–**	TSA at 30°C: Green pigment
161	11-12 mm	**–**	**+**	**–**	TSA at 30°C: Green pigment

Phenotype production was most prominent on TSA plated with *S. aureus* AH1710 at OD_{600} 0.003. Zones of Inhibition listed exceeded those of control strains Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519. Not all phenotypes noted were identified on all *V. paradoxus* EPS Δ4519 transposon mutants.

Table 6: Transposon mutants with Zone of Inhibition at RT & 30°C and with or without Mucoid phenotypes selected for Sanger Sequencing

	Zone of Inhibition formation		**Mucoid phenotype**		**Notes for *V. paradoxus* EPS Δ4519 transposon phenotypes**
	Tryptic Soy Agar		**Tryptic Soy Agar**		
	RT	**30°C**	**RT**	**30°C**	
***V. paradoxus* EPS Wild type (WT)**	10 mm	**–**	**–**	**–**	TSA at 30°C: Pigmented green and difficult to visualize. *S. aureus* viable under and outside spotted WT.
***V. paradoxus* EPS Δ4519**	7-10 mm	**–**	**+**	**–**	TSA at 30°C: Colonies are pigmented green
104	20 mm	5-6 mm	**+**	**+**	
167	14-15 mm	7-8 mm	**+**	**+**	
160	13-14 mm	12-13 mm	**+**	**+**	"Ghosts of staph" (**Figure 18**) YE at 30°C: Pigmented green
178	13-14 mm	13 mm	**–**	**+**	
88	13 mm	12-13 mm	**+**	**+**	TSA at 30°C Pigmented green

Phenotype production was most prominent on TSA plated with *S. aureus* AH1710 at OD_{600} 0.003. Zones of Inhibition listed exceeded those of control strains Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519. Not all phenotypes noted were identified on all *V. paradoxus* EPS Δ4519 transposon mutants.

Table 7: Transposon mutants with Zone of Inhibition less than Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 and with or without Mucoid phenotypes (RT & 30°C) selected for Sanger Sequencing

	Zone of Inhibition formation		**Mucoid phenotype**		**Notes for *V. paradoxus* EPS Δ4519 transposon phenotypes**
	Tryptic Soy Agar		**Tryptic Soy Agar**		
	RT	**30°C**	**RT**	**30°C**	
***V. paradoxus* EPS Wild type (WT)**	10 mm	**–**	**–**	**–**	TSA at 30°C: Pigmented green and difficult to visualize. *S. aureus* viable under and outside spotted WT
***V. paradoxus* EPS Δ4519**	7-10 mm	**–**	**+**	**–**	TSA at 30°C: Colonies are pigmented green
19	8 mm	**–**	**+**	**–**	
79	8-9 mm	**–**	**+**	**+**	

Phenotype production was most prominent on TSA plated with *S. aureus* AH1710 at OD_{600} 0.003. Zones of Inhibition listed were less than control strains Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519. Not all phenotypes noted were identified on all *V. paradoxus* EPS Δ4519 transposon mutants.

Table 8: Transposon mutants without Zone of Inhibition or Mucoid phenotypes (RT & 30°C) selected for Sanger Sequencing

Transconjugant number	Gene sequence identified (52)	Function (52)
26	Varpa_5734	ABC-type phosphate/phosphonate transport system, ATPase component
27	Varpa_5945	Transcriptional regulator, GntR family
37	Varpa_4681	UDP-glucose 4-epimerase
39	Varpa_0126	Hypothetical protein
47	Varpa_3786	Hypothetical protein
50	Varpa_3827	Xylose isomerase domain-containing protein TIM barrel; AP endonuclease 2 domain protein
54	Varpa_2772	GumN family protein
60	Varpa_5138	Hypothetical protein
86	Varpa_4382	HNH nuclease
87	Varpa_0777	glycosyl transferase family 2
90	Varpa_1031	transcriptional regulator, Crp/Fnr family

This subset of transposon mutants corresponds to those listed in **Table 4** above. Transposon mutants 39 and 90 are similar in sequence location within the *V. paradoxus* EPS genome while the remaining are more widespread throughout the genome. Functions within this subgroup include three hypothetical proteins and protein functions that are currently known.

Table 9: Transposon mutants with Zone of Inhibition greater than Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 and with or without Mucoid phenotypes (RT & 30°C) selected for Sanger sequencing

139	Varpa_4679	Sugar transferase
151	Varpa_2880	2-hydroxy-3-oxopropionate reductase
100	Varpa_3219	Polar amino acid ABC transporter, inner membrane subunit
162	Varpa_4272	Hypothetical protein
96	Varpa_4680	Glycosyl transferase group 1
84—Insert between	Vapra_4224	Patatin-like phospholipase
	Varpa_4223	Nuclease (SNase domain-containing protein
169	Varpa_3602	Protein of unknown function DUF6 transmembrane
91—Insert between	Vapra_4224	Patatin-like phospholipase
	Varpa_4223	Nuclease (SNase domain-containing protein
164	Varpa_4665	Integral membrane sensor signal transduction histidine kinase
161	Varpa_0944	NAD-dependent epimerase/dehydratase

This subset of transposon mutants corresponds to those listed in **Table 5** above. Transposon 164 corresponds to transposon 178 in **Table 10**. Transposon pairs 84 and 91 are identical in gene sequence while transposon mutants 96 and 139 are sequential. The remaining transposon mutants are more widespread throughout the genome.

Table 10: Transposon mutants with Zone of Inhibition at RT & 30°C and with or without Mucoid phenotypes selected for Sanger sequencing

104	Varpa_5846	Hypothetical protein
167	Varpa_3390	Hypothetical protein
160	Varpa_4127	Hypothetical protein
178	Varpa_4665	Integral membrane sensor signal transduction histidine kinase
88	Varpa_4548	Phosphoesterase

This subset of transposon mutants corresponds to those listed in **Table 6** above. Transposon 178 corresponds to transposon 164 in **Table 9**. Functions within this subgroup include hypothetical proteins that are currently unknown.

Table 11: Transposon mutants with Zone of Inhibition less than Wild type *V. paradoxus* EPS and *V. paradoxus* EPS Δ4519 and with or without Mucoid phenotypes (RT & 30°C) selected for Sanger sequencing

19	Varpa_5160	Protein of unknown function DUF2134, membrane
79	Varpa_2156	Polypeptide-transport-associated domain protein ShlB-type

This subset of transposon mutants corresponds to those listed in **Table 7** above and are unique in their gene sequence.