COMPARISON OF MULTIPLE ANTIBIOTIC RESISTANT *STAPHYLOCOCCUS AUREUS* GENOMES AND THE GENOME STRUCTURE OF *ELIZABETHKINGIA MENINGOSEPTICA*

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Abstract:

i. The aim of this study was to determine if methicillin-resistant *Staphylococcus aureus* (MRSA) strains could be identified in the milk of dairy cattle in a Paso Del Norte region dairy. Using physiological and PCR-based identification schemes, 40 *S. aureus* strains were isolated from 133 milk samples analyzed. This investigation also included the production of draft genome sequences of a MRSA and a methicillin-susceptible isolate. Genomic analysis of these strains demonstrate that strains H29 and PB32 represent novel clones of sequenced human and/or bovine-related strains of *S. aureus*.

ii. The draft genomes of a heterogeneous vancomycin-intermediate resistant *Staphylococcus aureus* (VISA) strain MM66 and strain MM66RVI-4 expressing reduced vancomycin-intermediate resistance harbored the same mutation in *graS* of a two-component regulatory system. MM66RVI-4 has also lost staphylococcal cassette chromosome, SCC*mec*, corroborating methicillin-resistance in this strain and harbored mutations present in *vraG,* encoding an ATP-binding cassette transporter. The observed genetic alterations in both strains have been shown to affect vancomycin resistance levels in VISA.

iii. *Elizabethkingia* species are environmental isolates that exhibit a multidrug-resistance phenotype and are a cause of life-threatening infections in immunocompromised individuals. To date, most studies have focused on the clinical aspects of this organism, and little is known regarding its antimicrobial resistance mechanisms. In addition, the phylogenetics and speciation of this genus is still highly controversial and has yet to be thoroughly defined. In an effort to clear up confusion surrounding the speciation of this genus as well as identifying possible antimicrobial resistance mechanisms, we report the draft genome sequence of the *E. meningoseptica* type strain ATCC 13253^{T}.

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CHAPTER I

INTRODUCTION

1.1 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive pathogen that is responsible for infections in both the hospital setting as well as in the community. *S. aureus* causes a wide array of infections ranging from moderate skin infections to more life-threatening infections such as endocarditis. In the early 1940s the β-lactam antibiotic penicillin was first made commercially available to treat *S. aureus* infections. Today, greater than 80% of *S. aureus* isolates are resistant to penicillin (113). The emergence of penicillin resistance led to introduction of the semi-synthetic penicillin, methicillin. However, in 1961, methicillin-resistant *S. aureus* (MRSA) were reported (83). The MRSA phenotype results from the acquisition of a mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (65, 78). To date, 11 different SCC*mec* types have been described and range from 21 kb to 58 kb in size (http://www.sccmec.org/). SCC*mec* is comprised of a *mec* region containing the *mecA* gene (encodes an alternative penicillin binding protein PBP2a), *mec* regulatory genes *mecR1* and *mecI*, two cassette

chromosome recombinase genes (*ccr*) responsible for site-specific integration/excision from the chromosome and a "junkyard" region consisting of variable genes (79).

S. aureus isolates demonstrate resistance to the majority of antimicrobials used to treat infections. *S. aureus* was reported to colonize the nasal nares of ~28% of the U.S. population in 2004 (54) and if it contaminates a normally sterile site, it can cause infection. It was estimated that in 2011, there were 80,461 invasive MRSA infections in the U.S. of which resulted in 11,285 deaths (33). Risk factors for hospital-acquired MRSA infections include MRSA colonization, prolonged or history of hospitalization, long-term care residence and history of surgery (94).

Several molecular typing techniques are used to determine the spread of specific *S. aureus* strains, which include multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). MLST uses sequencing of 7 housekeeping gene amplicons to group MRSA strains into clonal complexes (CC) using "based upon related sequence types" (BURST) analysis (43). The majority of *S. aureus* strains isolated worldwide belong to a handful of CCs that include CC1, CC5, CC8, CC9, CC15, CC22, CC25, CC30, CC45, and CC51 (37, 143) with the rest belonging to smaller, less common clonal complexes. To date, the largest clonal complex described is CC8 represented by 248 strains of both hospital-acquired and community-acquired human isolates as well as animal isolates (http://saureus.mlst.net/). PFGE is a method that uses specific infrequent cutting restriction endonuclease enzymes (eg. SmaI) to cleave genomic DNA into different sized fragments that give distinct banding patterns for specific strains. This allows for the capability of determining the possible gain or loss of mobile genetic elements and/or mutations via the introduction or removal of restriction enzyme sites as determined by the number of bands

seen on an agarose gel (183). Based on this method, the majority of *S. aureus* clones in the U.S. are from twelve lineages, PFGE types USA100 – USA1200, which are representative of hospital-acquired and community-acquired *S. aureus* strains (126).

S. aureus is also a pathogen of swine, horses, cattle and domestic animals. *S. aureus* is the major cause of bovine mastitis (infection within the mammary gland), infecting between 7% and 40% of all dairy cows in the United States (119). Bovine mastitis from MRSA was first reported in 1972 and MRSA transmission appears to occur between animals, and from animals to humans (39, 102, 114, 193). *S. aureus* mastitis causes scarring and fibrosis of the mammary gland along with excretion of pus, thereby reducing the volume and quality of the milk produced by the animal (12). In addition to the burden of reduced milk production, the difficulty of treating *S. aureus* infections means that the primary strategy for dealing with an infected animal is quarantine with antibiotic treatment, and in the case of treatment failure, the animal will be culled (162). The economic impact of bovine mastitis *S. aureus* infections is also difficult to estimate (~$2 billion in the US (192)) with losses coming from reduced milk yield, replacement of culled animals, additional labor, and veterinary and antibiotic costs (164). Carriage of MRSA by livestock has been shown to correlate with the MRSA colonization of farmers and farm families, veterinarians and healthcare workers (85, 193, 202). There is also concern about the potential of MRSA to be transmitted to humans through contaminated food products although what risk is actually posed remains a matter of debate (20, 35, 95). MLST analyses have shown that livestock-associated MRSA belong to a small number of CC with the most prevalent being CC398 and it is known that CC398 strains also cause infections in humans (115, 160, 193). A recent report suggests that CC398 strains emerged from a human evolved methicillin-susceptible *S. aureus* (MSSA) strain and that

livestock-associated MRSA sublineages arose during antimicrobial selection in livestock (152).

1.2 Vancomycin

The evolution of methicillin resistance led to the pursuit of alternative treatments for MRSA infections (43). In 1958, the glycopeptide antibiotic vancomycin was first used clinically to fight infections caused by Gram-positive bacteria (103). Vancomycin, a bactericidal drug, binds to the D-alanyl-D-alanine residues in growing peptidoglycan chains, preventing the cross-linking of these chains and thus inhibiting peptidoglycan synthesis. Vancomycin is currently the antibiotic of choice for treating serious MRSA infections in the United States (103, 111).

1.3 Vancomycin-intermediate resistant *Staphylococcus aureus*

In 1997, the first vancomycin-intermediate resistant *S. aureus* (VISA) strain was reported in Japan exhibiting a vancomycin minimum inhibitory concentration (MIC) of 8 μg/ml and since then, many more have been described globally (66). Heterogeneous vancomycin-intermediate resistant *S. aureus* (hVISA) are strains that demonstrate a vancomycin MIC within the susceptible range (MIC $\leq$ 2 μg/ml) but have a subpopulation of cells within the vancomycin-intermediate range (MIC = 4 - 8 μg/ml) that are not detected by standard broth and disk diffusion techniques. In 1996, Mu3 was the first hVISA strain described in Japan from a patient with unsuccessful vancomycin treatment (64). Mu3 exhibits a vancomycin MIC of 3 μg/ml with a subset of cells that demonstrate vancomycin-resistance up to 9 μg/ml vancomycin. In 2002, the first vancomycin-resistant *S. aureus* (VRSA, MIC $\geq$ 32 μg/ml) strain was isolated in the U.S. (23, 25). VRSA strains have been

shown to carry *van* genes acquired from vancomycin-resistant enterococci that exhibit high-level vancomycin resistance (197). The VISA mechanism in *S. aureus* differs from the *van*-mediated resistance mechanism of VRSA. Several phenotypic changes exhibited by hVISA/VISA strains have been described, which include reduced autolysis, thicker cell walls, reduced peptidoglycan cross-linking and overexpression of PBP2 and PBP2a (14, 15, 30, 31, 34, 47, 62, 71, 86, 96, 118, 133, 146, 155, 171, 172). Comparative genomic studies have identified several genetic changes associated with the hVISA/VISA phenotype, however, the exact mechanisms of resistance are still poorly understood. The mechanism behind vancomycin-intermediate resistance are sequential mutations (or a single mutation) that fully or partially changes the function of a specific gene(s) (73). Mutations in genes associated with the hVISA/VISA phenotype include *rpoB*, *walKR, vraSR, graRS, agr, yycH, yvqF, isdE* (29, 50, 74, 81, 90, 121, 136, 142, 168, 205). Mutations in regulatory genes involved in cell wall metabolism such as *vraSR*, *graRS* and *walKR* have most frequently been associated with the VISA phenotype, however, the exact mutations found within these genes can differ between *S. aureus* strains (32, 70, 72, 74, 89, 136, 137). Therefore, based on the reported VISA descriptions to date, the VISA phenotype can result from multiple mutations and/or mutation combinations.

1.4 *Staphylococcus aureus* genomics

The first *S. aureus* genomes were sequenced in 2001, which were MRSA strain N315 and VISA strain Mu50 (99). Sequencing of these genomes revealed that the majority of the antibiotic and virulence genes of *S. aureus* reside on mobile genetic elements, i.e. plasmids, bacteriophages, and pathogenicity islands (99). *S. aureus* genomes are ~2.8 to 2.9 Mbp in size and have similar chromosomal synteny. The majority of the genome (~75%), known as

the "core genome," is comprised of essential genes that encode proteins needed for cell growth, cellular metabolism and replication (110). The other ~25% of the *S. aureus* genome is considered the "accessory genome" which contains mobile genetic elements such as plasmids and bacteriophages that can be transferred between strains (110). As of March 2014, a total of 50 *S. aureus* genomes have been completely sequenced with another 2,710 genomes in the draft genome state. Sequencing of whole genomes allows for the comparison of genetic differences between organisms down to single nucleotide changes. Several studies have used comparative genomics of closely related *S. aureus* strains, such as a parent strain compared to the antimicrobial-resistant strain, to determine what mutations, if any, may be responsible for the variations in susceptibilities. For example, Mwangi *et al.* was able to use whole-genome sequencing to track the development of genetic mutations of a *S. aureus* strain originally isolated as MRSA, and after undergoing vancomycin therapy, evolving to VISA (136). The first *S. aureus* strain sequenced (JH1) was susceptible to vancomycin and was isolated prior to vancomycin treatment. After the start of vancomycin treatment, a second *S. aureus* strain (JH9) was isolated and exhibited a VISA phenotype. This method identified 35 mutations between the two *S. aureus* genomes that contributed to both the multidrug-resistant and VISA phenotype of JH9 (136). In another study, Neoh *et al*. used comparative genomics of two closely related hVISA/VISA strains (Mu3/Mu50) to determine which mutations contribute to their hVISA/VISA phenotype (137). In this study, 16 mutations were found between the Mu3 and Mu50 genomes including a mutation in *graR,* the response regulator of a two-component regulatory system, which shown to convert the hVISA strain Mu3 into a full VISA strain, similar to that of Mu50 (137). As a whole, these studies demonstrate the

ability of using whole-genome sequencing as a means to ascertain the genetic basis of antibiotic resistance of *S. aureus*.

1.5 *Elizabethkingia* species

The genus *Elizabethkingia* (formerly *Chryseobacterium* and *Flavobacterium*) belongs to the phylum Bacteroidetes, within the Flavobacteriaceae family. Currently, the genus consists of three species, *E. meningoseptica*, *E. miricola* and *E. anophelis*, with the latter being the most recent addition. *E. meningoseptica* was isolated in 1958 from a case of neonatal meningitis (17, 92), *E. miricola* from condensation water in the space station Mir in 2003 (105), and *E. anophelis* from the midgut of the mosquito, *Anopheles gambiae,* in 2011 (87).

Elizabethkingia species are Gram-negative, non-motile, non-spore-forming rods (17, 87, 91, 105). Colonies are circular, translucent, smooth, off-white in color and range from 1 μm to 2 μm in size (17, 87, 91, 105). Optimal growth is seen at 25 - 37°C but not at 5 or 42°C and growth on MacConkey agar is variable (17, 87, 91, 105). All species are oxidase, catalase, and indole positive. Acid is produced from glucose, lactose, mannitol, maltose and trehalose (17, 87, 91, 105). Acid is not produced from arabinose, xylose, adonitol, dulcitol, raffinose, and salicin (17, 87, 91, 105). Acid production from rhamnose, sucrose, and cellobiose is seen for *E. anophelis,* however no acid production is seen for *E. meningoseptica* and *E. miricola* (17, 87, 92, 105). Aesculin hydrolysis is positive and starch hydrolysis is negative for all three species, however urea hydrolysis is negative for *E. meningoseptica* and *E. anophelis* and positive for *E. miricola* (17, 87, 92, 105). H_2S production is positive for *E. miricola* and negative for *E. meningoseptica* and *E. anophelis* (17, 87, 92, 105).

1.6 Clinical aspects of *Elizabethkingia* species

Elizabethkingia are opportunistic pathogens that have been found to be the cause of many different types of infections in both adults and children. Infections are mainly found in neonates and immunocompromised adults. The majority of infections reported are presumably caused by *E. meningoseptica,* however, due to difficulties in speciation of the members of this genus, the exact organism causing the infection is most likely unknown. Infections reportedly caused by *E. meningoseptica* include meningitis (17, 92), sepsis (176), endocarditis (13), pneumonia (106), bacteremia (107), cellulitis (176), sinusitis (174), endophthalmitis (45), septic arthritis (97), peritonitis (145), keratitis (2), osteomyelitis (101), pericarditis (112), pyoventriculitis (187) and necrotizing fasciitis (100). Several different antibiotic therapies have been used in the treatment of infections caused by putative *E. meningoseptica* that include ciprofloxacin, levofloxacin, gentamicin, vancomycin, rifampin, piperacillin/tazobactam and linezolid (4, 24, 59, 60, 75). However, most infections are unsuccessfully treated with the above therapies due to a multidrug-resistant (MDR) phenotype exhibited by *E. meningoseptica*. To date, there has been only one report of an infection caused by *E. miricola* and in that case it was originally misidentified as *E. meningoseptica* (55). In 2013, the first case of infection caused by *E. anophelis* was reported in Africa and identified through the utilization of 16S rRNA gene sequence comparisons of type strains within the *Chryseobacterium, Bergeyella, Riemerella, Empedobacter* and *Elizabethkingia* genera (48). Soon after the initial description of the first *E. anophelis* infection, additional infections were reported in Singapore. These strains were originally thought to be *E. meningoseptica* on the basis of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) (184). However, whole genome

alignments and 16S rRNA gene sequencing demonstrated that the isolates were more closely related to *E. anophelis* than *E. meningoseptica* (184).

Elizabethkingia infections in humans have been linked to a wide variety of sources that include contaminated sinks (21, 68), pasteurizers (31), aerosolizers (167), garden hoses (167), humidifiers (174), chlorhexidine gluconate solution (28) and povidone iodine prep pads. It has also been found in the pharynx, nose and throat of healthy babies, suggesting the ability of humans to be asymptomatic carriers of *Elizabethkingia* (165, 186, 195). In addition to clinical sources, *Elizabethkingia* have been isolated from a variety of other environmental niches that include soil (188), water (188) and spent nuclear fuel pools (26).

Elizabethkingia are not only human pathogens but also have been isolated from many different species of diseased animals. The first documented case in an animal was in 1974 from meningitis in a cat (173). Since then, many more infections have been documented in animals including different species of frogs, freshwater fish, snakes, turtles, birds and dogs (52, 56, 80, 124, 128, 203). *Elizabethkingia* have also been isolated from several different species of insects that include the fowl tick *Argas persicus* and mosquitoes species *Aedes aegypti, Anopheles gambiae, Anopheles stephensi* and *Anopheles sinensis* (41, 104, 108, 131, 154, 185).

1.7 Speciation of *Elizabethkingia* species

Since the initial isolation of *E. meningoseptica*, there have been some uncertainties surrounding classification of new *Elizabethkingia* strains. Before the popularity/ease of whole-genome sequencing, the DNA-DNA hybridization technique was used for defining bacterial species. DNA-DNA relatedness of 70% or greater was recommended as the

standard in delineating species (196). Parallel studies by Ursing and Bruun (1987) used DNA-DNA hybridization and phenotypic characterization to separate several *E. meningoseptica* strains into two genomic groups (18, 188). Group I, which included 3 isolates, demonstrated 90% or greater hybridization to the type strain of *E. meningoseptica* (ATCC 13253^T), while group II demonstrated 36-48% hybridization to ATCC 13253^T. Despite these genetic differences seen with DNA-DNA hybridization, all strains were still considered *E. meningoseptica* in the databases and even in recent publications. In 1994, six *Flavobacterium* species, including *E. meningoseptica,* were reclassified into the *Chryseobacterium* genus based upon 16S rRNA similarities (189). In 2005, phylogenetic (16S rRNA) and phenotypic data demonstrated that *E. meningoseptica* and *E. miricola* could be differentiated from other *Chryseobacterium* species, therefore transferring these species to the new genus *Elizabethkingia* (91). Additionally, in that same study, DNA-DNA hybridizations of several *Elizabethkingia* strains were confirmed (91). However, in all studies to date, the decision to not separate the genetically different *E. meningoseptica* type strain from other *Elizabethkingia* strains is due to no distinct phenotypic variations or differences seen in 16S rRNA sequences among the strains. Today, 16S rRNA sequencing is continually being used for the speciation of *Elizabethkingia* strains despite the inability of this methodology to differentiate between the species of this genus. In an effort to demonstrate why 16S rRNA sequence comparison is a poor predictor for speciation of *Elizabethkingia*, a phylogenetic tree was constructed using 59 publicly available *Elizabethkingia* 16S rRNA gene sequences (1287 bp in length). Figure 1 clearly demonstrates that the species within this genus cannot be separated from each other on the basis of 16S rRNA sequence due to the fact that all three species within this genus are intermingled. In addition, the species names

already given may be incorrect due to speciation being determined primarily using the 16S rRNA sequences. Genomic differences seen with the DNA-DNA hybridization studies demonstrate that the *E. meningoseptica* type strain is clearly a different species from the majority of the other reported *E. meningoseptica* strains (91, 188). Since there is little, if any, discernible phenotypic differences between *Elizabethkingia* species and 16S rRNA sequencing does not effectively discern *Elizabethkingia* species, this has led us to complete and compare the draft genomes of *E. meningoseptica* and the recently characterized *E. anophelis* organisms in an effort to further delineate evolutionary relatedness.

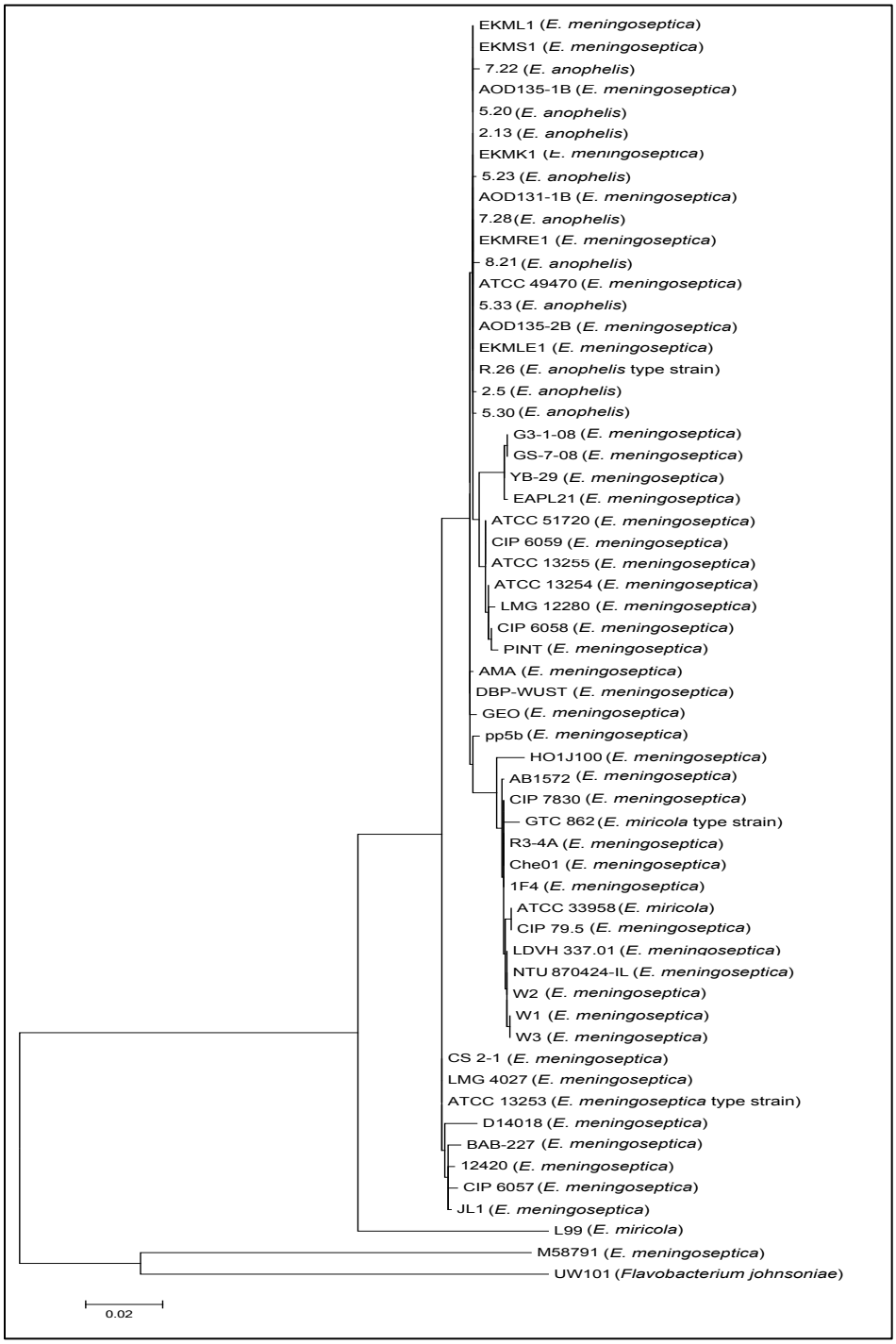

Figure 1. Maximum likelihood tree based on 16S rRNA gene sequences from sequenced *Elizabethkingia* in the NCBI database (http://www.ncbi.nlm.nih.gov/). *Flavobacterium johnsoniae* UW101 was used as an outgroup. Bar represents 0.02 substitutions per nucleotide position over 1287 bp.

1.8 Goals of these studies

i. The purpose of this study was to characterize numerous *S. aureus* strains isolated from raw milk samples collected from mastitic and healthy dairy cattle from a Paso Del Norte region dairy. This investigation also included the production of draft genome sequences of a MRSA and a MSSA isolate, and the comparison of these draft genomes with *S. aureus* genomic sequences present in the databases.

ii. Previously, a clinical hVISA strain, MM66, was isolated from the Memorial Medical Center in Las Cruces, New Mexico (38). In an effort to provide more information on the nature of the hVISA mechanism in MM66, we have completed the draft genomes of MM66 and a MM66 isolate (MM66RVI-4) expressing reduced vancomycin-intermediate resistance.

iii. *Elizabethkingia* species are environmental isolates that exhibit a multidrug-resistance phenotype and are a cause of life-threatening infections in immunocompromised individuals. To date, most studies have focused on the clinical aspects of this organism, and little is known regarding its antimicrobial resistance mechanisms. In addition, the phylogenetics and speciation of this genus is still highly controversial and has yet to be thoroughly defined. In an effort to clear up confusion surrounding the speciation of this genus as well as identifying possible antimicrobial resistance mechanisms, whole genome sequencing of the *E. meningoseptica* type strain was utilized.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial storage and preparation of stock plates

Stock cultures of *S. aureus* were maintained on either Mueller Hinton agar (MHA), Luria Broth agar (LBA) or Tryptic Soy Agar (TSA) and *E. meningoseptica* ATCC 13253^{T} were maintained on nutrient agar (NA) plates at 4°C. Single colonies were used to initiate growth in 2 ml of liquid media which were grown overnight at 30°C (*E. meningoseptica*) or 37°C (*S. aureus* strains) in a shaking incubator (200 rpm). Five hundred microliters of overnight cultures were transferred to a cryotube containing 500 µl of 50% glycerol to reach a final glycerol concentration of 25%. These glycerol stocks were then stored at -20°C and -80°C and were used to streak bacterial isolates onto working stock plates.

2.2 Isolation and characterization of *S. aureus* dairy strains

Samples of raw milk were collected from 33 hospital cows, some receiving antibiotic treatment, and 100 healthy cows in two milking parlors (A and B) at a local dairy. The isolation of *S. aureus* strains was carried out as follows.

Briefly, 100 µl of phosphate buffered diluted milk samples were spread onto Baird-Parker agar (Becton Dickinson and Co., Sparks, MD) plates and incubated at 37°C for 48 h. Resulting colonies were then subjected to Gram staining, a catalase test with 3% hydrogen peroxide and a coagulase test using rabbit plasma (Becton Dickinson and Co.). Presumptive *S. aureus* isolates were further scrutinized on mannitol salt agar (Acumedia Manufacturers, Inc., Lansing, MI) plates. Luria broth (LB) cultures of all presumptive isolates were then grown overnight and glycerol (20% v/v) was added to aliquots which were then stored at -80°C. Working cultures of these isolates were maintained on LBA plates at 4°C. Total DNA from 20 ml LB overnight cultures of 40 suspected *S. aureus* milk isolates, two positive control *S. aureus* lab strains (COL and ATCC 25923) and a negative control culture (*Staphylococcus epidermidis* ATCC 12228), were isolated as described below (157). These DNA samples were then scrutinized by a PCR protocol designed to detect the *S. aureus*-specific *nucA* nuclease gene or for *mecA* as previously described (16, 134).

2.3 Milk growth curves

The growth rate of ten *S. aureus* dairy strains plus one laboratory strain, COL in TSB and commercially available milk was compared. Briefly, overnight cultures were diluted in 1 X phosphate buffered saline (PBS) to an OD_{650nm} of 0.01. Five hundred microliters of diluted culture was then added to 50 ml of TSB or milk followed by incubation at 37°C with shaking (200 rpm). Serial dilutions (10-fold) were carried out and colony counts (CFU/ml) were determined on TSA plates at time-points 0, 1, 2, 3, 4, 6, 8 and 24 h following incubation at 37°C for 18-24 h.

2.4 Vancomycin-susceptible MM66 mutant isolation

Selection of the vancomycin-susceptible MM66 mutant, MM66RVI-4 was done using a replica plating method, by Mitchell Nelson (New Mexico State University). Briefly, a 5 ml LB culture of MM66 was maintained by passaging a 10% inoculum (final v/v) through drug-free LB everyday for 14 days. At days 7 and 14, the cultures were serially diluted to 10^{-6} and 100 µl aliquots of the diluted cultures were pipetted onto an LBA plate and spread across the surface using a sterile cotton swab, followed by incubation overnight at 37°C. A Kimwipe (Kimberly-Clark Professional, Dallas, Texas) was stretched across a PVC replica plating tool (Bel-art Scienceware, Wayne, NJ) and secured with a rubber band. The inoculated plates were then inverted and pressed onto the surface of the Kimwipe with slight pressure. The resulting imprinted Kimwipe was then used to transfer colony imprints onto drug-free LBA plates (control plates) and onto plates containing 1 µg/ml vancomycin. The plates were then examined for vancomycin susceptible mutant colonies that grew only on the control plates and not the vancomycin-containing plates. Single suspected vancomycin-susceptible mutant colonies were then picked off the drug-free plates and used to inoculate 3 ml of drug-free LB and grown overnight (37°C, 200 rpm). Inoculums from these overnight cultures were then used to create 20% glycerol freezer stocks and stored at -80°C.

2.5 Extraction of genomic DNA

Initially, strains were grown overnight in 25 ml of LB at 37°C (200 rpm) for *S. aureus* strains, 25 ml of nutrient broth (NB) at 30°C (200 rpm) for *E. meningoseptica* ATCC 13253^{T}. Cells were harvested by centrifugation at 7, 598 x *g* for 8 min. at 4°C and resuspended in 3 ml of lysis solution (20 mM Tris-HCl; 2 mM EDTA; 1.2% Triton X-

100; pH 8.0). Lysostaphin was added to the *S. aureus* isolates to a final concentration of 10 mg/l and lyzozyme was added to the *Elizabethkingia* isolates (final concentration of 2 mg/ml) and samples were incubated at 37°C or 30°C for 2 hr. After incubation, 0.3 ml of a 5% w/v SDS-50% v/v ethanol solution was added and vortexed for 10 s followed by the addition of 2 ml of phenol/chloroform/isoamyl alcohol (25:24:1; Sigma Aldrich). Samples were centrifuged at 47,488 x *g* for 10 min at 4°C and the supernatant was transferred to a new tube. The aqueous phase was extracted with an equal volume of chloroform (Sigma Aldrich), vortexed and centrifuged at 16,100 x *g* for 10 min at 4°C. The supernatant was then transferred to a new tube and two times the volume of 100% ethanol (Pharmco-Aaper, Brookfield, CT) was added and mixed well followed by centrifugation at 16,100 x *g* for 25 min at 4°C to pellet the DNA. The pellet was resuspended in 1 ml of TE buffer and RNAse-A (Sigma Aldrich) was added to a final concentration of 20 mg/l. Samples were incubated overnight at room temperature. Following overnight incubation, 0.2 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added, vortexed and centrifuged at 16,100 x *g* for 10 min at 4°C to get the aqueous phase. An equal amount of chloroform was added to each sample and again centrifuged at 16,100 x *g* for 10 min at 4°C. The aqueous phase was then transferred to a new tube and one times the volume of 3 M sodium acetate and two times the volume of 100% ethanol was added. The samples were centrifuged once again at 16,100 x *g* for 10 min at 4°C and the supernatants were decanted. The samples were allowed to dry at room temperature for 10 min then the DNA pellet was dissolved in 30 µl of 1X TE buffer. The purity and integrity of the DNA sample was determined by gel electrophoresis. Extracted DNA was stored at -20°C.

2.6 Draft genome sequencing

The draft genomes of *S. aureus* H29 and PB32 were sequenced (>20X overall coverage) using Roche 454 GS (FLX Titanium) pyrosequencing according to manufacturer's instructions at New Mexico State University (Las Cruces, NM). All reads were assembled into contigs utilizing the Newbler assembler (v 2.3; 454 Life Sciences). Draft genomes of *S. aureus* MM66 and MM66RVI-4 were produced with the Roche 454 GS (Junior) pyrosequencing platform at Oklahoma State University. Sequencing of MM66 generated 187,437 reads (>30 X coverage; average read length 455.4 bp) while MM66RVI-4 generated 213,277 reads (>30 X coverage; average read length 468.58 bp). Both genomes were assembled using the Roche GS *de novo* assembler (v 2.7). Contigs from *S. aureus* genomes were then compared to other genomes in the databases utilizing BLASTn (http://blast.ncbi.nlm.nih.gov/). Sequence types (ST) and allele numbers were determined with the *S. aureus* MLST website (http://saureus.mlst.net) using sequences obtained from 454 sequencing.

The draft genome of *E. meningoseptica* ATCC 13253^T was sequenced using the Roche 454 GS Junior pyrosequencing platform according to manufacturer's instructions at Oklahoma State University. Sequencing of ATCC 13253^T produced 223,447 reads (29.7 X coverage, average read length = 504.9 bp) that were assembled with Roche GS *de novo* assembler (v 2.7). All draft genomes were then uploaded into the Rapid Annotation using Subsystem Technology (RAST) server for annotation (6).

2.7 Pulsed-field gel electrophoresis (PFGE)

Initially, *S. aureus* strains were grown overnight in 5 ml of MHB at 37°C (200 rpm). The cultures (600 µl) were then transferred to microcentrifuge tubes and harvested by centrifugation (16,100 x *g*, 5 min, 25°C). The supernatant was then decanted and the pellet was resuspended in 400 µl PIV buffer (10 mM Tris-HCl; 1 M NaCl) and vortexed gently. The cells were reharvested (16,100 x *g*, 5 min, 25°C) and the microcentrifuge tubes were blotted to remove excess fluid. The pellet was resuspended in 200 µl of PIV buffer and mixed by pipetting. Each cell suspension was then diluted 1/200 with PIV buffer and the OD_{600nm} was read. To ensure that all samples have the same cell concentration, the OD_{600nm} value was put into the following dilution formula: (OD x 40 x 210) -210. A 1.5% low melting point agarose (Sigma Aldrich, St. Louis, MO) solution was then made up in 0.5X TBE (50 mM boric acid, 50 mM Tris, 1 mM EDTA) as required (150 µl of the agarose is required for each strain investigated). The solution was microwaved until the agarose dissolved and was then equilibrated in a 60°C water bath for 5 min. In a microcentrifuge tube at 60°C, initially 150 µl of the 60°C agarose and 150 µl of cells are added and then mixed via vortexing. Twenty five microliters of this cell suspension is then pipetted onto a flat parafilmed slide surface and covered with another parafilmed slide with 1 mm spacers and the disks are frozen at -20°C for 10 min. The lysis solution was prepared by adding 1 ml of EC buffer (1 M NaCl, 6 mM Tris-HCl; pH 8.0, 100 mM EDTA, 0.2% sodium deoxycholate, 0.5% sarkosyl), plus 2 mg/ml of lysozyme (Sigma Aldrich) powder and 8 µl of lysostaphin (Sigma Aldrich; 10 mg/ml stock) per strain to a microcentrifuge tube. All the disks from a single strain were then placed in 1 ml lysis solution in a microcentrifuge tube and allowed to lyse for 3 h at

37°C. After lysing, the tubes were placed on ice for 10 min. to firm the disks. The lysis buffer was then removed by pipetting and 1 ml of ES buffer (500 mM EDTA, 1% sarkosyl) and 10 µl of Proteinase K (Sigma Aldrich; 10 mg/ml stock) was added per strain and the samples were left overnight in a 50°C water bath. The next day, the samples were placed on ice for 10 min. and the buffer was removed and the disks were washed in TE buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA) at 37°C for 1 h. This was step was repeated five times with the last rinse being overnight for the total removal of the protease. At this stage the disks can be stored at 4°C indefinitely. The disks were placed on ice for 15 min prior to digestion. For each disk, 50 µl of restriction buffer (Buffer 4, New England Biolabs) was prepared for equilibration and 50 µl for digestion for a total of 100 µl per strain. Remove one disk and place into a tube containing 50 µl of restriction buffer and the disk was equilibrated for 30 min at 25°C (recommended temperature for the SmaI enzyme). The restriction mix was prepared by adding 1.2 µl of SmaI enzyme per strain to 50 µl of the diluted buffer 4 mix. The equilibration buffer was removed from the tubes containing the disks and the 50 µl of restriction mix was added and the tubes then sat overnight at 25°C. The pulsed field gel was a 1% agarose (BioRad, Hercules, CA) made in 0.5X TBE (45 mM Tris; 45 mM Boric acid; 1 mM EDTA, pH 8.0) and the running was also 0.5X TBE which was cooled in the pulsed field unit to 4°C approximated 30-40 min before loading the digested agarose disks. The disks were placed on wet ice for 10 min prior to loading the gel. Using a spatula, the disks were loaded against the bottom part of the well and once all disks were loaded, each well was sealed with 1.5% agarose. The gel was placed in the unit and the running conditions (6 volts/cm, initial switch time of 1 s, final switch time of 30 s, temperature of 11.3°C, and a

total run time of 23 h) were set. Once the gel was run, it was stained with ethidium bromide for 45 min and then washed with double distilled water for another 45 min. The image of the ethidium-stained SmaI bands was captured using the BioRad Gel Doc program and analyzed visually.

2.8 Antibiotic susceptibility testing

2.8.1 Kirby-Bauer disk diffusion

The antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method according to the CLSI (76). A single colony of each isolate was inoculated into 2 ml of MHB and incubated overnight at 30°C (*Elizabethkingia* strains) or 37°C (*S. aureus* strains), 200 rpm. Cultures were diluted to an OD_{625nm} of 0.1 in fresh MHB. Each adjusted suspension was then streaked onto MHA plates using a sterile cotton swab and antibiotic disks (Remel, Lenexa, KS) were put onto the bacterial lawn and incubated at appropriate temperature for 24 h. Zones of inhibition were then measured in mm.

2.8.2 Gradient plate analysis

Gradient plate analyses were performed as described previously (151). Briefly, gradient plates were prepared by pouring 40 ml of MHA into a 90 mm x 90 mm square petri dish raised at one side by a 1 ml pipette (6 mm) and left to dry overnight. After 24 h, 40 ml of MHA containing the correct antimicrobial concentration was then poured on top of the drug-free layer and allowed to solidify for 3 h at room temperature. Overnight MHB cultures grown at 37°C (*S. aureus* strains) or 30°C (*Elizabethkingia* strains), 200

rpm, were diluted in fresh MHB to an OD_{625nm} of 1.0. The resulting diluted cultures were used to streak the gradient plates three times (using a sterile cotton swab) for each row, in a concentration dependent manner (low concentration to high concentration). Plates were incubated for 48 h (37°C or 30°C) and distance of confluent growth along the gradient was measured and recorded in mm.

2.8.3 Vancomycin E-test

Vancomycin E-tests were performed as described by the manufacturer's instructions (AB Biodisk, New Jersey). Overnight *S. aureus* MHB cultures (37°C, 200 rpm) were diluted to an OD_{600nm} of 0.25. A cotton swab, dipped into the diluted cultures, was then used to spread an even inoculum onto MHA plates containing 2% NaCl. The plates were allowed to dry for 15 min and a single vancomycin E-test strip was placed in the center of the plate with tweezers flamed in 95% ethanol. After 24 h incubation (37°C), the MIC was read at the concentration at which growth was inhibited per manufacturer's instructions.

2.9 Vancomycin growth curves

Duplicate cultures (250 ml) were initiated with a 2 % (v/v) inoculum from LB overnights of MM66 and MM66RVI-4 (37°C, 200 rpm). Cultures were grown to an OD_{580nm} of 0.4 and then vancomycin was added (2.5 μg/ml final concentration) to one flask per strain. Sterile water was added to the second flasks as the control. Cultures were then incubated at 37°C, 200 rpm and the OD_{580nm} was read every h for a total of 8 h.

2.10 Vancomycin population analysis

Vancomycin population analyses were performed as previously described with slight modifications (151). Overnight MHB cultures of *S. aureus* strains MM66 and MM66RVI-4 (37°C, 200 rpm) were adjusted to an OD_{580nm} of 1.0 in fresh MHB and then serially diluted to 10^{-6}. A 10 µl aliquot of each dilution was inoculated onto MHA plates containing increasing concentrations of vancomycin (0 to 2.7 µg/ml). Following overnight incubation at 37°C, colony counts were then determined on plates containing dilution spots with 10 to 50 colonies.

2.11 Sequence analysis and phylogenetic tree construction

Sequence homology comparisons were done utilizing sequences within the GenBank databases using the BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and RAST programs (6). Nucleotide and protein sequence alignments and respective percent identities were produced using MacVector (v 12.6.0). Multiple sequence alignments for construction of the phylogenetic trees were completed using the Molecular Evolutionary Genetics Analysis (MEGA v5.2.1) software (182). The models used for the evolutionary distances were determined by MEGA (182).

In total, 59 partial 16S rRNA gene sequences (> 1000 bp) were obtained from the NCBI databases (http://www.ncbi.nlm.nih.gov/pubmed/). Alignment of the sequences was performed using the MUSCLE algorithm and trimmed to equal lengths (1287 bp). The evolutionary distances (phylogenetic tree) were computed using the Kimura 2-parameter method (Kimura, 1980) and *Flavobacterium johnsoniae* UW101 was used as an outlier.

Protein sequences of RpoB, GyrB, GroEL were obtained from the NCBI databases for 19 species of the *Chryseobacterium, Flavobacterium, Riemerella* and *Elizabethkingia* genera. Alignments were first performed for each protein individually and the resulting alignments were trimmed to equal lengths (GyrB, 228 amino acids (aa); RpoB, 249 aa; GroEL, 131 aa). The final trimmed protein sequences were combined end-to-end (GyrB, RpoB, GroEL; total size: 608 aa) for each species to obtain one concatenated protein sequence. A MUSCLE alignment was performed using the concatenated protein sequences and then used for construction of the phylogenetic tree. The evolutionary distances were computed using the Jones-Taylor-Thornton model (84).

Fourteen different protein sequences of previously characterized RND transporter genes were obtained from the NCBI databases and aligned to the 5 RND transporter sequences from *E. meningoseptica* ATCC 13253^{T}. Alignment of the proteins was performed using the MUSCLE algorithm and used for construction of the phylogenetic tree. The evolutionary distances were computed using the Whelan and Goldman (WAG) model (198).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation and characterization of *S. aureus* strains from a Paso Del Norte dairy

Growth on Baird Parker and mannitol salt agar, Gram stain and catalase reaction presumptively identified 40 *S. aureus* isolates from 29 milk samples. More than one isolated colony was chosen from samples that showed variable reactions on the selective media above (designated by strain number followed by a, b, or c). All 40 of these isolates were coagulase and *nucA* positive. These isolates included 7 from hospital cows (H strains), 18 from parlor A cows (PA strains) and 15 from parlor B cows (PB strains). None of the strains investigated demonstrated resistance to vancomycin by disk diffusion. All H strains except H15c were oxacillin-resistant, yet all 7 H strains were *mecA* positive. One H strain (H30) also demonstrated erythromycin resistance, while 3 H strains (H24, H26a, H26b) demonstrated resistance to ciprofloxacin and erythromycin. Strain H29 demonstrated resistance to both of these antimicrobials, as well as tetracycline and imipenem, and inducible clindamycin resistance. None of the PA and PB strains were

methicillin-resistant or *mecA*-positive. PA18 however demonstrated ciprofloxacin and erythromycin resistance, while PB26b demonstrated resistance to fusidic acid.

Contamination of milk by *S. aureus* has been previously linked to staphylococcal food-borne outbreaks (5, 46, 135). Studies have also revealed that virulence of *S. aureus* is significantly increased when grown in milk whey compared to growth in TSB (120). In addition, mastitis isolates have also demonstrated the ability to resist phagocytosis by bovine cells when grown in the presence of milk (180). A representative milk and TSB growth curve was produced to examine the ability of select dairy isolates (H29, H15a and PB32) and a common *S. aureus* laboratory strain (COL) to grow in commercially available milk compared to standard laboratory media (Figure 2). Both COL and H29 produced similar numbers of CFU/ml in milk as they did in TSB, however COL produced fewer CFU/ml in milk compared to H29 (Figure 2). Strains H15a and PB32 exhibited a decrease in CFU/ml when grown in milk compared to growth in TSB and both strains demonstrated differences in the levels of growth when compared to COL and H29 (Figure 2). All strains investigated demonstrated the ability to grow in milk and suggests the ability of these strains to be a source of staphylococcal food-borne intoxication outbreaks.

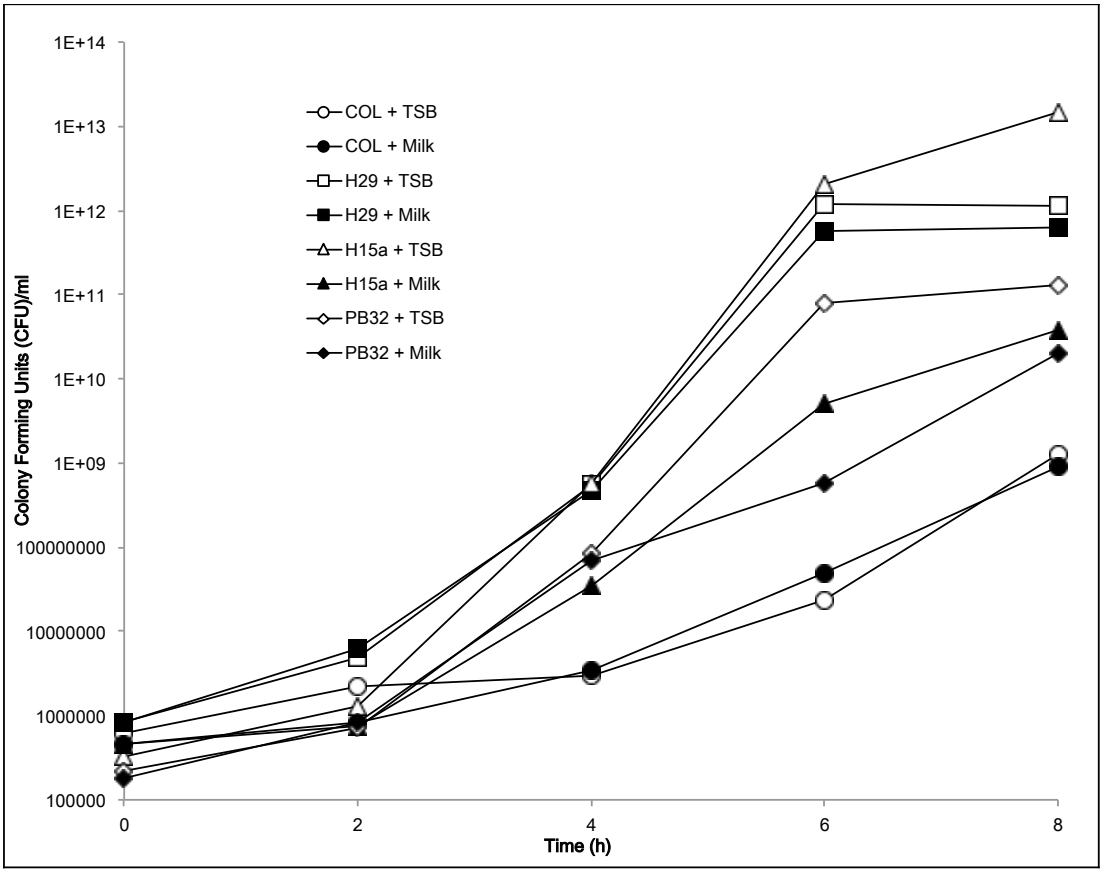

Figure 2. Representative growth curves for four *Staphylococcus aureus* strains (COL, H29, H15a and PB32) grown in milk and tryptic soy broth (TSB).

PFGE of SmaI-digested DNA separated the 40 strains into multiple pulsed-field types (PFT). PFT-A was the most frequently isolated PFT, representing 37.5% (n = 15) of all isolates (Figure 3). PFT-B contained the largest number of H strains (n = 5) and PA18, all of which demonstrated resistance to two or more of the antimicrobials investigated. PFT-C, -D and -E all contained two or more strains isolated from a single milk sample, which indicates the clonality of the colonies isolated from these milk samples. In two instances where more than a single *S. aureus* colony was chosen from

one milk sample (PA8a and PA8b; PA10a and PA10b), PFGE analysis revealed that the SmaI restriction fragment length polymorphisms were different (Figure 3). Both PA8b and PA10b clustered within the PFT-A strains, while PA8a and PA10a clustered close to PFT-A strains (Figure 3). This suggests that more than one *S. aureus* clone was present in each of these milk samples.

We next chose two strains for 454 sequencing. One was H29 which was a representative of the most widely distributed MRSA clone (PFT-B) and the other was PB32, which was a MSSA clone from PFT-C, that was most related to the PFT-B MRSA clones identified in this study.

The draft genome of MRSA strain H29 derived from 119 contigs (all >200 bp in length), consists of 2,844,315 bp, which encodes for 2,664 protein-coding genes. MLST revealed that strain H29 is ST5 (1,4,1,4,12,1,10), a sequence type that contains human MRSA isolates and is found within CC5. The presence of ST5 MRSA strains circulating in Paso Del Norte area hospitals located near the dairy investigated has previously been reported on (38, 141).

Overall, the H29 draft genome (accession no. PRJNA179361) proved to be 100% identical over 97% of the human MRSA strain JH1 genome (accession no. NC009632) by BLASTn comparisons. *S. aureus* JH1 is also an ST5 strain that was isolated from a patient with endocarditis and the JH lineage is capable of developing intermediate resistance to vancomycin (136, 170). A vancomycin E-test demonstrated that H29 does not demonstrate vancomycin-intermediate resistance.

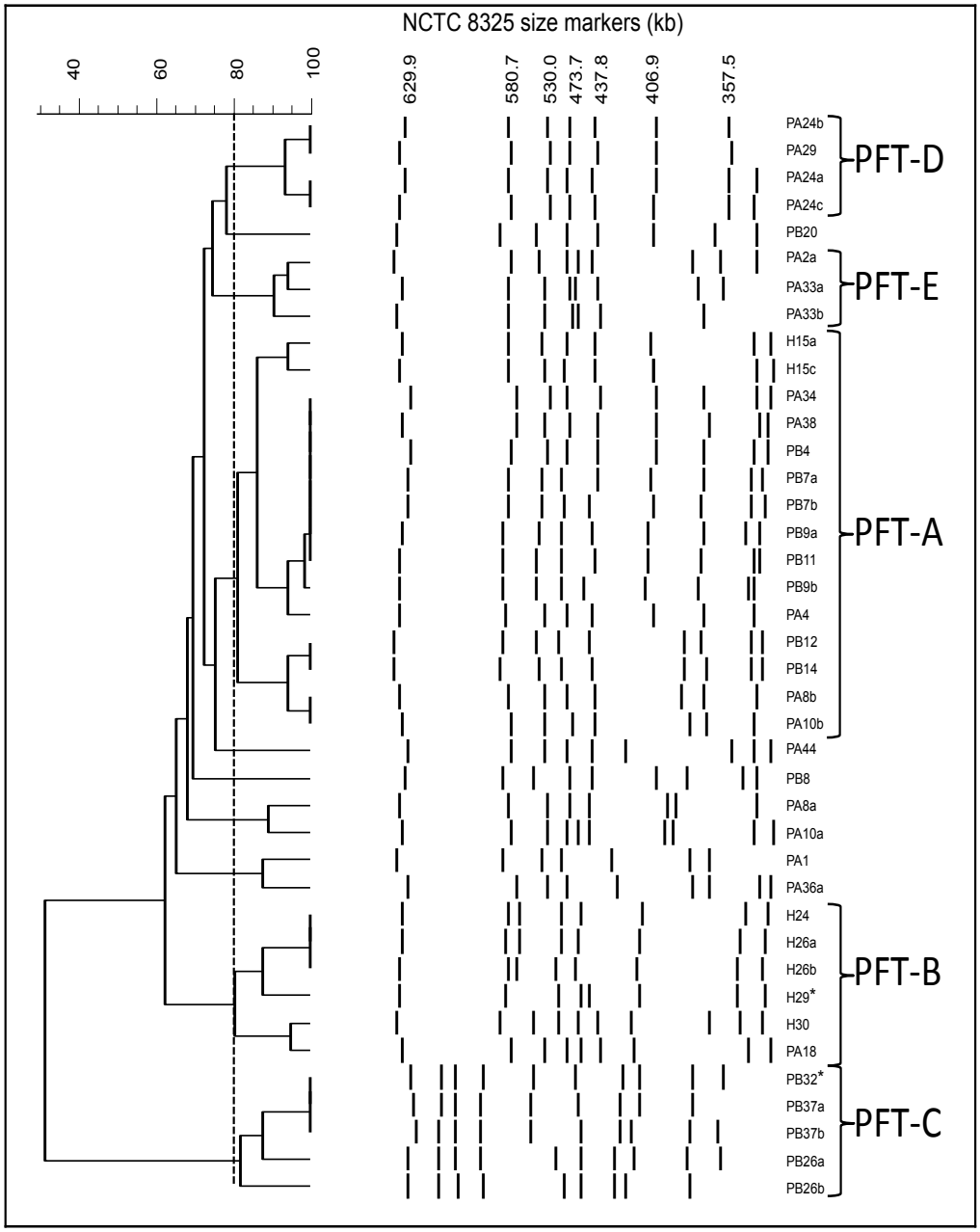

Figure 3. Pulsed-field gel electrophoresis (PFGE) patterns of SmaI-digested chromosomal DNA of *Staphylococcus aureus* strains investigated and dendrogram of percent relatedness derived from the patterns. Asterisks represent the two strains that were sequenced.

Contig 1 (27,139 bp) of the H29 draft genome represents a β-lactamase plasmid that had only 42% nucleotide similarity to plasmid pSJH101 (accession no. NC009619) found in JH1; yet was 99.5% identical to plasmid SAP048A (27,268 bp; accession no. GQ900406) found in *S. aureus* strain NE 3809, a human clinical blood isolate from Nebraska. SAP048A is a representative of pIB485-like plasmids that carry ΔTn552 encoding β-lactamase and a cluster of staphylococcal enterotoxin genes (*sed*, *sej*, and *ser*) that have contributed to outbreaks of *S. aureus* food-born illness (for review see Gustafson and Wilkinson, 2005; Shearer et al., 2011). In addition, contig 34 of H29 demonstrated 97% nucleotide identity to a previously described enterotoxin gene cluster (*egc*) operon that encodes five enterotoxin genes, *seo, sem, sei, sen, seg* and two pseudogenes, *ϕent1* and *ϕent2,* located on the νSaβ genomic island (82, 99). The H29 draft genome also contains the SCC*mec* sequence type II which harbors a erythromycin resistance gene (*erm*) (77), a Tn916-like transposon containing the tetracycline resistance gene *tetM* (36), and a mutation in the DNA gyrase gene (*gyrA*) that leads to a $^{84}S \rightarrow ^{84}L$ alteration in GyrA that has previously been tied to ciprofloxacin resistance (161).

The draft genome of MSSA strain PB32 (accession no. PRJNA179544) derived from 80 contigs consists of 2,808,519 bp which encodes 2,632 protein-coding genes. MLST sequences derived from the draft genome confirmed PB32 as a ST124 (3,1,1,37,1,5,3) strain of CC97, which is represented by bovine as well as shared bovine/human strains (175). There does not appear to be any readily identifiable plasmid sequences in the PB32 draft genome.

Overall, the PB32 draft genome (accession no. PRJNA179544) proved to be 100% identical over 94% of the human MRSA strain 04-02981 genome (accession no. CP001844.2) by BLASTn comparisons. Strain 04-02981 is a plasmid-less ST225 (1,4,1,4,12,25,10) multidrug-resistant SCC*mec* sequence type II strain that was isolated in Köln in 2004. (140). The ST225 sequence type first described in the 1990s in the USA represents a single locus variant of ST5 strains and the genome of 04-02981 is also co-linear with the JH1 genome (140).

S. aureus SCC*mec* insertion is carried out by site-specific recombination between the *attB* site on the chromosome and the *attS* site on SCC*mec* (78, 88). This recombination event results in copies of the *att* sites at each end of SCC*mec*, with *attR* within *orfX* and *attL* at the other end of the SCC*mec* (194). A 27,082 bp segment within contig 15 (215,480 bp) contains several genes previously described in SCC*mec* elements as well as the *attL* attachment site (Figure 4). These genes include the recombinase genes *ccrA* and *ccrB, pbp4*, and an arsenic resistance operon (*arsA-D*, *arsR*). It has been reported that all ST225 and JH strains carry the same *ccrB* gene (140). The *ccrB* gene of PB32 however only demonstrated 93% nucleotide identity to the *ccrB* of the ST225 strain 04-02981 and 39% nucleotide identity to the *ccrB* of the JH1 strain. *mecA* and *mecA* controlling genes (*mecI* and *mecRI*) were not present in the draft genome of PB32. In the PB32 draft genome, *orfX* is found on one end of contig 75 (130,864 bp) as well as the *attR* attachment site (Figure 4) suggesting that contig 15 and contig 75 belong together. *S. aureus* strains that possess chromosomally-located remnants of SCC*mec* determinants, including some with a *pbp4* gene, have been reported on (27, 42, 109, 116, 169, 200) and we suspect that sequences within contigs 15 and 75 described here represent a novel

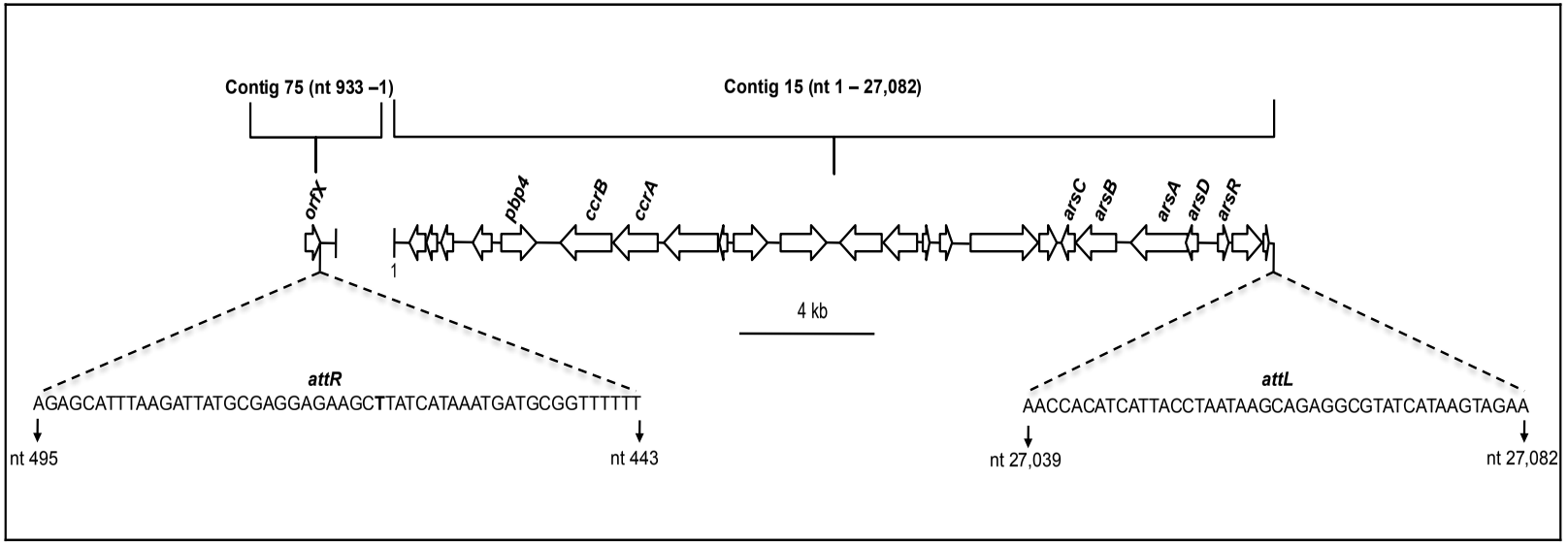

Figure 4. Cartoon representing open reading frames (ORF) of the SCC*mec* remnant found within contigs 15 and 75 *Staphylococcus aureus* strain PB32. Direction of the arrows represents the orientation of the ORF; attachment sites and their respective nucleotide sequences and positions are indicated. Abbreviations, nt is nucleotide.

SCC*mec* remnant. It is of interest to note that like PB32, ST225 MSSA isolates can also carry SCC*mec* remnants (140).

PB32 contains another mobile genetic element, a novel bovine staphylococcal pathogenicity island (SaPIbov) located within contig 66 (127,041 bp) which will be referred to as SaPIbovPB32. Sequence comparison of SaPIbovPB32 (nt 90,549 – 104,940) with other SaPIbov sequences, demonstrated the greatest nucleotide identity to SaPIbov5 (accession no. HM228919) at 79% identity followed by 66% identity to SaPIbov4 (accession no. HM211303) (191). SaPIbov5 (13,526 bp) was described in a *S. aureus* ST398 clone (191). *Staph. aureus* strains that contain SaPIbov5 have been isolated from different animals including cows, sheep and goats (191). SaPIbovPB32 is 14,391 kb in length and is flanked by 21 bp direct repeats that are found in other SaPIbov sequences (Figure 5). Of the 18 open reading frames (ORF) found in SaPIbov5, 11 were shared with SaPIbovPB32, which included the integrase, excisionase, primase-like genes as well as a von Willebrand factor-binding gene (*vwb*). PB32 also has a second *vwb* variant located on contig 12. Like PB32, it has been previously been reported that ruminant-associated *S. aureus* strains possess two variants of *vwb*, one located on SaPIbov and another located elsewhere on the chromosome (58, 191). The *vwb* gene contained within the SaPIbov is responsible for coagulation of ruminant-specific plasma and is suggested to play an important role in host-adaptation (58, 191). The genome of strain 04-02981 does not demonstrate the presence of a SaPIBov sequence. Lastly, a gene encoding enterotoxin A (*sea*) that produces a product with 91% amino acid identity to the *S. aureus* strain COL SeaA (51), was located on contig 22 of the PB32 draft genome.

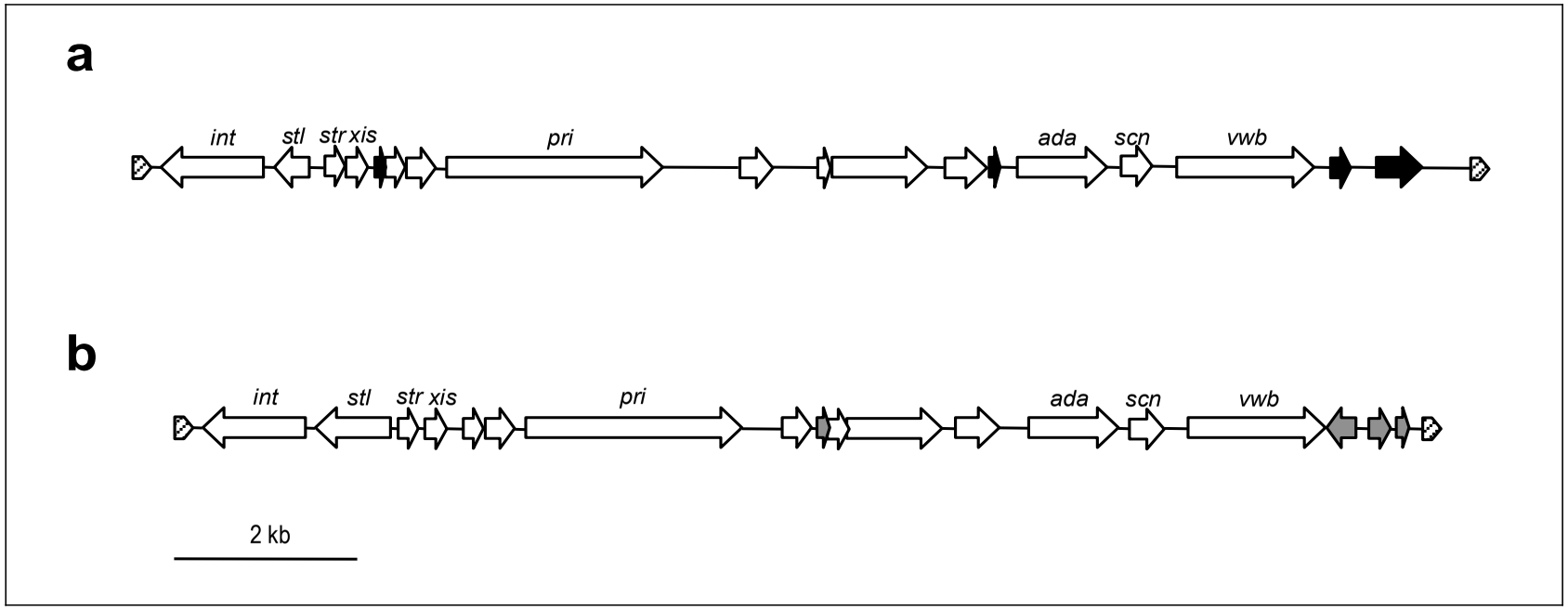

Figure 5. Comparison of SaPIbovPB32 (a) with SaPIbov5 (b). Direction of the arrows represents the orientation of the open reading frames (ORF). ORFs shared between SaPIbov5 and SaPIbovPB32 are indicated by white arrows. Black arrows in 3a indicate ORFs found only in SaPIbovPB32 but not in the SaPIbov5, while the grey arrows in 3b indicate ORFs found only in SaPIbov5. The hatched arrows at the ends of both sequences indicate the position of the 21 bp direct repeats (GAGTGGGAATAATTATATATA). Abbreviations, nt is nucleotide.

3.1.1 Conclusions

PFGE analysis revealed clonally-related *S. aureus* strains circulating within the dairy herd investigated. Strains represented by PFT-A, which included 13 MSSA strains and two *mecA*-positive strains (H15a and H15c), were the most widely distributed clones found within this herd. The presence of *mecA*-positive strains within PFT-A, including one that was also oxacillin-resistant (H15a), suggests that a PFT-A clone has either gained or lost the *mecA* determinant. Collectively, genomic analysis corroborates the multidrug resistance phenotype displayed by H29 and revealed the presence of both a novel SaPIbov sequence and SCC*mec* remnant in PB32. Genome analysis also demonstrated that H29 carried enterotoxin genes on both plasmid and chromosome locations, while PB32 harbored a chromosomally-located *sea* gene. This finding suggests that these strains are capable of causing staphylococcal food-poisoning. In addition, while the draft genome of PB32 was most related to MRSA strain 04-02981, based on differences in SCC*mec* and SaPIbov content, ST sequence types and *ccrB* sequences, PB32 and 04-02981 represent clearly diverged clones. Overall, our data demonstrates that H29 and PB32 represent novel clones of human and/or bovine-related strains of *S. aureus*. We speculate that the unique genomic features of these strains possibly reflect the evolutionary trajectory they have taken in the dairy herd examined.

3.2 Draft genomes of heterogeneous vancomycin-intermediate *Staphylococcus aureus* strain MM66 and MM66 variant exhibiting increased vancomycin susceptibility

3.2.1 Characterization of vancomycin resistance levels of MM66 and MM66RVI-4

Vancomycin is currently the antibiotic of choice for treating serious MRSA infections (103, 111) and resistance can now be found globally. Previously, a clinical hVISA strain MM66 was isolated from the Memorial Medical Center in Las Cruces, New Mexico (38). *S. aureus* strain MM66RVI-4 was obtained by passaging MM66 through drug-free media and selecting for MM66 colonies via replica plating that did not grow on plates containing 1 μg/ml of vancomycin. In order to determine the differences in vancomycin susceptibility between MM66 and MM66RVI-4, vancomycin E-test strips and vancomycin gradient plates were utilized. It was determined that the hVISA parent strain MM66 exhibits a vancomycin E-test MIC of 3 μg/ml while the vancomycin MIC of MM66RVI-4 was slightly decreased (2 μg/ml) in comparison to MM66 (Table 1). MM66RVI-4 demonstrated a significant decrease in the distance grown on a 0 → 3 μg/ml vancomycin gradient compared to MM66 (Table 1). Teicoplanin, another glycopeptide antibiotic, was used to determine whether the increase in susceptibility seen with MM66RVI-4 was unique to vancomycin or if it can be seen with other glycopeptide antibiotics. Teicoplanin gradient plates (0 → 3 μg/ml) of MM66RVI-4 demonstrated a similar increase in susceptibility to teicoplanin as vancomcyin, demonstrating that selection of MM66RVI-4 for increased susceptibility to vancomycin also confers increased susceptibility to other glycopeptide antibiotics (Table 1). It has been previously shown that selection for the VISA phenotype in the laboratory can also lead to the loss of SCC*mec* or demonstrate decreased levels of methicillin-resistance (1, 11, 139). Oxacillin

(methicillin) gradient plates were used to determine if there were differences in methicillin resistance levels upon selection of increased vancomycin susceptibility in MM66RVI-4. MM66RVI-4 demonstrated a significant decrease in methicillin resistance compared to MM66, as seen with the gradient plate technique (Table 1). This infers the possibility of either loss of the *mecA* gene responsible for methicillin resistance, or mutations within the *mecA* coding region leading to loss of function or decreased transcription of *mecA*. To determine if MM66RVI-4 lost the *mecA* gene, PCR was performed to amplify a 286 bp region of *mecA* from both MM66 and MM66RVI-4. Amplified MM66 DNA demonstrated a single 286 bp band while the DNA of MM66RVI-4 did not produce a *mecA* amplicon, confirming loss of the *mecA* gene and corroborates the decrease in methicillin resistance of MM66RVI-4.

Table 1. Antibiotic susceptibilities using gradient plates and vancomycin E-test

Strain	Vancomycin E-test (μg/ml)	Vancomycin Gradient	Teicoplanin Gradient	Oxacillin Gradient
		0 → 3 μg/ml	0 → 3 μg/ml	0 → 175 μg/ml
MM66	3	61.6 ± 4	30.6 ± 4	84.0 ± 2
		0 → 3 μg/ml	0 → 3 μg/ml	0 → 0.5 μg/ml
MM66RVI-4	2	41.6 ± 3*	16.0 ± 3*	73.0 ± 5

Numbers for gradient plates represent distances grown (mm) on 90 mm square petri plates with standard deviations (n=3)
*Denotes: MM66RVI-4 compared to MM66, p-value ≤ 0.05

A method that can be used to measure increased resistance to antimicrobials is bacterial growth curves in the presence of a specific antimicrobial. Growth of MM66 and MM66RVI-4 with and without the presence of 2.5 μg/ml vancomycin was measured by monitoring the optical density for an 8 h period. Without the presence of vancomycin, both MM66 and MM66RVI-4 exhibited similar growth. In the presence of 2.5 μg/ml vancomycin, MM66RVI-4 demonstrated a slight decrease in growth compared to MM66. As previously stated, hVISA are strains that have a subpopulation of cells resistant to vancomycin and are not detectable by standard broth and disk diffusion techniques. Therefore, vancomycin population analyses were then performed in an effort to determine if any minor differences in vancomycin resistance could be seen between the two strains. Differences in the number of surviving cells at various vancomycin concentrations were observed. It is important to note that at vancomycin concentrations of 1.5 μg/ml to 2.5 μg/ml, MM66RVI-4 was still able to produce colonies but not to the extent of the parent MM66 (Figure 6). This suggests that while MM66RVI-4 expresses an increased vancomycin susceptibility compared to MM66 utilizing vancomycin E-tests and gradient plates, it did not lose the ability to express some level of vancomycin resistance as seen with the vancomycin growth curves and population analyses.

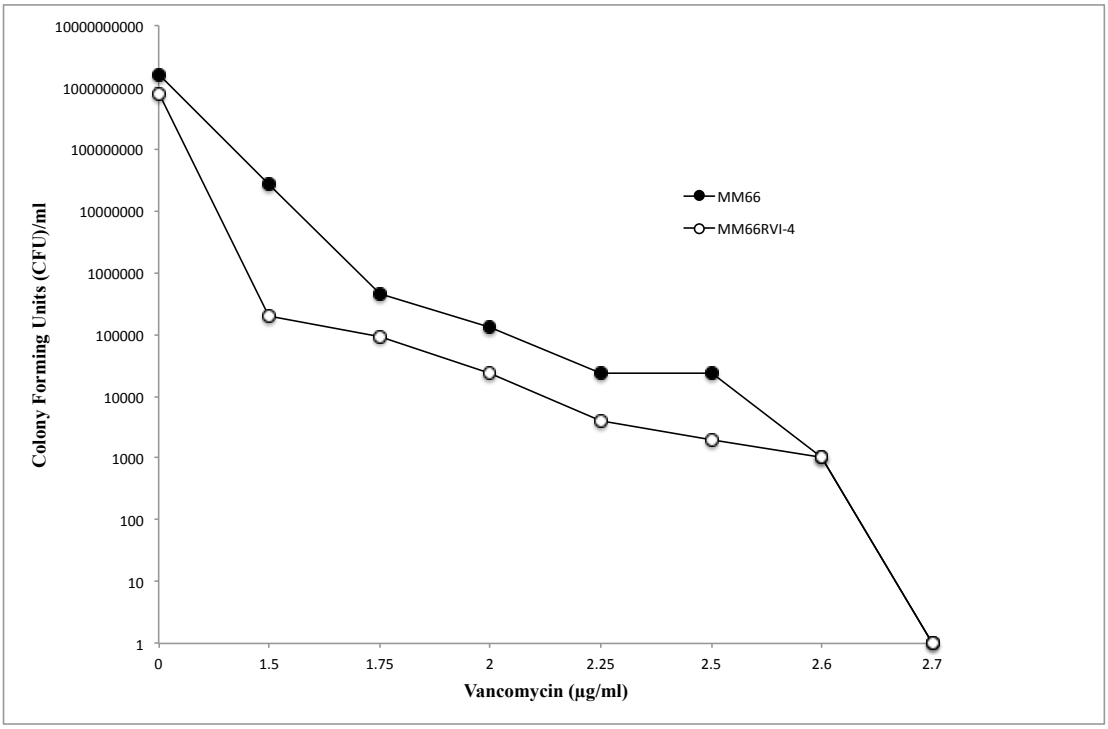

Figure 6. Vancomycin resistance population analysis profile of *Staphylococcus aureus* strains MM66 and MM66RVI-4.

3.2.2 MM66 and MM66RVI-4 draft genome analysis

In an effort to determine the genetic alterations, if any, that may be attributed to the phenotypic changes seen in MM66RVI-4 compared to MM66, in addition to genes involved with the hVISA mechanism, whole-genome sequencing was utilized. The MM66 draft genome sequence was 2,834,320 bp in length (32.9% G+C content) and included 2,684 protein-coding regions distributed in 114 contigs (>200 bp). The MM66RVI-4 draft genome sequence was 2,732,996 bp in length (33% G+C content) and included 2,563 protein-coding regions distributed in 197 contigs (>200 bp). MLST analysis of the drafts genomes demonstrated that both MM66 and MM66RVI-4 were sequence type 5 (ST 5), which is common for VISA strains (44). Interestingly, the

presence of ST 5 MRSA strains circulating among dairies in the Paso Del Norte area has previously been reported on (122, 141) and could suggest the clonal spread of these strains.

Initially, PCR confirmed deletion of *mecA* from MM66RVI-4, corroborating the loss of methicillin-resistance seen in this strain. However, further analysis of the MM66 and MM66RVI-4 draft genomes revealed that the majority (~46 kb) of the SCC*mec*II that is found in MM66 (~49 kb) was missing in MM66RVI-4 (Figure 7). It has been previously found that the passaging of MRSA strains through drug-free media can result in the spontaneous loss of methicillin-resistance (57, 67). Therefore, not only can selection for the VISA phenotype result in the loss of SCC*mec*, but selection for strains with increased susceptibility to vancomycin can as well. It has been argued that the loss of *mecA* is advantageous to the VISA mechanism (139) however, what role, if any, the loss of *mecA* plays in the selection for increased susceptibility to vancomycin requires further investigation.

It has been suggested that the VISA phenotype of *S. aureus* is the result of an accumulation of mutations over time or in some cases, a single mutation (22, 72, 136, 168). Mutations associated with the hVISA/VISA phenotype have been identified within a variety of *S. aureus* genes [for review see (70)]. Utilizing the draft genomes of MM66 and MM66RVI-4, sequence based comparisons with known hVISA/VISA genes were performed in an effort to determine which, if any, of these mutations are found in MM66 and MM66RVI-4. MM66 and MM66RVI-4 nucleotide/protein sequence comparisons were performed with *S. aureus* N315, a MRSA strain that is susceptible to vancomycin (99).

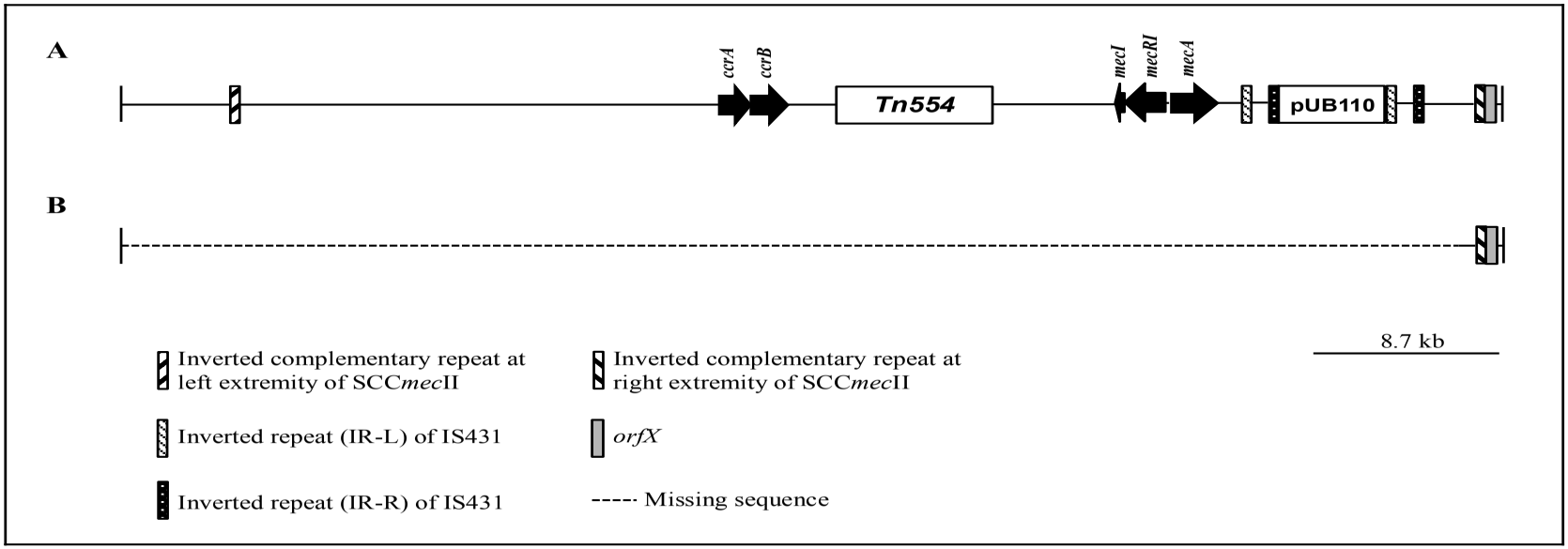

Figure 7. Comparison of the SCC*mec*II determinants from MM66 (a) and MM66RVI-4 (b). Direction of the arrows represents the orientation of the open reading frames.

No mutations in known genes responsible for the hVISA/VISA phenotype (*rpoB, walKR, vraR, graR, isdE, yycH*) were found within the MM66 and MM66RVI-4 draft genomes. This suggests that while these genes may be important for the VISA mechanism found in other VISA strains, they do not play a role in the hVISA phenotype exhibited by MM66 and MM66RVI-4.

The GraRS two-component regulatory system has been shown to control the expression of many genes (63) as well as playing a role in the VISA mechanism (32, 40, 74). For example, a single amino acid change in GraS (T136I) has been shown to contribute to the VISA phenotype of a clinical VISA strain (JKD6008) compared to a vancomycin-susceptible *S. aureus* (VSSA) strain (JKD6009) from the same patient (74). Introduction of the GraS T136I mutation into the VSSA strain JKD6009 demonstrated increased vancomycin resistance but not to the extent of the full VISA strain JKD6008 via population analyses (74). In this study, the GraS mutation was found to significantly contribute to the VISA phenotype, however, it was not the only mutation found and suggested that other mutations in addition to GraS may play a role in the VISA phenotype of these strains (74). Interestingly, a single mutation was also found within *graS* of both the MM66 and MM66RVI-4 draft genomes. Both MM66 and MM66RVI-4 harbored a novel mutation in *graS,* resulting in an amino acid change of S270N. PCR and Sanger sequencing were used to confirm the *graS* mutation found in MM66 and MM66RVI-4. Results from the vancomycin growth curves and population analysis assays demonstrated only minor phenotypic differences between MM66 and MM66RVI-4 in the presence of vancomycin. It can therefore be hypothesized that this *graS* mutation may contribute to the hVISA phenotype of MM66 and since the mutation is found in both

strains, may account for the similar levels of vancomycin resistance exhibited by MM66RVI-4.

In an effort to determine the genetic changes that may be associated with the minor increase in vancomycin susceptibility exhibited by MM66RVI-4, both draft genomes were aligned to identify single nucleotide polymorphisms (SNP) and/or insertions/deletions between the two strains. Alignment of the draft genomes produced a total of 9 nucleotide differences between MM66 and MM66RVI-4 (Table 2). Due to the fact that mutations linked to the hVISA/VISA phenotype have been found within cell wall-related genes, it is of no surprise that 5 of the alterations in MM66RVI-4 are found within genes known to be associated with the cell wall. It is of particular importance to point out the multiple changes found within the *vraG* gene of MM66RVI-4, which encodes an ATP-binding cassette (ABC) transporter permease and results in a premature stop codon at the 440th amino acid position (out of 629 amino acids). *vraG* is upregulated in the VISA phenotype however mutations within *vraG* have been shown to be responsible for increased vancomycin susceptibility in two different MRSA and VISA strain backgrounds (127). Based upon the mutations found within *vraG* it could be hypothesized that these mutations are responsible for the increased vancomycin susceptibility exhibited by MM66RVI-4 (MIC = 2 μg/ml) compared to MM66 (MIC = 3 μg/ml). Interestingly, *vraG* expression is under the control of *graRS* (127) and both MM66 and MM66RVI-4 contain a mutation within *graS*. We also hypothesize that the effects of the *graS* mutation on *vraG* expression or VraG activity might contribute to the vancomcyin-intermediate mechanism of MM66. Therefore, the mutations in *vraG* in MM66RVI-4 may reduce the effects of the vancomycin-intermediate supportive *graS*

mutation on *vraG* expression or VraG activity, and the *vraG* mutations in MM66RVI-4 may contribute to the reduced expression of the vancomycin-intermediate mechanism in this strain. It can also be postulated that the other genetic alterations found within MM66RVI-4 (Table 2) play a role in the increased vancomycin susceptibility of MM66RVI-4 as well. However, it should be noted that with the exception of the mutations found within *vraG,* the majority of these differences between MM66 and MM66RVI-4 are found within homopolymer regions of these genes. It has been previously found that homopolymer regions are known to be a source of sequencing errors within 454 pyrosequencing (208).

Table 2. Mutations detected in draft genome sequence of MM66RVI-4 compared to MM66

	Genetic Change MM66RVI-4	MM66	Gene	Product	Amino Acid Change
1	IN; Contig 126, A^{3100}	Contig 73		Hypothethical protein; *ssaA* homologue	No Change
2	DEL; Contig 36; A^{7921}	Contig 9	*ftsL*	Cell division protein	No Change
3	IN; Contig 138; A^{1503}	Contig 54	*sasG*	Virulence-associated cell-wall-anchored protein (LPXTG motif)	Met → stop (445^{th} amino acid of 859)
4	DEL; Contig 55; $A^{16,134}$	Contig 31	*vraG*	ABC transporter permease	Met → stop (440^{th} amino acid of 629)
5	DEL; Contig 55; $A^{16,528}$	Contig 31	*vraG*	ABC transporter permease	Met → stop (440^{th} amino acid of 629)
6	SNP; Contig 55; $T^{16,983}$	Contig 31; $A^{33,267}$	Intergenic		
7	DEL; Contig 55; $AT^{16,982}$	Contig 31	Intergenic		
8	DEL; Contig 89; A^{6613}	Contig 8	Intergenic		
9*	IN; Contig 44; $AC^{21,688}$	Contig 30		Hypothetical membrane protein	Truncation of protein (1^{st} Met at position 89 of 353 amino acids)

Abbreviations: IN, insertion; DEL, deletion; SNP, single nucleotide polymorphism
Numbers represent nucleotide positions of the mutations within given Contigs
* Contig 44 of MM66RVI-4 is missing the first 21 base pairs of this open reading frame

3.2.3 Conclusions and future directions

In this study, we were able to isolate MM66RVI-4, a MM66 clone that demonstrated increased susceptibility to vancomycin. MM66RVI-4 was able to demonstrate slightly reduced growth and a reduced ability to produce cells that survive on inhibitory concentrations of vancomycin. MM66RVI-4 also demonstrated an almost complete loss of the SCC*mec*II, corroborating the decrease in methicillin resistance of this strain. Investigations into the genetic differences between MM66 and MM66RVI-4 revealed mutations within genes previously associated with both the VISA phenotype and VISA reversion. The GraS mutation may account for the similar levels of vancomycin resistance exhibited by MM66 and MM66RVI-4 and that the mutations within *vraG* of MM66RVI-4 may be responsible for the slight increase in susceptibility to vancomycin. However, to confirm the significance of these mutations, re-sequencing and confirmation is needed to ascertain their role, if any, in the increased vancomycin susceptibility phenotype exhibited by MM66RVI-4.

3.3 Draft genome sequence of *Elizabethkingia meningoseptica*

3.3.1 Antimicrobial resistance mechanisms of *Elizabethkingia meningoseptica*

Bacteria have evolved several ways to overcome susceptibility to antibiotics which include alteration of drug targets, changes in membrane permeability, inactivation of drugs by degradation/modification and drug efflux from cells (148). In essence, all antimicrobial resistance mechanisms prevent the drug from binding to its target. Not much is known about the antimicrobial resistance mechanisms of *Elizabethkingia,* even though the organisms demonstrate a multidrug-resistant (MDR) phenotype (17, 92, 181). Utilizing the Kirby-Bauer disk diffusion method we have determined that *E. meningoseptica* strain ATCC 13253^{T} demonstrates resistance to 19 of 30 antibiotics tested confirming the MDR phenotype previously seen for this strain (Table 3) (17, 92). This strain is resistant to antibiotics representing the following antibiotic classes: aminoglycosides, cephalosporins, β-lactams, tetracyclines, lincosamides, streptogramin, oxazolidonone, and glycylcycline. To date, the antimicrobial regimens used for treating infections caused by supposed *E. meningoseptica* are quite variable (93). This variability might also be indicative of the inability of the attending physician to pick the best antimicrobial therapy because the species and their antimicrobial susceptibility profiles are undefined by the Clinical Laboratory Standards Institute (177). Due to the variability in antimicrobial susceptibility patterns seen amongst *Elizabethkingia* strains and 16S rRNA sequencing not effectively distinguishing between *Elizabethkingia* species, lead us to complete and compare the draft genomes of these organisms. Analysis of these draft genome sequences will provide a firm beginning to the process of correctly establishing the species of this genus, in addition, aiding in the identification of possible resistance

mechanisms responsible for the MDR phenotype of these organisms. Whole-genome sequencing of *E. meningoseptica* ATCC 13253^T produced a draft genome that was 3,797,222 bp (35.2% GC content) in length and included 3,486 protein-coding regions distributed in 115 contigs (>200 bp).

Table 3. Antibiotic susceptibility profile for *E. meningoseptica* ATCC 13253^T

Antibiotic	Susceptibility
Amikacin	R
Aztreonam	R
Cefamandole	R
Cefepime	R
Cefoxitin	R
Ceftazidime	R
Ceftriaxone	R
Chloramphenicol	R
Ciprofloxacin	S
Clindamycin	S
Erythromycin	I
Fusidic Acid	S
Gentamicin	R
Levofloxacin	S
Lincomycin	R
Linezolid	R
Minocycline	S
Neomycin	R
Oxacillin	R
Piperacillin	S
Piperacillin/Tazobactam	S
Quinupristin/Dalfopristin	R
Rifampin	S
Spectinomycin	R
Streptomycin	R
Tetracycline	R
Ticarcillin/Clavulanic Acid	R
Tigecycline	R
Trimethoprim/Sulfamethoxazole	S
Vancomycin	S

Abbreviations: R, resistant; S, susceptible; I, intermediate-resistance

The β-lactam class of antibiotics is often the first antimicrobial choice for the treatment of infections caused by Gram-negative bacteria (98). To date, the only mechanism of antibiotic resistance studied in the *Elizabethkingia* species revolves around its resistance to β-lactam antibiotics (7, 132, 158, 159, 201). The expression of β-lactam and related drug resistance by *Elizabethkingia* is attributed to the presence of at least three chromosomally located β-lactamase genes. This includes two different metallo-β-lactamase (MβL) genes (*blaB* and bla_{GOB}) and one extended-spectrum β-lactamase gene (ESBL; $blaA_{CME}$) (8, 9, 159). These genes produce enzymes that hydrolyze the β-lactam ring found in β-lactam antibiotics and related antimicrobials, therefore inactivating these drugs.

Metallo-β-lactamases belong to the Class B/Group 3 of the Amber/Bush-Jacoby-Medeiros classification on the basis of their functional properties (3, 19). Three subclasses (B1, B2, B3) for MβLs have been described and are based on sequence similarities between these enzymes (49). The first MβL found in *Elizabethkingia, blaB,* encodes a 249 amino acid polypeptide and is a Class B MβL that belongs to the Subclass B1 (159). Protein sequence comparison to other MβLs indicates *blaB* shares the greatest homology to the MβL enzymes Bc-II from *Bacillus cereus* (35% amino acid identity) and CcrA from *Bacteroides fragilis* (27% amino acid identity) (159). The second MβL gene found in *Elizabethkingia* species, bla_{GOB}, encodes a 290 amino acid polypeptide and is also a Class B MβL but belongs to the Subclass B3 (7). Protein sequence comparison indicates bla_{GOB} shares the greatest homology to FEZ-1 from *Legionella gromanii* (42% amino identity) and L1 from *Stenotrophomonas maltophilia* (18% amino acid identity)

(7, 69). The substrate profiles for both *blaB* and *bla*$_{GOB}$ have been shown to be broad, with the ability to hydrolyze the penicillin, cephalosporin, and carbapenem classes of β-lactam antibiotics (7, 53, 159, 190, 201). Alignment of the protein sequences of *blaB* and *bla*$_{GOB}$ confirms the two MβL genes found in *Elizabethkingia* are different as they only share 11% amino acid identity (7). The third β-lactamase, *blaA*$_{CME}$ found in *Elizabethkingia* species is a Class A serine β-lactamase within Group 2e of the Bush-Jacoby-Medeiros classification (3, 19, 158). The *blaA*$_{CME}$ gene encodes a 295 amino acid polypeptide that demonstrates the greatest homology to the Class A enzymes VEB-1 from *Escherichia coli* (46% amino acid identity), CblA from *Bacteroides uniformis* (40% amino acid identity) and PER-1 from *Pseudomonas aeruginosa* (39% amino acid identity) (158). Like the MβLs in *Elizabethkingia,* the ESBL *blaA*$_{CME}$ also exhibits a broad substrate profile, with the ability to hydrolyze cephalosporins, penicillins and monobactams (158). Taken together, the presence of these three chromosomally-located β-lactamases enables *Elizabethkingia* to effectively be resistant to most β-lactam antibiotics and emphasizes the difficulty in the treatment of infections caused by these organisms.

The β-lactamases found within *Elizabethkingia* exhibit heterogeneity adding to the complexity of the MDR phenotype. Two variants of *blaA*$_{CME}$ (9, 158), 13 variants of *blaB* (7, 159, 201, 207) and 18 variants of *bla*$_{GOB}$ (7, 132, 207) have been reported. Woodford et al., (2000), characterized and determined the distribution of the *blaB-1* and *blaA*$_{CME}$ genes from several reference strains of *E. meningoseptica* (National Collection of Type Culture [NCTC] collection), including the *E. meningoseptica* type strain ATCC 13253^{T} (201). In this study, the *blaB-1* gene was only detectable by PCR in 5 of the 15

NCTC strains and the *blaB-1* gene could not be detected in the type strain (201). However, resistance of the type strain to the carbapenem antibiotics, imipenem and meropenem, suggested the presence of a novel variant of the *blaB-1* carbapenemase (*blaB-3*) (201). Analysis of the 13 BlaB variant sequences described to date revealed 86 to 100% amino acid identity (81 to 100% nucleotide identity) amongst each other (Figure 8). It should be noted that BlaB-3 and BlaB-4 are in fact the same genes as they demonstrate 100% nucleotide and amino acid identity to each other, making the total number of *Elizabethkingia blaB* variants to be 12 (7, 201). Upon further examination of these BlaB variant sequences by our laboratory, ATCC 13253^T in fact demonstrates 99% amino acid identity not only to BlaB-3 (BlaB-4), but to BlaB-12 and BlaB-13 as well. In this same study, PCR detection for the ESBL *blaA*$_{CME}$ gene, which was found in other presumed *E. meningoseptica* strains, was negative for ATCC 13253^T (201). Utilizing our draft genome sequence of ATCC 13253^T, the inability to detect *blaA*$_{CME}$ is due to sequence differences that are found between strains of *E. meningoseptica* and that the primer sequences used were designed to detect specific β-lactamase sequences. For example, *blaA*$_{CME}$ and *bla*$_{CME-2}$ demonstrate 98% amino acid identity to each other while the β-lactamase found in ATCC 13253^T demonstrates 73% and 74% amino acid identity (73% nucleotide identity) to *blaA*$_{CME}$ and *bla*$_{CME-2}$, respectively (Figure 9). This suggests the possibility of ATCC 13253^T to either possess a novel variant of the *blaA*$_{CME}$ gene or that these genes are found within different *Elizabethkingia* species. In addition to the *blaB* and *blaA*$_{CME}$ variants, several *bla*$_{GOB}$ variants have been reported on, demonstrating 71 to 99% amino acid identity to each other (7, 207). The first description of *bla*$_{GOB-1}$ was in 2000 from a clinical *Elizabethkingia meningoseptica* isolate and that same study

described *E. meningoseptica* ATCC 13253^{T} as possessing a GOB-1-like sequence (7). However, a MUSCLE alignment of the two protein sequences demonstrates only 80% amino acid identity (Figure 10). More variants have been described since then and the ATCC 13253^{T} Bla_{GOB} sequence currently shares 98% and 99% amino acid identity to Bla_{GOB-16} and Bla_{GOB-17}, respectively (207).

Alignment of all the *Elizabethkingia* β-lactamase variants described to date clearly validates the heterogeneity previously found within these genes. The difficulty in PCR detection of these variants lies in the selection of correct primer sequences due to the wide range of nucleotide differences between the genes. In addition, these variations in β-lactamase sequences within this genus could in fact be due to the possession of different β-lactamase genes from multiple *Elizabethkingia* species, not just *E. meningoseptica.*

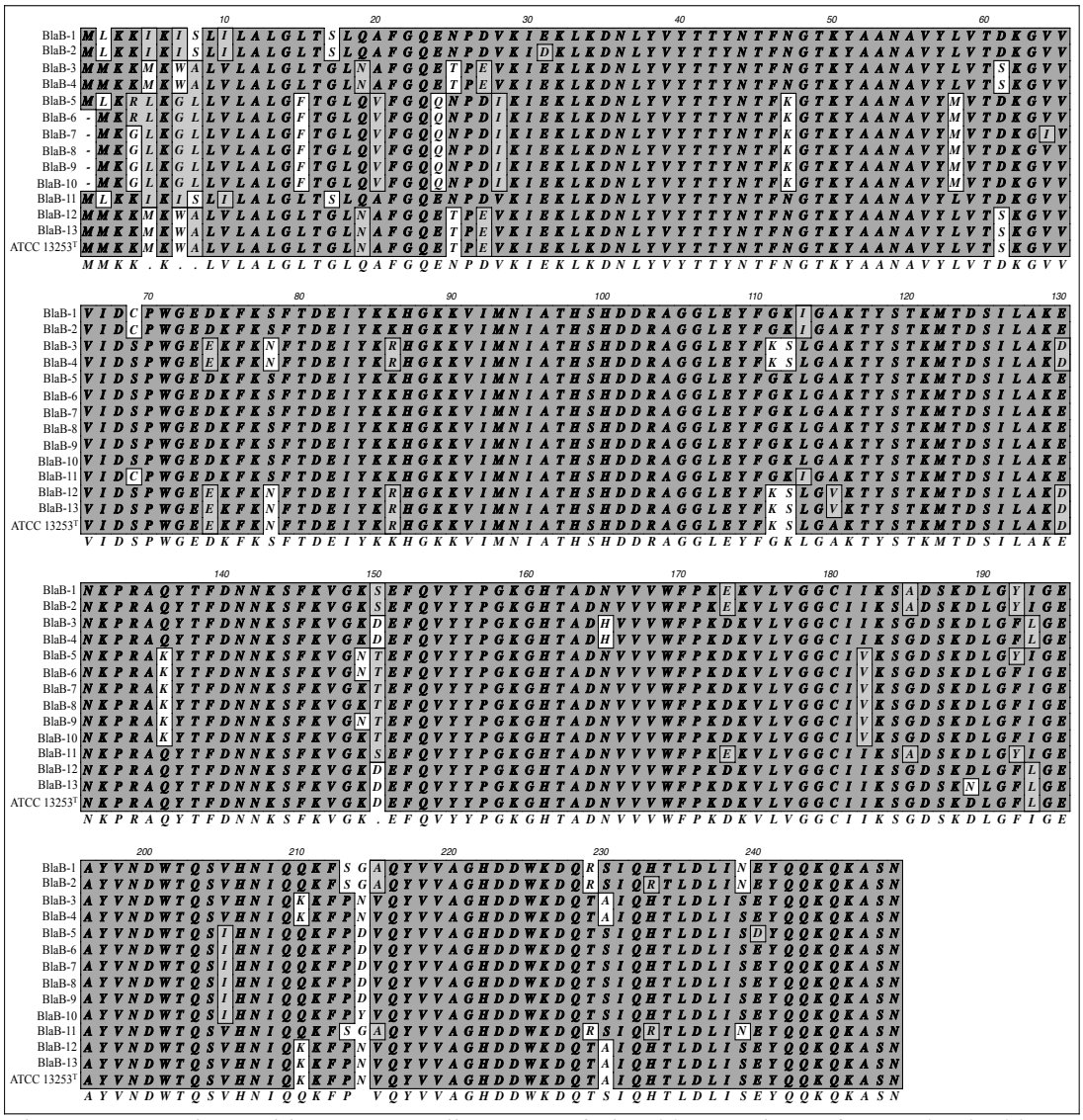

Figure 8. Amino acid sequence alignment of the *bla*B variants from *Elizabethkingia meningoseptica*. The alignment was generated using MUSCLE in MacVector (v 12.6.0). The dark gray boxes indicate identical amino acids and the light gray boxes indicate similar amino acids. Absence of gray indicates different amino acids. Dashes indicate gaps within the alignment.

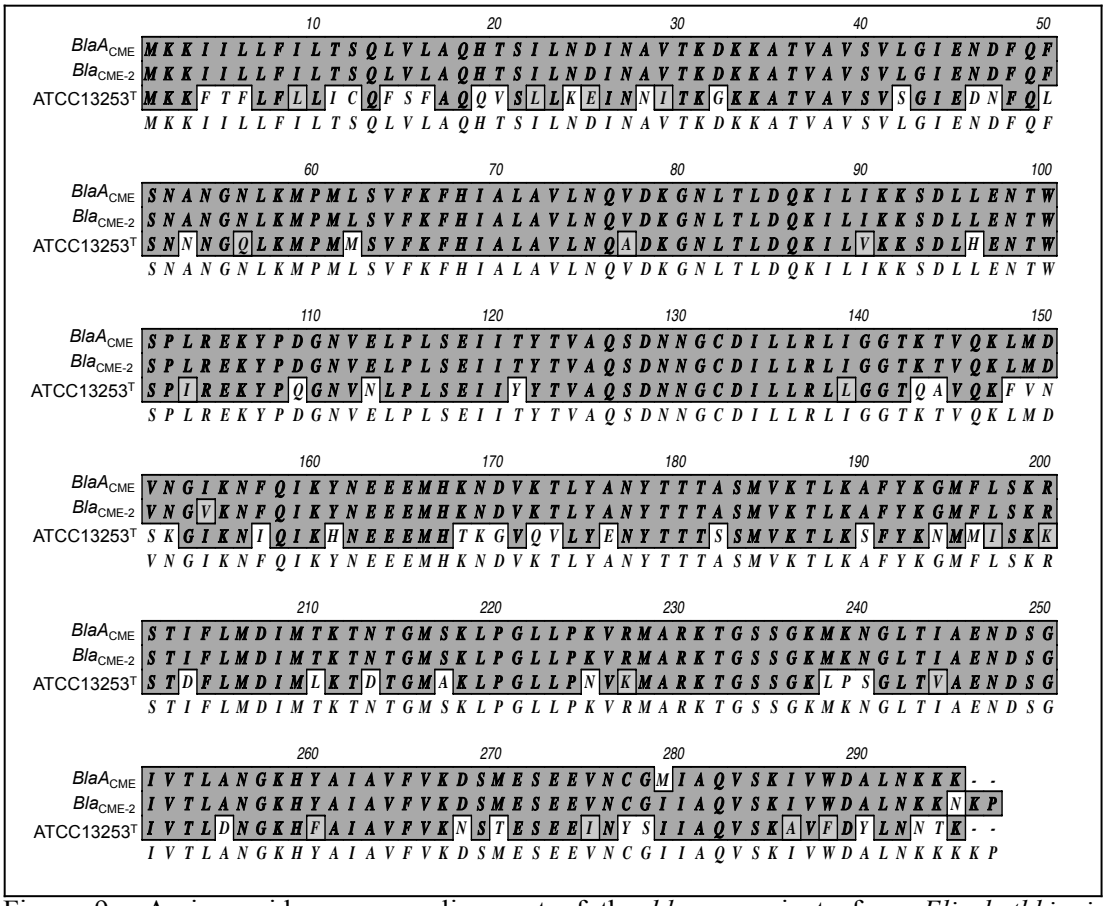

Figure 9. Amino acid sequence alignment of the *bla*$_{CME}$ variants from *Elizabethkingia meningoseptica*. The alignment was generated using MUSCLE in MacVector (v 12.6.0). The dark gray boxes indicate identical amino acids and the light gray boxes indicate similar amino acids. Absence of gray indicates different amino acids. Dashes indicate gaps within the alignment.

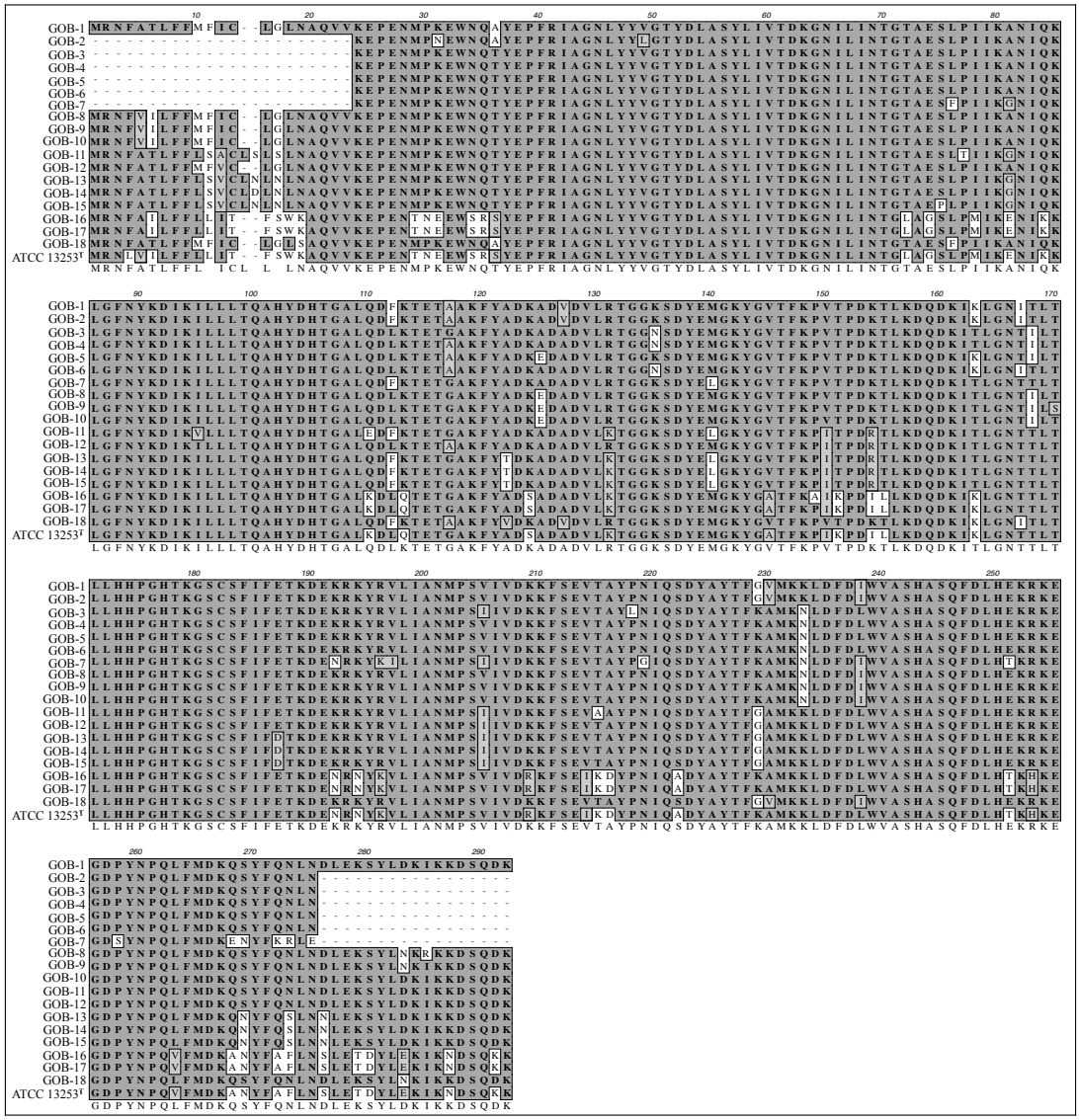

Figure 10. Amino acid sequence alignment of the *bla*$_{GOB}$ variants from *Elizabethkingia meningoseptica*. The alignment was generated using MUSCLE in MacVector (v 12.6.0). The dark gray boxes indicate identical amino acids and the light gray boxes indicate similar amino acids. Absence of gray indicates different amino acids. Dashes indicate gaps within the alignment.

The susceptibility/resistance patterns of clinical *E. meningoseptica* isolates have been shown to vary substantially (93). Besides the β-lactamase genes described above, the bioinformatic analysis of the draft genome of *E. meningoseptica* ATCC 13253^{T} did not reveal the presence of previously characterized horizontally transmitted antimicrobial resistance genes. One very important mechanism of "intrinsic" antimicrobial resistance is drug efflux, which is mediated by genes that encode single to multiple drug efflux pumps. The presence of these genes is in fact required for many acquired (either by mutation or gene acquisition) clinical antimicrobial resistance mechanisms. All the bacterial efflux pumps described to date fall into one of five groups: resistance-nodulation-division family (RND); major facilitator superfamily (MFS); ATP-binding cassette family (ABC); small multidrug-resistance family (SMR); and multidrug and toxic compound extrusion family (MATE). In Gram-negative bacteria, RND-type efflux pumps are known to play a significant role in resistance to a broad-spectrum of antimicrobial agents including classic antimicrobials, detergents, dyes and solvents (138, 147, 199). RND-type efflux pumps were first described in the 1990's in *Escherichia coli* (AcrAB-TolC) and *Pseudomonas aeruginosa* (MexAB-OprM) and have been shown to form tripartite membrane complexes (117, 149). These complexes consist of an outer membrane protein (OMP), a membrane fusion protein (MFP), and a resistance-nodulation-division transport protein (RND) that work together as a proton-drug antiport to pump antimicrobials from the cell interior into the external medium. The presence of intrinsic multidrug efflux pumps, particularly of the RND type may play a role in the intrinsic MDR mechanism expressed by this organism as they do in other well-characterized pathogens (61, 204, 206). Utilizing the ATCC 13253^{T} draft genome, 5 putative efflux gene operons belonging to

the RND-type family efflux pumps were identified (123). A phylogenetic analysis of the RND-type efflux transporter genes revealed that the transporter genes of *E. meningoseptica* ATCC 13253^{T} form their own clade and diverge away from other well-characterized Gram-negative RND efflux transporter genes (Figure 11) (61, 125, 129, 144, 153, 156, 163, 178, 179).

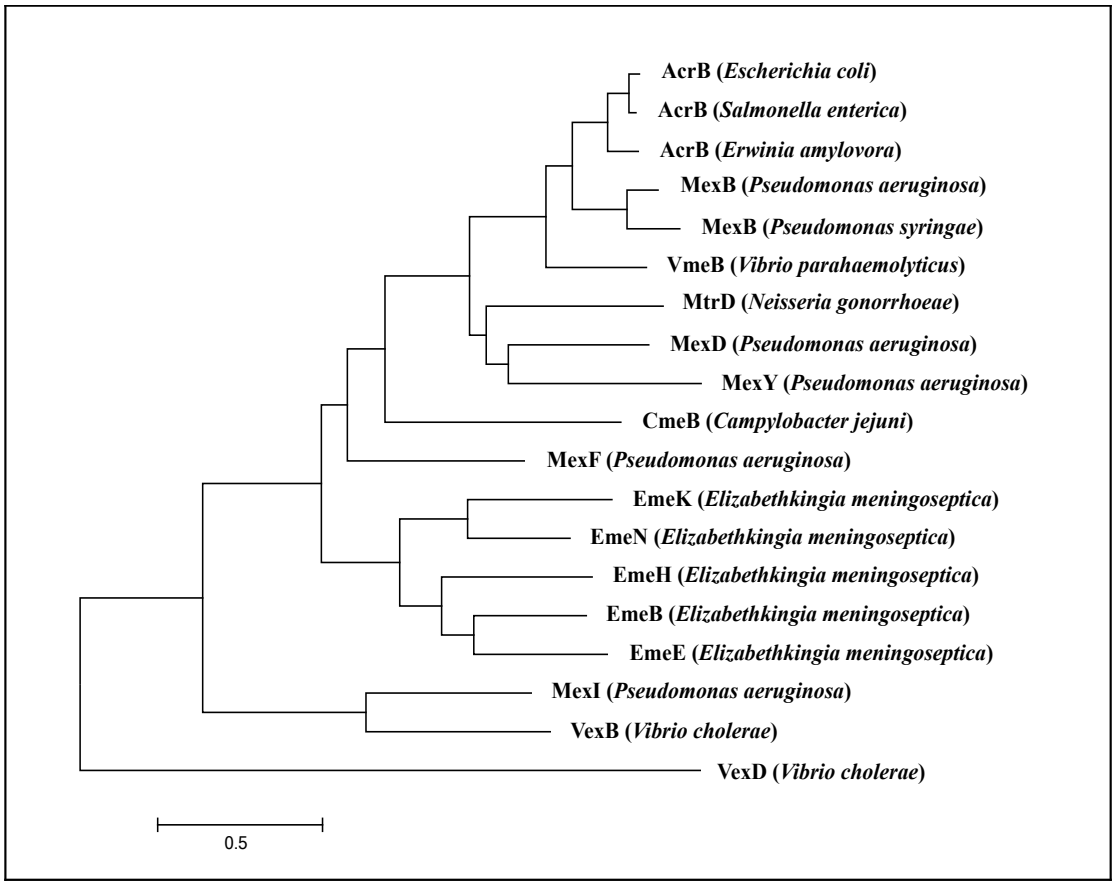

Figure 11. Phylogenetic relationship between representative RND-type efflux transporter proteins using a maximum-likelihood analysis. Bar represents 0.5 substitutions per amino acid position.

Figure 12 shows the alignment of the 5 putative *E. meningoseptica* RND efflux operons (*emeABC*, *emeDEF*, *emeGHI*, *emeJKL* and *emeMNO*) compared to the well-characterized *mexAB-oprM* operon from *P. aeruginosa* strain PAO1. Not only are these *eme* operons of similar size to *mexAB-oprM*, but they also have similar gene organization. The percent amino acid identity across the length of the MexAB-OprM proteins, were next compared to the Eme protein alignments (Figure 12). Alignment results revealed that the Eme proteins demonstrated 24% to 40% amino acid identity to the MexAB-OprM from *P. aeruginosa*. This data suggests the presence of RND-type efflux operons in *E. meningoseptica* ATCC 13253^{T}.

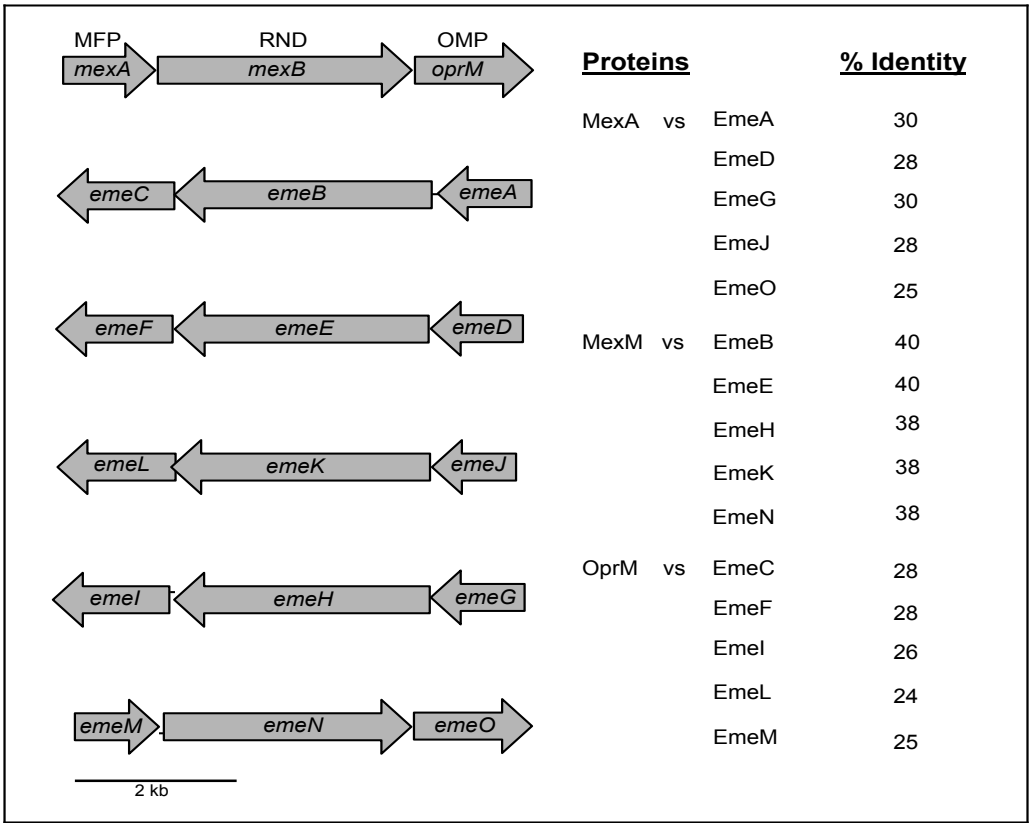

Figure 12. Organization of the RND-type efflux operons from *Elizabethkingia meningoseptica* ATCC 13253^{T} and amino acid identity to the MexAB-OprM operon in *Pseudomonas aeruginosa*.

One method used to determine if bacteria possess active efflux mechanisms is to grow them in the presence of substances known to induce efflux (e.g. salicylate) or reduce efflux (e.g. carbonylcyanide m-chlorophenylhydrazone, CCCP) and determine their relative resistance levels to various antimicrobials. For example, bacteria grown in the presence of salicylate can demonstrate increased resistance to ciprofloxacin, which is due to increased expression of efflux pump genes or increased efflux pump activity (10, 150). Therefore, we examined if the addition of salicylate (1 and 2 mM) to media utilized in the gradient plate technique affected the levels of ciprofloxacin (0 → 1 μg/ml gradient) resistance of *E. meningoseptica* ATCC 13253^T. No differences in ciprofloxacin susceptibility were detected with and without the addition of salicylate as the organism grew the entire length of the plate (90 mm) for all concentrations tested. Results of this limited induction experiment demonstrated that salicylate addition did not affect ciprofloxacin susceptibility levels at the concentrations tested, however salicylate could affect the susceptibility levels at different concentrations or with different antimicrobials. The Kirby-Bauer disk diffusion assay was also performed on *E. meningoseptica* ATCC 13253^T with and without the addition of the known efflux pump inhibitor CCCP which disrupts the proton motive force (130) required by RND efflux pumps to function. Of the 7 antimicrobials investigated (tetracycline, cefepime, levofloxacin, ciprofloxacin, aztreonam, gentamicin and ceftazidime), 20 μM CCCP addition to the Kirby-Bauer disk susceptibility media led to an increase in susceptibility to 2 known RND efflux pump substrates, tetracycline and levofloxacin (Figure 13), suggesting that active efflux may be involved with the intrinsic resistance of *E. meningoseptica* ATCC 13253^T to these drugs.

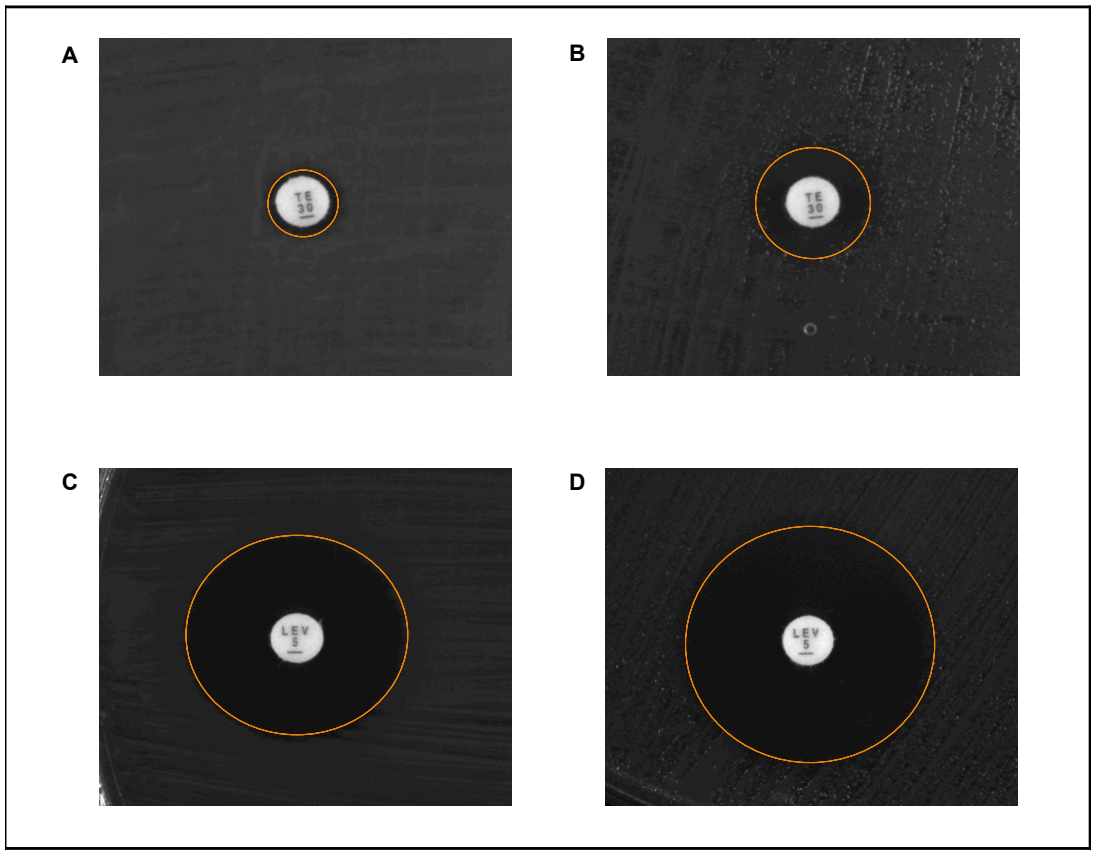

Figure 13. Susceptibility of *Elizabethkingia meningoseptica* ATCC 13253^{T} to tetracycline and levofloxacin without (A & C) and with (B & D) the addition of 20 μM CCCP.

3.3.2 Potential multi-locus sequence analysis for speciation of *Elizabethkingia* strains

Based on the papers published to date, the vast majority of *Elizabethkingia* strains given a species name is based on 16S rRNA sequencing, which we have already brought to question (Figure 1). For example, the 16S rRNA sequences of *E. meningoseptica* ATCC 13253^{T} and *E. anophelis* R26^{T} demonstrate 98% nucleotide identity (87). Several housekeeping gene sequences from the *E. meningoseptica* and *E. anophelis* type strains were used for phylogenetic and multi-locus sequence analysis in an

effort to identify new genes that can be used for speciation of the *Elizabethkingia* genus. Nucleotide alignment of several highly conserved genes from the *E. meningoseptica* ATCC 13253^T draft genome and *E. anophelis* R26^T draft genome (gene and bp identities = *gln,* 86%; *gyrB*, 87%; *recA*, 88%; *atpD*, 92%; *dnaK*, 92%; groEL, 93%; *rpoB,* 92%) supports previous findings that *E. anophelis* is at least a separate species and suggests the possibility of using these genes for further speciation (87). With this data, sequencing of these conserved genes with slow molecular clocks from new and previously identified *Elizabethkingia* strains may then be utilized to clear up the confusion surrounding the speciation of this genus. Figure 14 demonstrates the possibility of using the protein sequences of three conserved genes, *gyrB*, *rpoB* and *groEL,* as a means to phylogenetically separate the type strains of *E. meningoseptica* and *E. anophelis*. However, to move the process of characterizing the species within this genus forward, more genome sequences and species-specific allele identification of many more strains is required. An additional question that could be asked is whether or not the β-lactamase variants and their detection can be utilized for the speciation of this genus based on the heterogeneity of these genes? Comparison of the β-lactamase orthologues from these two species revealed 74% to 85% amino acid identity suggesting the possibility of each species possessing unique β-lactamases. However, as seen with the housekeeping genes, until the species can be correctly defined, we are unable to demonstrate that these β-lactamases genes are *E. meninogoseptica*-specific variants or if these variants are composed of multiple *Elizabethkingia* species.

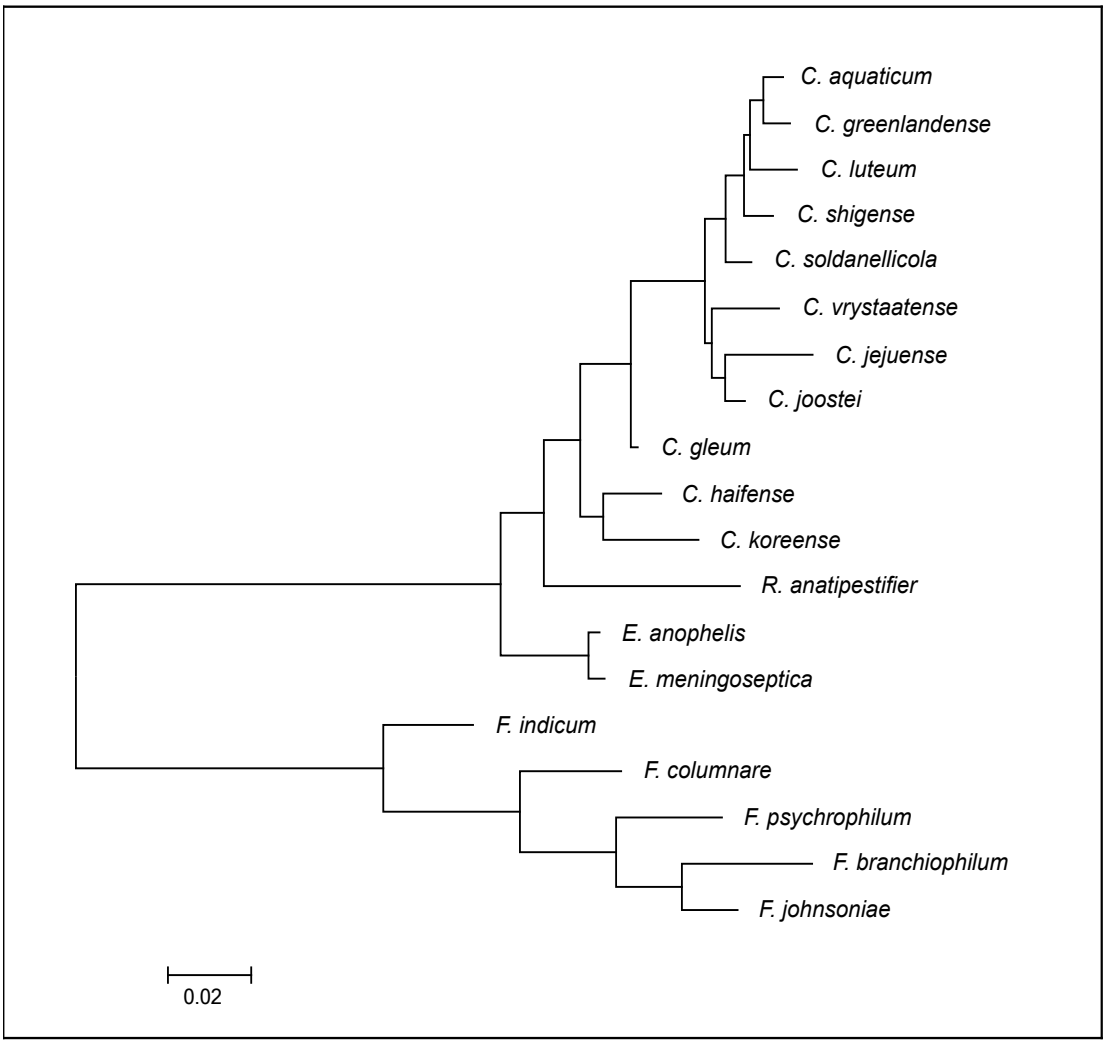

Figure 14. Phylogenetic relationships between representative species of the *Chryseobacterium, Flavobacterium, Riemerella* and *Elizabethkingia* genera based on maximum-likelihood analysis of partial protein sequences of the genes *gyrB, rpoB* and *groEL* analyzed end-to-end. Bar represents 0.02 substitutions per amino acid position over 608 amino acids.

3.3.3 Conclusions and future directions

There are several complications surrounding the speciation of members within the *Elizabethkingia* genus. The first complication deals with the continued use of 16S rRNA sequencing as a means for identifying/naming these species. As established in Figure 1, there is not enough differences between the 16S rRNA sequences to accurately name new *Elizabethkingia* isolates and brings us to question the identity of already identified strains within this genus. The second complication deals with the variation seen within the three β-lactamase genes found within this genus. Either the heterogeneity seen within these genes is truly found within a single *Elizabethkingia* species or these variants are different β-lactamase genes isolated from different *Elizabethkingia* species. A third complication is the variability with chosen antimicrobial therapies in the treatment of infections caused by *Elizabethkingia* species. If 16S rRNA sequencing continues to be the standard for the identification of *Elizabethkingia* species, this could lead to a majority of strains being misidentified as well as the incorrect antimicrobial therapy being chosen for the treatment of these infections. Clarification of the complexities associated with this genus is ultimately dependent upon correctly classifying and characterizing the species that compose the *Elizabethkingia* genus. Deciphering the correct species within this genus, as well as completing the draft genome sequences, will also provide information to individuals studying the natural ecology and evolution of these organisms.

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