β-Lactam Resistance and Novel Therapeutics for *Staphylococcus aureus*

By

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A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Infectious Diseases & Immunity in the Graduate Division of the University of California, Berkeley

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Abstract

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Staphylococcus aureus is an important human pathogen capable of causing disease in otherwise healthy individuals. It causes mostly skin and soft tissue infections but can cause more invasive diseases. Treatment for S. aureus has become a problem due to increasing resistance and limited new therapeutics, particularly for more serious infections. Methicillin-resistant S. aureus (MRSA) displays class resistance to β-lactam antibiotics through the presence of penicillin-binding protein 2a (PBP2a), encoded by mecA. Chapter 1 contains a thorough literature review on MRSA and antibiotic resistance, including epidemiology, therapeutic options, mechanisms of antibiotic resistance, modes of resistance acquisition and S. aureus animal models. MRSA has a great capacity to rapidly develop antibiotic resistance, defined as the ability of bacteria to resist a drug to which it was originally sensitive. Limited antibiotics are approved for severe MRSA infections including vancomycin, daptomycin, linezolid and ceftaroline. Resistance to these drugs almost always occurs in MRSA backgrounds rather than methicillin-sensitive S. aureus (MSSA) backgrounds. Invasive MRSA infections have been associated with treatment failure and increased risk of mortality. The development of new antibiotics is of utmost importance. This dissertation encompasses the following areas of research: i) ability of S. aureus to develop resistance to new β-lactam antibiotics, ii) potential pathways to prevent antibiotic resistance and iii) efficacy studies of a new antibiotic as a potential alternative for MRSA treatment.

Ceftobiprole and ceftaroline, members of a new class of β-lactams, target PBP2a with high affinity, the core component of β-lactam resistance in MRSA strains. Ceftobiprole is in phase 3 clinical trials while ceftaroline has been FDA approved. The goal of Chapters 2 and 3 was to identify mechanisms of resistance and likely targets associated with resistance. Chapter 2 determined resistance could be generated to ceftobiprole and ceftaroline by passaging MRSA strains in increasing concentrations of antibiotic. The mechanism of resistance in these mutants was mutagenesis of mecA and mutations in other PBPs. Chapter 3 analyzed mecA-independent mechanisms of resistance to determine the affect of these antibiotics in the absence of PBP2a. MRSA strains cured of SCCmec were passaged in ceftaroline and ceftobiprole, resulting in mutants with high-level, broad-
spectrum β-lactam resistance. These mutants have mutations in \( pbp4 \) and other genes as well as upregulated \( pbp4 \) mRNA levels in some mutants. Knowledge gained from these studies will provide information on novel mechanisms of β-lactam resistance and will guide development of new antibiotics.

Chapter 4 is dedicated to exploring the role of the SOS stress response in antibiotic resistance. The SOS response is a stress response regulated by LexA, a transcriptional repressor, and RecA, activator of LexA. When bacteria are exposed to stimuli that break DNA (e.g., UV, antibiotics, etc.), RecA activates LexA, causing derepression of the SOS genes, including error-prone polymerases that increase mutational frequencies. β-lactams have been shown to activate the SOS response in certain strains but not in a prevalent community-acquired MRSA (CA-MRSA) background. Given the increasing prevalence of CA-MRSA strains and β-lactam resistance, exposure of MRSA to β-lactams could potentially activate the SOS response resulting in increased antibiotic resistance. The goal of this chapter is to test the role of the SOS response in antibiotic resistance with β-lactam induction in CA-MRSA. To test this hypothesis, a non-cleavable \( lexA \) mutant was created, which constitutively represses the SOS response. The results of this study indicate that, in the USA300 background, the SOS response was not solely responsible for increased antibiotic resistance and suggests that this pathway might not be a good therapeutic target to decrease emergence of resistance in CA-MRSA strains.

Chapter 5 explores recently discovered options in the presence of ceftaroline-resistance or β-lactam intolerance. Given the emergence of strains resistant to antibiotics used for severe MRSA infections including vancomycin, daptomycin, linezolid and ceftaroline, new therapeutics are needed. Tedizolid phosphate is a second-generation oxazolidinone in late stage clinical development with activity against MRSA. The goal of this chapter is to test the efficacy of tedizolid phosphate in an animal model of invasive MRSA infection. Tedizolid phosphate was compared to standard of care antibiotics used to treat MRSA bacteremia, vancomycin and daptomycin, in a rabbit model of endocarditis. At high doses, tedizolid phosphate was non-inferior to vancomycin. At doses achieving serum concentrations similar to human doses, tedizolid phosphate was not as efficacious as vancomycin or daptomycin. Our results suggest tedizolid phosphate is ineffective at treating severe infections, such as MRSA endocarditis, compared to vancomycin.

The dissertation confirms the ability of \( S. aureus \) to develop resistance to new antibiotics and further describes the difficulty in developing efficacious new therapies. Discovering mechanisms of resistance will provide knowledge for potential new anti-MRSA therapeutics.
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Chapter 1:

Introduction to *Staphylococcus aureus* and Antibiotic Resistance
**Staphylococcus aureus** and Antibiotic Resistance

*Staphylococcus aureus* is a Gram-positive bacterium that can cause disease in otherwise healthy individuals. It mostly causes skin and soft tissue infections but can cause more invasive diseases such as endocarditis, osteomyelitis and bacteremia. Treatment for *S. aureus* infections is becoming increasingly difficult due to increasing antibiotic resistance. Based upon resistance to the antibiotic, methicillin, *S. aureus* is divided into two groups: methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA). Approximately 25% of the population are persistent carriers of *S. aureus* and about 5% are carriers of MRSA [4-6]. Even though prevalence of MRSA is lower compared to MSSA, MRSA is of great concern due to widespread antibiotic resistance [7, 8]. Historically, MRSA strains were associated with hospitals (HA-MRSA), but recently community-associated MRSA (CA-MRSA) with different antibiotic resistance trends and geographical locations have emerged (figure 1.1). The following sections will discuss the differences between MRSA strains in more detail.

![HA-MRSA, CA-MRSA and overall MRSA prevalence in the United States](image)

**Antibiotic resistance** is defined as the inability of an antibiotic to control the growth of bacteria. Fundamentally, resistance is due to an insufficient drug concentration at the target. Acquired resistance is the consequence of selective pressure resulting in the natural selection of a target [1, 9]. Mechanisms underlying antibiotic resistance can result from biochemical alteration of target sites, decreased permeability of the cell wall/membrane for the antibiotic, acquisition of or upregulated expression of efflux pumps or enzymatic inactivation of the antibiotic. The mode of transmission for resistance can be vertical (e.g. mutations passed from parents) or horizontal (e.g. mobile genetic elements acquired from other bacteria and passed to progeny). *S. aureus*, especially MRSA, has shown resistance to numerous antibiotic classes (tables 1.4-1.6), which have healthcare professionals...
concerned about the “post-antibiotic era”, where no effective antibiotic options are left to treat *S. aureus* infections.

In the following sections, various aspects of MRSA resistance will be discussed including epidemiology, general mechanisms of resistance in bacteria, mechanisms of action for and resistance to specific anti-staphylococcal antibiotics and animal models for MRSA infections and treatment.

### 1.1 MRSA Epidemiology

The first antibiotic, penicillin, a β-lactam antibiotic, was discovered by Alexander Fleming in 1928 [10]. Mass production of penicillin started in 1943. By 1947, just four years after the widespread use of penicillin, *S. aureus* was the first bacterium to become resistant [11]. Resistance was attributed by an acquired enzyme, β-lactamase, which inactivates the penicillin. In 1960, the development of methicillin, a β-lactamase insensitive penicillin-derivative, was used clinically but just one year later, methicillin-resistant *S. aureus* (MRSA) emerged [12]. Over the next 30 years, more β-lactams were created, such as cephalosporins, but these antibiotics were ineffective towards MRSA. Numerous antibiotics against *S. aureus* have been introduced, but MRSA has the ability to develop resistance to virtually any antibiotic. The misuse of antibiotics in the healthcare setting and food industry contributes to the growing problem of antibiotic resistance [13-19].

There are various antibiotics to treat *S. aureus* infections. Recommended antibiotics are listed in tables 1.1 and 1.2. β-lactams are arguably the best antibiotic for *S. aureus* infections. They have broad-spectrum activity, great tissue penetration, and fewer adverse effects, making β-lactams the preferred drug choice, especially for invasive infections such as endocarditis [20, 21]. However, MRSA displaying class resistance to β-lactams has rendered this class of antibiotics ineffective. Vancomycin is the first choice for severe MRSA infections. Daptomycin, linezolid and telavancin are the only other available alternatives. Tedizolid phosphate, a second-generation oxazolidinone in late stage clinical development, may be an alternative antibiotic for severe MRSA infections.
Table 1.1. Recommended antibiotic treatments for *S. aureus* infections.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>First line antibiotics</th>
<th>Alternatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methicillin–susceptible <em>Staphylococcus aureus</em></td>
<td>Oxacillin/nafcillin</td>
<td>Cephalosporin, Vancomycin, Teicoplanin, Clindamycin, Ceftaroline</td>
</tr>
<tr>
<td>Methicillin-resistant <em>Staphylococcus aureus</em> (Healthcare-associated)</td>
<td>Vancomycin, linezolid</td>
<td>Teicoplanin, Linezolid, Daptomycin, Telavancin, Ceftaroline</td>
</tr>
<tr>
<td>Methicillin-resistant <em>Staphylococcus aureus</em> (Community-associated)</td>
<td>TMP-SMX, doxycycline, minocycline</td>
<td>Clindamycin</td>
</tr>
<tr>
<td>Methicillin-resistant <em>Staphylococcus aureus</em> (Community-associated)</td>
<td>Vancomycin, Linezolid</td>
<td>Teicoplanin, Daptomycin</td>
</tr>
</tbody>
</table>

*Adapted from Table 2 (3) from The Sanford Guide to Antimicrobial Therapy 2013 43rd Edition.

Table 1.2. Recommended antibiotic treatments for resistant *S. aureus* infections.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Resistant to:</th>
<th>Primary treatment options</th>
<th>Alternatives</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Vancomycin (VISA or VRSA) and all β-lactams (except ceftaroline)</td>
<td>Daptomycin, Ceftaroline, (telavancin for VISA)</td>
<td>Linezolid</td>
</tr>
</tbody>
</table>

*Adapted from Table 5a from The Sanford Guide to Antimicrobial Therapy 2013 43rd Edition.

When MRSA first emerged in 1961, it was known as a nosocomial pathogen only found in healthcare settings. However, since the late 1990s, MRSA rates have been increasing in the community due to CA-MRSA (figure 1.1) [22, 23]. There are many differences between HA-MRSA and CA-MRSA including fitness (e.g. growth rate), antibiotic resistance, disease outcome, etc. The following sections will describe the emergence of MRSA and the main differences between HA-MRSA and CA-MRSA.

1.1.1 History of MRSA

Before the discovery of antibiotics, *S. aureus* bacteremia mortality rates were extremely high, approximately 80% [24, 25]. Penicillin, the miracle drug of the early 20th century, reduced mortality rates for many bacterial infections [26]. Penicillin-resistant *S. aureus* emerged in 1947, just four years after the mass production of penicillin, carrying a gene encoding β-lactamase, an enzyme that inactivates penicillin. In 1960, methicillin, a β-lactamase-insensitive derivative of penicillin, was produced. By 1961, the first methicillin-resistant *S. aureus* (MRSA) strain was discovered [12]. From then, *S. aureus* strains were categorized as methicillin-sensitive and methicillin-resistant *S. aureus*. MRSA strains have since been associated with more invasive disease and harbor resistance to multiple drugs compared to MSSA.
Historically, MRSA strains were considered nosocomial bacteria found mostly in hospital settings. These strains tend to harbor resistance to multiple drugs due to the constant selective pressure of antibiotic use in hospitals. Recently, MRSA strains started emerging in the community with increased fitness and less antibiotic resistance. The new community strains were named community-associated MRSA (CA-MRSA) to differentiate them from nosocomial strains, which were called healthcare-associated MRSA (HA-MRSA). Healthcare-associated risk factors differentiating HA-MRSA from CA-MRSA infections include recent hospitalization or surgery, dialysis, residence in a long-term care facility and the presence of a permanent indwelling device at the time of culture [23]. HA-MRSA strains tend to harbor resistance to non-β-lactam antibiotics, contain fewer genes encoding toxins, and cause more invasive disease, probably due to the high risk of hospitalized patients for infection [27]. Conversely, patients with CA-MRSA infections lack the risk factors for patients with HA-MRSA. CA-MRSA outbreaks have been associated with specific populations such as prison inmates [28], athletes [29, 30], injection drug users [31], children [32], military personnel [33, 34], and men who have sex with men [35]. CA-MRSA strains are less likely to carry resistance genes for non-β-lactam antibiotics [36], often harbor toxin genes, such as Panton-Valentine leukocidin (PVL), and usually cause skin and soft tissue infections although they can also cause severe infection [27].

MRSA strains can differ in fitness and trends of antibiotic resistance. HA-MRSA strains tend to be less fit: residing in the hospital setting and harboring resistance to non-β-lactam antibiotics, likely due to the selective pressure in hospitals [27]. Conversely, CA-MRSA strains have increased fitness, originated in the community, although are increasingly prevalent in hospital settings, and usually harbor fewer resistance genes. Antibiotic resistance in HA-MRSA and its capacity to cause invasive infections poses a major public health issue. There are limited antibiotics approved for the treatment of invasive MRSA infections including vancomycin, televancin, daptomycin, and linezolid. MRSA strains resistant to each of these antibiotics have been found clinically [37-41]. With the rapid emergence of antibiotic resistance by MRSA, the need for the development of new antibiotics is greater than ever.

Differences between HA-MRSA and CA-MRSA have been used to guide treatment choices. HA-MRSA strains are more likely to be resistant to non-β-lactam antibiotics, prompting treatment with intravenous drugs such as vancomycin. CA-MRSA strains are less likely to harbor non-β-lactam resistance genes, thus there are several therapeutic options (Table 1.1). However, the epidemiologic definitions for HA-MRSA and CA-MRSA have started to blur. HA-MRSA strains have been found in the community due to an increase in patients being cared for in outpatient settings and increased delivery of healthcare in the home setting [42, 43]. CA-MRSA strains have also started appearing in hospital settings as well, comprising 30% or more of MRSA infections detected in the hospital setting [44-47]. The change in epidemiology between HA-MRSA and CA-MRSA may complicate treatment
prescriptions. In areas where prevalence of MRSA is high, empiric treatment of *S. aureus* infections usually consists of more expensive antibiotics that target MRSA, increasing healthcare costs.

### 1.1.2 Staphylococcal chromosome cassette *mec* (SCCmec)

Methicillin resistance emerged from the acquisition of the staphylococcal chromosomal cassette *mec* (SCCmec). SCCmec is a mobile element integrated into the 2.8 megabase MRSA genome. It carries the *mecA* gene, a homologue of penicillin-binding protein 2, which has a reduced capacity to bind penicillin. Recently, a new *mec* variant was found, *mecC*. All MRSA strains harbor SCCmec, a mobile genetic element that is transmissible across different staphylococcal species and has been found in other species of coagulase-positive and -negative staphylococci [48-51] and closely related bacteria [52]. Eleven SCCmec elements are recognized, SCCmec I-XI [48, 53, 54], and all types contain two main elements: 1) the *mec* gene complex, which contains *mecA* or *mecC*, and 2) the *ccr* gene complex, which encodes site-specific recombinases, which allow movement of SCCmec [55]. The *ccr*-encoded recombinases allow for excision of the element and insertion into the 3′ end of *orfX* at the *attBSCC* site [51, 55, 56]. Many groups have speculated that MRSA SCCmec was derived from *S. sciuri*, a staphylococcal species associated with animals that often tests positive for the presence of *mecA* [57-59]. However, the *mecA* homologue in *S. sciuri* only shares 88% homology with *mecA* from MRSA. Tsubakishita et al. performed genomic analyses of *S. sciuri* and species from the same group, *S. vitulinus*, *S. lentus* and *S. fluerettii*, to find the origin of *mecA*. They determined SCCmec originates from the interaction of different staphylococcal species of animal origin: the genomic *mecA* gene from *S. fluerettii*, which is identical to *mecA* from MRSA, combined with a mobile SCCmec element lacking *mecA* from another staphylococcal species that coexisted with *S. fluerettii*. The original host of the SCCmec genetic element is unknown and may consist of more than one staphylococcal species [60] [61]. The origin of SCCmec suggests that this antibiotic resistant determinant evolved before *S. aureus* was exposed to β-lactams in humans.

HA-MRSA and CA-MRSA typically carry different SCCmec types. HA-MRSA usually harbor SCCmec types I, II and III [62-64] while CA-MRSA harbor types IV, V, VI, VII and VIII [65-67] and SCCmec types IX and X are found in livestock [53]. A strain containing SCCmec type XI was recently found in bovine milk in the UK and have been found in human infections [68, 69]. This new SCCmec contained *mecC* rather than *mecA*. SCCmec type IV, the most prevalent type in CA-MRSA, is relatively small compared to I, II and III, which may have resulted in increased mobility and capacity to spread to different *S. aureus* backgrounds [70-72]. SCCmec types are differentiated by the *ccr* and *mec* gene complexes (table 1.3).
MRSA strains can be further differentiated by multilocus sequence typing (MLST) [48] (table 1.3). MLST consists of sequencing about 500 base pairs from seven house-keeping genes to determine the alleles present at each locus. Sequence types (ST) are defined by the combination of alleles found at the seven loci. Sequence types can be further grouped into clonal complexes, sets of sequence types differing from each other at only one or two loci. Clonal lineages can be assigned to both HA-MRSA and CA-MRSA strains. Another useful technique to differentiate strains is the typing of S. aureus protein A (spa), a cell wall protein that exhibits extensive sequence polymorphisms. Spa typing is useful in outbreak settings with its good turnaround time and sensitivity [73]. Spa typing and MLST correlate well. Epidemiological studies analyzing clonal complexes can also answer important fundamental questions such as how strains spread, the number of major clones circulating the globe and the ancestry of modern MRSA clones [74].

Two hypotheses have been proposed for the emergence of MRSA: 1) a single MSSA clone acquired an ancestral SCCmec element and differentiated into different clonal lineages, and 2) multiple MSSA clones independently acquired SCCmec from other staphylococcal species [75]. Recent studies have strengthened the latter hypothesis, suggesting different MSSA strains acquired SCCmec and gave rise to different MRSA clones [76-78]. Likewise, CA-MRSA strains are genetically more similar to MSSA strains, suggesting they evolved from MSSA strains that acquired SCCmec [79]. CA-MRSA strains carrying type IV SCCmec (USA300) have a faster doubling time than HA-MRSA, which likely contributes to its overall fitness [71, 80]. Initially, CA-MRSA strains were more susceptible to non-β-lactam antibiotics [81] but strains of multidrug-resistant CA-MRSA have been reported [82-86]. CA-MRSA strains cause mostly skin and soft tissue infections [87], but they certainly can cause more invasive infections [80, 88-92].
1.2 Antibiotic resistance: Horizontal vs. Vertical Transfer

Bacteria have numerous methods for antibiotic resistance. Common mechanisms include enzymatic inactivation of the antibiotic (e.g. β-lactamase), target site mutagenesis, alteration of target genes (e.g. SCCmec), and decreased drug concentrations (e.g. efflux pump). Resistance can be acquired through mutations (vertical transfer) or acquisition of resistance determinants through mobile genetic elements (horizontal transfer). The following sections discuss plasmids found in S. aureus and how mutations mediate resistance.

1.2.1 Plasmids (horizontal transfer)

Plasmids are small pieces of mobile DNA that is easily transferrable between hosts and often harbor resistance genes. The CA-MRSA strain, USA300, harbor plasmids that contain tetracycline, macrolide, lincosamide and streptogramin (MLS), erythromycin and mupirocin resistance [86]. Plasmid-borne resistance genes are of particular concern since these genetic elements can be transferred across different species of bacteria. Recently, plasmid-borne resistance factors have been found for antibiotics commonly used in the healthcare setting. Resistance to vancomycin, empiric therapy for HA-MRSA and severe CA-MRSA infections [21], is found on a plasmid-borne Tn1546 element containing vanA, which originated from vancomycin-resistant enterococcus (VRE) strains [93, 94]. Even though the prevalence of vancomycin-resistant S. aureus (VRSA) is low, the potential for MRSA to acquire the vanA plasmid from VRE remains a huge concern. Another concerning plasmid is the pSCFS1 plasmid harboring the cfr gene, which is resistant to chloramphenicol, flornicol and linezolid. Plasmid pSCFS1 was first described in S. sciuri, a staphylococcal strain associated with cattle [95], and is transmissible between staphylococci [96]. This plasmid was responsible for the linezolid outbreak in Spain [97, 98]. Since plasmids can be transferred to S. aureus from other staphylococcus and enterococcus strains, the potential for multidrug resistant S. aureus strains harboring resistance to many frontline antibiotics is a reality.

1.2.2 Mutations (vertical transfer)

Mutations usually confer resistance by altering the target site or other genes involved in the resistance pathway and are the first detected mechanisms of resistance to new antibiotics. Additionally, clonal diversification of S. aureus occurs 15-fold more frequently from point mutations compared to recombination [99]. For example, vancomycin-intermediate S. aureus (VISA) phenotypes are mediated through altered production of penicillin-binding proteins, thickened cell wall, reduced autolytic activity and decreased agr function [100-104]. The emergence of VISA strains occurred before the detection of VRSA strains (table 1.4). Ciprofloxacin resistance is mediated through mutations in DNA gyrase, gyrA [86] and was found
naturally occurring in the USA300 CA-MRSA strain. More examples of resistance through mutations are provided in section 1.3.

1.3 Antibiotics for S. aureus treatment: bacterial targets and mechanisms of resistance

Antibiotics have four main targets in bacteria: cell wall synthesis, the cell membrane, protein synthesis, and nucleic acid synthesis. β-lactams and glycopeptides target cell wall synthesis (figure 1.2). Lipoglycopeptides and lipopeptides target the cell wall synthesis and the cell membrane. Protein synthesis inhibitors target ribosomal RNA (rRNA), either the 30S or 50S subunits. Aminoglycosides and tetracyclines target the 30S subunit. Macrolides, lincosamides, streptogramins and oxazolidinones target the 50S subunit. Fluoroquinolones, rifamycin, monoxycarbolic acid (mupirocin) and sulfonamides target nucleic acid synthesis. Vancomycin is the first choice for severe MRSA infections. Daptomycin, linezolid, telavancin and ceftaroline are the only other available alternatives. Tedizolid phosphate, a second-generation oxazolidinone in late stage clinical development, may be an alternative antibiotic for severe MRSA infections. All other antibiotic classes of protein and nucleic acid synthesis inhibitors are not as applicable for severe MRSA infections due to widespread resistance, low efficacy, adverse reactions or have not been tested. The mechanism of action and resistance for each class will be discussed in more detail in the following sections.

Figure 1.2. Antibiotic targets. (From Lewis et al. 2013 [2])
1.3.1 Antibiotics that target cell wall synthesis and the cell membrane

There are four major classes of cell wall synthesis inhibitors: β-lactams, glycopeptides, lipoglycopeptides and lipopeptides. β-lactams and glycopeptides function by inhibiting peptidoglycan synthesis. Lipoglycopeptides and lipopeptides inhibit cell wall synthesis and alter cell membrane stability. β-lactams covalently bind and inactivate enzymes that catalyze cell wall synthesis. Glycopeptides bind cell wall precursors preventing chain elongation. Lipopeptides destabilize cell membranes and interrupt cell wall synthesis. Lipoglycopeptides destabilize cell membranes and binds cell wall precursors. The antibiotics for each class are listed in table 1.4 and are discussed in further detail in the following section.

Cell Wall Synthesis Inhibitors

1.3.1.1 β-lactams

The β-lactam class of antibiotics used to treat staphylococci includes penicillins, cephalosporins, and β-lactamase inhibitors. There are two classes of penicillins: β-lactamase-unstable and β-lactamase-resistant penicillins. Five generations of β-lactamase-resistant cephalosporins exist, 1st through 5th generations. Generations 2-4 were developed primarily for Gram-negative bacteria and will not be discussed [105, 106]. Fifth generation cephalosporins are the only cephalosporins to have anti-MRSA activity.

β-lactams inhibit cell wall synthesis by covalently binding penicillin-binding proteins (PBP). S. aureus has four native PBPs, PBP1-4, that enzymatically crosslink muropeptides in the cell wall to form peptidoglycan, a major component of Gram-positive bacterial cell walls. They bind the D-ala-D-ala terminal ends of peptidoglycan precursors, creating crosslinks between neighboring peptide sidechains [107]. β-lactams function as a substrate analog of the D-ala-D-ala terminus of peptidoglycan precursors, forming a noncovalent bond with the binding site (Michaelis complex). In this complex, the β-lactam-PBP bond is close to the nucleophilic serine. The serine of the PBP then attacks the β-lactam-PBP bond, creating a covalent acyl-enzyme complex (at rate $k_2$). The complex can be dissociated by the nucleophilic attack of water (at rate $k_3$) to release the PBP, but this reaction is extremely slow for β-lactam antibiotics bound to PBPs (decreased $k_3$ resulting in inactivated PBPs). The low rate of $k_3$ can also be attributed to the β-lactam ring occupying the position for the adjacent peptidoglycan sidechain, preventing the enzyme from undergoing deacylation [107-111]. The interaction of PBP with β-lactams can be described by the reaction below from Lovering et al. [3].
S. aureus resistance to β-lactams is mediated by PBPs through two main mechanisms: production of β-lactamases and the presence of low affinity PBPs, encoded by meca or meCc. Staphylococcal β-lactamase, which is also a PBP, functions by rapidly hydrolyzing the β-lactam ring and inactivating the drug. The mechanism of action is similar to PBPs (see reaction above), where β-lactamase forms a covalent bond with β-lactams (at rate k₂) and rapidly hydrolyzes the antibiotic (at increased rate k₃). The enzymatic hydrolysis of β-lactams by β-lactamase is 100-fold faster than regular PBPs and decreases antibiotic concentrations at the target site. This resistance mechanism can be overcome by the addition of β-lactamase inhibitors to the penicillin or using penicillinase-resistant β-lactams such as methicillin [112]. All MRSA strains are resistant to β-lactams through the presence of meca or meCc, which encodes an extra PBP, PBP2a. PBP2a has low affinity for β-lactams, allowing the cells to function in the presence of β-lactams, thereby enabling survival [113]. PBP2a have similar k₄ and k₃ to penicillin susceptible PBPs, but the nucleophilic serine in PBP2a is located in a narrow, extended cleft that is inaccessible to the β-lactam-PBP bond, preventing the formation of the covalent acyl-enzyme complex (decreased k₂) [114].

Fifth generation cephalosporins, such as ceftobiprole and ceftaroline, bypass both mechanisms of β-lactam resistance [115, 116]. The structure of these two antibiotics is shown in figure 1.3. The two R groups confer an advantage over regular cephalosporins: i) confer β-lactamase stability and ii) enhance binding of PBP2a. Ceftobiprole’s R1 group is an oxyimino aminothiazole substituent linked to the cephalosporin nucleus, conferring stability and resistance to β-lactamases. The R2 group is a vinylpyrrolidinone moiety that facilitates interaction with the narrow groove of the PBP2a active site and favors acylation. Ceftobiprole binding causes conformational changes in PBP2a, with the backbone residues of helix α2 twisting to further expose the active site serine. Structural studies have shown ceftobiprole connected to the active site serine, indicating an acyl-enzyme formation [3]. This interaction suggests ceftobiprole’s mechanism of action is similar to other β-lactams and not of a completely novel mechanism. Ceftaroline is presumed to act similarly.
1.3.1.2 Glycopeptides

Glycopeptides, such as vancomycin and teicoplanin, inhibit cell wall formation by inhibiting peptidoglycan chain elongation. Vancomycin binds the D-ala-D-ala terminus of peptide chains (the substrate for PBPs), inhibiting peptidoglycan polymerization (transglycosylation) and preventing chain elongation [117]. Vancomycin non-susceptibility in S. aureus can be generated through several mechanisms, resulting in vancomycin-intermediate S. aureus (VISA) and vancomycin-resistant S. aureus (VRSA) strains. VISA strains are more prevalent and genetically complicated [100-102, 104, 118-120]. One mechanism of resistance is the downregulation of PBP4, which results in an accumulation of muropeptide monomers, providing more substrate to sequester vancomycin molecules [121]. VRSA isolates are less prevalent [37, 38, 122-126] and have high-level resistance to vancomycin through the acquisition of the vanA gene from enterococcus. vanA encodes an ATP-dependent ligase that replaces the D-alanine-D-alanine terminus of peptidoglycan chains with D-alanine-D-lactate, a change resulting in the loss of a hydrogen bond, which decreases vancomycin binding by approximately 100-fold [127].

Cell Membrane Disruptors

1.3.1.3 Lipoglycopeptide

Lipoglycopeptides are dual function antibiotics with more potency compared to glycopeptides [128-130]. Telavancin, dalbavancin and oritavancin are derived by addition of hydrophobic and hydrophilic groups to the glycopeptide core structure [131]. The mechanism of action is similar to vancomycin, binding the D-ala-D-ala
terminus of peptide chains, preventing elongation of peptide chains by blocking transglycosylation [129, 132]. They also trigger specific rapid dissipation of the bacterial cell membrane by, presumably, binding lipid intermediate molecules resulting in membrane pores followed by leakage of cytoplasmic adenosine triphosphate and potassium ions [129-132]. Oritavancin can bind to both D-ala-D-ala and D-ala-D-lac (produced by VanA) terminals of peptidoglycan chains, allowing it to function in strains containing the \textit{vanA} resistance gene [133, 134]. Resistance is mediated through the presence of wild-type or mutated \textit{vanA} gene [131, 135-137].

1.3.1.4 Lipopeptide

Daptomycin acts by binding to and oligomerizing the cytoplasmic membrane, resulting in an efflux of potassium and membrane depolarization [138-140]. Decreased susceptibility to daptomycin has been associated with various phenotypes [141-144]. The best-defined mechanism of resistance is from single-nucleotide polymorphisms (SNPs) in the \textit{mprF} gene, presumably through a gain of function phenotype. \textit{MprF} controls the production of lysyl-phosphatidylglycerol, which has a uniquely positive charge. The SNPs in \textit{MprF} are hypothesized to result in a charge repulsion mechanism of resistance [145].

Cell Wall Synthesis Inhibitors and Cell Membrane Disruptors Role in Therapy

Cell wall synthesis inhibitors and cell membrane disruptor classes of antibiotics provide the best treatment options for \textit{S. aureus} infections. β-lactams are the best treatment option for \textit{S. aureus} infections due to good bioavailability and penetration, minimal adverse effects and low cost. However, MRSA with PBP2a, displays class resistance to β-lactams, rendering this class of antibiotics ineffective against MRSA. Vancomycin is the empiric antibiotic for severe MRSA infections, but increasing prevalence of VISA and VRSA strains could reduce its usefulness in the future. Daptomycin is an effective alternative antibiotic for severe MRSA infections. However, daptomycin non-susceptibility has been associated with vancomycin non-susceptibility and may not be a good alternative for VISA and VRSA strains [104, 146-149]. Lipoglycopeptides are non-inferior to vancomycin but have serious adverse effects and have not shown to be efficacious in severe infections [129, 150, 151]. Recently developed 5\textsuperscript{th} generation cephalosporins, with the benefits of β-lactams and anti-MRSA activity, are attractive alternative antibiotics. Ceftobiprole is in phase III clinical trials and ceftaroline was FDA approved for clinical use in October 2010. However, despite the high PBP2a affinity of this antibiotic and the attractive broad-spectrum activity, resistance has been found in clinical isolates [61] and \textit{in vitro} resistance has been described in laboratory strains [152, 153]. Alternative therapies for severe MRSA infections are needed.
Table 1.4. Anti-staphylococcal antibiotic that target cell wall synthesis and cell membrane.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Class</th>
<th>Year FDA Approved</th>
<th>Mechanism of Action</th>
<th>Year Resistance Developed</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins Penicillin Ampicillin</td>
<td>β-lactam (Penicillins)</td>
<td>1943</td>
<td>Binds covalently to PBPs</td>
<td>1947</td>
<td>β-lactamase production that inactivates the penicillin</td>
</tr>
<tr>
<td>Methicillin Nafcillin Oxacillin</td>
<td>β-lactam (β-lactamase insensitive penicillins)</td>
<td>1960</td>
<td>Binds covalently to PBPs</td>
<td>1961</td>
<td>Acquisition of meca, which encodes the low-affinity PBP, PBP2a</td>
</tr>
<tr>
<td>Cephalosporins Cefazolin</td>
<td>β-lactam (1st generation cephalexin)</td>
<td>1973</td>
<td>Binds covalently to PBPs</td>
<td>1961</td>
<td>Acquisition of meca, which encodes the low-affinity PBP, PBP2a</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>β-lactam (2nd generation cephalexin)</td>
<td>1993</td>
<td>Binds covalently to PBPs</td>
<td>1961</td>
<td>Acquisition of meca, which encodes the low-affinity PBP, PBP2a</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>β-lactam (3rd generation cephalexin)</td>
<td>2006</td>
<td>Binds covalently to PBPs</td>
<td>1961</td>
<td>Acquisition of meca, which encodes the low-affinity PBP, PBP2a</td>
</tr>
<tr>
<td>Ceftaroline Ceftobiprole</td>
<td>β-lactam (5th generation cephalexin)</td>
<td>2010</td>
<td>Binds covalently to PBPs, including PBP2a</td>
<td>2008</td>
<td>*Mutations in meca; mutations in pbp4 and gdpP</td>
</tr>
<tr>
<td>Glycopeptides Vancomycin</td>
<td>Glycopeptide</td>
<td>1986</td>
<td>Binds peptide chains at D-ala-D-ala site</td>
<td>1996 (VISA) 2002 (VRSA)</td>
<td>Reduction of cell wall penetration; Acquisition of the vanA gene</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>Glycopeptide</td>
<td>NA</td>
<td>Binds peptide chains at D-ala-D-ala site</td>
<td>2008</td>
<td>Acquisition of the vanA gene</td>
</tr>
<tr>
<td>Lipoglycopeptide Telavancin Dalbavancin Oritavancin</td>
<td>Lipoglycopeptide</td>
<td>2009</td>
<td>Binds peptide chains at D-ala-D-ala site and depolarizes cell membranes</td>
<td>--</td>
<td>Acquisition of the vanA gene; altered expression or mutation of vanA or vanB genes</td>
</tr>
<tr>
<td>Lipopeptide Daptomycin</td>
<td>Lipopeptide</td>
<td>2003</td>
<td>Cell membrane depolarization and inhibition of cell wall synthesis</td>
<td>2005</td>
<td>Mutations to mprF</td>
</tr>
</tbody>
</table>

*In vitro resistance studies. NA= Not FDA approved in the United States.
1.3.2 Protein synthesis inhibitors

*S. aureus* protein synthesis inhibitors function by interacting with the 50S or 30S ribosomal subunit or tRNA synthesis. Macrolides, lincosamide (clindamycin), streptogramin and oxazolidinone target the 50S ribosomal subunit. Aminoglycosides and tetracyclines target the 30S ribosomal subunit. Mupirocin inhibits isoleucyl-tRNA synthetase. A list of antibiotics that inhibit protein synthesis can be found in table 1.5.

30S rRNA Protein Synthesis Inhibitors

1.3.2.1 Aminoglycosides

Aminoglycosides inhibit the 30S subunit rRNA. There are various mechanisms of aminoglycoside resistance including methylation and/or mutation of specific residues on the 30S subunit [154], plasmid encoded aminoglycoside-modifying enzymes resulting in deactivation [155], decreasing membrane permeability and/or active efflux of the drug [156].

1.3.2.2 Tetracyclines and Glycylcycline

Tetracyclines and glycylcyclines inhibit protein synthesis by binding the 30S subunit of rRNA [157, 158]. Resistance can arise from the tet(A-D, K) efflux pumps or ribosomal protection resistance determinant tet(M,O) that bind ribosomes, blocking the binding site for tetracyclines [157, 159-163] [164] [165].

50S rRNA Protein Synthesis Inhibitors

1.3.2.3 Macrolides, lincosamides, and streptogramins (MLS)

Macrolides, lincosamides and streptogramins (MLS) cause dissociation of peptidyl-tRNAs from the ribosome by binding to the 50S ribosome, blocking the entrance to the ribosomal tunnel, resulting in dissociation of the peptidyl-tRNA complex from the rRNA [166]. Resistance is [167] mediated through plasmid-encoded *erm* genes, which methylates adenosine 2058 of 23S rRNA, causing a conformational change and decreased binding affinity for MLS. Resistance is also conferred through the plasmid-encoded *msrA* gene, which encodes an ABC transporter that pump out macrolides [168-171]. Inducible clindamycin resistance is mediated through the 14-aa peptide upstream of *ermC*. It contains a set of 4 inverted repeats at the 5’ end creating a stem-loop structure, resulting in inaccessibility of *ermC* by the ribosome. In the presence of erythromycin, the ribosome stalls, resulting in rearrangement of the mRNA and displacement of the stem-loop allowing for translation of *ermC* [169, 172].
1.3.2.4 Amiphenicols

Chloramphenicol, an amiphenicol used to treat *S. aureus* infections, inhibits protein synthesis by preventing peptidyl transferase activity of bacterial ribosome. It specifically binds to A2451 and A2452 residues in the 23S rRNA of the 50S ribosomal subunit. Resistance to chloramphenicol is mediated through three mechanisms: decreased drug permeability, mutation of the 50S ribosomal subunit or acquisition of chloramphenicol acetyltransferase that inactivates the drug [173]. The presence of the *cfr* gene, which encodes a methylase conferring resistance to several classes of antibiotics including oxazolidinones, phenicols, streptogramin compounds, and lincosamidins, is now found in MRSA [174]. More details on the *cfr* gene can be found in the next section.

1.3.2.5 Oxazolidinones

Linezolid, an oxazolidinone with activity against MRSA, inhibits protein synthesis by binding the 23S rRNA of the 50S subunit of bacterial ribosome [175]. Since linezolid is a synthetic compound, natural reservoirs of resistance were not expected. Initial resistance to linezolid consisted of point mutations on the 50S subunit rRNA that resulted in decreased antibiotic binding [176-179]. However, a natural transferrable resistance gene, *cfr* (chloramphenicol-florfenicol resistance) gene, originating from *Staphylococcus sciuri* in animals, was found on plasmids capable of horizontal transfer across staphylococci [19, 96-98, 180]. It encodes a methyltransferase that methylates A2503 in the 23S rRNA gene, resulting in resistance to chloramphenicol, florfenicol, clindamycin, and linezolid.

Tedizolid phosphate (TR-701), prodrug of tedizolid (TR-700), is a second-generation oxazolidinone with activity against MRSA and linezolid-resistant MRSA. It is in late-stage clinical development and is a promising alternative to treat severe MRSA infections. *In vitro* passage of tedizolid showed lower mutational frequencies compared to linezolid, suggesting a decreased risk for the emergence of resistance through point mutations of the 23S rRNA [181].

**Protein Synthesis Inhibitor Role in Therapy**

Due to widespread resistance and/or low efficacy, most protein synthesis inhibitors are not used to treat severe MRSA infections, except linezolid. Aminoglycosides lack efficacy and have severe side effects [182] [183-186]. Tetracyclines and MLS are efficacious antibiotics but widespread resistance has limited their use [21, 160, 187] [188-190]. Linezolid, one of the few alternatives to vancomycin, is approved for complicated skin and skin structure and pneumonia [191]. The dissemination of the *cfr* gene may limit its use. Tedizolid phosphate may serve as an alternative antibiotic for MRSA severe infections. It is 4-16 times more
potent against staphylococci compared to linezolid [192, 193] and 8-16 fold more potent against linezolid non-susceptible staphylococci strains, including cfr-containing strains [194, 195]. Tedizolid was non-inferior compared to linezolid for the treatment of acute bacterial skin and skin structure infections and had 94-98% cure rates in complicated skin and skin structure infections [196, 197]. Three doses, 200 mg, 300 mg, and 400 mg once daily, were tested in phase II clinical trials for cSSSI infections. The 200 mg dose was as effective as the higher doses, which reduces the probability of adverse events compared to the 600 mg dose of linezolid [197].
Table 1.5. Anti-staphylococcal antibiotics that target protein synthesis

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Class</th>
<th>Year FDA Approved</th>
<th>Mechanism of Action</th>
<th>Year Resistance Developed</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>30S rRNA inhibitor</td>
<td>Gentamicin</td>
<td>Aminoglycoside</td>
<td>1988</td>
<td>1969</td>
<td>Methylation/mutation of residues on 30S rRNA</td>
</tr>
<tr>
<td></td>
<td>Doxycycline</td>
<td>Tetracycline</td>
<td>1983</td>
<td>1974</td>
<td>Efﬂux pumps Tet(A-D,K); Ribosomal protection Tet(M,O)</td>
</tr>
<tr>
<td></td>
<td>Minocycline</td>
<td>Prior 1982</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tigecycline</td>
<td>Glycylcycline</td>
<td>2005</td>
<td>2013</td>
<td>MATE efﬂux pumps MepA overexpression*</td>
</tr>
<tr>
<td>50S rRNA inhibitor</td>
<td>Erythromycin</td>
<td>Macrolide</td>
<td>1952</td>
<td>1957</td>
<td>Methylation of 23S rRNA encoded by erm genes</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>Lincosamide</td>
<td>1968</td>
<td>1971</td>
<td>Methylase ErmA that alters the MLS binding sites</td>
</tr>
<tr>
<td></td>
<td>Quinupristin</td>
<td>Streptogramin</td>
<td>1999</td>
<td>1998</td>
<td>vatA/B/C genes encoding acetytransferase; vgaA/B genes encoding an ATP transporter efflux pump</td>
</tr>
<tr>
<td></td>
<td>Dalfopristin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>Amphenicol</td>
<td>1982</td>
<td>1958</td>
<td>Reduced membrane permeability; mutations in 50S rRNA; inactivation by chloramphenicol acetytransferase</td>
</tr>
<tr>
<td></td>
<td>Linezolid</td>
<td>Oxazolidinone</td>
<td>2000</td>
<td>2002</td>
<td>Mutations in 50S rRNA; methylation of 23S rRNA encoded by cfr gene</td>
</tr>
</tbody>
</table>

*In vitro resistance studies. NA= Not FDA approved in the United State
Nucleic acid inhibitors

*S. aureus* nucleic acid inhibitors target enzymes that synthesize DNA or RNA. Fluoroquinolones target DNA gyrase. Rifampicin targets RNA polymerase. Mupirocin targets isoleucyl-tRNA synthetase. Trimethoprim and sulfamethoxazole inhibits folic acid synthesis, a necessary component of thymidine and uridine synthesis. A list of antibiotics that target nucleic acid production can be found in table 1.6.

1.3.2.6 DNA Synthesis Inhibitors

Fluoroquinolones target DNA gyrase, inhibiting DNA synthesis [198-202]. Resistance to fluoroquinolones is mediated through mutations in DNA gyrase and topoisomerase [202] and NorA/B/C-mediated efflux. NorA production can be upregulated via mutations in the promoter site [202].

1.3.2.7 RNA Synthesis Inhibitors

Rifampicin is a bacterial RNA initiator inhibitor that targets the β-subunit of RNA polymerase. However, when mRNA synthesis has started, rifampicin cannot inhibit synthesis [203, 204]. Resistance to rifampicin is mediated through point mutations of the *rpoB* gene of the rifampicin binding site [205] and through inactivation of the drug by ribosylation [206-209].

Mupirocin acts by inhibiting RNA synthesis by inhibiting isoleucyl-tRNA synthetase [210]. Clinical isolates with intermediate resistance to mupirocin have been found with altered isoleucyl-tRNA synthetase [211, 212]. Isolates with high levels of mupirocin resistance contain plasmid-encoded mupA that is transferrable to other staphylococci [213, 214].

1.3.2.8 Folate Synthesis Inhibitors

Trimethoprim-sulfamethoxazole (TMP-SMX) is a sulfonamide antibiotic that inhibits folate synthesis. Folate is essential in the synthesis of nucleotides thymidine and uridine [215]. Trimethoprim resistance is conferred through plasmid encoded dihydrofolate reductase (DHFR) with mutations in the trimethoprim binding site [216-220].

Nucleic Acid and Metabolic Pathway Inhibitors Role in Therapy

Anti-staphylococcal antibiotics that target nucleic acid synthesis are generally not recommended for MRSA severe infections due to widespread resistance or lack of efficacy studies. Ciprofloxacin has broad-spectrum activity, efficacy and good bioavailability against *S. aureus*, but resistant strains were described before widespread use [198] [202, 221]. Of even greater concern is that
fluoroquinolone exposure can induce bacterial stress responses, leading to increased mutations and an increased frequency of antibiotic resistance as mentioned in section 1.2.2 [222-224]. Rifampicin is not recommended for use in monotherapy since resistance easily arises during treatment. Rifampicin resistant S. aureus strains were first found on patients treated with rifampicin for M. tuberculosis infections [225]. Mupirocin is a topical antibiotic and has no role in invasive therapy of invasive disease [211, 226] but widespread use has resulted in increasing resistance [227]. Trimethoprim-sulfamethoxazole (TMP-SMX) has good activity against S. aureus, but resistance has been reported as early as 1964 [228, 229]. TMP-SMX has been indicated for skin and soft tissue infections caused by community-acquired MRSA (CA-MRSA) but has not been shown to be efficacious in severe MRSA infections [230].
Table 1.6. Anti-staphylococcal antibiotics that target nucleic acid synthesis.

<table>
<thead>
<tr>
<th>Nucleic Acid Inhibitors</th>
<th>Antibiotic</th>
<th>Class</th>
<th>Year Marketed</th>
<th>Mechanism of Action</th>
<th>Year Resistance Developed</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA synthesis inhibitor</td>
<td>Ciprofloxacin</td>
<td>Fluoroquinolone</td>
<td>1983</td>
<td>DNA gyrase inhibitor</td>
<td>1985</td>
<td>Mutations in DNA gyrase; upregulated expression of norA, a drug efflux pump</td>
</tr>
<tr>
<td>RNA synthesis inhibitor</td>
<td>Rifampcin</td>
<td>Rifamycin</td>
<td>1967</td>
<td>RNA synthesis inhibitor</td>
<td>1975</td>
<td>Mutations in rpoB</td>
</tr>
<tr>
<td></td>
<td>Mupirocin</td>
<td>Monoxycarbolic acid</td>
<td>1995</td>
<td>Inhibits isoleucyl-tRNA synthesis</td>
<td>1987</td>
<td>Acquisition of mupA encoding a mupirocin-resistant isoleucyl-tRNA synthetase</td>
</tr>
<tr>
<td>Antifolates</td>
<td>Trimethoprim-</td>
<td>DHFR Inhibitor/</td>
<td>2005</td>
<td>Folate synthesis inhibitor</td>
<td>1964</td>
<td>Mutations in DHFR</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td>Sulfonamides Combination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Anti-staphylococcal antibiotics summary**

Anti-staphylococcal antibiotics target four main characteristics of bacteria: cell wall synthesis, the cell membrane and protein and nucleic acid synthesis. However, *S. aureus* is capable of developing resistance to most antibiotics within a few years of widespread use (tables 1.4-1.6). This phenomenon can be attributed to bacteria harboring resistance genes even before the antibiotic is used in humans due to competition with other microorganisms. In the early days of antibiotic discovery, most antibiotics were purified from other microorganisms (e.g. penicillin from the *Penicillium* fungi, vancomycin from Acetinobacteria and erythromycin from Streptomyces). Newer generation antibiotics are synthesized from biochemical structures of known antibiotics (e.g. teicoplanin from glycopeptides). Common mechanisms of resistance include acquisition of genes encoding functional alternative proteins (e.g. meca, vanA), inactivation of the antibiotic (e.g. β-lactamase), target site mutagenesis (e.g. mutations in mprF, rpoB), acquisition or upregulation of efflux pumps (e.g. tet) and mutations in the promoter site that leads to overexpression (e.g. pbp4). Widespread resistance, lack of efficacy and adverse events has limited the use of most protein and nucleic acid synthesis inhibitors, except for linezolid.

The best antibiotics to treat *S. aureus* are β-lactams because of good bioavailability, good penetration and less adverse effects. However, the high prevalence of MRSA in hospitals combined with its ability to cause severe infections has limited its use. Only four antibiotics are FDA approved for severe MRSA infections: vancomycin, telavancin, daptomycin, and linezolid. These treatment options often require intravenous administration and have more serious adverse effects. Ceftaroline and ceftobiprole, a new class of β-lactam antibiotics with anti-MRSA activity, are promising new antibiotics for severe MRSA infections. Tedizolid phosphate, a second-generation oxazolidinone in late stage clinical development, is another promising alternative for severe MRSA infections. Each chapter of this dissertation will explore the role of antibiotics in MRSA, particular.
1.4 Staphylococcus aureus animal models

In the following section, we discuss S. aureus animal models used to study staphylococcal infections, including their advantages and disadvantages (outline in table 1.7).

Table 1.7. S. aureus animal models: advantages and disadvantages for studying antibiotic efficacy.

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Best animal model to use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin and soft tissue infections</td>
<td>Non-lethal infection</td>
<td>Difficult to study antibiotic</td>
<td>Mouse, rabbit and guinea</td>
</tr>
<tr>
<td></td>
<td>Easy to establish infection</td>
<td>efficacy</td>
<td>pig</td>
</tr>
<tr>
<td></td>
<td>Mouse, rabbit, guinea pig and pig models available</td>
<td>Non-lethal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>May be self-curing</td>
<td></td>
</tr>
<tr>
<td>Bacteremia</td>
<td>Easy to establish infection</td>
<td>Narrow window for CFU required for</td>
<td>Mouse, rabbit and guinea</td>
</tr>
<tr>
<td></td>
<td></td>
<td>infection</td>
<td>pig</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>Easy to establish infection</td>
<td>Acute infection/short time frame</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Good model for treatment of infections with biofilm or chronic infection</td>
<td>Difficult to establish infection</td>
<td>Rat and rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poor model of acute infection</td>
<td></td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Easy to establish infection in rabbits and rates</td>
<td>Difficult to establish infections</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>Lethal model</td>
<td>in mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Good mimic of human disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rabbit is most severe model to test efficacy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.4.1 Skin and Soft Tissue Infections

In the community, MRSA causes mainly skin and soft tissue infections such as abscesses. Mouse models are used to study pathogenesis due to reagent availability and ability to genetically manipulate the mouse [231-241]. However, mice metabolize drugs more rapidly compared to other animals and humans, so this model may not be suitable for testing new drug efficacy although it is useful to screen for drug activity. Rabbits and guinea pigs are better models to determine the efficacy of new antimicrobials since they metabolize drugs more similarly to humans compared to mice [242-246]. There have been studies on the pig abscess model for studying S. aureus abscesses but have been limited by cost [247].

1.4.2 Bacteremia

Bacteremia, or septicemia, is the presence of bacteria in the blood and is the eleventh leading cause of death in the US in 2009 [248]. In humans, bacteremia can be difficult to treat because of metastatic infections and a fulminant and rapidly fatal course of illness. Common bacterial sites of metastatic infections include infected bones and joints (osteomyelitis), heart valves (endocarditis) and organs, such as kidney and spleen [249].

Mouse models are used to screen for the activity of new drugs in bacteremia with advantages such as different genetic background and low cost [250-252]. In addition to determining drug efficacies, mouse models can be used to evaluate the role of S. aureus
virulence factors [253-257]. Other animal models used to study *S. aureus* bacteremia include rabbits, guinea pigs, rats, pigs and even chickens [258-261]. There are few bacteremia studies using these other models, probably due to the advantages of using mice. *S. aureus* bacteremia can be used to study sites of metastatic infection such as kidney infection, osteomyelitis and endocarditis, although the latter two require injury [255, 256, 261, 262].

### 1.4.3 Pneumonia

Pneumonia is an inflammatory condition of the lung and can be caused by bacteria, virus or fungi. Community-acquired MRSA (CA-MRSA) has been known to cause necrotizing pneumonia, mediated by toxins [263-266]. Rabbit models are used to study the effect of toxins in MRSA pneumonia but limited for drug efficacy. The rabbit pneumonia model has a narrow inoculum window between a lethal and non-lethal dose. When an infection is established, its acute course renders rescue by antibiotics difficult. Mouse models are used to screen for efficacy and pharmacokinetics of new drugs [267-271]. Advantages include different genetic background and low cost [272]. Other models for MRSA pneumonia have been used including rats, sheep and piglets, but cost is a limitation in these studies [273-275].

### 1.4.4 Osteomyelitis

Osteomyelitis, an infection of the bone or bone marrow, can lead to persistent disease. Antibiotics used to treat MRSA osteomyelitis may have poor bone penetration [276, 277]. New antibiotics for osteomyelitis must have good bone and bone tissue penetration since biofilm formation in implants and poor vascularization in bone structures may impede normal drug delivery. Rats and rabbits are common models used to determine the efficacy of new drugs or drug combinations and testing implants containing antimicrobials for the treatment of *S. aureus* osteomyelitis [278-292]. These models are a great way to study chronic infections, especially with biofilm formation. Rat and rabbit models of MRSA osteomyelitis are popular probably due to the larger size and low cost [293, 294]. Larger animals, such as sheep and pig, have been useful in studying the pathophysiological impact of MRSA osteomyelitis on the host, but are limited by cost [295, 296].

### 1.4.5 Endocarditis

*S. aureus* endocarditis, inflammation of the endocardium, usually involves colonizing lesions and biofilm formation on the endocardium, leading to the generation of vegetations. A mouse model of endocarditis has been used but its small size makes it difficult to use for endocarditis [297]. Two models are mainly used: rabbits and rats. Endocarditis in animal models is induced by inserting a catheter from the carotid artery into the heart, causing lesions on the heart valves, followed by intravenous inoculation of *S. aureus* resulting in colonization of the heart valves and vegetation formation. The rabbit model is useful in evaluating the role of bacterial virulence factors in pathogenesis [298-302], but is mainly used to study drug efficacy. The rabbit model is the preferred model to study MRSA and
antibiotic efficacy for the following reasons: i) it mimics human pharmacokinetics (PK) and pharmacodynamics (PD) making it the best model for human disease, ii) it is the most severe model to test efficacy in acute infections and iii) it is the gold standard for testing bactericidal activity of antibiotics [303-310].

1.4.5 Animal models summary

Various animal models are used to study different staphylococcal diseases, each with their own pros and cons. Mouse models are useful in screening for antibiotic activity but their small size and different drug metabolism rate make it hard to model human disease. Larger models such as pigs and sheep are useful in studying pathophysiology, but cost limits their use. Rats and rabbits are used often to test efficacy of S. aureus infections. The rabbit endocarditis model is arguably the best model for testing antibiotic efficacy to S. aureus infections for the following reasons: i) the rabbit is larger in size compared to rats, resulting in more consistent infections and larger bacterial vegetations, ii) it is the gold standard for testing bactericidal activity of antibiotics, iii) it most closely mimics human disease and iv) drug metabolism in rabbits is more similar to humans allowing PK and PD to be modeled. Overall, the rabbit endocarditis model is the best model to study human disease.
Research hypotheses to be addressed in this dissertation:

Chapter 2 hypothesis: Ceftobiprole and ceftaroline select for high-level, broad-spectrum β-lactam resistance in mecA-positive COL and USA300 backgrounds. Resistance to ceftobiprole and ceftaroline is mediated through mutations in mecA.

Chapter 3 hypothesis: Ceftobiprole and ceftaroline select for high-level, broad-spectrum β-lactam resistance in mecA-negative COL and USA300 backgrounds. By identifying mutant genes associated with resistance, new targets and mechanisms of resistance can be found.

Chapter 4 hypothesis: β-lactam antibiotics induce the SOS response in USA300, a prevalent CA-MRSA strain; SOS induction results in increased mutation frequencies leading to resistance.

Chapter 5 hypothesis: Tedizolid phosphate is non-inferior to vancomycin for the treatment of MRSA endocarditis in rabbits.

Knowledge to be gained:

Antibiotic resistance in *S. aureus* is a major public health problem. There are limited new antibiotics available for the treatment of severe MRSA infections. Ceftobiprole and ceftaroline are two new β-lactams with anti-MRSA activity. The goals of Chapters 2 and 3 are to determine mechanisms of resistance for ceftobiprole and ceftaroline and identify potential targets. Chapter 2 focuses on resistance in MRSA while Chapter 3 analyzes antibiotic resistance in strains lacking mecA. Determining resistance mechanisms in MRSA backgrounds will give insight on possible outcomes with MRSA treatment in the hospitals. Identifying mecA-independent mechanisms may lead to a better understanding of resistance and new targets.

Chapter 4 explores the role of antibiotic-mediated SOS response induction and resistance. The SOS response mediates resistance through increased mutational frequencies. Studies in *E. coli* and rare strains of *S. aureus* have shown β-lactams can induce the SOS response [222, 224, 311]. β-lactams, although ineffective at killing MRSA, may induce the SOS response, resulting in increased frequency of resistant mutants. Testing the role of the SOS response in antibiotic resistance in USA300 could serve as a therapeutic target to prevent the emergence of antibiotic resistance.

Chapter 5 determines the efficacy of tedizolid phosphate, a potential antibiotic for severe MRSA infections. With a limited number of antibiotics and increasing resistance, there is a need for new antibiotics. Testing tedizolid phosphate in a rabbit model of endocarditis compared to vancomycin and daptomycin will be useful in assessing whether tedizolid phosphate is an appropriate therapy option for invasive MRSA infections.
Chapter 2:

Ceftobiprole- and Ceftaroline-resistant Methicillin-resistant *Staphylococcus aureus*
2.1 Abstract

Background: *Staphylococcus aureus* is an important pathogen that can cause disease in otherwise healthy individuals. Treatment for *S. aureus* infections is becoming increasingly difficult due to a rise in antibiotic resistance. Methicillin-resistant *S. aureus* (MRSA) produce a low-affinity penicillin-binding protein 2a (PBP2a), which confers broad class resistance to the β-lactam class of antibiotics. Newly developed fifth generation cephalosporins target PBP2a with high affinity, as well as other PBPs. The purpose of this study is to determine whether in vitro resistance to fifth generation cephalosporins can be developed in MRSA. Method: COL and SF8300, a USA300 strain, were serially passaged in increasing concentrations of ceftobiprole and ceftaroline until resistance ceased to increase. Results: A single mutation in *mecA*, E447K, was sufficient to confer broad-spectrum β-lactam resistance in COL but only provided resistance to ceftobiprole in SF8300. In one strain lacking mutations in *mecA*, mutations in other genes, *php4* and *gdpP*, were present. Conclusion: Resistance to fifth generation cephalosporins can be selected in vitro in two difference MRSA backgrounds. Mutations in *mecA* were sufficient, but not necessary for resistance. Resistance in a ceftaroline-passaged mutant of COL was associated with mutations in *php4* and *gdpP*.
2.2 Introduction

*Staphylococcus aureus* is a serious human pathogen that can cause disease in otherwise healthy individuals. β-lactam antibiotics are the mainstay in therapy of staphylococcal infections but widespread β-lactam class resistance of methicillin-resistant *S. aureus* (MRSA) has forced clinicians to rely on second-line agents, which are less efficacious and more toxic. Vancomycin, standard therapy for invasive MRSA infections, has been associated with nephrotoxicity and increased resistance [312-316]. Patients with intolerance to vancomycin have been given linezolid and daptomycin. Linezolid is approved for SSSIs and pneumonia but is associated with haematological toxicity [317, 318]. Daptomycin is effective for treatment of bacteremia and endocarditis but not pneumonia and its bone penetration may not be adequate for osteomyelitis [319-323]. Linezolid is susceptible to the chloramphenicol-florfenicol resistance gene (*cfr*) and daptomycin non-susceptible strains of MRSA have emerged, some of which are associated with vancomycin treatment failure [324-329]. MRSA infections are associated with increased hospital stay, cost and mortality [188, 330-332].

MRSA strains display broad class resistance to β-lactams by virtue of an extra penicillin-binding protein 2a (PBP2a), encoded by *mecA*. *S. aureus* contains four native PBPs, PBP1-4, which crosslink muropeptides in the cell wall to create peptidoglycan, a major component of Gram-positive bacteria cell walls. β-lactams bind PBPs covalently at the active site, inactivating the protein thereby inhibiting cell wall synthesis leading to cell death [110]. PBP2a, unlike PBP1-4, have low affinity for β-lactams, allowing cell wall synthesis to continue in the presence of the antibiotic [113]. The binding site in PBP2a is located in a narrow, extended cleft that is inaccessible to β-lactams, preventing the formation of the covalent acyl-enzyme complex, while still maintaining transpeptidase activity and allowing survival in otherwise lethal β-lactam concentrations [114]. A new class of cephalosporins, including ceftobiprole (BPR) and ceftaroline (TRL), targets PBP2a with high affinity. Ceftobiprole’s mechanism of action involves two R groups: one R group protects the molecule from β-lactamase degradation while the other R group extends deep into the cleft of PBP2a, accessing the binding site. Ceftaroline’s mechanism of action is presumed to be similar to ceftobiprole. They have been evaluated in phase III clinical trials and ceftaroline (TRL) is FDA approved for community-acquired bacterial pneumonia and acute bacterial skin infections [333-338].

Previous studies have shown that *in vitro* passage of a laboratory MRSA strain, COL, in ceftobiprole generates resistant mutants as a result of mutations in *mecA*. COLnex, a variant of COL lacking SCCmec, with *mecA* on a plasmid was passaged in increasing concentrations of ceftobiprole. After 28 days of passage, mutants developed ceftobiprole resistance to 128 µg/ml. These mutants had numerous mutations in *mecA*. Curing the plasmid in the mutant resulted in susceptibility to β-lactams, including ceftobiprole. Conversely, introducing mutated *mecA* on a plasmid into susceptible parent, COLnex, conferred resistance, demonstrating that mutations in *mecA* were responsible for resistance [152]. This chapter broadens the scope of the previous study by determining
whether ceftaroline-resistant mutants can be generated in COL and USA300 community-acquired MRSA (CA-MRSA) backgrounds.

There are many differences and similarities between COL and USA300 MRSA backgrounds. COL was the original MRSA strain discovered in 1961, but is no longer present in hospital or community settings [12]. It emerged as a nosocomial strain, but is now considered a lab strain due to passages incurred throughout the years. It is Ridom spa type t008, multilocus sequence type 250, contains SCCmec type I, belongs to the clonal complex 8 group and harbors few virulence factors. COL is homogeneously resistant to β-lactams. USA300, on the other hand, is the most prevalent community-associated MRSA strain in North America [339]. It first emerged around 2000 and has been increasing in prevalence ever since. It is Ridom spa type t008, multilocus sequence type 8, contains SCCmec type IV, and belongs to the clonal complex 8 group. Similar to other CA-MRSA strains, USA300 contains many virulence factors, particularly Panton-Valentine leukocidin (PVL), toxins, and the arginine catabolic mobile element (ACME), and it can be multidrug resistance [35]. USA300 is heterogeneously resistant to β-lactams. Even though COL and USA300 differ in epidemiologic characteristics, they both belong to clonal complex 8 with only 678 single nucleotide polymorphisms (SNPs), 70% of which resulted in amino acid substitutions [35]. Studies from this dissertation utilizes the CA-MRSA USA300 strain rather than other CA-MRSA strains, such as USA400, because USA300 is the most prevalent CA-MRSA lineage in the North America [340].

Ceftobiprole and ceftaroline are similar in structure, with a cephalosporin core, a β-lactamase insensitive R1 group and a PBP binding R2 group (figure 1.3). Previously published studies had shown resistance to ceftobiprole in COL was achieved through mutations in mecA [152]. This chapter aims to determine whether resistance to ceftaroline can be achieved in COL and USA300. COL and USA300 were passaged in increasing concentrations of active drug ceftaroline (kindly provided by Forest Labs). Resistance to ceftobiprole in USA300 was not assessed due to limitations in drug availability. Results indicate that resistance to both ceftobiprole and ceftaroline can be achieved through mutations in mecA as well as other genes (pbp4 and gdpP).
2.3 Methods and Materials

2.3.1 Bacterial strains and plasmids.

All strains were grown on trypticase soy agar (TSA), in trypticase soy broth (TSB) with aeration, or on blood agar (Remel) at 37°C. Strains used are listed in table 2.1. COLnlex and SF8300ex are the mecA-negative derivative strains of COLn and SF8300, in which chromosomal staphylococcal cassette chromosome mec has been precisely excised by the introduction of plasmid pSR [55]. This plasmid carries site-specific recombinases, ccrA and ccrB that precisely excise chromosomal SCCmec [341]. pYK20, a pAW8 plasmid encoding wild-type mecA from COL [342], was used to reintroduce mecA into COLnlex and SF8300ex.

2.3.2 Antibiotics.

Ceftobiprole solution was prepared fresh daily from ceftobiprole powder (provided by Johnson and Johnson Pharmaceutical Research and Development) at a concentration of 4 mg/mL. Ceftaroline solution was prepared from ceftaroline powder (provided by Forest labs) at a concentration of 10 mg/mL in 30% DMSO and 0.9% saline. Nafcillin, ampicillin, cefazolin, cefoxitin and ceftriaxone were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

2.3.3 Multipassage selection.

COLnlex pYK20 and SF8300ex pYK20 were serially passaged in increasing concentrations of ceftaroline as previously described [152]. Briefly, strains were passaged in 10 mL preparations of TSB containing ceftobiprole and 10 µg/mL tetracycline. Overnight cultures were diluted 1:100 in increasing concentrations of ceftobiprole.

2.3.4 Antibiotic resistance measurement.

MICs were determined according to CLSI standards. Briefly, 1x10^5 CFU were incubated 18-24 hours in 0.2 mL cation-adjusted Mueller Hinton broth (CAMHB) containing increasing concentrations of antibiotic. MIC levels were recorded as the lowest concentration with the absence of growth.

2.3.5 Plasmid studies.

Parent pYK20 (mecA wild-type) plasmids and pYK20 passaged plasmids were introduced into susceptible COLnlex and SF8300ex parent strains. Plasmids were transduced from host to recipient using bacteriophage Φ11. Plasmids used in this study are listed in table 2.2.
2.3.6 Curing passaged mutants

Passaged mutants were cured of plasmid by passaging for 5 days in TSB without drug at 37°C and 150 rpm. Single colonies were isolated at day 5 and analyzed for the loss of plasmid by two methods: i) isolates were replica-plated onto TSA and TSA containing 10 μg/mL tetracycline and ii) PCR analyzed for the absence of the mecA gene.
2.4 Results

2.4.1 Isolation of mutants with ceftobiprole and ceftaroline resistance.

Isolates with resistance to ceftobiprole and ceftaroline were generated by serial passage in tryptic soy broth. After 28 days of passage, two mutants were isolated. COLnex pYK20 and SF8300ex pYK20 passaged in ceftaroline resulted in mutants COLnex_{pT} pYK20_{COLT*} and SF8300ex_{pT} pYK20_{8300T*}, respectively (table 2.1). As previously described, selection of COLnex pYK20 in ceftobiprole generated mutant COLnex_{pB} pYK20_{COLB*} [152]. MICs for this strain exhibited high-level resistance to ceftobiprole and ceftaroline (MIC 64 μg/mL). Similarly, COLnex_{pT} pYK20_{COLT*} (COLnex pYK20 passaged in ceftaroline) had high-level ceftaroline and ceftobiprole resistance (MIC 64 μg/mL and 32 μg/mL, respectively). SF8300ex_{pT} pYK20_{8300T*} (SF8300ex pYK20 passaged in ceftaroline) remained at 8 μg/mL after 24 passages and exhibited low-level resistance to ceftobiprole and ceftaroline (table 2.3).

2.4.2 Mutation analysis

To determine which genes may contribute to resistance, sequencing of selected genes was used to identify mutations. Based on the previous study, mecA was sequenced for mutations [152]. Additionally, since PBPs are usually the target of β-lactams, pbp1-4 were sequenced. Lastly, based on a previous study reporting ceftobiprole resistance in a mecA-negative strain containing mutations in gdpP and acrB, these genes also were sequenced [343]. SF8300ex_{pT} pYK20_{8300T*} had only one mutation in mecA, E447K; no mutations were present in the other genes that were screened. Of note ceftobiprole passage also selected for the E447K mutation (among others) in the COL background [152]. COLnex_{pT} pYK20_{COLT*} had no mutations in mecA, but contained one mutation in gdpP, H443Y, and two mutations in pbp4, T201A and F241L (table 2.4).

2.4.3 Plasmid studies

To assess the contribution of wild-type and mutated mecA to resistance, two types plasmid studies were performed, elimination and transformation. First, each mutant was cured of its plasmid and MICs were determined. Curing COLnex_{pB} pYK20_{COLB*} and SF8300ex_{pT} pYK20_{8300T*} of their plasmids decreased MICs for all β-lactams tested. Curing COLnex_{pT} pYK20_{COLT*} of its plasmid did not decrease MICs (table 2.5 and 2.6). Plasmids with mutated mecA, pYK20_{COLB*} and pYK20_{8300T*}, were transduced into susceptible parents, SF8300ex and COLnex, and MICs were determined. Transforming pYK20_{COLB*} into susceptible COLnex parent (COLnex pYK20_{COLB*}) resulted in high-level ceftaroline and ceftobiprole resistance at 32 μg/mL and 64 μg/mL, respectively. COLnex transformed with pYK20_{8300T*} (COLnex pYK20_{8300T*}) resulted in high-level resistance to ceftobiprole but low-level resistance to ceftaroline (table 2.5). Transforming pYK20_{COLB*} into SF8300ex (SF8300ex pYK20_{COLB*}) resulted in high-level resistance to ceftaroline and ceftobiprole (MIC 32 μg/mL) (table 2.6). Transformations of pYK20_{8300T*} into SF8300ex were unsuccessful.
2.5 Discussion

Previous studies described ceftobiprole-resistant mutants that were selected, in vitro, using the COL strain. This study expands on the previous study by exploring ceftaroline resistance in two different MRSA backgrounds, COL and SF8300, a USA300 strain. Results suggest that both ceftobiprole and ceftaroline are capable of selecting high-level, broad-spectrum β-lactam resistant mutants in both MRSA backgrounds in vitro through mutations in mecA or other genes (pbp4 and gdpP).

COLnex pYK20 and SF8300ex pYK20 passaged in ceftaroline reached the highest level of resistance by day 21. In the COL background, ceftaroline selected for higher resistance more quickly compared to ceftaroline, with both mutants exhibiting high-level resistance to both drugs. Of note, COLnexpT pYK20COLT* was passaged in 10 mL volumes while COLnexpb pYK20COLB* was passaged in 300 mL volumes, suggesting passage volumes do not affect the selection of resistant mutants. Even though COLnexpT pYK20COLT* was passaged to 256 µg/ml, MICs were stable at 64 µg/ml. SF8300ex pYK20 passaged in ceftaroline did not develop high-level resistance but remained at 8 µg/mL with stable MICs of 4 µg/mL.

To determine what mutations may contribute to resistance, selected genes were sequence analyzed. Both SF8300expT pYK208300T* and COLnexpb pYK20COLB* had the mecA E447K mutation and no mutations in other genes analyzed, suggesting this mutation is accounts for resistance. COLnexpT pYK20COLT*, did not have any mutations in mecA but contained mutations in gdpP, H443Y, and pbp4, F241L and T201A. Interestingly, pbp4 and gdpP mutations are also found in ceftobiprole and ceftaroline passaged mecA-negative strains described in Chapter 3. PBP4 is a low-molecular weight, non-essential PBP responsible for synthesizing long-chain muropeptides from monomers and dimers [103, 344]. Since it is non-essential, there are fewer studies analyzing the role of PBP4 in resistance. GdpP is a recently discovered protein with a degenerate GGDEF domain (with unknown function) and a phosphodiesterase domain. GdpP phosphodiesterase functions in breaking down a small signaling molecule, cyclic di-AMP, which plays a role in the stress response in S. aureus and host immune response in L. monocytogenes [345, 346]. The gdpP mutation occurs in the phosphodiesterase domain and likely affects enzymatic function. However, further analysis of c-di-AMP levels in mutants is needed to confirm enzymatic function. This suggests that, even in the presence of mecA, ceftaroline can select for mutations in other genes, resulting in broad-spectrum β-lactam resistance. Whole genome sequencing of this mutant may identify additional genes that contribute to resistance.

To determine the contribution of mecA mutations on β-lactam resistance, plasmid studies were performed. Curing SF8300expT pYK208300T* and COLnexpb pYK20COLB* of the plasmid decreased MICs, suggesting resistance is mediated by plasmid determinants. As expected, curing COLnexpT pYK20COLT* of the plasmid did not reduce MICs, suggesting that mutations in other genes are responsible for resistance. Since plasmid pYK208300T* only had one mutation in mecA, plasmid studies were used to determine the contribution of this single mutation on resistance. When this plasmid was transduced into COLnex, the
resultant mutant had high-level resistance to ceftobiprole but low-level resistance to ceftaroline, suggesting the E447K mutation is sufficient for ceftobiprole resistance in this background but not ceftaroline. This result implies that, even though the structures of ceftobiprole and ceftaroline are similar, the structural differences may result in different antibiotic-PBP2a interactions. To determine whether additional mutations in mecA would contribute to ceftaroline resistance, plasmid studies with COLnexpB pYK20COLB* (with 5 additional mecA mutations) were performed. Transforming both COLnex and SF8300ex with COLnexpB pYK20COLB* resulted in high-level ceftobiprole and ceftaroline resistance, suggesting that additional mecA mutations are required for ceftaroline resistance. Structurally, E447K in PBP2a is next to Y446, which is hypothesized to interact with the R2 group of ceftobiprole. Since E447K conferred resistance to ceftobiprole but not ceftaroline, this suggests that the two antibiotics may interact with different sites on PBP2a. Crystal structures of PBP2aE447K bound to ceftaroline and ceftobiprole would provide more information on possible structural differences contributing to resistance.

Limitations in this study include the single passage experiment for each strain and antibiotic. Due to the limited amount of active drug available, it was not feasible to repeat the passages. However, the E447K mutation in mecA emerged from both strains passaged in different antibiotics, suggesting this mutation in important in resistance for both antibiotics. Another limitation is that only vertically transmitted resistance (mutations) was analyzed. It is possible genetic elements carrying determinants conveying resistance to ceftobiprole and ceftaroline exist, similar to the cfr-carrying plasmid for resistance to linezolid.

In conclusion, ceftaroline-resistant mutants can be generated in vitro using two different MRSA backgrounds. Ceftaroline selects for both mecA and non-mecA mutations. The single mutation E447K mecA mutation emerged from both ceftobiprole and ceftaroline selection. This single mutation conferred high-level resistance to ceftobiprole and only low-level resistance to ceftaroline in the COL background. Unexpectedly, a mutant with high-level resistance was generated with mutations in pBP4 and gdpP, even in the presence of PBP2a. Thus, it is important to determine mecA-independent mutations in ceftobiprole and ceftaroline resistance, which is discussed in Chapter 3.
2.6 Figures and Tables

Figure 2.1. Ceftobiprole and ceftaroline passaged *mecA*-positive strains. Resistance to BPR and TRL were generated by passaging strains in subinhibitory concentrations of BPR or TRL in broth. The highest concentration of drug in which strains grew each day is shown on the y axis.
Table 2.1. List of parental and mutant strains used in *mecA*-positive passage studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLn</td>
<td>Parental strain</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex</td>
<td>SCC<em>mec</em> excision strain derived from COLn</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex pAW8</td>
<td>COLnex with an empty plasmid</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex pYK20</td>
<td>COLnex with <em>mecA</em> on a plasmid</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex&lt;sub&gt;pB&lt;/sub&gt; pYK20&lt;sub&gt;COLB&lt;/sub&gt;*</td>
<td>COLnex pYK20 passaged in BPR strain</td>
<td>BPR&lt;sup&gt;r&lt;/sup&gt;; Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex&lt;sub&gt;pT&lt;/sub&gt; pYK20&lt;sub&gt;COLT&lt;/sub&gt;*</td>
<td>COLnex pYK20 TRL passaged strain</td>
<td>TRL&lt;sup&gt;r&lt;/sup&gt;; Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex&lt;sub&gt;pB&lt;/sub&gt;</td>
<td>COLnex&lt;sub&gt;pB&lt;/sub&gt; pYK20&lt;sub&gt;COLB&lt;/sub&gt;* cured of <em>mecA</em> plasmid</td>
<td>Mc&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>COLnex&lt;sub&gt;pT&lt;/sub&gt;</td>
<td>COLnex&lt;sub&gt;pT&lt;/sub&gt; pYK20&lt;sub&gt;COLT&lt;/sub&gt;* cured of <em>mecA</em> plasmid</td>
<td>Mc&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300</td>
<td>USA300 MRSA clinical isolate</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt; Em&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300ex</td>
<td>SCC<em>mec</em> excision strain derived from SF8300 ES</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt; Em&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300ex pAW8</td>
<td>SF8300ex with an empty plasmid</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt; Em&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300ex pYK20</td>
<td>SF8300ex with <em>mecA</em> on a plasmid</td>
<td>Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300ex&lt;sub&gt;pT&lt;/sub&gt; pYK20&lt;sub&gt;8300T&lt;/sub&gt;*</td>
<td>SF8300ex pYK20 TRL passaged strain</td>
<td>TRL&lt;sup&gt;r&lt;/sup&gt;; Mc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF8300ex&lt;sub&gt;pT&lt;/sub&gt;</td>
<td>SF8300ex&lt;sub&gt;pT&lt;/sub&gt; pYK20&lt;sub&gt;8300T&lt;/sub&gt;* cured of <em>mecA</em> plasmid</td>
<td>Mc&lt;sup&gt;s&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* The table lists parent and mutant strains.
Table 2.2. Plasmids used in *mecA*-positive passage studies.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAW8</td>
<td>Empty plasmid</td>
</tr>
<tr>
<td>pYK20</td>
<td>pAW8 containing <em>mecA</em></td>
</tr>
<tr>
<td>pYK20_{COLB}*</td>
<td>pYK20 in COLnex passaged in BPR</td>
</tr>
<tr>
<td>pYK20_{COLT}*</td>
<td>pYK20 in COLnex passaged in TRL</td>
</tr>
<tr>
<td>pYK20_{8300T}*</td>
<td>pYK20 in SF8300ex passaged in TRL</td>
</tr>
</tbody>
</table>

*Parent and passaged plasmids isolated during ceftobiprole and ceftaroline selection in COL and SF8300 backgrounds.*
Table 2.3. MIC (µg/mL) for mecA-positive passaged mutant strains.

<table>
<thead>
<tr>
<th></th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLn</td>
<td>256</td>
<td>16</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
<td>2</td>
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<tr>
<td>COLnex</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>COLnex pAW8</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>COLnex pYK20</td>
<td>128</td>
<td>8</td>
<td>256</td>
<td>256</td>
<td>&gt;256</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>COLnex_pYK20_col_T*</td>
<td>128</td>
<td>64</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>COLnex_pYK20_col_B*</td>
<td>&gt;256</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>SF8300</td>
<td>32</td>
<td>128</td>
<td>32</td>
<td>64</td>
<td>128</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SF8300ex</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300ex pAW8</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300ex pYK20</td>
<td>32</td>
<td>8</td>
<td>128</td>
<td>128</td>
<td>&gt;256</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>SF8300_pYK208300_T*</td>
<td>128</td>
<td>16</td>
<td>128</td>
<td>128</td>
<td>256</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

NAF = nafcillin, AMP = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BPR = ceftobiprole, TRL = ceftaroline
Table 2.4. Mutations in *mecA*-positive mutant strains passaged in ceftobiprole and ceftaroline.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid mecA</th>
<th><em>ppb1</em></th>
<th><em>ppb2</em></th>
<th><em>ppb3</em></th>
<th><em>ppb4</em></th>
<th><em>gdpP</em></th>
<th>acrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLnex_pBpYK20_COLB*</td>
<td>E150K Y446L E447K F467Y R589K S649A</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>COLnex_pTpYK20_COLT*</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>T201A F241L</td>
<td>H443Y</td>
<td>Wild-type</td>
</tr>
<tr>
<td>SF8300ex_pTpYK20_8300T*</td>
<td>E447K</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Table 2.5. MIC (µg/mL) for *mecA*-positive passaged mutant strains cured of plasmid and parental strains transduced with plasmids from passaged strains in the COL background.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLn</td>
<td>256</td>
<td>16</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>COLnex</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>COLnex pAW8</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>COLnex pYK20</td>
<td>128</td>
<td>8</td>
<td>256</td>
<td>256</td>
<td>&gt;265</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>COLnex pYK20_{830T}</td>
<td>&gt;256</td>
<td>32</td>
<td>&gt;256</td>
<td>64</td>
<td>32</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>COLnex pYK20_{COLB}</td>
<td>&gt;256</td>
<td>32</td>
<td>&gt;256</td>
<td>128</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>COLnex_{pB} pYK20_{COLB}</td>
<td>&gt;256</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>COLnex_{pB} no plasmid</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>COLnex_{pT} pYK20_{COLT}</td>
<td>128</td>
<td>64</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>COLnex_{pT} no plasmid</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>16</td>
<td>&gt;256</td>
<td>64</td>
<td>32</td>
</tr>
</tbody>
</table>

*NAF = nafcillin, AMP = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BPR = ceftobiprole, TRL = ceftaroline*
Table 2.6. MIC (µg/mL) for meca-positive passaged mutant strains cured of plasmid and parental strains transduced with plasmids from passaged strains in the USA300 strain SF8300 background.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SF8300</strong></td>
<td>32</td>
<td>128</td>
<td>32</td>
<td>64</td>
<td>128</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>SF8300ex</strong></td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>SF8300ex pAW8</strong></td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>SF8300ex pYK20</strong></td>
<td>32</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>256</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td><strong>SF8300ex pYK20 ColB</strong></td>
<td>32</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td><strong>SF8300ex pYK20 a300T</strong></td>
<td>128</td>
<td>16</td>
<td>128</td>
<td>128</td>
<td>256</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>SF8300ex no plasmid</strong></td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

*NAF = nafcillin, AMP = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BPR = ceftobiprole, TRL = ceftaroline
Chapter 3:

Ceftobiprole- and Ceftaroline-resistant *mecA*-negative *Staphylococcus aureus*
3.1 Abstract

Background: Methicillin-resistant S. aureus (MRSA) are resistant to the β-lactam class of antibiotics through the presence of penicillin-binding protein 2a (PBP2a). Ceftobiprole and ceftaroline, target PBP2a with high affinity. From Chapter 2, two of three mutants isolated with high-level ceftobiprole and ceftaroline resistance had mutations in meca. A third mutant lacked mutations in meca but had mutations in pbp4 and gdpP. This suggests that, even in the presence of meca, mutations in other genes can occur conferring high-level resistance. Additionally, a previous passage study revealed, in the absence of meca, ceftobiprole passage of S. aureus resulted in broad-spectrum β-lactam resistance and only had mutations in three genes: pbp4, gdpP and acrB. The purpose of this study is to explore meca-independent ceftobiprole and ceftaroline resistance. Method: COLnex and SF8300ex, meca-negative derivatives of COL and SF8300, a USA300 strain, were passaged in increasing concentrations of ceftobiprole and ceftaroline. Mutants were isolated and analyzed for resistance. Pbp1-4, gdpP and acrB were sequenced for mutations. Results: Passaged mutants had high-level, broad-spectrum β-lactam resistance. All mutants contained mutations in pbp4 and gdpP or pbp4 and pbp2. pbp4 is essential for resistance in 3 of 4 mutants. When mutations were introduced in SF8300ex parent, pbp4 and gdpP mutations enabled resistance to ceftobiprole and ceftaroline. Conclusion: Selection of COLnex and SF8300ex in ceftobiprole and ceftaroline can generate mutants with high-level β-lactam resistance. PBP4 is important in the resistance mechanism.
3.2 Introduction

*Staphylococcus aureus* is among the most important of human pathogens. Treatment for *S. aureus* has become increasingly difficult due to resistance and limited new antibiotics. β-lactams are the best treatment option for *S. aureus* infections due to bioavailability, good penetration and minimal adverse effects [335, 347]. However, methicillin-resistant *S. aureus* (MRSA) is resistant to the β-lactam class forcing clinicians to utilize less efficacious second-line antimicrobial agents with more adverse effects.

MRSA displays class resistance to β-lactams through the presence of an extra penicillin-binding protein 2a (PBP2a), encoded by *mecA*. Ceftobiprole (BPR) and ceftaroline (TRL) target PBP2a with high affinity [334-338]. Previous studies analyzing COL passaged in ceftobiprole revealed two mechanisms of resistance: *mecA*-dependent and *mecA*-independent. *In vitro* passage of a laboratory MRSA strain, COL, generated ceftobiprole resistance via mutations in *mecA*. However, even in the presence of *mecA*, mutations in *pbp4* and *gdpP* emerged. COLnex, an SCCmec excised derivative of COL, passaged in ceftobiprole resulted in high-level β-lactam resistance with mutations in only 3 genes: *pbp4*, *gdpP* and *acrB* [152, 153].

Since COLnex passaged in ceftobiprole resulted in mutations in only three genes, it suggests that the mutations, alone or in combination, are responsible for resistance. PBP4 and GdpP have been implicated in *S. aureus* β-lactam resistance in published studies [344, 346, 348-350]. AcrB is part of a drug efflux pump in Gram-negative bacteria [351, 352]. In *S. aureus*, it is a putative transporter with no known function.

As discussed in Chapter 2, there are many similarities and differences between COL and USA300. Since previously published studies observed mutations in the COL background, this study will broaden the results by analyzing *mecA*-negative ceftobiprole- and ceftaroline-resistant mutants generated in both COL and USA300 backgrounds. The objective of this study is to determine mechanisms of resistance and identify genes that may contribute to β-lactam resistance. Given the observation that non-*mecA* mutations can emerge in a MRSA background, it is important to identify *mecA*-independent mechanisms of resistance. Identifying *mecA*-independent mechanisms may lead to a better understanding of resistance and to the development of new targets.
3.3 Methods and Materials

3.3.1 Bacterial strains and plasmids.

All strains were grown on trypticase soy agar (TSA), in trypticase soy broth (TSB) with aeration or on blood agar (Remel) at 37°C. Strains used are listed in table 3.1. COLnex and SF8300ex are the mecA-negative derivative strains of COLn and SF8300 from which staphylococcal cassette chromosome mec (SCCmec) has been precisely excised by the introduction of plasmid pSR, which carries site specific recombinases, ccrA and ccrB [341]. pASP was derived from pALC2073, a tetracycline inducible expression plasmid for S. aureus (a generous gift from Dr. Ambrose Cheung). The pbp4 construct in the anti-sense direction was inserted into pALC2073.

3.3.2 Antibiotics.

Ceftobiprole solution was prepared fresh daily from ceftobiprole powder (provided by Johnson and Johnson Pharmaceutical Research and Development) at a concentration of 2 mg/mL. Ceftaroline solution was prepared from ceftaroline active powder (provided by Forest labs) at a concentration of 1 mg/ml. Nafcillin (Sigma Cat. #N3269-5G), ampicillin (Sigma Cat. #A9393), cefazolin (Sigma Cat. #C5020), cefoxitin (Sigma Cat. #C4786) and ceftriaxone (Sigma Cat. #C5793) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

3.3.3 Multipassage selection.

SF8300ex was serially passaged in ceftobiprole as previously described [152]. Briefly, 300 mL preparations of TSB containing various concentrations of ceftobiprole were inoculated at a 1:100 dilution with overnight cultures containing 10⁹ CFU/mL. The ceftrobiprole concentration was doubled at each passage, as tolerated. COLnex and SF8300ex were serially passaged in ceftaroline as described in Chapter 2.

3.3.4 Antibiotic resistance measurement.

MICs were determined according to CLSI standards as described in Chapter 2. Population analyses were done by the agar method. A 10µl volume of serially diluted culture was spotted onto agar plates containing various concentrations of antibiotic. The plates were incubated at 37°C for 48-72 hours. Plates were read and expressed as CFU/ml.

3.3.5 Insertional Inactivation of pbp4.

Plasmid pNL155, which contains truncated pbp4, was used as a suicide plasmid to inactivate pbp4 in mutant strains as previously described [341]. Briefly, pNL155 was transduced into each mutant strain, passaged in TSB containing erythromycin at 42°C for
three days, plated onto TSA containing erythromycin and incubated at 42°C. Colonies were purified and MICs tested.

3.3.6 Allelic exchange.

Plasmid pKOR1 was used to conduct allelic exchanges as previously described [353]. Briefly, constructs for allelic exchange were created via splice overlap extension polymerase chain reaction (SOE-PCR). The PCR fragment was cloned into the pKOR1 plasmid using BP Clonase II (Invitrogen Cat. #11789-020). Constructs were transformed into *E. coli* Top10 cells (Invitrogen C404003) and plated onto ampicillin 100 µg/mL TSA plates. Colonies were purified and sequenced for construct confirmation. Plasmids were purified from *E. coli* using QIAprep Spin Miniprep kits (Qiagen Cat. #27104). Purified plasmids were electroporated into competent RN4220 *S. aureus*. Plasmids were transduced from RN4220 to host *S. aureus* strains. Strains were grown in TSB containing chloramphenicol 10 µg/mL at 30°C and 42°C for allelic exchange followed by passage at 30°C without selection. Cultures were plated onto 0.5 µg/mL and 1 µg/mL anhydrotetracycline plates for counter selection. Resultant strains were sequenced for allelic exchange.

3.3.7 mRNA measurement and qRT-PCR.

Overnight cultures were diluted 1:100. One mL of cell suspension was collected during exponential phase, mixed with 4 mL RNAlater and stored at 4°C overnight. RNA was isolated using Lysing Matrix B (MP Biomedical Cat. # 116911100) and Qiagen RNeasy mini kit (Qiagen Cat. # 74104). RNA was treated with Turbo DNasefree (Invitrogen Cat. #AM1907). cDNA was synthesized using High Capacity RNA-to-cDNA Mastermix (Invitrogen # 4390776). Relative mRNA levels were measured using SYBR green mastermix (Invitrogen # 4309155) and primers listed in table 3.1.
3.4 Results

3.4.1 Isolation of mutants with ceftobiprole and ceftaroline resistance

Isolates with resistance to ceftobiprole and ceftaroline were generated by serial passage in tryptic soy broth containing increasing concentrations of ceftobiprole and ceftaroline. SF8300ex, a clinically relevant CA-MRSA strain, USA300, lacking mecA, was passaged in increasing subinhibitory concentrations of ceftobiprole or ceftaroline while COLnex, a mecA-negative variant of COL, was passaged in ceftaroline until the increase in resistance reached 256 µg/mL. The highest concentration of growth was plotted daily until the maximum concentration tolerated (figure 3.1).

The initial MICs of both strains were <0.25-0.5 µg/mL. As described previously, COLnex passaged in ceftobiprole resulted in mutant CRB, a mecA-negative, high-level, broad-spectrum β-lactam resistant mutant [152]. CRB reached 256 µg/mL ceftobiprole by day 21 with a stable MIC at 64 µg/mL. This mutant had high-level cross-resistance to ceftaroline (32 µg/mL). Passage of SF8300ex in ceftobiprole generated a β-lactam resistant mutant, SRB. It reached 256 µg/mL ceftobiprole by day 42 (data not shown) with a stable MIC at 64 µg/mL. SRB had low-level cross-resistance to ceftaroline (MIC 4 µg/mL). COLnex and SF8300ex passaged in ceftaroline generated mutants CRT and SRT, respectively. CRT reached 256 µg/mL by day 17 with stable a MIC at 64 µg/mL. It had low-level cross-resistance to ceftobiprole (MIC 4 µg/mL). SRT reached 256 µg/mL ceftaroline by day 15 with a stable MIC at 64 µg/mL. It had low-level cross-resistance to ceftobiprole (MIC 4 µg/mL) (table 3.3).

3.4.2 Mutation analysis of passaged mutants

As previously published, whole-genome sequencing of CRB revealed mutations in 3 genes: pbp4, gdpP and acrB [343]. Since CRB has high-level resistance to β-lactams, it suggests that these mutations, alone or in combination, are responsible for resistance. To determine whether mutations in these genes were also present in SRB, CRT and SRT, each strain was sequence analyzed for pbp4, gdpP and acrB. Additionally, since PBPs are the target of β-lactams, pbp1-3 were also sequence analyzed for mutations. SRB has 4 mutations in pbp2, 1 mutation in pbp3 and 1 mutation in pbp4. CRT contains one mutation in pbp2 and two mutations in pbp4. SRT contains 2 mutations in pbp3, 2 mutations in pbp4 and a stop mutation in gdpP (table 3.4).

3.4.3 pbp4, gdpP and acrB mRNA expression

Since there were multiple mutations in pbp’s, gdpP and acrB and increased PBP production has been associated with low-level β-lactam resistance, mRNA levels were analyzed to determine if expression of these genes were altered. mRNA levels of pbp1-4, gdpP and acrB were measured during exponential phase. CRB, CRT and SRT had
upregulated \textit{pbp4} mRNA levels but not SRB (figure 3.2). To determine whether or not \textit{pbp4} mRNA levels were important for resistance, a plasmid expressing anti-sense \textit{pbp4} mRNA (pASP) was constructed and introduced into the mutants that overexpressed \textit{pbp4} mRNA (table 3.5). SRT MICs decreased with the introduction of pASP but CRB and CRT MICs were not affected.

3.4.4 Insertional inactivation of \textit{pbp4}

Since all mutants contained mutations in \textit{pbp4} and three of four mutants had upregulated \textit{pbp4} mRNA levels, insertional inactivation of \textit{pbp4} was performed to determine whether it is essential for resistance in each strain. Inactivating \textit{pbp4} in CRB and SRT rendered the mutants susceptible, comparable to \textit{mecA}-negative parental levels. However, inactivating \textit{pbp4} in CRT did not reduce MIC levels. Inactivating \textit{pbp4} in SRB decreased MICs to SF8300ex parental levels. But replacing mutant with wild-type \textit{pbp4} only decreased MICs 2-4 fold (table 3.6)

3.4.5 CRB mutations in SF8300ex

Since CRB is known to have only three mutations, one or more of these mutations were probably sufficient for high-level, broad-spectrum \textit{β}-lactam resistance. To determine the individual contribution of each mutation, allelic exchange was performed in a parental background. Allelic exchange in COLn proved to be difficult. Instead, these mutations were introduced into SF8300ex, and different measures of resistance were evaluated. Single, double and triple mutations in SF8300ex did not increase MICs (table 3.7). In population analyses, the single \textit{gdpP\textsuperscript{N182K}} mutation had a slight advantage compared to wild-type and \textit{pbp4} single mutant (figure 3.3). When grown in subinhibitory concentrations of nafcillin, SF8300ex \textit{gdpP\textsuperscript{N182K}} had an initial growth advantage over wild-type, but growth decreased after 26 hours. SF8300ES \textit{gdpP\textsuperscript{N182K}} grown in subinhibitory concentrations of nafcillin had a consistent growth advantage compared to wild-type over 48 hours (figure 3.4).

Since CRB has high-level, broad-spectrum \textit{β}-lactam resistance and has mutations in only three genes, SF8300ex with the same mutations was hypothesized to exhibit \textit{β}-lactam resistance as well. However, SF8300 is a heterogeneously \textit{β}-lactam resistant strain whereas COL exhibits homogeneous resistance. Heterogeneously resistant strains become homogeneously resistant with passage in subinhibitory concentrations of \textit{β}-lactams [113]. To determine if the heterogeneous phenotype of SF8300ex contributed the lack of resistance with the mutations, wild-type, single, double and triple mutants were passaged in increasing concentrations of oxacillin. When wild-type and mutants were passaged in subinhibitory concentrations of oxacillin, resistance developed more quickly in the double and triple mutants (figure 3.5). When these passaged strains were tested for MICs, all passaged strains had increased MICs to most \textit{β}-lactams tested. However, increased MICs in ceftobiprole and ceftaroline were only present in the double and triple mutants passaged in oxacillin. In addition to ceftobiprole and ceftaroline resistance, there was a decrease in cefoxitin resistance, similar to CRB, SRB and SRT (table 3.8).
3.5 Discussion

Banerjee et al. generated [354] ceftobiprole-resistant mutants that were selected in vitro using the COL background. They reported MRSA passaged mutants with high-level resistance to ceftobiprole and other β-lactams tested through mutations in mecA. In Chapter 2, resistance to ceftaroline emerged via mutations in pbp4 and gdpP even in the presence of mecA. Thus, it is important to determine mecA-independent mechanisms of resistance. As previously published, CRB, the mecA-negative variant of COL passaged in ceftobiprole, exhibited resistance similar to mecA-positive COL [152]. The objective of this study was to determine mecA-independent mechanisms of ceftobiprole and ceftaroline resistance.

Whole genome analysis of CRB revealed mutations in only 3 genes: pbp4, gdpP and acrB [153]. PBP4 is a low molecular weight, non-essential PBP. It is responsible for secondary transpeptidase activity, important in the synthesis of long peptidoglycan chains. Deletion of pbp4 leads to an accumulation of short chain muropeptides and a decrease in long chain muropeptides [344, 355]. Overproduction of PBP4 results in increased resistance to β-lactams and decreased resistance to vancomycin [119, 121, 349, 356-358]. PBP4 interacts with PBP2 and PBP2a to function in cell wall synthesis [359]. It is required for resistance in community but not hospital MRSA strains [341, 344].

GdpP is associated with antibiotic tolerance but there is limited knowledge regarding the pathways associated with resistance [346, 348, 360]. The most studied aspect of gdpP is the phosphodiesterase activity. GdpP is a transmembrane protein containing a degenerate GGDEF domain and a DHH/DHHA1 domain with phosphodiesterase activity, similar to B. subtilis [346, 361]. The phosphodiesterase domain regulates the level of cyclic di-AMP (c-di-AMP), a small signaling molecule. Point mutations in the GGDEF domain can decrease phosphodiesterase activity, resulting in increased c-di-AMP [346]. c-di-AMP levels and gdpP mutations have been implicated in resistance and cell wall stress [346, 360, 362-364]. AcrB is a well-known drug efflux pump associated with resistance in Gram-negative bacteria [365, 366]. However, the role of AcrB in Gram-positive bacteria has not been reported.

This study broadens the scope of the previously published study by analyzing whether ceftobiprole and ceftaroline is capable of selecting high-level, broad-spectrum β-lactam resistant mutants in COLnex and SF8300ex. SRB, CRT and SRT mutants were generated by passage in ceftobiprole and ceftaroline. Of note, ceftobiprole passage was conducted in 300 mL cultures, as previously published, while ceftaroline passage was conducted using 10 mL cultures due to limited ceftaroline drug availability. Even though passage volumes differed for the two antibiotics, strains passaged in ceftaroline developed resistance earlier compared to ceftobiprole passage, suggesting the volume used for passage does not affect the generation of resistance.

Whole genome sequencing of CRB revealed mutations in only three genes. To determine whether mutations in these genes were present in other mutants, pbp4, gdpP
and acrB were sequence analyzed. Additionally, since PBPs are the targets of β-lactams, \textit{pbp1-3} were also sequence analyzed. All mutants had mutations in \textit{pbp4}. CRB and SRB had almost identical mutations in \textit{pbp4}, suggesting these amino acids were specific for resistance to ceftobiprole. CRT had a mutation in \textit{pbp4} at T201. The same amino acid was mutated in COL passaged in ceftaroline (from Chapter 2), which suggests this amino acid may interact specifically with ceftaroline and may be important in ceftaroline resistance.

Navratna et al. described functional domains of \textit{S. aureus} PBP4 by crystalizing PBP4 with β-lactams. They reported the existence of a Ω-like loop (Gly181 to Glu199) that is essential for the degradation of the acyl-enzyme complex [367]. Mutations in Glu183, present in CRB and SRB, may change the conformation of the Ω-like loop. They also noted that the hydrolyzed β-lactam is surrounded by a number of residues, including F241. It is possible ceftobiprole interacts with these residues directly. T201, although not specifically discussed in the study by Navratna et al., resides adjacent to the Ω-like loop and may affect the structure of the loop as well. Crystallizing wild-type and mutant PBP4 with ceftobiprole and ceftaroline would reveal more information for PBP4-antibiotic interactions.

SRB has four mutations in \textit{pbp2}. Replacing mutant with wild-type \textit{pbp4} in SRB reduced MICs 2-4 fold for SRB, but remained high. This might be explained by the interaction between PBP4 and the transpeptidase domain of PBP2 for β-lactam resistance. The interaction of PBP4 with PBP2 has been reported, although the precise mechanism of interaction is not known [359]. Further analysis of PBP enzymatic interactions would reveal more information on the role of the specific mutations and the interactions that contribute to resistance.

In addition to \textit{pbp4}, CRB and SRT both have mutations in \textit{gdpP}. Interestingly, the interaction of PBP4 with GdpP, directly or indirectly, has never been described, although GdpP has been implicated in cell wall balance [345, 346, 360, 368, 369]. The \textit{gdp}^{\text{PN182K}} mutation from CRB is located in the degenerate GGDEF domain while the \textit{gdp}^{\text{Y306STOP}} mutation from SRT truncates the protein, eliminating the phosphodiesterase domain. It is possible that the \textit{gdp}^{\text{PN182K}} mutation in the degenerate GGDEF domain negatively affects the phosphodiesterase domain, thereby decreasing enzymatic activity. Truncating GdpP in SRT abolishes phosphodiesterase activity. If the mutations alter phosphodiesterase activity, the resulting change in c-di-AMP levels may signal to other pathways to regulate PBP levels.

In the study by Corrigan et al., knocking out the phosphodiesterase domain of \textit{gdpP} resulted in an accumulation of short chained muropeptides; a phenotype concordant with overexpression of PBP4 [356]. Future studies measuring c-di-AMP levels in \textit{gdpP} mutants and cell wall analysis will give more information on the effects of \textit{gdpP} on cell wall composition.

To determine whether PBP4 was essential for resistance, insertional inactivation of \textit{pbp4} was performed for all mutants. Disrupting \textit{pbp4} in CRB, SRB and SRT decreased MICs to parental COLnex and SF8300ex levels, suggesting \textit{pbp4} is important in resistance. Replacing mutant with wild-type \textit{pbp4} in SRB decreased MICs 2-4 fold, but still retained high levels of resistance, suggesting that mutations in \textit{pbp4} contributes to resistance, but an
intact pbp4 also plays a role. Disrupting pbp4 in CRT did not influence MICs. This suggests the high-level β-lactam resistance observed in CRT is independent of pbp4. Whole genome sequencing of CRT will reveal alternate mechanisms of mecA-independent β-lactam resistance.

PBP levels have been associated with β-lactam resistance in other studies [349, 370]. In addition to sequence analysis of select genes, mRNA levels were analyzed in mutant strains. CRB, CRT and SRT had upregulated levels of pbp4 mRNA. To determine whether pbp4 mRNA levels were important for resistance in these mutants, pbp4 mRNA knock-down was performed using plasmid pASP expressing anti-sense pbp4. Decreasing pbp4 mRNA in CRB and CRT did not decrease MICs, but SRT MICs decreased to parental levels. In concordance with the pbp4 knock-out data, decreasing pbp4 mRNA in CRT did not have an effect on MICs. This supports the hypothesis that β-lactam resistance in CRT is not dependent on pbp4. In CRB, knocking out pbp4 decreased MICs but reducing pbp4 mRNA levels did not reduce MIC levels, suggesting β-lactam resistance in CRB is dependent on an intact, pbp4 but not on elevated pbp4 mRNA. However, since it was difficult to introduce the pbp4 mutations into the COLnex background, it was difficult to determine whether the mutations played a role in CRB resistance. In SRT, knocking out pbp4 and reducing pbp4 mRNA levels both reduced MICs to parental levels. This suggests that β-lactam resistance in SRT is dependent on the upregulated levels of pbp4 mRNA.

Since whole genome sequencing of CRB revealed mutations in only 3 genes, it suggests that these genes, individually or together, are responsible for pbp4 mRNA upregulation in CRB. Allelic replacement of wild-type with mutant genes in parental COLnex proved to be difficult, so these mutations were introduced into SF8300ex. Single, double and triple mutations did not increase MICs. However, the gdpP<sup>DN182K</sup> mutation alone showed a minor increase in resistance via population analysis and growth curve in subinhibitory concentrations of nafcillin.

Passaging the allelic replacement mutants in subinhibitory concentrations of oxacillin resulted in high-level, broad-spectrum β-lactam resistant mutants. The presence of at least one mutation generated resistance to oxacillin faster than SF8300ex wild-type. Additionally, the double and triple mutants developed resistance even faster than the single mutants. When the MICs were analyzed for the passaged mutants, all strains had increased resistance to most β-lactam tested. However, only the double and triple mutants had high MICs towards ceftaroline and ceftobiprole, drugs to which these strains have never been exposed. This suggests that the CRB mutations, alone, are not enough to confer high-level resistance in SF8300ex. Mutations in pbp4 and gdpP together enable resistance to ceftobiprole and ceftaroline. Future experiments will determine the relationship of pbp4 and gdpP with ceftobiprole and ceftaroline.

In conclusion, ceftaroline and ceftobiprole resistance can be generated in vitro using two different S. aureus backgrounds. Resistance in the mecA-independent backgrounds consisted of pbp4-dependent and independent mechanisms. Mutations in pbp4, gdpP and acrB are sufficient for resistance in the COL background but are not sufficient for resistance
in SF8300ex, although they are enabling mutations. *mecA*-independent β-lactam resistance mechanisms are particularly important in this new era of antibiotic development that targets PBP2a with great affinity.
3.6 Figures and Tables

Figure 3.1. BPR and TRL passaged *meca*-negative strains. Resistance to BPR and TRL were generated by passaging strains in subinhibitory concentrations of BPR or TRL in broth. The highest concentration of drug in which strains grew each day is shown on the y axis. The legend indicates the parental strain and the drug used for each passage.
Figure 3.2. mRNA levels of mecA-negative mutant strains from ceftobiprole and ceftarolene passaged mutants. (A) Fold change of CRB mRNA levels compared to parental COLn levels. (B) Fold change of CRT mRNA levels compared to parental COLnex levels. (C) Fold change of SRB mRNA levels compared to parental SF8300 levels. (D) Fold change of SRT mRNA levels compared to parental SF8300 levels. mRNA levels are expressed as arbitrary units (fold change compared to parent).
Figure 3.3. Population analysis of strains with CRB mutations in SF8300ex background. Population analysis was performed via the agar method with increasing concentrations of oxacillin with 2% NaCl TSB. SF8300ex wild-type, single pbp4 or gdpP, double pbp4 and gdpP and triple pbp4, gdpP and acrB mutants were tested for oxacillin population analysis.
Figure 3.4. Growth curve of wild-type and gdpP(N182K) mutants in subinhibitory concentrations of nafcillin. A. *mecA*-negative SF8300ex wild-type and *gdpP*(N182K) mutants grown with and without nafcillin 0.5 µg/mL over 48 hours. B. *mecA*-positive SF8300ES wild-type and *gdpP*(N182K) mutants grown with and without nafcillin 4 µg/mL over 48 hours.

A.

B.
Figure 3.5. Oxacillin passage of strains with CRB mutations in SF8300ex background. Strains from figure 3 were passaged in increasing concentrations of oxacillin until it reached 256 µg/ml. The highest concentration of oxacillin culture with growth was plotted daily.
Table 3.1. Parental and mutant strains used in the *meca*-negative passage study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLn</td>
<td>Homogeneously methicillin-resistant HA-MRSA strain</td>
<td>Mc⁺ Tc³</td>
</tr>
<tr>
<td>COLnex</td>
<td>SCC<em>mec</em> excision strain derived from COL, tetracycline sensitive</td>
<td>Mc⁺ Tc³</td>
</tr>
<tr>
<td>CRB</td>
<td>COLnex resistant to BPR passaged strain</td>
<td>BPR resistant; Mc⁺</td>
</tr>
<tr>
<td>CRT</td>
<td>COLnex resistant to TRL passaged strain</td>
<td>TRL resistant; Mc⁺</td>
</tr>
<tr>
<td>SF8300</td>
<td>Heterogeneously methicillin-resistant CA-MRSA strain (USA300)</td>
<td>Mc⁺ Em⁺</td>
</tr>
<tr>
<td>SF8300ex</td>
<td>SCC<em>mec</em> excision strain derived from Em⁺ derivative of SF8300</td>
<td>Mc⁺ Em⁺</td>
</tr>
<tr>
<td>SRB</td>
<td>SF8300ex resistant to BPR passaged strain</td>
<td>BPR resistant; Mc⁺</td>
</tr>
<tr>
<td>SRT</td>
<td>SF8300ex resistant to TRL passaged strain</td>
<td>TRL resistant; Mc⁺</td>
</tr>
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Table 3.2. Real-time primers for mRNA measurement.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer Sequence</th>
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<td>PBP1-F</td>
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</tr>
<tr>
<td>PBP1-R</td>
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<tr>
<td>PBP2-F</td>
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<td>PBP2-R</td>
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</tr>
<tr>
<td>PBP3-F</td>
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</tr>
<tr>
<td>PBP3-R</td>
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<td>PBP4-F</td>
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</tr>
<tr>
<td>rrsA-R</td>
<td>cattcaccgctacacatgg</td>
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### Table 3.3. MICs (μg/ml) for mecA-negative passaged strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>meca</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
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<td>+</td>
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<td>16</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<td>0.5</td>
</tr>
<tr>
<td>CRB</td>
<td>-</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
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<td>-</td>
<td>&gt;256</td>
<td>256</td>
<td>&gt;256</td>
<td>64</td>
<td>&gt;256</td>
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<td>128</td>
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<td>1</td>
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<tr>
<td>SF8300ex</td>
<td>-</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>SRB</td>
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<td>2</td>
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<tr>
<td>SRT</td>
<td>-</td>
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<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>64</td>
<td>4</td>
</tr>
</tbody>
</table>

Naf = nafcillin, Amp = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BRP = ceftobiprole, TRL = ceftaroline
Table 3.4. Mutations in pbp1, pbp2, pbp3, pbp4, gdpP and acrB in CRB, SRB, CRT and SRT.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pbp1</th>
<th>pbp2</th>
<th>pbp3</th>
<th>pbp4</th>
<th>gdpP</th>
<th>acrB</th>
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<tbody>
<tr>
<td>CRB</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>F241R</td>
<td>N182K</td>
<td>I960V</td>
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<td></td>
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<td></td>
<td></td>
<td>E183A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRB</td>
<td>--</td>
<td>Y437C</td>
<td>D683N</td>
<td>F241R</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V445L</td>
<td></td>
<td>E183V</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q453R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M559I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRT</td>
<td>--</td>
<td>G631S</td>
<td>--</td>
<td>T201S</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N196I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRT</td>
<td>--</td>
<td>--</td>
<td>V45I</td>
<td>N138L</td>
<td>Y306(STOP)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D683N</td>
<td>H270Q</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutations in pbp1-4, gdpP and acrB for each strain was sequence analyzed for mutations.
Table 3.5. MIC (µg/mL) of meca-negative passaged mutant strains containing pASP.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF8300</td>
<td>8</td>
<td>128</td>
<td>16</td>
<td>32</td>
<td>64</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>CRB</td>
<td>64</td>
<td>128</td>
<td>256</td>
<td>8</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>CRB pASP</td>
<td>64</td>
<td>128</td>
<td>128</td>
<td>8</td>
<td>&gt;256</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>CRT</td>
<td>128</td>
<td>64</td>
<td>&gt;256</td>
<td>32</td>
<td>&gt;256</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>CRT pASP</td>
<td>128</td>
<td>128</td>
<td>&gt;256</td>
<td>32</td>
<td>&gt;256</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>SRT</td>
<td>8</td>
<td>64</td>
<td>256</td>
<td>16</td>
<td>256</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>SRT pASP</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

*Anti-sense pbp4 mRNA was expressed from plasmid pASP in mutant strains that overexpressed pbp4 mRNA as indicated in figure 1.2. Naf = nafcillin, Amp = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BRP = ceftobiprole, TRL = ceftaroline
Table 3.6. MIC (µg/mL) with insertional inactivation of \( \textit{pbp4} \) in ceftobiprole and ceftaroline passaged \( \textit{mecA} \)-negative mutants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \textit{mecA} )</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLn</td>
<td>+</td>
<td>256</td>
<td>32</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>COLnex</td>
<td>-</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>CRB</td>
<td>-</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>CRB(Δ( \textit{pbp4} ))</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CRT</td>
<td>-</td>
<td>&gt;256</td>
<td>256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>CRT(Δ( \textit{pbp4} ))</td>
<td>-</td>
<td>128</td>
<td>128</td>
<td>&gt;256</td>
<td>64</td>
<td>&gt;256</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>SF ES</td>
<td>+</td>
<td>64</td>
<td>8</td>
<td>128</td>
<td>64</td>
<td>&gt;256</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300ex</td>
<td>-</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>2</td>
<td>2</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>SRB</td>
<td>-</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>2</td>
<td>32</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>SRB(( \textit{pbp4}^{\text{wt}} ))</td>
<td>-</td>
<td>2</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>16</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>SRB(Δ( \textit{pbp4} ))</td>
<td>-</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>2</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>SRT</td>
<td>-</td>
<td>32</td>
<td>256</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>256</td>
<td>64</td>
</tr>
<tr>
<td>SRT(Δ( \textit{pbp4} ))</td>
<td>-</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>2</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
</tr>
</tbody>
</table>

\*\( \textit{PBP4} \) was inactivated through the insertion of a suicide plasmid, \( \textit{pNL155} \), into genomic \( \textit{pbp4} \) as previously published.\[341\] Naf = nafcillin, Amp = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BRP = ceftobiprole, TRL = ceftaroline.
Table 3.7. MIC (µg/mL) of strains with CRB mutations in the SF8300ex background.

<table>
<thead>
<tr>
<th>Strains</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF8300ex wild-type</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300ex pbp4(E183A, F241R)</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
<tr>
<td>SF8300ex gdpP(N182K)</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300ex pbp4(E183A, F241R), gdpP(N182K)</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>2</td>
</tr>
<tr>
<td>SF8300ex pbp4(E183A, F241R), gdpP(N182K), acrB(I960V)</td>
<td>0.5</td>
<td>&lt;0.25</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>&lt;0.25</td>
<td>1</td>
</tr>
</tbody>
</table>

*Allelic replacement of wild-type pbp4, gdpP and/or acrB was done in SF8300ex. MICs were measured for wild-type SF8300ex, single, double and triple mutants. Naf = nafcillin, Amp = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BPR = ceftobiprole, TRL = ceftaroline*
Table 3.8. MIC (µg/mL) of strains with CRB mutations in the SF8300ex background passaged in oxacillin to 256 µg/mL.

<table>
<thead>
<tr>
<th>Strains</th>
<th>NAF</th>
<th>AMP</th>
<th>CFZ</th>
<th>FOX</th>
<th>CTX</th>
<th>TRL</th>
<th>BPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF8300ex</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>SF8300ex <em>pbp4</em>(E183A, F241R)</td>
<td>256</td>
<td>128</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>SF8300ex <em>gdpP</em>(N182K)</td>
<td>128</td>
<td>64</td>
<td>256</td>
<td>256</td>
<td>&gt;256</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>SF8300ex <em>pbp4</em>(E183A, F241R), <em>gdpP</em>(N182K)</td>
<td>64</td>
<td>64</td>
<td>256</td>
<td>8</td>
<td>&gt;256</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>SF8300ex <em>pbp4</em>(E183A, F241R), <em>gdpP</em>(N182K), <em>acrB</em>(I960V)</td>
<td>64</td>
<td>128</td>
<td>&gt;256</td>
<td>8</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
</tr>
</tbody>
</table>

*Mutants were passaged in increasing concentrations of oxacillin until it reached 256 µg/ml. Colonies were isolated, purified and MICs were measured. Naf = nafcillin, Amp = ampicillin, CFZ = cefazolin, FOX = cefoxitin, CTX = ceftriaxone, BRP = ceftobiprole, TRL = ceftaroline
Chapter 4:

Role of the LexA SOS Response and β-lactam resistance in *Staphylococcus aureus*
4.1 Abstract

**Background:** The SOS response is a global response to DNA damage. The bacterial SOS response regulates the expression of SOS genes, including error-prone polymerases, which increase mutation rates leading to adaptive selection. Inhibiting the SOS response in *E. coli* prevents the emergence of ciprofloxacin resistance. In addition to fluoroquinolones, β-lactams can also induce the SOS response. Since MRSA are resistant to almost all β-lactams, MRSA exposure to β-lactams may induce an SOS response, generating adaptive mutations, which under the appropriate conditions of selection have a role in antibiotic resistance. To test this hypothesis, the SOS response was disrupted in USA300, a prevalent community-associated MRSA strain, via a lexA mutation. **Methods:** A noncleavable lexA mutant was constructed via allelic exchange. MICs, population analyses, killing assays and antibiotic selection assays were performed to assess the role of lexA in antibiotic resistance. **Results:** In USA300, lexA does not contribute to the emergence of antibiotic resistance. **Conclusion:** LexA in USA300 is not a good target to prevent the emergence of antibiotic resistance. There are probably other factors that play a role in antibiotic resistance in addition to LexA.
4.2 Introduction

Global changes in gene expression occur in bacteria upon exposure to stressful environmental conditions. A stressful condition to which MRSA are particularly well adapted is exposure to β-lactam antibiotics. Exposure of bacteria to β-lactam antibiotics activates the SOS stress response genes [371-374]. SOS response is an important mechanism for adaptation to environmental change. β-lactams may paradoxically facilitate adaptive mutations enabling resistant bacteria to survive and adapt under pressure of otherwise lethal drug exposure.

The SOS response regulates expression of SOS genes, including error-prone polymerases that increase mutational frequencies and enhance genetic variability, which may confer a survival advantage under selective pressure, a process that has been termed adaptive mutation [371]. One potential outcome of genetic variation is the evolution of antibiotic resistance. The SOS response is regulated by two proteins: LexA, a transcriptional repressor, and RecA, a regulator of LexA. LexA forms a dimer on the promoter site of SOS genes, repressing transcription. RecA, activated by binding single-stranded DNA, induces LexA autocleavage by promoting serine-130 to cleave the alanine-83 glycine-84 bond. Cleaved LexA is released from the promoter site, allowing transcription of SOS genes, including error-prone polymerases [222, 373]. In E. coli, LexA regulates an error-prone DNA polymerase, PolV (encoded by the imuDC operon). S. aureus carries an error-prone polymerase in the ImpB/MucB/SamB family protein [222, 223, 375].

β-lactam induced SOS response mitigates antimicrobial lethality in E. coli and facilitates phage induction and horizontal transfer of virulence factors in S. aureus [374]. Cirz et al. showed that blocking induction of the SOS response by interfering with the protease activity of LexA in E. coli prevented emergence of fluoroquinolone resistant mutants in vitro and in vivo [222]. LexA mutation in S. aureus 8325, a methicillin sensitive S. aureus (MSSA) strain, prevents the evolution of antibiotic resistance after exposure to UV irradiation [223]. Blocking the SOS response in an atypical strain of S. aureus prevents heterogeneous to homogeneous resistance [311]. Thus, inhibiting the SOS response may serve as an appealing strategy to prevent the emergence of antibiotic resistance. Even though two studies have looked at the role of the SOS response in lab strains or some clinical isolates of S. aureus, the results are not directly applicable to commonly found MRSA, especially CA-MRSA strains. This study will determine if exposure of CA-MRSA, strain SF8300, to β-lactams can help explain the emergence of high-level resistance that occurs clinically.

There are two goals to this chapter: i) to determine whether β-lactams can induce the SOS response in CA-MRSA and ii) to determine if the SOS response regulates mutational frequencies leading to the evolution of antibiotic resistance. β-lactams, although ineffective for MRSA, may trigger the SOS response leading to adaptive mutations and evolution of resistance, including cross-resistance to other antibiotics. Blocking SOS induction may prevent emergence and selection of highly resistant mutants in CA-MRSA.
4.3 Methods and Materials

4.3.1 Allelic exchange.

Plasmid pKOR1 was used to conduct allelic exchanges as previously described [353]. Briefly, constructs for allelic exchange were created via splice overlap extension polymerase chain reaction (SOE-PCR). The PCR fragment was cloned into the pKOR1 plasmid using Gateway BP Clonase II (Invitrogen Cat. #11789-020). Constructs were transformed into E. coli Top10 cells (Invitrogen C404003) and plated onto ampicillin 100 µg/mL TSA plates. Colonies were purified and sequenced for construct confirmation. Plasmids were purified from E. coli using QIAprep Spin Miniprep kits (Qiagen Cat. #27104). Purified plasmids were electroporated into competent RN4220 S. aureus. Plasmids were transduced from RN4220 to host S. aureus strains. Strains were grown in TSB containing chloramphenicol 10 µg/mL at 30°C and 42°C for allelic exchange followed by passage at 30°C without selection. Cultures were plated onto 0.5 and 1.0 µg/mL anhydrotetracycline plates for counter selection. Resultant strains were sequenced for allelic exchange.

A noncleavable lexA mutant was created by allelic exchange as described above. LexA contains a DNA binding domain, an active site and a cleavage site. The active site contains a serine at position 130 that donates a proton for autocleavage targeted at the alanine and glycine bond between amino acid positions 93 and 94. By mutating the glycine at position 94, the protein becomes non-cleavable, causing constitutive repression of SOS genes [376]. Primers used to create the lexA mutant are found in table 4.1.

4.3.2 Mutation rate analysis.

Overnight cultures were diluted 1:100 in TSB or TSB containing ciprofloxacin 0.25 µg/mL and 0.5 µg/mL and grown overnight at 37°C at 120 rpm. Cultures were serially diluted and plated onto TSA blood agar plates and TSA plates containing 0.1 µg/mL rifampicin with overnight growth at 37°C. Mutations rates are displayed as the number of CFU grown on rifampicin plates per 10^8 CFU grown on blood agar.

4.3.3 Antibiotics.

Oxacillin (Sigma Cat. #28221), ceftriaxone (Sigma Cat. #C5793), rifampicin (Sigma Cat. #R3501), ciprofloxacin (Sigma Cat. #17850), vancomycin (Sigma Cat. #75423) and Nafcillin (Sigma Cat. #N3269) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

4.3.4 Antibiotic resistance measurement.

Growth curves were performed by diluting overnight cultures 1:100 in TSB with and without subinhibitory concentrations of nafcillin (4 µg/ml). OD_{600} of the cultures were measured at specified intervals. MICs were determined according to CLSI standards and as
described in Chapter 2. Population analyses were done by the agar method as described in Chapter 3.

4.3.5 Antibiotic induction

Overnight cultures were diluted 1:100 in 10 mL TSB and grown to the beginning of exponential phase ($OD_{600} = 0.1$) at which point sublethal concentrations of antibiotic were added.

4.3.6 Growth curves

Overnight cultures were induced with antibiotic as described above. Cultures were grown at 37°C and 150 RPM. Growth curves were measured at $OD_{600}$ every hour for 5 hours.

4.3.7 mRNA isolation and measurement

Overnight cultures were induced with antibiotic as described above. 1 mL of culture was harvested 2 hours post induction and placed in 4 mL RNAlater. mRNA was harvested and real-time PCR was performed as mentioned in Chapter 3.
4.4 Results

4.4.1 Antibiotic induction of the SOS response

Fluoroquinolones are known inducers of the SOS response for *E. coli* and *S. aureus*, but have not been described for CA-MRSA strains [377]. To determine whether ciprofloxacin can induce the SOS response in USA300, mRNA levels were analyzed after exposure to sublethal concentrations of ciprofloxacin. A previously published microarray study reported genes regulated by the SOS response in *S. aureus* lab strain, RN8325, after induction with sublethal concentrations of ciprofloxacin [223]. Based on their findings, mRNA levels of *impB, lexA* and *recA* were measured after ciprofloxacin induction. *impB* (SACOL1400) is a part of the *umuC* family of error-prone polymerases, which have been shown to be regulated by LexA and facilitate increased mutation rates and antibiotic resistance in *E. coli* [375]. *lexA* and *recA* are regulators of the SOS response and were also shown to be upregulated. When treated with ciprofloxacin 0.5 µg/mL, mRNA levels of SF8300 were increased 57, 3 and 9 fold for *impB, lexA* and *recA*, respectively, over untreated controls. When the *lexA* mutant was treated with ciprofloxacin, mRNA levels were 2, 1 and 2 fold higher for *impB, lexA* and *recA*, respectively, compared to untreated controls (figure 4.1a).

To determine whether β-lactams can induce the SOS response in a CA-MRSA strain, SF8300 and SF8300 *lexA*(G94E) were induced with 1 µg/mL oxacillin. Since *impB* was highly upregulated with ciprofloxacin, a known inducer of the SOS response, it was used as a measure of SOS activation. Additionally, β-lactams are known to inducers of *mecA* expression [378]. When treated with 1 µg/mL oxacillin, there was no difference between SF8300 and SF8300 *lexA*(G94E) in *impB* or *mecA* mRNA levels (figure 4.1b).

4.4.2 Mutation rate analysis

To determine if mutating *lexA* resulted in decreased adaptive mutation, mutations rates were measured for wild-type and *lexA* mutant strains. Briefly, both strains were grown with and without ciprofloxacin and plated on blood agar plates or rifampicin containing TSA plates. Mutation rates were measured as the number of CFU growing on rifampicin plates compared to the number of CFU growing on blood agar plates. For no treatment, ciprofloxacin 0.25 µg/ml and 0.5 µg/ml inductions, mutation rates were 39, 361 and 556 mutations/10^8 CFU for wild-type SF8300 and 24, 57, and 81 for *lexA* mutant, respectively. When induced with vancomycin, there was no difference in mutation rates between mutant and wild-type strains. When induced with 1 µg/mL oxacillin, there was no difference (p > 0.05) (figure 4.2).

To determine whether the heterogeneous phenotype of SF8300 was altered in the mutant, population analysis was performed to detect differences in resistance levels in subpopulations. There was no difference in population analysis profiles for SF8300 *lexA*(G94E) mutant compared to SF8300 (figure 4.3).
4.4.3 Antibiotic resistance phenotype of SF8300 wild-type versus \textit{lexA}(G94E) mutant.

Since the \textit{lexA} mutant has decreased expression of error-prone polymerase \textit{impB} and decreased mutation rates with ciprofloxacin induction, it may also have a decreased capacity to develop antibiotic resistance. The first measure of resistance tested was minimum inhibitory concentration (MIC) assays. MICs of oxacillin (penicillin \(\beta\)-lactam), ceftriaxone (cephalosporin \(\beta\)-lactam), rifampicin (rifamycin), ciprofloxacin (fluoroquinolone), and vancomycin (glycopeptide) were measured for wild-type SF8300 and \textit{lexA} mutant. There was no difference in MIC for any of the drugs tested (table 4.2). To determine whether there was a phenotype that may have been missed with the MIC assay, SF8300 and SF8300 \textit{lexA}(G94E) were grown in broth with various concentrations of oxacillin, ceftriaxone, ciprofloxacin and vancomycin (figure 4.4). There was no difference between SF8300 and SF8300 \textit{lexA}(G94E) when comparing growth in the presence of antibiotics.
4.5 Discussion

The SOS response is important for adaptive selection for antibiotic resistance and is regulated by LexA and RecA. To determine whether the SOS response would be a good therapeutic target to prevent the emergence of antibiotic resistance in CA-MRSA, this chapter answers two questions: 1) can β-lactams induce the SOS response in CA-MRSA and 2) does induction of the SOS response lead to adaptive mutations leading to the evolution of antibiotic resistance?

To determine if β-lactams induce the SOS response in SF8300, a USA300 strain, mRNA of select genes were measured after ciprofloxacin and oxacillin induction. Ciprofloxacin induced the SOS response in wild-type SF8300 but not in SF8300 lexA(G94E), suggesting the mutant has constitutive repression of the SOS response. When induced with oxacillin, there was no difference between wild-type and mutant impB levels, suggesting β-lactams do not activate the SOS response in USA300 strains.

To determine if lower levels of impB mRNA correlate with decreased mutation rates, SF8300 and SF8300 lexA(G94E) were grown with or without antibiotic and plated onto blood agar or TSA plates containing 0.1 µg/mL rifampicin. Rifampicin resistance occurs easily through a single mutation in the rpoB gene. Thus, rifampicin resistance used as an indicator of mutational frequencies. When grown in sublethal concentrations of ciprofloxacin, SF8300 had a higher mutation rate than SF8300 lexA(G94E). Vancomycin, a control antibiotic not known to induce the SOS response, did not have an effect on either wild-type or mutant. Concordant with the mRNA results, there was no difference between SF8300 or SF8300 lexA(G94E) mutation rates.

Since SF8300 lexA(G94E) has impaired expression of SOS genes and a lower mutation rate compared to SF8300, this may translate to decreased levels of antibiotic resistance. To determine whether SF8300 lexA(G94E) has an impaired capability to develop resistance, various assays for antibiotic resistance were measured. SF8300 and SF8300 lexA(G94E) were induced with 1 µg/mL oxacillin and MICs were performed. There was no difference in oxacillin, ceftriaxone, rifampicin, ciprofloxacin and vancomycin MICs. Since MIC levels are the result of overnight growth, differences occurring during exponential growth might have been missed. To determine if there was a growth difference in the presence of sublethal concentrations of antibiotic, SF8300 and SF8300 lexA(G94E) were grown in TSB with and without various concentrations of antibiotic. Similar to the last results, there was no difference. Finally, population analysis was performed to determine whether there were resistant subpopulations not detected with MIC or growth assays. There was no difference between SF8300 and SF8300 lexA(G94E) in population analysis. The results of these experiments suggest that, despite differences in impB mRNA and mutation rates, it did not translate into antibiotic resistance differences between SF8300 and SF8300 lexA(G94E). Thus, inhibiting the SOS response in SF8300 did not prevent the emergence of antibiotic resistance.
The findings in this study were unexpected given the results of previously published studies that showed blocking the SOS response in lab strains and some clinical isolates of *S. aureus* reduced emergence of antibiotic resistance [223, 311]. However, this study shows that the SOS response, although capable of regulating mutation rates, is not directly responsible for the emergence of antibiotic resistance. Taken together, the results suggest that targeting the SOS response to prevent the emergence of antibiotic resistance in USA300 is not a good therapeutic strategy.
4.6 Figures and Tables

**Figure 4.1. mRNA levels of SF8300 wild-type and lexA(G94E) mutant after antibiotic induction.** [A] mRNA levels of *impB, lexA* and *recA* were measured in SF8300 and SF8300 *lexA*(G94E) after 0.5 µg/mL ciprofloxacin induction. Fold change of mRNA for ciprofloxacin 0.5 µg/mL treatment over untreated was calculated. Each column represents an average of 8 replicates. Each column represents an average of 8 replicates. [B] mRNA levels of *impB* and *mecA* were measured for SF8300 and SF8300 *lexA*(G94E) after 1 µg/mL oxacillin induction. Fold change of mRNA of 1 µg/mL treatment over untreated was calculated. Each column represents an average of 8 replicates. (** = p < 0.001; ns = not significant)
Figure 4.2. Mutation rate analysis with or without antibiotic induction in SF8300 and SF8300 lexA(G94E). [A] SF8300 and SF8300 lexA(G94E) were grown in TSB without antibiotic (TSB) or TSB with ciprofloxacin (Cipro). Overnight cultures were plated onto blood again and TSA with 0.1 µg/mL rifampicin, an indicator or mutations. Mutation rate was determined by growth on rifampicin 0.1 µg/mL TSA divided by growth on TSA blood agar plates. Results were expressed as mutations per 10^8 CFU. Each column represents an average of 3 experiments. [B] SF8300 and SF8300 lexA(G94E) were grown in TSB without antibiotic or TSB with vancomycin (Vanco). Each column represents an average of 3 experiments. [C] SF8300 and SF8300 lexA(G94E) were grown in TSB without antibiotic or TSB with oxacillin (Oxa). Each column represents an average of 5 experiments. (* = p < 0.05; ns = not significant)
Figure 4.3. Population analysis for SF8300 wild-type and lexA(G94E) mutant to oxacillin. Overnight cultures were serially diluted and 10 µL was spotted onto 2% NaCl TSA with increasing concentrations of oxacillin. The graph represents an average of 3 experiments.
Figure 4.4. Growth curve of SF8300 and SF8300 lexA(G94E) in various concentrations of antibiotic. Overnight cultures of SF8300 and SF8300 lexA(G94E) were diluted 1:100 in TSB containing increasing concentrations of oxacillin (Oxa), ceftriaxone (Cef), ciprofloxacin (Cip) or vancomycin (Van). OD$_{600}$ was measured every hour for 5 hours.
Table 4.1. Primers Used for Inactivation of *lexA*.*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lexA-X1</td>
<td>GCGAACCAGAAACAGAGATTTG</td>
</tr>
<tr>
<td>lexA-X2</td>
<td>GTAAAGTCACAGCagagTTTCTATTACCGCAGTAGAAAAATTGAAG</td>
</tr>
<tr>
<td>lexA-X3</td>
<td>GAACcTCTGCTGTGACTTTACCAATACTG</td>
</tr>
<tr>
<td>lexA-X4</td>
<td>GATTGTCGATACCTTAGTC</td>
</tr>
<tr>
<td>lexA-X5</td>
<td>GGGGACAAGTTTTGACAAAAAAGCACGCCCCGTAGAGTCGTTCTACAAAC</td>
</tr>
<tr>
<td>lexA-X6</td>
<td>GGGGACCACTTTGTCACAAGAAATGGTGGTCTTTGTTACTCTTCCCTT</td>
</tr>
</tbody>
</table>

*Underlined are 5’ extensions on X2 primers that correspond to reverse complements of X3 primers and used to spliced together the PCR products of X1-X2 and X3-X4. Boxed are attB sites for recombination to plasmid pKOR1. Lower case triplets indicate introduced mutation in *lexA*, resulting in change from Gly (GGT) to Glu (GAG) at amino acid position 94.
Table 4.2. MIC (µg/mL) of SF8300 wild-type and lexA mutant.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Oxacillin</th>
<th>Ceftriaxone</th>
<th>Rifampicin</th>
<th>Ciprofloxacin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF8300</td>
<td>32</td>
<td>64</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>SF8300 lexA(G94E)</td>
<td>32</td>
<td>64</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Chapter 5:

Comparative Efficacy of TR-701 (Prodrug of Tedizolid), Vancomycin and Daptomycin in a Rabbit Model of Methicillin-Resistant \textit{Staphylococcus aureus} Endocarditis
5.1 Abstract

**Background:** Tedizolid (TR-700), the active component of the prodrug tedizolid phosphate, TR-701 (TDZ), is an investigational oxazolidinone that is 8-10x more potent than linezolid against *S. aureus* in vitro. **Method:** The in vivo efficacy of tedizolid phosphate (2, 4, 8, and 15 mg/kg IV twice daily) was compared to that of vancomycin (30 mg/kg IV twice daily) and daptomycin (18 mg/kg IV once daily) in a rabbit model of aortic valve endocarditis caused by the methicillin-resistant *Staphylococcus aureus* strain COL. **Results:** Vegetation titers of daptomycin-treated rabbits were significantly lower than those of rabbits treated with tedizolid phosphate 15 mg/kg bid (P=0.003), whereas titers for vancomycin-treated compared to tedizolid-treated rabbits were not different (P=0.599). The numbers of organisms in spleen and kidney tissues were similar for all treatment groups. To determine the lowest efficacious dose, the experiment was repeated with tedizolid 2 mg/kg, 4 mg/kg and 8 mg/kg compared to vancomycin (30 mg/kg) using a higher inocula (10^8 CFU). **Conclusion:** Tedizolid 2 mg/kg was not efficacious. Tedizolid 4 mg/kg and 8 mg/kg had vegetation titers significantly lower compared to control but only tedizolid 8 mg/kg was comparable to vancomycin.
5.2 Introduction

Tedizolid (TR-700), the active component of prodrug tedizolid phosphate, TR-701 (TZP), is an investigational oxazolidinone in late stage clinical development for treatment of infections caused by Gram-positive bacteria, including methicillin-resistant strains of Staphylococcus aureus. Tedizolid phosphate is a second-generation oxazolidinone that is cleaved in the bloodstream to yield the active component, tedizolid. It is 8-10x more potent than linezolid against S. aureus in vitro, is 16x more potent against linezolid-resistant staphylococci in vitro and has some bactericidal activity [193]. To determine whether this potency translates into a bactericidal effect in vivo, tedizolid was compared to vancomycin and daptomycin in a rabbit model of aortic valve endocarditis caused by the MRSA strain COL.
5.3 Methods

5.3.1 Bacterial strains.

*S. aureus* strain COL is a homogeneous, methicillin-resistant strain. COL inoculum was prepared by diluting a frozen stock in 0.9% injectable sodium chloride. The frozen stock was prepared from an overnight culture grown in tryptic soy broth and washing and resuspending cells in 1xPBS with 10% glycerol which was stored at -80°C.

5.3.2 Susceptibility studies.

Susceptibility studies were done with broth dilutions to determine the minimum inhibitory concentrations (MIC) using standard CLSI methods as described in Chapter 2.

5.3.3 Time-kill studies.

Time-kill studies were conducted at 37°C in 10 ml of CAMHB broth at a starting inoculum of 10^6 CFU/ml containing vancomycin 5 µg/mL, daptomycin 5 µg/mL, or tedizolid 2 µg/mL.

5.3.4 - Rabbit model of endocarditis.

New Zealand White rabbits (2.5 to 3 kg) were used. Endocarditis was established by standard methods [379]. Briefly, a cutdown was made over the right carotid artery. A polyethylene catheter was introduced via carotid arteriotomy, positioned into the left ventricle, and sutured in place. Forty-eight hours later, a 1-ml suspension of 10^7-10^8 CFU of *S. aureus* in 0.9% NaCl was injected intravenously. On postinoculation day 1, approximately 16 to 18 h after infection, untreated control rabbits were sacrificed to determine pretreatment bacterial counts. The hearts, spleens, and kidneys were harvested. Aortic valves and endocardial vegetations and approximately 0.2 g samples of spleen and kidney were placed in 1.0 ml of 0.9% NaCl and homogenized with a tissue grinder. Tenfold serial dilutions of homogenate were prepared, and 0.1-ml volumes were inoculated onto blood agar medium, which was incubated for 24 to 48 h at 37°C. The numbers of CFUs were counted to determine the tissue burdens of organisms. The lower limit of detection is approximately 1 log_{10} CFU/g.

Antibiotic-treated rabbits were administered 15 mg/kg, 8 mg/kg, 4 mg/kg or 2 mg/kg tedizolid phosphate intravenously bid (twice daily) for 4 days; or 30 mg/kg vancomycin intravenously bid (twice daily for 4 days); or 18 mg/kg daptomycin intravenously qd (once daily) for 4 days. The first dose of drug was administered 18 to 20 h after inoculation.

Treated rabbits were sacrificed on postinoculation day 5 approximately 18 to 24 h after the last dose of daptomycin and 12 to 18 h after the last dose of vancomycin and tedizolid phosphate. Bacterial burdens in endocardial vegetations, spleens, and kidneys
were determined as described above for controls. Rabbits that died prior to sacrifice on day 5 or those sacrificed because of moribund condition had quantitative tissue cultures performed and were included in the data analysis only if they survived to day 2 of therapy.

5.3.5 Statistical analysis.

The number of organisms in tissues was expressed as \( \log_{10} \) CFU/gram; tissues with no growth were assigned a value of 1 CFU/tissue sample weight in grams. Statistically significant differences in bacterial burden in tissues among control and antibiotic treatment groups were determined by student's t-test without correction for multiple comparisons. Statistically significant differences were defined as those with \( P < 0.05 \).

5.3.6 Plasma drug concentrations.

Blood was obtained 1h, 9h and 18h (at time of sacrifice) after intravenous injection of tedizolid phosphate. Blood was obtained 1h and 18h after intravenous injection of daptomycin, and vancomycin. Plasma was isolated and stored at -80°C for determination of drug concentrations, which was assayed by HPLC. Pharmacokinetic assays were performed by Trius. Area under the curve (AUC) was calculated using the trapezoidal rule assuming first order kinetics [380].
5.4 Results

5.4.1 MIC and time-kill

The MIC of tedizolid, vancomycin and daptomycin against the COL strain were 0.125 µg/mL, 1 µg/mL, and 1 µg/mL, respectively. Tedizolid achieved a 0.69 log\(_{10}\) cfu reduction of the starting inoculum after 24h in time-kill studies, compared to 2.8 log\(_{10}\) for vancomycin and 3.1 log\(_{10}\) for daptomycin (figure 5.1).

5.4.2 Comparative study of tedizolid 15 mg/kg IV bid, vancomycin 30 mg/kg IV bid, and daptomycin 18 mg/kg IV qd, at an inoculum of 10\(^7\) CFU.

Fifty-five rabbits were included in this study. Two rabbits randomized to the vancomycin group died before 24 hours of treatment and were thus excluded from data analysis. Two additional deaths occurred during treatment, both on day 2, one from the vancomycin group and the other from the daptomycin group. Mortality rates were not significantly different between treatment groups.

The burden of organisms in endocardial vegetations was significantly lower in the tedizolid-treated rabbits compared to the no treatment control with a mean 1.7 log\(_{10}\) CFU/g decrease in titer (p=0.030) (table 5.1). There was no difference between tedizolid phosphate- and vancomycin-treated rabbits in which mean titers were 6.0 and 5.5 log\(_{10}\) CFU/g in vegetations at the end of therapy, respectively. The mean vegetation titer for daptomycin-treated rabbits was 4.2 log\(_{10}\) CFU/g lower than untreated controls, and significantly lower than that achieved with tedizolid phosphate (p=0.003).

The burden of organisms in the spleen was significantly lower in the tedizolid-treated rabbits compared to the no treatment control with a 1.6 log\(_{10}\) CFU/g decrease (p=0.004). Results for tedizolid-treated rabbits were similar to those for vancomycin- and daptomycin-treated rabbits. The burden of organisms in the kidneys was similar in tedizolid-treated rabbits compared to vancomycin- or daptomycin-treated rabbits.

Mean plasma concentrations (± standard deviation) of tedizolid achieved were 9.7±1.8 mg/ml (n=3) 1 h after dosing and 5.8 ± 2.2 mg/ml (n=3) 9h after dosing. Concentrations 1h after dosing were 34 ± 10 mg/ml (n=3) for vancomycin and 93 ± 13 mg/ml (n=3) for daptomycin. The AUC\(_{0-24}\) of tedizolid was 168.3 mg x h/L, approximately 8 times the AUC for the 200 mg qd human dose, 22.5 ± 6.5 mg x h/L, which was the lowest efficacious dose used in clinical trials [381]. Accordingly, a dose-ranging study was conducted to determine in vivo activity of tedizolid at concentrations producing AUC\(_{0-24}\) values approximating those in humans (table 5.2).
5.4.3 Comparative study of tedizolid phosphate 2 mg/kg IV bid versus tedizolid 4 mg/kg IV bid versus tedizolid 8 mg/kg IV bid versus vancomycin 30 mg/kg bid, at an inoculum of 10⁸ CFU.

Forty-four rabbits were included in this study. One rabbit randomized to the tedizolid phosphate 2 mg/kg treatment group died on day 4 of treatment. Three rabbits randomized to the tedizolid phosphate 8 mg/kg treatment group died early, two on day 3 and one on day 4 of treatment.

The burden of organisms in vegetations was significantly lower in tedizolid phosphate-treated rabbits at 4 mg/kg and 8 mg/kg with mean 0.9 and 1.2 log₁₀ CFU/g decreases in titers compared to untreated controls, respectively (table 5.3). However, tedizolid phosphate treatment at 2 mg/kg did not significantly decrease bacterial counts compared to untreated control (p=0.945). Tedizolid phosphate treatment at 8 mg/kg was not different from vancomycin treatment (p=0.078). Despite efficacy compared to untreated control, tedizolid phosphate at 4 mg/kg was not as efficacious as vancomycin (p=0.026). When compared to tedizolid phosphate treatment at 2 mg/kg, treatments at 4 mg/kg and 8 mg/kg significantly decreased mean titers in vegetations (table 5.4).

The burden of organisms in the spleen and kidneys was significantly lower in vancomycin treated rabbits (table 5.2). Tedizolid phosphate treated rabbits at 4 mg/kg and 8 mg/kg did not have statistically significant decreases in titers for spleen or kidneys. When compared to tedizolid phosphate treatment at 2 mg/kg, treatments at 4 mg/kg and 8 mg/kg were not different in the spleens but were statistically lower in kidneys (table 5.4).
5.5 Discussion

Daptomycin was the most effective antibiotic for the treatment of aortic valve endocarditis in rabbits infected with the COL MRSA strain. Results with vancomycin and tedizolid-phosphate (15 mg/kg bid) were comparable in the low inoculum model with a modest reduction in \( \log_{10} \) CFU/g in vegetations compared to untreated control of 2.2 and 1.7 \( \log_{10} \) CFU/g, respectively. All three antibiotics had similar efficacy in eradication of organisms from spleen and kidney. The tedizolid phosphate 15 mg/kg bid dose, however, produced plasma concentrations about 5-fold the level achieved with once daily dosing of 200 mg in humans, which is the dose used in clinical trials. To determine whether tedizolid phosphate at lower doses is comparable to vancomycin, a second experiment with a range of doses of tedizolid phosphate was compared to vancomycin in a higher inoculum model. Tedizolid phosphate treatment at 2 mg/kg and 4 mg/kg bid resulted in serum levels similar to human doses of 200 mg qd and 400 mg qd, respectively. Tedizolid phosphate treatment at 8 mg/kg and 4 mg/kg significantly reduced bacterial loads in vegetations compared to untreated controls. Tedizolid phosphate treatment at 8 mg/kg was similar to vancomycin in reducing bacterial counts in vegetations. Lower tedizolid phosphate doses were less efficacious in reducing bacterial burden in the spleen or kidneys compared to vancomycin.

In conclusion, tedizolid phosphate had modest bactericidal activity in vivo and overall was less active than either vancomycin or daptomycin. Daptomycin was the best antibiotic for endocarditis treatment. At the lower inoculum, the high dose of tedizolid phosphate (15 mg/kg) was as effective as vancomycin. At the higher inoculum, tedizolid phosphate at 8 mg/kg was as effective as vancomycin. However, plasma relevant tedizolid phosphate doses (2 mg/kg and 4 mg/kg) were less efficacious than vancomycin. The 2 mg/kg dose was no different from control while the 4 mg/kg dose was better than control but not as effective as vancomycin. This activity is similar to that for linezolid in experimental endocarditis models [304, 382]. These results suggest that tedizolid phosphate is unlikely to be effective as a single agent or as primary for treatment of endocarditis. Whether there is a role for tedizolid phosphate in treatment of bacteremia from extracardiac sources was not addressed in these experiments. Recent reports that sub-inhibitory concentrations of tedizolid slow emergence of daptomycin non-susceptibility suggest that tedizolid could have a role as a companion agent with daptomycin for treatment of invasive \textit{S. aureus} infections [383].
5.6 Figures and Tables

Figure 5.1. Time-kill studies of COL in vancomycin, daptomycin or tedizolid. $10^6$ CFU/ml COL were grown in TSB alone or with 5 µg/ml vancomycin (Van), 5 µg/ml daptomycin (Dap) or 2 µg/ml tedizolid (TZP). Samples were taken at 0, 4 and 24 hours post treatment and results are expressed as log$_{10}$ CFU/ml.
Table 5.1. Comparative study of tedizolid phosphate (TZP) 15 mg/kg IV bid versus daptomycin 18 mg/kg IV qd versus vancomycin 30 mg/kg IV bid.

<table>
<thead>
<tr>
<th>Treatment (n rabbits)</th>
<th>Mean Organism Titer ± SD (log_{10} CFU/g)</th>
<th>P-values vs. Control</th>
<th>P-values vs. Tedizolid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg.</td>
<td>Spleen</td>
<td>Kidneys</td>
</tr>
<tr>
<td>Control (n=9)</td>
<td>7.7 ± 1.2</td>
<td>4.6 ± 0.7</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>TZP 15 mg/kg bid (n=14)</td>
<td>6.0 ± 2.0</td>
<td>3.0 ± 1.4</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>Daptomycin 18 mg/kg qd (n=16)</td>
<td>3.5 ± 2.0</td>
<td>2.3 ± 1.5</td>
<td>2.2 ± 1.0</td>
</tr>
<tr>
<td>Vancomycin 30 mg/kg bid (n=16)</td>
<td>5.5 ± 2.6</td>
<td>3.2 ± 1.5</td>
<td>2.6 ± 1.2</td>
</tr>
</tbody>
</table>
Table 5.2. Pharmacokinetics of tedizolid phosphate (TZP) administered orally in normal humans and intravenously in infected rabbits.

<table>
<thead>
<tr>
<th>Group, dose</th>
<th>Cmax (n, time)</th>
<th>Cmin (n, time)</th>
<th>T1/2 (h)</th>
<th>AUC0-24h mg x h/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human, 200 mg qd</td>
<td>1.8 ± 0.4 (8, peak)</td>
<td>0.3 ± 0.1 (8, trough)</td>
<td>10 ± 2.0 (8)</td>
<td>22.5 ± 6.5 (8)</td>
</tr>
<tr>
<td>Human, 400 mg qd</td>
<td>4.5 ± 0.9 (8, peak)</td>
<td>0.8 ± 0.2 (8, trough)</td>
<td>8.4 ± 1.0 (8)</td>
<td>53.2 ± 7.9 (8)</td>
</tr>
<tr>
<td>Rabbit, 2 mg/kg bid</td>
<td>1.5 ± 0.7 (6, 1h)</td>
<td>0.1 ± 0.1 (5, 18h)</td>
<td>4.8</td>
<td>19.0</td>
</tr>
<tr>
<td>Rabbit, 4 mg/kg bid</td>
<td>3.3 ± 1.0 (8, 1h)</td>
<td>0.3 ± 0.2 (10, 18h)</td>
<td>5.1</td>
<td>42.8</td>
</tr>
<tr>
<td>Rabbit, 8 mg/kg bid</td>
<td>4.9 ± 1.2 (6, 1h)</td>
<td>1.1 ± 0.5 (6, 18h)</td>
<td>7.8</td>
<td>76.3</td>
</tr>
<tr>
<td>Rabbit, 15 mg/kg bid</td>
<td>9.7 ± 1.8 (3, 1h)</td>
<td>5.8 ± 2.2 (3, 9h)</td>
<td>11.8</td>
<td>168.3</td>
</tr>
</tbody>
</table>

Human serum concentrations were obtained from Prokokimer et al. [196]. Rabbit blood was collected at the indicated peak and trough time points (hours post dosing) and serum was sent to Trius therapeutics to determine Cmax and Cmin.
Table 5.3. Comparative study of tedizolid phosphate (TZP) 2 mg/kg IV bid versus tedizolid 4 mg/kg IV bid versus tedizolid 8 mg/kg IV bid versus vancomycin 30 mg/kg IV bid.

<table>
<thead>
<tr>
<th>Treatment (n rabbits)</th>
<th>Mean Organism Titer ± SD (log₁₀ CFU/g)</th>
<th>P-values vs. Control</th>
<th>P-values vs. Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg.</td>
<td>Spleen</td>
<td>Kidneys</td>
</tr>
<tr>
<td>Control (n=12)</td>
<td>9.2 ± 0.7</td>
<td>5.0 ± 0.7</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>TZP 2 mg/kg (n=7)</td>
<td>9.3 ± 0.2</td>
<td>5.7 ± 0.4</td>
<td>4.9 ± 0.6</td>
</tr>
<tr>
<td>TZP 4 mg/kg (n=9)</td>
<td>8.3 ± 0.5</td>
<td>5.0 ± 0.9</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>TZP 8 mg/kg (n=8)</td>
<td>8.0 ± 0.5</td>
<td>5.3 ± 0.9</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Vancomycin (n=8)</td>
<td>6.5 ± 2.2</td>
<td>2.5 ± 1.1</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>
Table 5.4. Statistical comparison of tedizolid phosphate (TZP) dosed at 2 mg/kg IV bid versus 4 mg/kg IV bid or 8 mg/kg IV bid.

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Mean Organism Titer ± SD (log$_{10}$CFU/g)</th>
<th>P-values for Each Treatment vs. 2 mg/kg dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veg.</td>
<td>Spleen</td>
</tr>
<tr>
<td>TZP 2 mg/kg (n=7)</td>
<td>9.3 ± 0.2</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>TZP 4 mg/kg (n=9)</td>
<td>8.3 ± 0.5</td>
<td>5.0 ± 0.9</td>
</tr>
<tr>
<td>TZP 8 mg/kg (n=8)</td>
<td>8.0 ± 0.5</td>
<td>5.3 ± 0.9</td>
</tr>
</tbody>
</table>
Chapter 6:

Antibiotic Resistance and *Staphylococcus aureus*: Conclusions and Future Directions
Conclusions

Antibiotic resistance in *S. aureus* is a major public health problem. Limited new antibiotics have been developed with anti-MRSA activity. This dissertation explored MRSA antibiotic resistance and its role in antibiotic development. Ceftaroline, a new β-lactam antibiotic with activity against MRSA, has been FDA approved for treating complicated skin and skin structure infections and community-acquired pneumonia since 2010. Ceftobiprole, another new β-lactam with anti-MRSA activity, is in phase 3 clinical trials. Tedizolid phosphate, a second-generation oxazolidinone, is in late stage clinical development.

Chapter 2 focused on the role of *mecA* in mediating resistance to ceftobiprole and ceftaroline. *mecA* is a key target for mutation. One mutation in particular, E447K, was found in both ceftobiprole and ceftaroline passaged mutants. It was sufficient for high-level resistance to ceftobiprole but provided only low-level resistance to ceftaroline. Despite the presence of *mecA*, one mutant harbored mutations in *pbp4* and *gdpP* and not in *mecA*. The results of this study have important implications for the use of ceftobiprole and ceftaroline for MRSA. Future studies analyzing the interaction of mutated PBP2a with ceftobiprole and ceftaroline would provide more information on how the E447K mutation in *mecA* results in resistance.

Chapter 3 focused on ceftaroline and ceftobiprole resistance using *mecA*-negative MRSA backgrounds. Three mutants had *pbp4*-dependent mechanisms of resistance while one mutant had *pbp4*-independent mechanisms of resistance. Mutations in *gdpP* and *pbp4* in SF8300ex appear to be resistance-enabling mutations. Further experiments analyzing mutant PBP4 function via transglycosylation assays would reveal whether the mutations result in a gain-of-function or loss-of-function phenotype. Experiments analyzing cell wall composition, enzymatic activity of mutant PBP4 and crystal structures of PBP4-ceftobiprole/ceftaroline interactions will greatly enhance our understanding of how *pbp4* plays a role in mediating β-lactam resistance in these mutants.

Chapter 4 focused on the role of the SOS response and β-lactams in the emergence of antibiotic resistance in CA-MRSA. The results indicate β-lactams are not able to induce the SOS response in CA-MRSA. Although constitutive repression of the SOS response decreased the mRNA for error-prone polymerases and mutation rates, it had no affect on the emergence of antibiotic resistance. These findings suggest the SOS response would not be a good therapeutic target to prevent the emergence of antibiotic resistance in USA300.

Chapter 5 focused on the efficacy of tedizolid phosphate compared to vancomycin and daptomycin in the treatment of a rabbit model of endocarditis. Daptomycin was the most efficacious antibiotic. Tedizolid phosphate had modest bactericidal activity in vivo and overall was less active than either vancomycin or daptomycin. These results suggest that tedizolid phosphate is unlikely to be effective as a single agent or as primary for treatment of endocarditis.
Future Directions

Overall, this dissertation further confirms the capability of *S. aureus* to develop resistance to new antibiotics and the difficulty in developing new therapies. The development of new anti-MRSA therapeutics is important for treatment when antibiotic resistance is on the rise. The work described in the dissertation provides some information on future directions, including deciphering mechanisms of non-*mecA* β-lactam resistance and determining which *S. aureus* disease models would be suitable for treatment with tedizolid phosphate.

*In vitro* ceftobiprole and ceftaroline resistance is mediated through mutations in *pbp4* and *gdpP*, which have never been associated with high-level β-lactam resistance in *S. aureus*, presenting a novel mechanism of resistance. Given the development and clinical use of antibiotics that target PBP2a, *mecA*-independent mechanisms of resistance are sure to emerge. Studies focusing on the interaction of PBP4 with ceftobiprole/ceftaroline could reveal detailed mechanisms of action and resistance, leading to the development of new drugs. Additionally, *gdpP*, although its role in resistance is unknown, may be a novel mediator for the emergence of resistance. If it plays a role in mediating the emergence of resistance, blocking GdpP may serve as a potential therapeutic target. These studies will provide important information on potential therapeutic targets for the development of new antibiotics.

Tedizolid phosphate was not as efficacious as vancomycin or daptomycin at clinical doses for the treatment of rabbit endocarditis. However, the role of tedizolid phosphate in other invasive infection models was not assessed. Future experiments testing the efficacy of tedizolid phosphate to empiric therapies in other invasive disease models may reveal an important indication for this antibiotic. Additionally, recent reports that sub-inhibitory concentrations of tedizolid slow emergence of daptomycin non-susceptibility suggest that tedizolid could have a role as a companion agent with daptomycin for treatment of invasive *S. aureus* infections [383]. Future experiments analyzing the efficacy of combination therapy may provide a role for tedizolid in the treatment of invasive MRSA infections.

Given the increasing rates of antibiotic resistance in MRSA, new antibiotics are essential in providing effective treatment. The number of new antibiotics for MRSA treatment has been limited. Efforts to determine resistance mechanisms are essential for identifying targets and designing new, efficacious antibiotics.
References

25. TH M: Staphylococccic septicemia—a review of thirty-five cases, with six recoveries, twenty-nine deaths and sixteen autopsies. *Arch Intern Med* 1939, **63**:1068-1083.


78. Musser JM, Kapur V: Clonal analysis of methicillin-resistant Staphylococcus aureus strains from intercontinental sources: association of the mec gene with


164. Dabul AN, Camargo IL: Molecular characterization of methicillin-resistant Staphylococcus aureus resistant to tigecycline and daptomycin isolated in a hospital in Brazil. Epidemiol Infect 2013:1-5.


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280. Ozturan KE, Yucel I, Kocoglu E, Cakici H, Guven M: Efficacy of moxifloxacin compared to teicoplanin in the treatment of implant-related chronic


314. Appelbaum PC: MRSA--the tip of the iceberg. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2006, **12 Suppl** 2:3-10.


325. Sader HS, Fritsche TR, Jones RN: Daptomycin bactericidal activity and correlation between disk and broth microdilution method results in testing of *Staphylococcus aureus* strains with decreased susceptibility to vancomycin. *Antimicrobial Agents and Chemotherapy* 2006, **50**(7):2330-2336.


Emergence of Daptomycin non-resistant Staphylococcus aureus.


Locke JB, Zuill DE, Shaw KJ: Sub-Inhibitory Concentrations of Tedizolid Slow the Emergence of Daptomycin Non-Susceptibility in Methicillin-Resistant
**Staphylococcus aureus.** 53rd Interscience Conference for Antimicrobial Agents and Chemotherapy 2013.