Antibiotic resistance, antibiotic tolerance and the stringent response

by

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B.Sc. (Hons.), University of Victoria, 2021

A Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biochemistry and Microbiology

Supervisory Committee

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Abstract

Bacterial infections are a major global cause of mortality, and antibiotics are critical to their treatment. However, effective antibiotic therapy is threatened by antibiotic resistance and antibiotic tolerance. Tolerance is distinct from resistance as tolerant bacteria are still susceptible to antibiotics, but the rate of killing is significantly reduced. While resistance is a well-understood antibiotic evasion strategy, tolerance is an underappreciated bacterial phenomenon that greatly impacts treatment outcomes. Unlike resistance (which is identified by routine testing in laboratories), tolerance is not tested for in clinical laboratories, in part because there is no simple test available. Consequently, the clinical prevalence and significance of tolerance is unknown. Recently, the stringent response (SR) has emerged as a clinically-relevant mechanism implicated in both resistance and tolerance. The SR is a universal bacterial stress response that acts as a master regulator of bacterial physiology and virulence. In most bacteria, activation of the SR is controlled by the protein Rel. In this study, the SR was used as a model system in *Staphylococcus aureus* to better understand the wider consequences of SR-activating mutations for antibiotic therapy, and the magnitude of the problem of tolerance. Here, I demonstrate that SR activation promotes conjugal transfer of multidrug resistance plasmids between strains of *S. aureus via* elevated plasmid copy number. SR activation increased the transmission of plasmids from diverse families, suggesting that the SR plays a significant role in the dissemination of resistance. SR-activated mutants were also used in the development and validation of a simple screen to detect genotypic tolerance. This screen uses ATP as a proxy for viability and eliminates the need for enumeration of colony-forming units. In a pilot study, I applied the screen to a library of 39 *S. aureus* isolates from cystic fibrosis lung infections and detected tolerance in ~8% of isolates. This study demonstrates that, while Rel mutations may arise during infection and confer tolerance, they have a secondary, coincidental consequence of promoting the dissemination of multidrug resistance plasmids. Furthermore, it demonstrates that the SR is a useful and much-needed model system for studying antibiotic evasion strategies in general. Ultimately, this study highlights the multifaceted implications of clinical Rel mutations and demonstrates that SR activation has significant consequences for antibiotic therapy.

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Abbreviations

ATP	Adenosine triphosphate
CA-MRSA	Community-acquired MRSA
CDA	Cyclic diadenosine monophosphate
CF	Cystic fibrosis
cfu	Colony forming units
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ETC	Electron transport chain
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HGT	Horizontal gene transfer
ICE	Integrative conjugative element
MBC	Minimum bactericidal concentration
MDK	Minimum duration for killing
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
OD_{590}	Optical density at 590 nm
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PBP	Penicillin-binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ppGpp	Guanosine tetraphosphate
pppGpp	Guanosine pentaphosphate
(p)ppGpp	Collective term for ppGpp and pppGpp
qPCR	Quantitative PCR
RJH	Royal Jubilee Hospital

Abbreviations

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAS	Small alarmone synthetases
SCV	Small colony variant
SEM	Standard error of the mean
SR	Stringent response
tRNA	Transfer RNA
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VISA	Vancomycin-intermediate *S. aureus*
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*

Acknowledgements

First and foremost, I would like to thank my co-supervisors Dr. Joanne Hobbs and Dr. Alisdair Boraston for giving me the opportunity to conduct research as a graduate student. Further, I would like to express my deepest gratitude to Dr. Hobbs for being such a supportive and encouraging mentor. I am incredibly thankful for the time and effort that went into this project. I would also like to thank my committee members Dr. Lisa Reynolds and Dr. Jürgen Ehlting for their support throughout my graduate studies. Additionally, I would like to thank Dr. John Galbraith, Dr. Pamela Kibsey and Dr. Victor Yuen for providing clinical isolates that were invaluable to this project. My time in the Boraston lab has been a fantastic experience and I'm grateful to all of the current and former lab members, many of whom have become close friends. I would like to thank Claire Stevens for making me hundreds (probably thousands) of agar plates and for being a great friend outside of work. A special thanks to Brendon Medley for all of the laughs, the daily walks, and for sharing the many highs and lows of grad school with me. Finally, I would like to thank my family for their unwavering love and support in all of my endeavours.

CHAPTER 1
Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a common colonizer of the human population that is both a commensal bacterium and an opportunistic pathogen. Approximately 20-30% of the population hosts *S. aureus* asymptomatically, with the primary site for colonization being the nasopharynx[1,2]. The skin also serves as a major reservoir for the organism[3]. Recent studies suggest that the commensal-to-pathogen transition is highly influenced by the composition of the microbiota[4]. Competition for nutrients, epithelial attachment sites and the production of antimicrobials by other commensal bacteria often impedes *S. aureus* colonization[4]. Although colonization is harmless for *S. aureus* carriers, it is a risk factor for subsequent infections caused by the organism[5]. *S. aureus* does not normally cause infection in healthy individuals; however, if it gains access to underlying internal tissues or the bloodstream, the pathogen can cause a variety of infections[6]. Invasion of sterile tissues can occur as a result of damage to epithelial barriers (*e.g.* wounds and surgery) or a compromised immune system[4]. As an opportunistic pathogen, *S. aureus* can also exploit virus-mediated damage to the respiratory system and cause secondary infections[7]. *S. aureus* infections range in severity from mild skin and soft tissue infections to severe life-threatening conditions, including bacteremia, endocarditis, and osteomyelitis[8]. Many of the systemic infections caused by *S. aureus* arise from complications associated with an indwelling medical device[9]. Globally, *S. aureus* is associated with more deaths than any other bacterial pathogen and is the leading cause of fatal bloodstream infections[10]. In 2019, *S. aureus* was the only bacterium associated with over one million deaths, yet there are few public health initiatives that address the burden of *S. aureus* infections[10]. In the pre-antibiotic era, the case fatality rate for *S. aureus* bacteremia was ~80%; however, it was reduced to 15-50% following the introduction of penicillin[11,12]. This rate has remained steady over the last several decades due to the plateau in antibiotic efficacy and the increasing age of the population.

Despite the achievements of the antibiotic era, *S. aureus* continues to be a leading human pathogen that is renowned for its ability to evade the host immune response. In contrast

to other bacterial pathogens that rely on a few specific factors to promote infection, *S. aureus* produces an arsenal of virulence factors that includes hemolysins, enterotoxins, proteases and immune-modulatory factors[13,14]. The accessory genome encodes many of these virulence determinants and comprises ~25% of the 2.8 Mb genome of *S. aureus*[15]. It consists of mobile genetic elements (MGEs) such as pathogenicity islands, chromosomal cassettes, transposons, and plasmids that are acquired by horizontal gene transfer. These elements often confer adaptive traits that increase bacterial fitness. Most clinical isolates of *S. aureus* harbour one to three plasmids that often carry multiple antibiotic resistance genes[16]. The accessory genome contributes to the genetic diversity of *S. aureus* strains and can act as a reservoir for virulence factors. Many virulence factors inhibit components of the immune system and may contribute to a lack of protective immunity towards the pathogen[17]. Consequently, chronic, and recurrent *S. aureus* infections are common throughout life[18].

Persistent and recurrent *S. aureus* infections are associated with serious challenges. Persistent infections are defined as infections that do not resolve with appropriate antibiotic therapy[19]. Common persistent infections include bacteremia and chronic airway infections[19,20]. Persistent *S. aureus* bacteremia occurs in 6% to 38% of cases and is associated with significantly higher mortality rates compared to nonpersistent bacteremia[21]. Furthermore, the likelihood of metastatic complications such as endocarditis and osteomyelitis increases with the duration of bacteremia, leading to complicated infections with worse outcomes[21,22]. In cases of *S. aureus* bacteremia, the frequency of recurrence has been reported to be as high as 20%[23]. The majority of recurrent infections are caused by strains that are identical or genetically closely related to the initial infection[23,24]. Recurrent infections are also associated with higher mortality rates and longer hospital stays compared to non-recurrent infections[23,25].

Chronic *S. aureus* infections also occur in the airways of cystic fibrosis (CF) patients and are associated with a decline in lung function, and an increased risk of death. Due to defects in the cystic fibrosis transmembrane conductance regulator, the airways of CF patients are lined with viscous mucus. This reduces mucociliary clearance and makes the airways particularly susceptible to bacterial colonization. The thick mucus layer creates hostile environmental conditions that are unfavourable for many bacterial pathogens; however, *S. aureus* has adapted

to colonize different microenvironments in the CF lung and displays remarkable genetic diversity. Genome alterations have been shown to occur at significantly higher frequencies in *S. aureus* isolates derived from CF patients as opposed to healthy individuals[26]. Consistent with higher mutation rates, *S. aureus* tends to form phenotypically heterogeneous populations in the CF lung to fit into different niches[27]. Interestingly, phenotypic diversity appears to promote persistent colonization rather than infection. Colonization does not cause immediate clinical consequences, whereas infection can cause pulmonary exacerbations requiring therapeutic intervention[28]. One phenotype associated with persistence that is frequently observed in airways of CF patients is the small colony variant (SCV). SCVs are marked by slow growth and reduced virulence[29]. It has been estimated that the growth rate of SCVs is approximately nine times slower than that of the parental strain[30]. The basis of this slow-growing phenotype appears to be auxotrophy and defects in the electron transport chain (ETC)[31]. Prevalent auxotrophies include thymidine, hemin, thiamine and menadione[29]. Hemin, menadione and thiamine are all required for the ETC; therefore, impaired biosynthesis of these molecules contributes to reduced electron transport. In turn, this results in a decreased electrochemical gradient and reduced adenosine triphosphate (ATP) synthesis[32]. Due to their reduced metabolism and slow replication, SCVs are intrinsically "tolerant" (see section 1.3 for definition) to a range of antibiotics. Furthermore, SCVs have been found to exhibit antibiotic resistance as decreased membrane potential leads to ineffective transport of aminoglycosides and antifolate antibiotics into the bacterium[33]. Evidence suggests that treatment with certain antibiotics, including gentamycin and trimethoprim/sulfamethoxazole, can select for SCVs[33]. This is of particular concern for CF patients receiving long-term suppressive antibiotic therapy. Given their severe growth defects, SCVs are often overlooked by conventional diagnostic methods, and consequently, antibiotic therapy ceases before SCVs are cleared from an infection[33]. Persistence of SCVs despite antibiotic therapy presents a significant threat because these variants can revert to their wildtype phenotype and regain virulence once selective pressure is removed. Successful treatment of life-threatening *S. aureus* infections, including CF lung infections, is dependent on the effective use of antibiotics. Despite considerable effort to develop a preventative *S. aureus* vaccine, no vaccine candidates have proven successful[17]. The lack of a vaccine against *S. aureus* infections further emphasizes the importance of antibiotic treatment and the crucial need to preserve antibiotic efficacy.

1.2 Antibiotic resistance in *S. aureus*

1.2.1 Burden of resistance

Pathogenic bacteria such as *S. aureus* use a number of strategies to evade the activity of antibiotics. The most recognized and well-studied evasion strategy is antibiotic resistance. Although the mechanisms behind resistance are largely well-understood, it continues to pose a growing threat to antibiotic efficacy. As such, resistance has been deemed one of today's greatest global public health challenges[34]. Resistance is the inheritable ability of bacteria to grow in the presence of antibiotic for an indefinite period of time[35]. It is readily detected in clinical laboratories as a minimum inhibitory concentration (MIC) above the clinically determined breakpoint[35]. In 2019, there were an estimated ~5 million deaths associated with resistance globally[36]. Current estimates suggest in the United States alone there are over 2.8 million antibiotic resistant infections each year and over 35,000 deaths as a result[37]. Resistant infections are associated with higher healthcare costs and higher mortality rates compared with non-resistant infections[38]. In Canada, it is estimated that resistant infections will kill 400,000 individuals by 2050 and cost the economy $388 billion[39]. Following *Escherichia coli*, *S. aureus* is the leading bacterium for deaths associated with resistance worldwide[36]. In particular, methicillin-resistant *S. aureus* (MRSA) poses a tremendous challenge as the leading pathogen-drug combination for deaths attributed to resistance[36]. Recently, the rate of MRSA in Canada has steadily increased with nearly one in six cases of MRSA bloodstream infection resulting in death within 30 days of diagnosis[40] The propensity of *S. aureus* to develop resistance further complicates effective antibiotic therapy and contributes to prolonged infections that are a tremendous burden on the healthcare system[41]. As such, *S. aureus* has been identified as a priority organism for resistance surveillance[42].

1.2.2 Endogenous resistance

Selective pressure is an important factor that promotes the development of resistance. Endogenous resistance is the result of chromosomal mutations that arise with selective pressure such as prolonged antibiotic exposure[43]. Resistance can occur due to a single mutation or develop gradually through the accumulation of multiple mutations[43]. Common mechanisms of resistance include alteration of the antibiotic target, increased expression of efflux pumps, and inactivation of the antibiotic[44]. One well-characterized example of endogenous resistance is

the development of rifampin resistance. Rifampin exerts bactericidal activity by inhibiting DNA-dependent RNA polymerase through binding to the beta subunit (encoded by *rpoB*) within the DNA/RNA channel[45]. Rifampin binding is thought to interrupt transcription by blocking the path of nascent RNA[45]. Single point mutations that result in amino acid substitutions in *rpoB* have been shown to confer high-level rifampin resistance by reducing the affinity of the antibiotic for its target[46]. Rifampin is no longer used as a monotherapy for *S. aureus* infections because resistance emerges rapidly at rates of 10^{-7}; however, rifampin can still be effective when used in combination with β-lactams or glycopeptides[47,48]. In contrast to rifampin resistance, high-level resistance to fluoroquinolones requires the stepwise emergence of multiple mutations[49]. Fluroquinolones interact with DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*), resulting in the formation of lethal double-stranded DNA breaks[50]. Mutations that reduce antibiotic binding affinity can occur in *parC*, conferring low-level resistance, followed by mutations in *gyrA* that confer high-level resistance[49]. Clinical isolates that exhibit high levels of fluoroquinolone resistance have also been found to overexpress chromosomally-encoded efflux pumps[51]. Efflux systems drive the translocation of compounds across the membrane and are one of the primary mechanisms used to withstand antibiotic exposure. The chromosomally encoded multidrug efflux pump NorA is the most well-studied pump in *S. aureus*. Several studies have shown that NorA can extrude a broad range of substrates including fluoroquinolones such as ciprofloxacin and norfloxacin, biocides, and antiseptics[52]. Overexpression of the NorA efflux pump can be constitutive *via* mutations in the *norA* promoter or inducible through the action of regulatory proteins[53]. The *norA* gene has been found to be overexpressed in 43% of *S. aureus* strains, particularly in MRSA strains[54]. In 2020, the Canadian Nosocomial Infection Surveillance Program found that 65.3% of healthcare-associated MRSA isolates from bloodstream infections were resistant to ciprofloxacin[40]. Of these isolates, 34.2% were resistant to clindamycin and 71.2% were resistant to erythromycin[40]. Multi-drug resistance limits treatment options and often requires the use of last resort antibiotics such as vancomycin.

The emergence of vancomycin resistance is the most concerning *S. aureus* adaptation to date. Vancomycin disrupts cell wall synthesis by binding to the terminal D-Ala-D-Ala residue on peptide chains, preventing further elongation and cross-linking of the peptidoglycan

matrix[55]. The surge in MRSA led to widespread reliance on vancomycin treatment and created intensive selective pressure that promoted two types of resistance: vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). The MIC susceptibility breakpoint for vancomycin is defined as 2 μg/mL for *S. aureus*[56]. VISA strains exhibit moderate resistance with an MIC of 4-8 μg/mL and have been associated with poor clinical outcomes[57]. These variants tend to emerge from a heterogeneous population following sustained selective pressure such as prolonged or repetitive courses of vancomycin treatment[15]. Multiple point mutations can accumulate over time and lead to successively higher vancomycin MICs[58]. The majority of these mutations affect peptidoglycan synthesis and result in a thickened cell wall with irregular architecture[57]. VISA strains exhibit reduced cross-linking of peptidoglycan and increased exposure of free D-Ala-D-Ala residues that act as false targets to sequester vancomycin[44]. This prevents diffusion of vancomycin to the cytoplasmic membrane where the antibiotic is active. Recent estimates suggest that the global prevalence of VISA is 4.3%[59]. Compared to 2010, this represents a 3.6-fold increase in prevalence[59]. One of the main risk factors appears to be prior vancomycin exposure, suggesting that unnecessary vancomycin use should be limited in order to minimize selective pressure[60].

1.2.3 Exogenous resistance

In addition to the development of endogenous resistance, *S. aureus* can acquire exogenous resistance determinants encoded on MGEs such as transposons, chromosomal cassettes, and plasmids through horizontal gene transfer (HGT). The importance of MGEs to the dissemination of resistance was first evidenced with the introduction of penicillin. Although the discovery of penicillin was a tremendous breakthrough in therapeutic medicine, resistance emerged rapidly following widespread use in the 1950s. By the late 1960s, more than 80% of staphylococcal isolates from both healthcare-acquired and community-acquired infections were resistant to penicillin[61]. Penicillin functions by inhibiting the transpeptidase activity of penicillin-binding proteins (PBPs) that is essential for cell wall synthesis[62]. Penicillin resistance is mediated by the *blaZ* gene that encodes for a β-lactamase. The *blaZ* gene is carried by a transposon that can be located on large plasmids or integrated into the chromosome[44]. β-lactamase inactivates penicillin through hydrolysis of the β-lactam ring. Following the rise of penicillin resistance, semisynthetic derivatives such as methicillin were developed to

circumvent β-lactamase activity. Unfortunately, clinical isolates of methicillin-resistant *S. aureus* were identified less than one year after the first clinical use of methicillin[15]. The genetic basis of methicillin resistance in *S. aureus* is associated with the staphylococcal cassette chromosome *mec* (SCC*mec*)[63]. This cassette integrates into the chromosome and carries the *mecA* gene that confers methicillin resistance. The product of *mecA* is the transpeptidase penicillin-binding protein 2a (PBP2a) that is involved in peptidoglycan cross-linking[61]. Compared to native PBPs, the active site of PBP2a is less accessible to β-lactams. As a result, PBP2a has a lower affinity for β-lactams and remains active in the presence of methicillin while native PBPs are inhibited[64]. Different β-lactam subclasses all have the β-lactam ring as the functional core; therefore, PBP2a confers resistance to almost all β-lactams, with the exception of fifth generation cephalosporins[65]. The expression of β-lactam resistance is typically heterogenous with the majority of the population exhibiting low-level resistance and small subpopulations demonstrating high-level resistance[66]. The conversion from heterogenous to homogenous expression of high-level resistance occurs as a result of chromosomal mutations. A large number of functionally diverse genes appear to be involved in this transition, suggesting that expression of *mecA* is quite complex[67].

The most alarming instance of exogenous resistance is plasmid-borne vancomycin resistance. In contrast to chromosomally-mediated resistance in VISA, high-level vancomycin resistance (MIC ≥ 16 μg/mL) is conferred by the *vanA* gene cluster encoded on transposon Tn*1546*[68]. Tn*1546* is found on plasmids carried by vancomycin-resistant enterococci (VRE) and can be acquired by *S. aureus* through conjugation[57]. The *vanA* gene cluster encodes seven proteins that are responsible for replacing native D-Ala-D-Ala peptides with D-Ala-D-Lac[68]. The modified D-Ala-D-Lac moieties reduce the binding affinity of vancomycin by ~1000-fold[69]. The prevalence of VRSA remains low at 2.4%[59], likely due to the infrequency of horizontal gene transfer from enterococci. Even when conjugation does occur, replication of enterococcal plasmids in *S. aureus* is often suboptimal[70]. However, one case study found the *vanA* gene cluster on a highly transferable staphylococcal plasmid carried by a clinical isolate of MRSA[71]. This plasmid was readily transferred to other strains of *S. aureus* through conjugation, suggesting that dissemination of MRSA containing *vanA* is a worrisome possibility.

1.2.4 Conjugation

The three main mechanisms of HGT in *S. aureus* are conjugation, transduction, and natural transformation[72]. Although phage transduction had been considered to be the primary mechanism of HGT, recent findings suggest that conjugation plays a greater role in the dissemination of resistance genes than previously recognized[73]. Most clinical isolates of *S. aureus* carry at least one plasmid ranging in size from 1 to 60 kb[74]. Small plasmids often confer resistance to erythromycin, chloramphenicol, or tetracycline, whereas larger plasmids can carry multiple resistance genes[75]. In large conjugative plasmids, resistance genes are often encoded by transposons and integrated into the plasmid. Conjugative plasmids are considered self-transmissible as they carry all of the genes required for mating-pore formation, DNA processing, single-stranded DNA replication and recruitment[76]. An endonuclease referred to as the relaxase recognizes, cleaves, and covalently attaches to the origin of transfer (*oriT*). This forms a nucleoprotein complex that is referred to as the relaxasome. The relaxasome is recruited to the mating-pore by accessory proteins and is then transferred to the recipient cell through a type-IV secretion system (Figure 1A). Relaxases can also be involved in replication of plasmid DNA in the recipient cell[76]. This is also the mechanism of transfer used by integrative conjugative elements (ICEs) that can integrate into the chromosome[73]. In contrast to conjugative plasmids, mobilizable plasmids contain limited conjugation genes and lack genes required for mating pore formation[76]. As a result, mobilizable plasmids are not self-transmissible. However, mobilizable plasmids can exploit the mating pores of conjugative plasmids resident in the same cell for horizontal transfer. The majority of mobilizable plasmids encode their own relaxase that is compatible with the coupling protein of the conjugative plasmid (Figure 1B)[77]. Mobilizable plasmids can also encode a mimic sequence of the *oriT* that is recognized by the relaxase of the co-resident conjugative plasmid (Figure 1C) or encode a replicative relaxase that is compatible with the coupling protein used by the conjugative plasmid (Figure 1D)[78]. While only 5-6% of *S. aureus* plasmids are self-transmissible, recent work suggests that most non-conjugative plasmids are potentially mobilizable if co-resident with a conjugative plasmid[76].

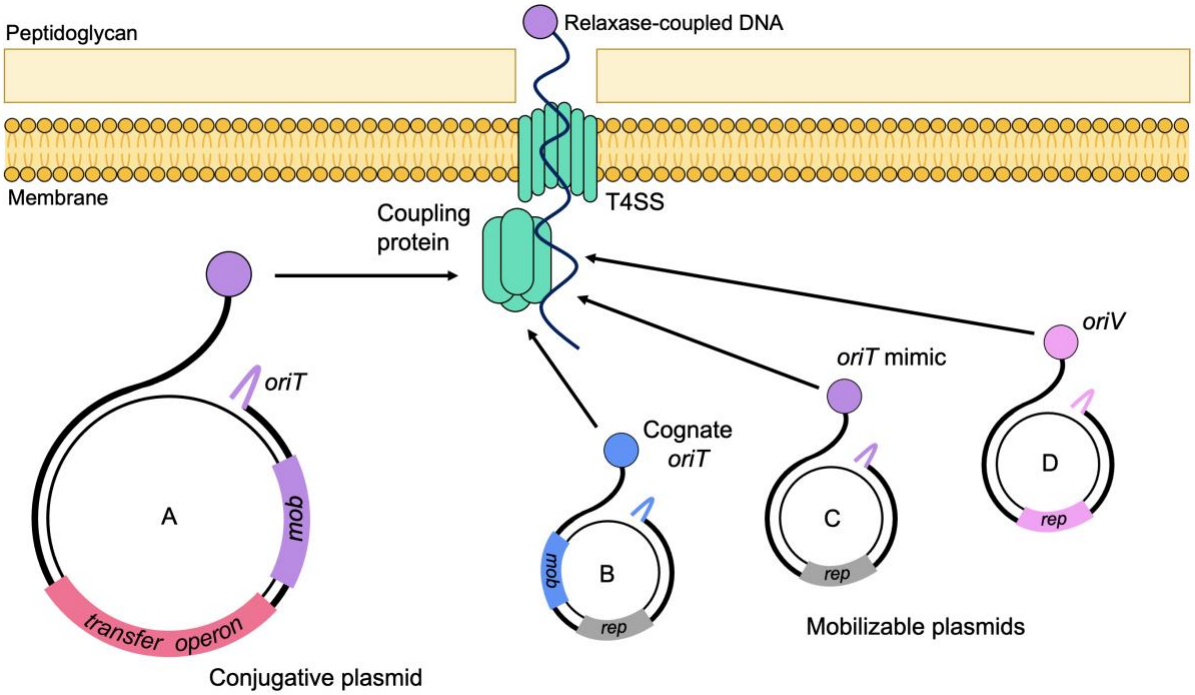

Figure 1. Mechanisms of conjugation and mobilization in *S. aureus*. (A) Conjugative plasmids encode an origin of transfer (*oriT*) that is nicked by a compatible relaxase (*mob*) and recruited to the mating-pore through interactions with a coupling protein. Mobilizable plasmids can exploit the conjugative-plasmid mating pore by: (B) encoding their own relaxase/*oriT* that is compatible with the coupling protein of the conjugative plasmid; (C) encoding a mimic *oriT* sequence that is recognized by the relaxase of the conjugative plasmid; or (D) encoding a replicative relaxase (*rep*) that recognizes the origin of replication (*oriV*) and it compatible with the coupling protein of the conjugative plasmid. (Figure based on Ramsay *et al.*, 2016)[76].

Staphylococcal conjugative plasmids are categorized into three distinct families based on their replication initiation genes[76]. The pSK41/pGO1 family was first identified in the early 1980s following a drastic increase in the frequency of aminoglycoside resistance among clinical isolates of *S. aureus*[79]. The pGO1 plasmid has since been deemed to be a prototype for staphylococcal conjugative plasmids and is used as a model for studying plasmid transfer. pGO1 carries a cluster of genes required for conjugation referred to as the conjugative transfer region (*trs*)[80]. The *trs* region contains 14 open reading frames designated *trsA*-*trsN*. *trsA* to *trsM* are transcribed from one DNA strand, whereas *trsN* is transcribed from the opposite strand and regulates the other *trs* genes[81]. In addition to aminoglycoside resistance, pGO1 derivatives have been found to confer resistance to trimethoprim, penicillins, tetracycline, and macrolides[76]. Notably, the pGO1 backbone has been associated with high-level vancomycin resistance in a clinical isolate of VRSA[82]. Furthermore, pGO1 can mobilize some smaller

plasmids such as pC221 when co-resident in the same cell[83]. While pGO1 is the prototypical conjugative plasmid in *S. aureus*, the pWBG4 and pWBG749 families are also important for the dissemination of resistance. The pWBG4 family is widely distributed and has been associated with resistance to aminoglycosides, macrolides, lincosamides, and linezolid[76]. pWBG707 is an important member of the pWBG4 family that confers trimethoprim resistance[84]. Unlike the pGO1 and pWBG4 families, the majority of plasmids in the pWBG749 family do not carry resistance genes; however, pWBG749-like plasmids are capable of transferring co-resident mobilizable plasmids that encode resistance genes[78]. Sequences that closely resemble the *oriT* of pWBG749 have been identified on over 50% of all sequenced *S. aureus* plasmids, further suggesting that most plasmids can be mobilized[78].

Although conjugation appears to play a major role in the dissemination of resistance genes, we know very little about the factors affecting the frequency of conjugal transfer in *S. aureus*. Studies with *E. coli* have shown that environmental conditions such as temperature and nutrient levels affect the rate of plasmid transfer; however, this has not been examined with *S. aureus*[85]. In *S. aureus*, biofilm formation has been shown to significantly increase the frequency of plasmid transfer by both conjugation and mobilization[86]. Compared to conjugation frequencies in planktonic cultures, conjugal transfer of pGO1 increased by ~7000-fold in biofilms[86]. While biofilm formation is a significant factor contributing to the spread of resistance genes *via* conjugation, but it is important to investigate other factors that may increase the rate of conjugal transfer in *S. aureus*.

1.3 Antibiotic tolerance

In addition to antibiotic resistance, the emerging phenomenon of antibiotic tolerance threatens effective antibiotic therapy. Tolerance describes the ability of a bacterial population to withstand transient exposure to an otherwise lethal concentration of antibiotic, without exhibiting an elevated MIC[87] (Figure 2). Tolerant bacteria are killed at a slower rate compared to their susceptible counterparts. As such, the "minimum duration for killing" (MDK) has been proposed as a metric for quantifying tolerance[87]. In the context of an infection, a slower rate of killing means tolerant bacteria will survive the typical course of antibiotic treatment, leading to persistent and recurrent infections with higher mortality rates[19,20]. Further complicating

treatment, tolerance has been shown to provide protection against multiple classes of antibiotic[88]. Alarmingly, there is also evidence that suggests tolerance promotes the development of endogenous resistance *in vitro*[89]. Tolerance should not be confused with the phenomenon of bacterial persistence that is observed when the majority of the population dies at the same rate as a susceptible strain, but a small subpopulation (typically less than 1%) is killed at a slower rate[87]. Unlike resistance, tolerance is not detected by routine susceptibility tests in clinical laboratories; therefore, its current clinical prevalence is largely unknown. However, a few small historic studies identified tolerance in 40-60% of *S. aureus* clinical isolates[90–93]. More recent small-scale studies have focused specifically on vancomycin tolerance. Among clinical isolates of *S. aureus*, the rate of vancomycin tolerance has been reported to range from 6% to 43%[94,95]. One retrospective study of *S. aureus* bacteremia found that 26.7% of patients were infected with a vancomycin-tolerant isolate[96]. Clinical failure occurred in 68.3% of cases involving tolerance[96]. Interestingly, there was still an association between vancomycin tolerance and clinical failure among methicillin-susceptible *S. aureus* patients treated with a β-lactam[96]. This suggests that vancomycin tolerance lowers the efficacy of other antibiotics that are active against the cell wall. Furthermore, reduced bactericidal activity of vancomycin due to tolerance has been associated with an increased risk of mortality[97]. Tolerance has also been observed in other pathogenic bacteria, including *E. coli*, enterococci, and streptococci. Among a library of clinical isolates that caused endocarditis, vancomycin tolerance was identified in 57% of streptococci and 100% of enterococci isolates[98]. Recently, tolerance was detected in 28.6% of *E. coli* bacteremia isolates and was associated with an increased risk of recurrence[99]. Although these studies demonstrated that tolerance is a widespread bacterial phenomenon associated with poor treatment outcomes, only a limited number of isolates were tested, and tolerance detection methods varied between studies. Without routine tolerance detection in clinical laboratories, our understanding of the magnitude and clinical significance of tolerance remains limited to data provided by small-scale studies.

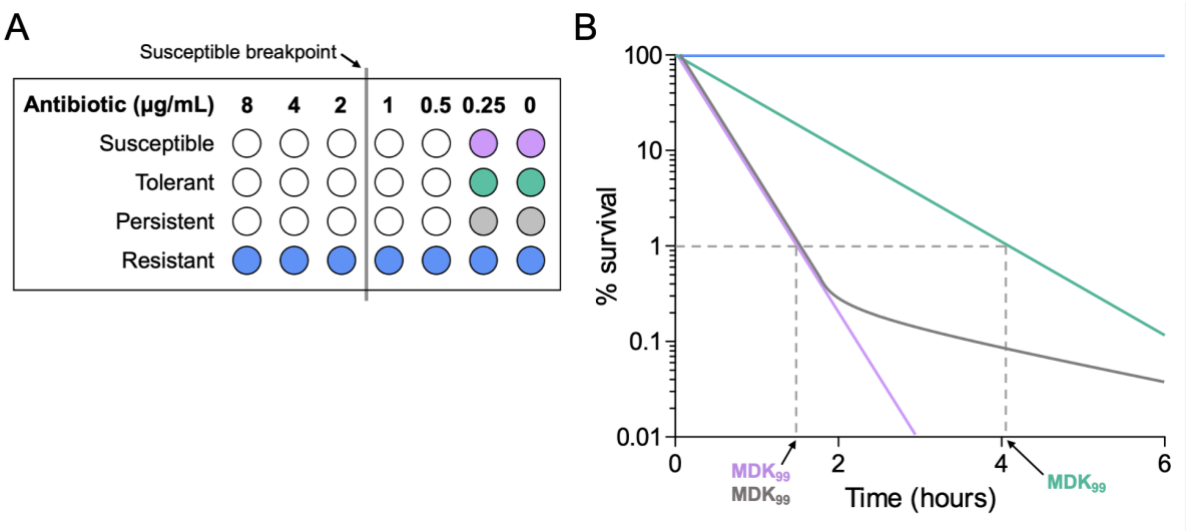

Figure 2. Schematic depiction of resistance, tolerance, and persistence. (A) A resistant strain exhibits growth at antibiotic concentrations above the breakpoint, whereas a tolerant and persistent strain exhibit a susceptible MIC. (B) A tolerant strain exhibits a slower rate of killing in a time-kill assay compared to a susceptible strain. For a tolerant strain, the duration of antibiotic exposure must be longer to achieve the same level of killing (*e.g.* MDK_{99}, the time required to kill 99% of the population). A persistent strain exhibits a similar initial rate of killing to that of a susceptible strain, but a small subpopulation dies at a slower rate. A resistant strain is resistant to killing in a time-kill assay. (Figure based on Hobbs and Boraston, 2019)[100].

Tolerance is currently overlooked by routine susceptibility testing because the MIC is unchanged compared to that of a susceptible isolate. Time-kill assays are the current gold standard for tolerance detection[87]. In this method, bacteria are exposed to a lethal concentration of a bactericidal antibiotic and viable count determinations are performed over time. The MDK has been proposed as a quantitative metric used to measure tolerance in time-kill assays[87]. The underlying assumption for the MDK is that killing activity reaches saturation with the concentration of antibiotic used in the assay; therefore, the rate of cell death is only dependent on time. Although the MDK can be a useful metric to compare levels of tolerance between strains, there are no standards to define what MDK constitutes tolerance. Due to the absence of criteria to define tolerance, time-kill assays always require side-by-side comparison with a known non-tolerant strain. Given the lack of standards and the labor-intensive protocol, time-kill assays are not used in clinical laboratories. Another method that has been proposed to identify tolerance is the MBC/MIC ratio. The minimum bactericidal concentration (MBC) is the lowest antibiotic concentration required to kill ≥99.9% of cells after 24 hours. In cases of tolerance, there is a marked discrepancy between the MBC and the MIC. The elevated MBC

produces a large MBC/MIC ratio compared to that of a susceptible strain. Isolates are defined as tolerant if they exhibit an MBC/MIC ratio ≥ 32[101]. In early tolerance studies, MBC testing was frequently used to screen libraries of clinical isolates; however, it has since been shown that MBC results are inconsistent and difficult to reproduce[102]. Recently, a modified disk-diffusion assay was developed to detect tolerance. In step one, the tolerance disk test (TDtest) follows the protocol for the standard Kirby-Bauer disk diffusion assay with an antibiotic disk forming a zone of inhibition. In step two, the antibiotic disk is removed, and a nutrient disk is added for overnight incubation. The addition of nutrients promotes the growth of tolerant bacteria that survived antibiotic exposure in the zone of inhibition[103]. Tolerant bacteria form detectable colonies while susceptible bacteria are killed. The TDtest can provide a qualitative evaluation of tolerance as low, medium, or high-level based on the number of colonies inside the inhibition zone. Although the TDtest is a simple, low-cost tolerance detection method, the multi-day protocol is not suited for clinical microbiology labs. A rapid tolerance detection method is needed in order to improve the diagnosis of persistent infections and identify isolates for further examination in research laboratories.

Since tolerance is not readily identified in clinical laboratories, investigation of the underlying molecular mechanisms has been relatively limited; however, the central themes appear to be slow growth and reduced metabolism[104,105]. As most antibiotics target active metabolic processes, slowing down these processes can provide protection from antibiotic activity. The two main forms of tolerance that have been identified are tolerance by lag and tolerance by slow growth[87]. The lag phase is the period of non-replication prior to exponential growth that allows bacteria to adapt to their environment. A lag phase can happen after stationary phase or following transitions between growth conditions. Growth-arrest during the lag phase provides protection against the action of antibiotics[106]. In contrast to the specificity of resistance, tolerance by lag confers protection against a broad spectrum of antibiotics[106,107]. Bacteria that are tolerant by lag adapt to the duration of antibiotic treatment rather than the chemical composition of the antibiotic. From a clinical perspective, this presents a major challenge for antibiotic treatment. Notably, tolerance by lag is a transient phenotype induced by stress or starvation that is not sustained when bacteria resume growth. Despite this, tolerance by lag is an effective strategy used to evade antibiotic killing, with some bacteria demonstrating

an MDK of many hours or even days[87]. One study found that cyclic exposure of *E. coli* to a β-lactam can select for a lag phase 10 times longer than that of the ancestral strain[106]. Furthermore, the duration of the lag phase evolved to match the duration of the antibiotic-exposure interval. Several mutations were selected for in the evolved population, giving rise to inherited tolerance by lag. Interestingly, the MIC remained unchanged, suggesting that tolerance mutations may emerge faster than resistance mutations. Mutations associated with tolerance by lag have been identified in genes encoding toxin-antitoxin systems, amino-acyl-transfer RNA synthetases, and phosphoribosylpyrophosphate synthetase[106] (Table 1).

A slow growth rate can also confer multidrug tolerance. Unlike tolerance by lag, which is transient, tolerance by slow growth occurs at a steady state. Growth rate has long been known to influence antibiotic efficacy. For example, it has been shown that the rate of bacterial killing by β-lactams is proportional to growth rate[105]. β-lactams target nascent peptidoglycan in the dividing cell; therefore, the number of antibiotic targets increases with growth rate. Similarly, exposure to fluoroquinolones, which target DNA replication, results in killing at a rate that is proportional to growth rate[108]. These studies demonstrate a strong correlation between slow growth and tolerance to several different classes of antibiotic. Tolerance can be genotypic or phenotypic. The classic example of phenotypic tolerance occurs when cells are protected within a biofilm, whereas known mechanisms of genotypic tolerance include defects in electron transport and thymidine metabolism as observed in SCVs[33,109]. Activation of bacterial stress responses has also been implicated in tolerance *in vitro*[104]. Interestingly, many of the genes associated with tolerance are involved in processes such as translation and ribosome biogenesis that have been shown to feed into stress response pathways (Table 1). In particular, induction of the stringent response (SR) appears to be frequently linked to tolerance[100,110–112].

Table 1. Examples of genes associated with tolerance-conferring mutations

Cellular pathway/process	Gene	Protein function
Electron transport chain	*hemB*[113]	Hemin synthesis
Electron transport chain	*menD*[113]	Menadione synthesis
Protein quality control	*clpX*[114]	ClpP protease ATP-binding subunit
Purine synthesis	*prs*[89]	Phosphoribosylpyrophosphate synthetase
Purine synthesis	*purR*[114]	Purine repressor
Ribosome biogenesis	*rsgA*[115]	Small ribosomal subunit biogenesis GTPase
Stringent response	*rel*[e.g. 88,116,117]	Guanosine tetra- and pentaphosphate synthesis/hydrolysis
Toxin-antitoxin system	*vapB*[106]	Antitoxin
Transcription	*rpoC*[114]	RNA polymerase β' subunit
Translation	*alaS*[118]	Alanyl-tRNA synthetase
Translation	*argS*[118]	Arginyl-tRNA synthetase
Translation	*aspS*[119]	Aspartyl-tRNA synthetase
Translation	*ileS*[120]	Isoleucyl-tRNA synthetase
Translation	*leuS*[117]	Leucyl-tRNA synthetase
Translation	*metG*[89]	Methionyl-tRNA synthetase
Translation	*thrS*[119]	Threonyl-tRNA synthetase
Tricarboxylic acid cycle	*citZ*[111]	Citrate synthase

1.4 The stringent response

The SR is a universal bacterial stress response that was originally observed in response to amino acid starvation in *E. coli*[121]. In the classical description of SR activation, the presence of uncharged tRNAs causes ribosome stalling, which is sensed by the SR controller RelA (Figure 3). RelA responds by synthesizing guanosine tetra- and pentaphosphate (known as (p)ppGpp) from GTP/GDP and ATP. Aided by the transcription factor DksA, (p)ppGpp binds to RNA polymerase, resulting in the downregulation of most metabolic genes and cessation of growth[122]. The accompanying hydrolase protein and RelA paralogue, SpoT, degrades (p)ppGpp once the stress has been alleviated. While this is the classical description of SR

induction, we now know that (p)ppGpp synthesis can be induced by a number of different stimuli and basal levels are required for viability[123]. Furthermore, (p)ppGpp has emerged as a master regulator of nearly all aspects of bacterial physiology, including biofilm formation and virulence[123–125].

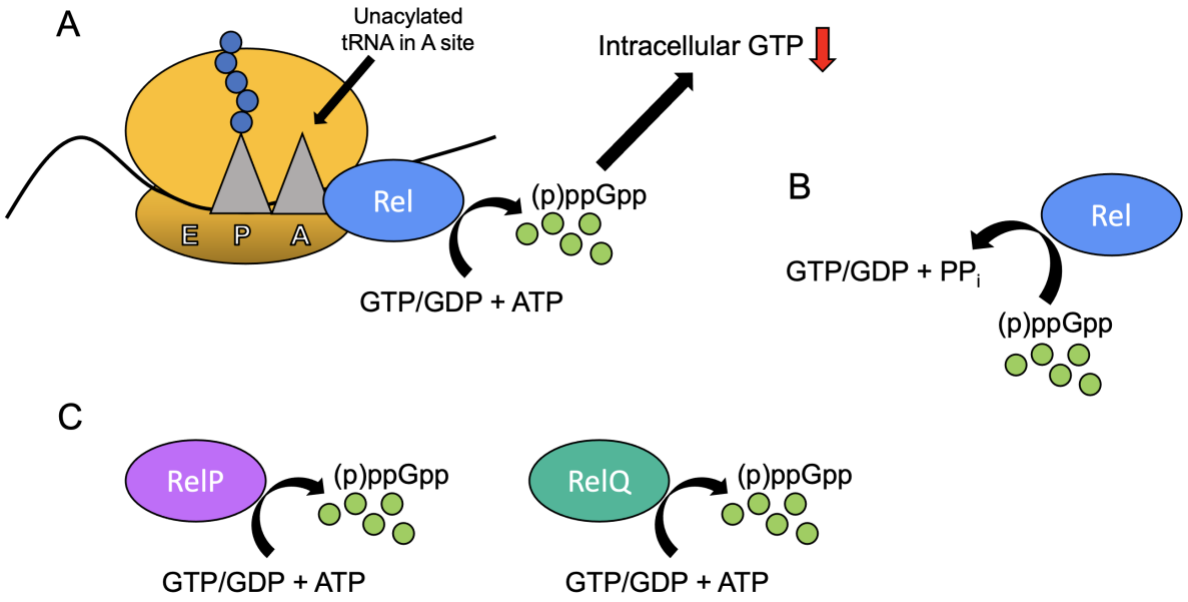

Figure 3. Stringent response in *S. aureus*. (A) Deprivation of an amino acid leads to the presence of an uncharged tRNA in the A site of the ribosome, causing the ribosome to stall. Ribosome stalling is sensed by Rel, which responds by synthesizing (p)ppGpp from GTP/GDP and ATP. Synthesis of (p)ppGpp depletes intracellular GTP pools, resulting in downregulated transcription of most metabolic genes and cessation of growth. (B) When amino acid starvation has been alleviated, Rel hydrolyses (p)ppGpp to GTP/GDP and pyrophosphate. (C) The small alarmone synthetases RelP and RelQ can synthesize (p)ppGpp in response to cell wall stress. (Figure adapted from Hobbs and Boraston, 2019).

While the canonical arrangement of RelA and SpoT applies to *E. coli* and other β- and γ-proteobacteria, in most bacteria, including *S. aureus*, the cellular level of (p)ppGpp is controlled by a single bifunctional enzyme known as Rel. The enzymatic N-terminal region of Rel consists of a hydrolase domain, which degrades (p)ppGpp, and a synthetase domain, which synthesizes (p)ppGpp[126] (Figure 4). A central three-helix bundle linker region joins the two enzymatic domains and is thought to be important for the regulation of the opposing catalytic activities[127]. The two antagonistic activities of Rel are controlled through conformational changes with synthetase-off/hydrolase-on being the resting activity state of the protein[127]. In the absence of stalled ribosomes, the regulatory C-terminal region interacts with the synthetase

domain to limit synthetase activity and prevent the accumulation of high levels of (p)ppGpp that are toxic to the cell[128]. *S. aureus* also possesses two small alarmone synthetases (SAS), RelP and RelQ, that consist solely of an isolated synthetase domain[126]. While Rel is the predominant contributor to (p)ppGpp production, the SASs are thought to synthesize (p)ppGpp in response to cell wall stress and play an important role in maintaining homeostasis[123,129].

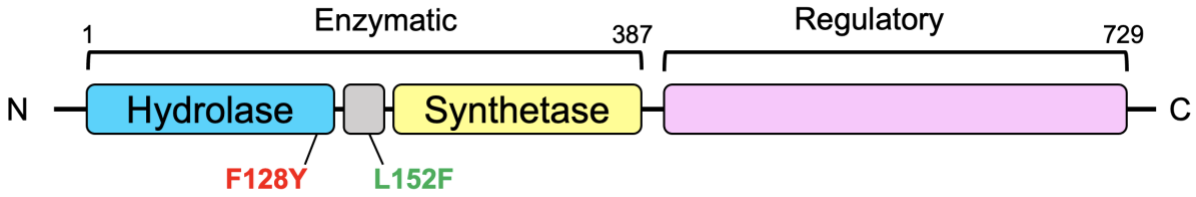

Figure 4. Domain architecture of Rel. The enzymatic N-terminal region of Rel consists of a hydrolase and synthetase domain joined by a short linker region (shown in grey). Two clinical Rel mutations (shown in red and green) occur within this enzymatic half of the protein. The regulatory C-terminal region of Rel is involved in ribosome binding and regulation of the two opposing catalytic activities. Amino acid number is shown on the top.

In contrast to Proteobacteria, (p)ppGpp does not bind to RNA polymerase in *S. aureus*. Instead, the effects of SR activation occur indirectly through modulation of intracellular GTP levels[123]. (p)ppGpp has been shown to affect GTP synthesis through a variety of mechanisms, including inhibition of GTPases such as RsgA[115]. RsgA binds to the 30S ribosomal subunit and acts as a chaperone for ribosome assembly[130]. Binding of (p)ppGpp inhibits the GTPase activity of RsgA, leading to decreased ribosome assembly and reduced growth[115]. Depletion of GTP pools leads to reduced transcription of mRNAs with GTP as the initiating nucleotide[131]. In Gram-positive bacteria, this includes most rRNA promoters[131]. GTP levels are also important for the function of CodY, a global transcriptional regulator[132]. In a GTP-bound state, CodY binds to DNA and represses the transcription of genes involved in adaptation to nutrient limitation[133]. When (p)ppGpp is synthesized, GTP levels decrease and CodY is released from the DNA, thereby allowing transcription to proceed. Although (p)ppGpp does not bind to RNA polymerase in *S. aureus*, a number of diverse proteins have been identified as binding targets[134]. Interestingly, a common (p)ppGpp binding site has not been identified among these proteins.

Activation of the SR has long been associated with tolerance *in vitro*. The relationship between the SR and tolerance has been investigated using a variety of methods such as tRNA synthetase inhibitors, amino acid-deficient media and gene knockouts[100]. Although classical induction of the SR leads to a state of quiescence, it has recently been demonstrated that activation of the SR is not binary, and the cellular levels of (p)ppGpp can be finely tuned to the needs of the bacterium[135,136]. Partial SR activation results in reduced, rather than arrested growth, that confers antibiotic tolerance[88]. Mutations that partially activate the SR can be found in Rel, and other proteins involved in the wider SR network. For example, nonsynonymous mutations in the *leuS* and *ileS* genes that encode for tRNA synthetases have been found in slow-growing isolates of *S. aureus* that exhibit multidrug tolerance[117,120]. In 2010, the first Rel mutation identified in a clinical isolate was reported[136]. This *S. aureus* isolate was identified in a case of persistent and recurrent bacteremia that required over 100 days of antibiotic treatment. Whole genome sequencing revealed a F128Y mutation in Rel that caused elevated (p)ppGpp levels, a SCV phenotype and a reduced growth rate. In another recent case of persistent bacteremia, an isolate of *Enterococcus faecium* bearing a L152F Rel mutation was identified[135]. This isolate also exhibited elevated (p)ppGpp levels consistent with partial SR activation. Bryson *et al.* recently introduced these two mutations into a model strain of *S. aureus*, Newman, and demonstrated that they confer multidrug tolerance as a result of (p)ppGpp-mediated growth defects[88]. A structural model of Rel predicted by AlphaFold2 indicated that F128Y is located in the hydrolase domain and L152F is in the linker region. Deventer *et al.* showed that the F128Y mutation caused a dramatic decrease in hydrolase activity, leading to an overall shift in favour of (p)ppGpp synthesis[137]. The L152F mutation favours (p)ppGpp synthesis by reducing hydrolase activity and slightly increasing synthetase activity[137]. In recent years, additional Rel mutations have been identified in *S. aureus* isolates associated with persistent infections[110,111]. It is now clear that the SR is a clinically-relevant mechanism of tolerance, and that the F128Y and L152F mutant strains generated by Bryson *et al.* represent a useful and much-needed model system of tolerance and SR activation in *S. aureus.*

As well as its role in tolerance, the SR has been linked to the expression, development, and acquisition of antibiotic resistance genes[100]. In most clinical isolates of MRSA, the expression of β-lactam resistance is heterogeneous; however, activation of the SR has been

implicated in the transition from hetero- to homogenous resistance[138]. Whole genome sequencing of the two populations (low- and high-level resistance) within an MRSA strain identified two *rel* mutations in the highly resistant cells[139]. One of the mutations introduced a premature stop codon that resulted in a truncated Rel lacking the regulatory region. The truncated Rel mutant strain exhibited high-level β-lactam resistance, slow growth, and elevated levels of (p)ppGpp. Other truncating mutations have since been identified in highly resistant subpopulations of clinical MRSA isolates[140,141]. In addition to truncating mutations, Deventer *et al.* have recently demonstrated that the clinically-relevant F128Y and L152F Rel mutations also induce high-level, homogeneous β-lactam resistance[137]. Furthermore, chemical induction of the SR with mupirocin (an iseoleucyl tRNA synthetase inhibitor) or the serine analog serine hydroxamate has been shown to convert heterogenous MRSA isolates to homogenous, highly resistant forms[142]. Interestingly, the SR-activated strains express higher levels of PBP2a compared to their counterparts with low-level resistance. Other mutations associated with homogenous β-lactam resistance have been identified in genes involved in indirect routes of SR activation. This includes genes involved in guanine metabolism, transcription, and translation.

The SR has also been linked to the dissemination of resistance genes within *E. coli* biofilms[143]. The SR was shown to induce the SOS response and increase the expression of SOS-regulated genes. Induction of the SOS response resulted in increased expression of a class 1 integron integrase[143]. Integrases catalyze the insertion of gene cassettes, such as resistance cassettes, into integrons that express them. Induction of the SOS response has also been shown to promote the spread of resistance genes through conjugative transfer of ICEs and prophage induction[144,145]. Conjugative plasmids and ICEs use the same mechanism of transfer, thus raising the question of whether the SR promotes conjugal transfer of staphylococcal resistance plasmids.

1.5 Research objectives

Antibiotic tolerance represents a currently undiagnosed and underappreciated threat to effective antibiotic therapy, and the SR constitutes a clinically-relevant molecular mechanism underlying this phenomenon. Given the global burden of persistent *S. aureus* infections, and

the lack of new antibiotics coming into use, it is critical to better understand: a) the potential wider consequences of SR-activating mutations for antibiotic therapy; and b) the magnitude of the problem of tolerance in general. I hypothesize that clinical SR-activating mutations promote the dissemination of resistance by increasing the transmission of conjugative multidrug resistance plasmids (Hypothesis 1). I also hypothesize that tolerance is prevalent among clinical isolates of *S. aureus* associated with persistent infections (Hypothesis 2). As such, I have four main aims (two related to Hypothesis 1 and two related to Hypothesis 2): 1.1) investigate the effects of SR activation and cellular (p)ppGpp levels on conjugal transfer of staphylococcal plasmids from diverse families and; 1.2) explore the potential mechanism(s) behind the relationship between the SR and conjugation; 2.1) establish and validate a simple screen to detect genotypic tolerance in *S. aureus* using the known tolerant Rel mutant strains; 2.2) use the screen to conduct a pilot study into the prevalence of tolerance among clinical isolates derived from CF lung infections.

CHAPTER 2

Materials and methods

2.1 Bacterial strains and vectors

Table 2 lists all the standard bacterial strains and engineered mutants used throughout this study. The library of 39 clinical isolates derived from CF lung infections was provided by Dr. John Galbraith at the Royal Jubilee Hospital (RJH), Victoria, BC.

Table 2. Bacterial strains used in this study

Strain	Description	Source/Reference
Newman	MSSA clinical isolate	Dr. Michael Murphy, University of British Columbia[146]
MW2	CA-MRSA isolate	Dr. Paul Kubes, University of Calgary[147]
N315	CA-MRSA isolate	Dr. Paul Kubes, University of Calgary[148]
COL	MRSA	Dr. Paul Kubes, University of Calgary[140]
LAC*	CA-MRSA LAC isolate that has been cured of its resistance plasmid	Dr. Rebecca Corrigan, University of Sheffield[149]
USA300	CA-MRSA isolate	Dr. Paul Kubes, University of Calgary[150]
SH1000	Laboratory strain	Dr. Paul Kubes, University of Calgary[151]
RN4220	Laboratory strain	Dr. Michael Murphy, University of British Columbia[152]
WBG4515	RN450 with chromosomal resistance to streptomycin and novobiocin	Dr. Josh Ramsay, Curtin University[153]

Table 2. continued

Strain	Description	Source/Reference
WBG541	RN450 with chromosomal resistance to fusidic acid and rifampicin	Dr. Josh Ramsay, Curtin University[153]
Newman *rel* F128Y	Tolerant *rel* mutant	Dr. Joanne Hobbs, University of Victoria[88]
Newman *rel* F128Y comp.	Wildtype *rel* reintroduced; silent mutation for identification	Dr. Joanne Hobbs, University of Victoria[88]
Newman *rel* L152F	Tolerant *rel* mutant	Dr. Joanne Hobbs, University of Victoria[88]
Newman *rel* L152F comp.	Wildtype *rel* reintroduced; silent mutation for identification	Dr. Joanne Hobbs, University of Victoria[88]
Newman *rel* E169G	Non-tolerant *rel* mutant	Matthew Shortill, University of Victoria
Newman Δrel_{syn}	*rel* 308-310 (YQS) deletion	Dr. Joanne Hobbs, University of St Andrews
Newman Δrel_{syn}Δ*relP*Δ*relQ*	*rel* 308-310 (YQS) deletion *relP* deletion *relQ* deletion	Dr. Joanne Hobbs, University of Victoria
Newman Δ*codY*	*codY* deletion	Dr. Joanne Hobbs, University of Victoria
Newman *ileS* Y723H	Non-tolerant mutant	Ashley Deventer, University of Victoria
DAR6247 ICE*6013*	Mock wildtype strain; resistant to erythromycin	Dr. Ashley Robinson, University of Mississippi[154]

Abbreviations: Methicillin-sensitive *S. aureus* (MSSA); Community-acquired methicillin-resistant *S. aureus* (CA-MRSA)

Table 3. Bacterial plasmids used in this study

Plasmid	Description	Resistance marker	Source/Reference
pGO1	Natural plasmid	Gentamicin, trimethoprim	Dr. Alex O'Neil, University of Leeds[155]
pWBG707	Natural plasmid	Trimethoprim	Dr. Josh Ramsay, Curtin University[84]
pWBG749e	pWBG749 with Tn551 inserted	Erythromycin	Dr. Josh Ramsay, Curtin University[156]
pC221	Natural plasmid	Chloramphenicol	Dr. Josh Ramsay, Curtin University[157]
pJB185:*nes*	*nes* promoter-*lacZ* fusion construct	Chloramphenicol	Dr. Joanne Hobbs, University of Victoria
pJB185:*trsA*	*trsA* promoter-*lacZ* fusion construct	Chloramphenicol	Dr. Joanne Hobbs, University of Victoria
pJB185:*trsL*	*trsL* promoter-*lacZ* fusion construct	Chloramphenicol	Dr. Joanne Hobbs, University of Victoria

2.2 Antibiotics and reagents

Daptomycin was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) and vancomycin was purchased from BioBasic (Markham, ON, Canada). All other antibiotics and reagents were purchased from MilliporeSigma (Burlington, MA) unless otherwise stated. Table 4 lists all antimicrobial agents used in this study and their solvents.

Table 4. Antibiotics used in this study

Antibiotic	Abbreviation	Concentration* (μg/mL)	Solvent
Ciprofloxacin	CIP	n/a	H_2O + NaOH
Chloramphenicol	CHL	10.0	50% EtOH
Daptomycin	DAP	n/a	H_2O
Erythromycin	ERY	5.0	50% EtOH
Flucloxacillin	FLC	n/a	H_2O
Fusidic acid	FUS	1.0	50% EtOH
Gentamicin	GEN	5.0	H_2O
Mupirocin	MUP	n/a	H_2O
Novobiocin	NOV	1.0	H_2O
Rifampicin	RIF	5.0	50% DMSO
Streptomycin	STR	50.0	H_2O
Trimethoprim	TMP	10.0	DMSO
Vancomycin	VAN	n/a	H_2O

*Concentrations used to select for resistance

2.3 General microbiological techniques

2.3.1 Routine culture and storage of *S. aureus*

Broth cultures were grown aerobically in tryptic soy broth (TSB) with shaking (180 rpm) at 37°C for 12-18 hours and plate cultures were growth aerobically on tryptic soy agar (TSA) at 37°C for 12-24 hours. All bacterial strains were stored long-term as broth cultures containing a final concentration of 8% (v/v) glycerol at -80°C.

2.3.2 Growth curves

Growth curves were determined in 5 mL of TSB in 16-mm test tubes incubated at 37°C in a shaking water bath. Overnight cultures (10 μL) derived from three different colonies were used to inoculate triplicate test tubes. Absorbances at 600 nm were read periodically in a test tube spectrophotometer.

2.3.3 Minimum inhibitory concentration determination

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method of the Clinical Laboratory Standards Institute (CLSI) in Mueller-Hinton broth (MHB) or MHB supplemented with 50 μg/mL Ca^{2+} (MHB-Ca^{2+}; for daptomycin)[56]. Two-fold serial dilutions of antibiotic were added to MHB with an inoculum of ~10^4 CFU/mL in a round-bottom 96-well plate. Plates were sealed with Breathe-Easy® sealing membranes and incubated at 37°C overnight with shaking at 200 rpm. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth after overnight incubation.

2.3.4 Time-kill assays

Time-kill assays were performed in triplicate at 37°C in MHB or MHB-Ca^{2+}, as appropriate, according to CLSI guidelines[101]. Overnight cultures were diluted 1/500 in 5 mL fresh MHB and incubated at 37°C for 80 minutes with shaking at 200 rpm. Cell density at the point of antibiotic addition were in the range of 10^5 to 10^7 CFU/mL. All antibiotics were tested at 4 × MIC. Samples were taken from each culture immediately following the addition of antibiotic, and at time intervals thereafter, and viable counts were performed. Samples were diluted in phosphate-buffered saline (PBS) and plated on TSA in duplicate. TSA plates were incubated at 37°C overnight and colonies enumerated either manually or using an aCOLyte 3 automated colony counter (Synbiosis Ltd., Cambridge, UK). Minimum duration for killing (MDK) values were calculated in triplicate using the linear regression and interpolation functions in GraphPad Prism 6.07. A minimum of four data points in the linear range of the MDK were used in the calculation and all R^2 values were >0.9.

2.3.5 Induction of the stringent response

The stringent response was induced in *S. aureus* using mupirocin, an isoleucyl tRNA synthetase inhibitor[158]. Different concentrations of mupirocin were tested in order to determine the highest subinhibitory concentration that allowed growth. Bacteria were grown at 37°C overnight on TSA containing 0.04 μg/mL mupirocin.

2.3.6 Introducing plasmids into resistant strains of *S. aureus* Newman

The protocol for filter matings was adapted from a method previously described by Savage *et al.* (2013). Donor and recipient strains bearing chromosomal resistance markers were generated as described in section 2.4.2. These strains were grown on TSA (containing antibiotic, as appropriate) at 37°C overnight and resuspended in TSB to an OD_{600} of 1.0. Strains were combined at a 1:1 ratio (500 μL each) and pushed through a syringe onto a 0.2 μm pore-size nylon filter using 13 mm Swinnex filter holders. Filters were placed bacteria side down on TSA and incubated at 37°C for 24 hours. Following incubation, cells were resuspended in 1 mL TSB and serially diluted in PBS. Serial dilutions were plated on TSA containing the appropriate antibiotics for selection of transconjugants and incubated at 37°C for 16-24 hours. Donor and recipient strains were mated as shown in Table 5.

Table 5. Filter matings to introduce plasmids into resistant strains of *S. aureus* Newman

Donor strain	Recipient strain	Transconjugant strain
SH1000 (pGO1)	Newman WT NOV^R	Newman WT NOV^R (pGO1)
SH1000 (pGO1)	Newman F128Y NOV^R	Newman F128Y NOV^R (pGO1)
SH1000 (pGO1)	Newman F128Y comp. NOV^R	Newman F128Y comp. NOV^R (pGO1)
SH1000 (pGO1)	Newman L152F NOV^R	Newman L152F NOV^R (pGO1)
SH1000 (pGO1)	Newman L152F comp. NOV^R	Newman L152F comp. NOV^R (pGO1)
SH1000 (pGO1)	Newman Δ*codY* NOV^R	Newman Δ*codY* NOV^R (pGO1)
SH1000 (pGO1)	Newman Δrel_{syn} NOV^R	Newman Δrel_{syn} NOV^R (pGO1)
SH1000 (pGO1)	Newman Δrel_{syn}Δ*relP*Δ*relQ* NOV^R	Newman Δrel_{syn}Δ*relP*Δ*relQ* NOV^R (pGO1)
pWBG4515 (pWBG707)	Newman F128Y comp. FUS^R	Newman F128Y comp. FUS^R (pWBG707)
Newman F128Y comp. FUS^R (pWBG707)	Newman WT NOV^R	Newman WT NOV^R (pWBG707)
Newman F128Y comp. FUS^R (pWBG707)	Newman F128Y NOV^R	Newman F128Y (pWBG707) NOV^R
Newman F128Y comp. FUS^R (pWBG707)	Newman F128Y comp. NOV^R	Newman F128Y comp. NOV^R (pWBG707)
Newman F128Y comp. FUS^R (pWBG707)	Newman L152F NOV^R	Newman L152F NOV^R (pWBG707)

Table 5. continued

Donor strain	Recipient strain	Transconjugant strain
Newman F128Y comp. FUS^R (pWBG707)	Newman L152F comp. NOV^R	Newman L152F comp. NOV^R (pWBG707)
WBG541 (pWBG749e) FUS^RFIF^R	Newman WT NOV^R	Newman WT NOV^R (pWBG749e)
WBG541 (pWBG749e)	Newman F128Y NOV^R	Newman F128Y NOV^R (pWBG749e)
WBG541 (pWBG749e)	Newman F128Y comp. NOV^R	Newman F128Y comp. NOV^R (pWBG749e)
WBG541 (pWBG749e)	Newman L152F NOV^R	Newman L152F NOV^R (pWBG749e)
WBG541 (pWBG749e)	Newman L152F comp. NOV^R	Newman L152F comp. NOV^R (pWBG749e)
Newman WT NOV^R (pWBG749e)	Newman Δ*rel*$_{syn}$ NOV^R	Newman Δ*rel*$_{syn}$ NOV^R (pWBG749e)
Newman WT NOV^R (pWBG749e)	Newman Δ*rel*$_{syn}$Δ*relP*Δ*relQ* NOV^R	Newman Δ*rel*$_{syn}$Δ*relP*Δ*relQ* NOV^R (pWBG749e)
SH1000 (pGO1)	Newman *lexA* S130A NOV^R	Newman *lexA* S130A NOV^R (pGO1)
DAR6247 ICE*6013*	Newman WT NOV^R	Newman WT NOV^R ICE*6013*
DAR6247 ICE*6013*	Newman F128Y NOV^R	Newman F128Y NOV^R ICE*6013*

All mutations are in *rel* unless stated otherwise.

2.3.7 Determination of conjugation/mobilization frequency

Donor and recipient strains with chromosomal antibiotic resistance markers were grown on TSA (containing antibiotic, as appropriate) at 37°C overnight and resuspended in TSB to an OD_{600} of 1.0. Wildtype and complemented strains were combined with SH1000 at a 1:2 ratio (250 μL of wildtype or complemented strain and 500 μL of SH1000). Mutant strains were

combined with SH1000 at a 1:1 ratio (500 μL each). Mating mixtures were pushed through a syringe onto a 0.2 μM pore-size nylon filter using 13 mm Swinnex filter holders. Filters were placed bacteria side down on TSA and incubated at 37°C for 4 hours. Following incubation, cells were resuspended in 1 mL TSB and serially diluted in PBS. Serial dilutions were plated on TSA containing the appropriate antibiotics for selection of donors, recipients or transconjugants, and incubated at 37°C for 16-24 hours. Colonies were enumerated either manually or using an aCOLyte 3 automated colony counter (Synbiosis Ltd., Cambridge, UK). Conjugation/mobilization frequency was expressed per donor.

2.3.8 Static biofilm assay

The static biofilm assay was adapted from methods previously described[159]. Overnight cultures were diluted to an OD_{600} of 0.05 in TSB + 1% glucose. A 24-well polystyrene cell culture plate was inoculated with 1 mL of diluted culture per well, sealed with a Breathe-Easy® sealing membrane, and incubated at 37°C for 24 hours. Following static incubation, the wells were washed three times with 1 mL PBS and the biofilm was heat fixed by incubation at 80°C for 30 minutes. Biofilm was stained with 800 μL of 1% crystal violet per well for 15 minutes. Wells were then washed with distilled water and dried at room temperature. Biofilm was quantified by OD_{590} measurements using a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA). To account for differences in biofilm density within a single well, 37 measurements were taken per well and the average was calculated.

2.4 Molecular biology techniques

2.4.1 Colony polymerase chain reaction and sequencing

Each colony was resuspended in 5 μL of dH_2O, and streaked on TSA containing antibiotic, as appropriate. TSA plates were incubated at 37°C overnight. Lysis buffer was prepared with 855 μL *S. aureus* lysis buffer (20 mM Tris, 3 mM MgCl2, 0.5 % Tween-20, pH 8.0) and 4.5 μL proteinase K (Life Technologies, Carlsbad, CA), and 95 μL of this mixture was added to each resuspended colony. Cells were incubated at 56°C for one hour. Proteinase K was inactivated by incubation at 95°C for 15 minutes. Tubes were cooled on ice and cell debris was pelleted by centrifugation at 12,000 × g for 3 minutes. DNA extracted from each colony was used as

template in polymerase chain reactions (PCR) using CloneAmp HiFi PCR premix (TaKaRa Bio USA, Mountain View, CA). PCR cycling conditions used were an initial denaturation cycle at 95°C for one minute followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 68°C for 10 seconds/kb, and a final elongation cycle at 68°C for 3 minutes. PCR products were visualized with SYBR™ Safe DNA Gel Stain (ThermoFisher, Waltham, MA) on a 1% agarose gel to confirm the size of amplification products. PCR products were purified using a NucleoSpin® Gel and PCR clean-up kit according to the manufacturer's instructions. All sequencing was performed by Sequetech Corporation (Mountain View, CA).

2.4.2 Selection and sequencing of resistant strains

Novobiocin-resistant (NOV^R) and rifampicin-resistant (RIF^R) derivatives of Newman and SH1000 strains were selected by plating ~10^{10} cells on TSA containing 1 μg/mL novobiocin or 5 μg/mL rifampicin and incubating at 37°C for 48 hours. Mutations conferring NOV^R and RIF^R were identified by colony PCR and sequencing of *gyrB* and *rpoB*, respectively, as described above. Fwd and rev primers (Table 6) were used for amplification and sequencing, while "seq" primers were used for sequencing only.

Table 6. Primers used for PCR amplification and sequencing

Primer	Sequence (5' to 3')
rel out fwd	AGGAATAGTATACAAATTAAACTCGC
rel out rev	GTAGAGTTCTGACCGATACC
rel seq fwd	TGAACGTGAAGCGTATATCG
gyrB fwd	GAAATTATAAAGTAACAGAAAGCGATGG
gyrB rev	GTAATTCAGCCATCAAGAGTTCC
rpoB fwd	TTGGCAGGTCAAGTTGTCCAATATG
rpoB rev	GCTCCTGAAACACAAAAAGAAGTTACTGATTAA
rpoB seq fwd	ATACAGATGATATTGACCATTTAGG
rpoB seq rev	GCTATCACTGATCAAATTGACTATTTA

2.4.3 β-galactosidase assay with pJB185 reporter construct

pGO1 was introduced into strains bearing pJB185 reporter constructs as described in section 2.3.6. The promoter activity of *nes*, *trsA* and *trsL* was measured using *lacZ* fusion reporter constructs (Table 3). β-galactosidase assays were performed in quadruplicate. For each reporter construct, five isolated colonies were picked from TSA plates containing antibiotic and used to inoculate 5 mL of TSB containing antibiotic. Cultures were incubated at 37°C with shaking at 200 rpm until an OD_{600} of 0.6 was reached; exact OD_{600} values were recorded for data normalization. In a white opaque 96-well plate, 50 μL of each culture was added to 50 μL Beta-Glo® reagent (Promega Corporation, Madison, WI) and the plate was incubated at room temperature for 30 minutes. At 25°C, the plate was shaken for 10 seconds in a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA) and three endpoint luminescence reading were taken with an integration time of 1 ms. The three endpoint readings for each sample were averaged and expressed relative to the OD_{600} of the starting culture.

2.4.4 Quantitative polymerase chain reaction

2.4.4.1 Sample preparation

Genomic DNA and plasmid DNA from Newman NOV^R (pGO1) and Newman *rel* F128Y NOV^R (pGO1) was used for qPCR. In quadruplicate for each strain, 5 mL of TSB containing gentamycin and novobiocin was inoculated with a single colony. Cultures were incubated overnight at 37°C with shaking. 3 mL of each overnight culture was centrifuged at 5000 × g for 10 minutes. Total DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany) with the following modifications for Gram-positives. Cells were resuspended in 180 μL enzymatic lysis buffer (20 mM Tris pH 8.0, 2 mM EDTA, 1-2% Triton X-100 and 10 μL lysostaphin at 0.5 mg/mL) and incubated at 37°C for 30 minutes. RNase A (4 μL from 100mg/mL stock) was added, and cells were incubated at room temperature for 5 minutes, followed by 25 μL of proteinase K and 200 μL of Buffer AL was added. Cells were incubated at 56°C for 30 minutes and the protocol was continued following the manufacturer's instructions.

2.4.4.2 Real-time qPCR using SYBR Green dye

DNA primers were designed using Primer3 (Table 7)[160]. Chromosomal DNA target genes were previously reported to be single copy number[161,162]. Primer sets were validated using a two-tiered stringent quality control protocol[163]. Tier one established optimal thermocycle parameters to yield a target-specific amplicon with minimal background noise. The specificity of each primer set was examined for a single amplification signal representative of one targeted DNA product. Different annealing temperatures were tested for optimal primer specificity. Control qPCR reactions containing no sample DNA were performed to assess background/non-specific signal. Amplified DNA products generated in the qPCR assay were run on a 1.5% agarose gel with SYBR™ Safe DNA Gel Stain (ThermoFisher, Waltham, MA) and confirmed to be the correct predicted size. *sspB* and *Aac-AphD* primers produced the best target-specific amplification with minimal background noise; therefore, these primers were further validated in tier two.

Tier two evaluated the amplification efficiency of each primer set to ensure similar performance in the qPCR assay. DNA concentrations were determined using a Nanodrop ND-1000 (ThermoFisher, Waltham, MA) and samples were diluted to 12.5 μg/mL in UltraPure™ distilled water (ThermoFisher, Waltham, MA). These DNA stock solutions were diluted five-fold and then two-fold serially diluted. The final dilution series was a 5, 10, 20, 40, 80, and 160-fold dilution. For each dilution, the average cycle threshold (C_T) of the triplicates was calculated. For each sample, the average C_T was plotted against $\log_2$ of the dilution. The trendline equation was assessed to ensure a slope between 0.9 and 1.1 and $R^2 > 0.98$. The ΔC_T was calculated by subtracting the average C_T of the chromosomal gene from the average C_T of the plasmid gene. The ΔC_T values were plotted against $\log_2$ of the dilution to ensure a trendline slope between -0.1 and 0.1.

Based on primer validation and optimization, qPCR reactions were performed using the following protocol. Quadruplicate reactions were performed for each condition in a transparent 96-well full skirt PCR plate (SARSTEDT, Inc., Nümbrecht, Germany) sealed with a Microseal® 'B' sealing film (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Each reaction consisted of 2 μL of diluted DNA, 7.5 μL of 2× PowerUp™ SYBR™ Green Master

Mix (ThermoFisher, Waltham, MA), 0.525 µL of each primer (10 µM), and 4.45 µL of UltraPure™ distilled water (ThermoFisher, Waltham, MA). Reactions were analyzed on a CFX Connect real-time system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Parameters for qPCR were set at 50°C for two minutes, 95°C for two minutes, followed by 40 cycles of 95°C for 15 seconds, 62°C for 15 seconds and 60°C for 30 seconds.

Table 7. Primers used for qPCR

Target	Sequence (5' to 3')	Primer position (nt)	Product size (bp)
sspB (gDNA)	F: AATTCGATAACTCATGGTGTGCAGG R: GAAGGTCTTGCTCACTTACTTCAGG	710-839	129
ebpS (gDNA)	F: TGGATAAATCACATCCAGAACCAATCG R: TTGATGTTTCTGATTGGTTAGCACC	374-541	168
scpA (gDNA)	F: ATGTGCAAGTTAATGTCGAGG R: AAGGTTCACCAAGTGTATACG	113-250	138
repA (pGO1)	F: AGATAAGGCTGAAAATGAAGTTGAGG R: TCAATAAAATCAGTATCATTTGTGTGTCC	369-518	150
dfrA (pGO1)	F: GTTATACGAAGCAATGATTGACCAGG R: ACCTTCTACTGAAGATTCGACTTCC	291-421	130
qacC (pGO1)	F: GAAGTGCATTTCTTAAATCTTCAGAAGG R: ACGGTTGTTAAGACTAAACCTAGTCC	47-216	169
AacA-AphD (pGO1)	F: CAAGAGCAATAAGGGCATACC R: TTCATTGCCTTAACATTTGTGG	425-570	145

2.4.4.3 Relative plasmid copy number determination

Average (C_T) and SD values of quadruplicates were calculated for each dilution. For each sample, all dilutions were used as the SD of C_T values was < 0.5. An average C_T value was calculated for genomic and plasmid amplicons in each sample. An amplification efficiency (E) of 100% (E = 2) was used for all calculations. For each sample, ΔC_T was calculated as the difference between the average C_T values for the genomic and plasmid amplicons. Relative plasmid copy number (PCN) was calculated using the following equation[164]: $PCN = (E)^{\Delta CT}$.

2.4.5 ATP-based assay to detect tolerance

ATP assays were performed in triplicate in MHB. Overnight cultures were inoculated from three different starting colonies. Overnight cultures were diluted 1/50 in 5 mL MHB and incubated at 37°C for one hour with shaking at 200 rpm. Vancomycin was tested at 20 μg/mL, which is 10 × the MIC at the susceptibility breakpoint[56]. Samples (30μL) were taken from each culture immediately following the addition of antibiotic, and at time intervals thereafter. Samples removed from each culture were immediately added to DMSO at a 1:1 ratio in a flat-bottom 96-well polystyrene plate and stored at 4°C. In each well of a white opaque 96-well plate, 30 μL of the sample in DMSO was added to 20 μL of dH_2O. In each well, 50 μL of CellTiter-Glo® reagent (Promega Corporation, Madison, WI) was added, and the plate was incubated at room temperature for 10 minutes. The plate was shaken for 10 seconds in a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA) and three endpoint luminescence reading were taken with an integration time of 500 ms. The three endpoint readings for each sample were averaged and luminescence values at each time point were expressed relative to the time zero luminescence value.

2.4.6 ATP stability

The ATP assay was performed as described above. At time zero, two samples were taken from each culture and added to DMSO. In one sample, the ATP content was measured immediately and in the other sample, ATP content was measured after 90 minutes of storage in DMSO at 4°C. ATP content was compared between each pair of samples.

2.5 Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.07. To analyze the difference between the means of three or more groups, one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used. An unpaired t-test was used to compare the means between two groups. All statistical analysis used a cut-off of $p \leq 0.05$ for significance.

2.6 Ethical approval

The use of retrospective chart reviews in this study was reviewed and approved as a harmonized minimal risk clinical study by the Island Health Clinical Research Ethics Board

and the University of Victoria Human Research Ethics Board (REB number: H19-03377). Following the identification of tolerant clinical isolates, isolate accession numbers were linked back to patients and chart reviews were performed by Dr. Victor Yuen. Anonymized data were then provided to Dr. Joanne Hobbs.

CHAPTER 3

Stringent response activation promotes conjugal transfer of staphylococcal plasmids

3.1 Introduction

Antibiotic resistance is a common strategy used by bacterial pathogens to evade antibiotic action. Resistance can spread rapidly due to the acquisition of resistance determinants through horizontal gene transfer. Until recently, phage transduction was thought to be the primary mechanism of HGT in *S. aureus*; however, we now know that conjugation plays a much greater role than previously thought[76]. Most clinical isolates of *S. aureus* carry at least one plasmid and many of these are large conjugative plasmids. Conjugative plasmids often encode multiple resistance genes, meaning that with a single conjugation event, a bacterium can gain resistance to multiple classes of antibiotic. In addition to resistance genes, conjugative plasmids carry all of the genes required for autonomous transfer. Notably, conjugative plasmids can also facilitate the transfer of co-resident mobilizable plasmids. Recent work suggests that nearly all *S. aureus* plasmids can be mobilized; therefore, conjugation is an important factor in the dissemination of resistance genes[76]. Although conjugation represents a major mechanism of HGT in *S. aureus*, little is known about the factors regulating the frequency of plasmid transmission. Induction of the SR has been shown to promote the dissemination of resistance genes in *E. coli* biofilms *via* induction of the SOS response[143]. Furthermore, induction of the SOS response has been shown to increase the frequency of conjugative transfer of a staphylococcal integrative conjugative element (ICE)[144]. Given the apparent prevalence of Rel mutations among clinical isolates, SR activation is potentially a clinically-relevant mechanism underlying the spread of resistance genes. I hypothesize that SR activation promotes the dissemination of resistance by increasing the transmission of conjugative multidrug resistance plasmids. This hypothesis will be investigated in this chapter.

3.2 Results and Discussion

3.2.1 SR activation increases the donation of pGO1

The F128Y and L152F Rel mutations were first identified in clinical isolates associated with persistent infections. These mutations were recently introduced into a laboratory strain of *S. aureus*, Newman, and have been shown to confer elevated cellular (p)ppGpp levels

consistent with SR activation[88]. To determine whether SR activation promotes the dissemination of resistance genes through conjugation, filter matings were performed using the SR-activated strain, *S. aureus* Newman Rel F128Y, and wildtype Newman, both bearing the same chromosomal resistance marker. Donor and recipient strains were combined on nylon filters to increase cell-to-cell contact and promote conjugation. Following mating, donor, recipient and transconjugant populations were quantitated based on resistance profiles. Donor-recipient relatedness has been shown to effect the frequency of conjugation; therefore, the recipient strain remained constant when the donor strain was varied, and the donor strain remained constant when the recipient strain was varied[165]. The prototypical multidrug resistance plasmid pGO1 was used for initial investigation[155]. Interestingly, SR activation in the F128Y mutant resulted in a ~10-fold increase in the donation frequency of pGO1 compared to wildtype Newman (Figure 5A). The frequency of receipt of pGO1 remained unchanged in the F128Y mutant compared to wildtype Newman (Figure 5B). These results suggest that SR activation increases the donation of pGO1, but not the receipt.

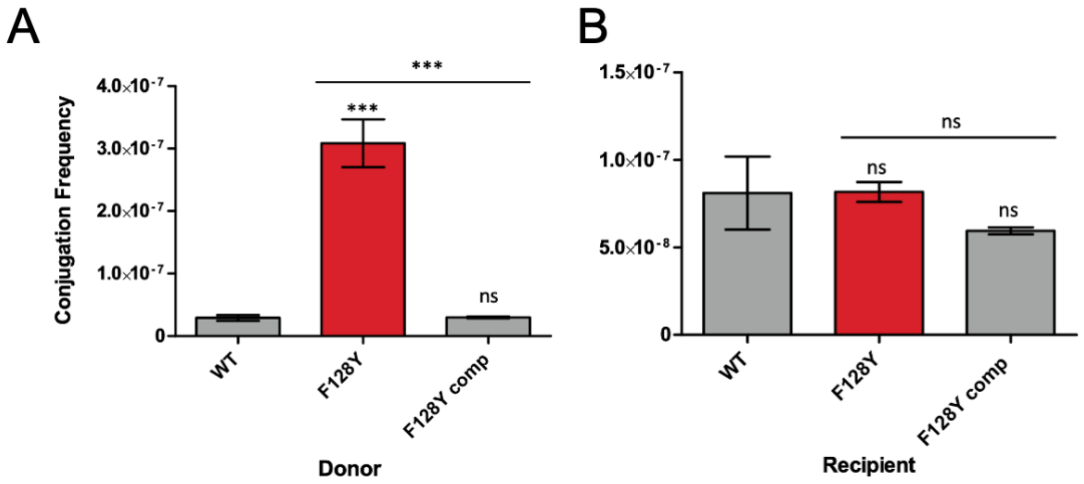

Figure 5. SR activation in the donor strain increases conjugation frequency with pGO1. (A) Conjugation frequency increases when the SR is activated in the donor strain. SH1000 was used as the recipient strain. (B) SR activation in the recipient strain does not affect conjugation frequency. SH1000 was used as the donor strain. Conjugation frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Asterisks indicate a statistically significant difference from the wildtype strain as determined by one-way ANOVA with Tukey's multiple comparisons test (*, ** and *** indicate $p = 0.01$ to 0.05, 0.001 to 0.01 and < 0.001, respectively; ns, not significant).

3.2.2 SR activation increases the donation of plasmids from diverse families

The distinct families of staphylococcal conjugative plasmids have been identified[76]. The conjugative plasmids pGO1, pWBG707 and pWBG749e are classified as members of different plasmid families based on their conjugation gene clusters. To investigate whether the increase in conjugation frequency is specific to pGO1 or general across different families of staphylococcal plasmids, filter matings were performed using wildtype Newman and the F128Y mutant bearing pWBG707 or pWBG749e. SR activation resulted in a ~2.8-fold increase in the donation of pWBG707 (Figure 6A) and a ~2.2-fold increase in the donation of pWBG749e (Figure 6B). To further examine the generality of this trait, filter matings were performed using the non-conjugative plasmid pC221 co-resident with pWBG749e. The F128Y mutant also mobilized pC221 at a frequency ~2.7-fold higher than wildtype Newman (Figure 6C). These results suggest that the mechanism underlying increased plasmid donation is widespread across diverse families of plasmids.

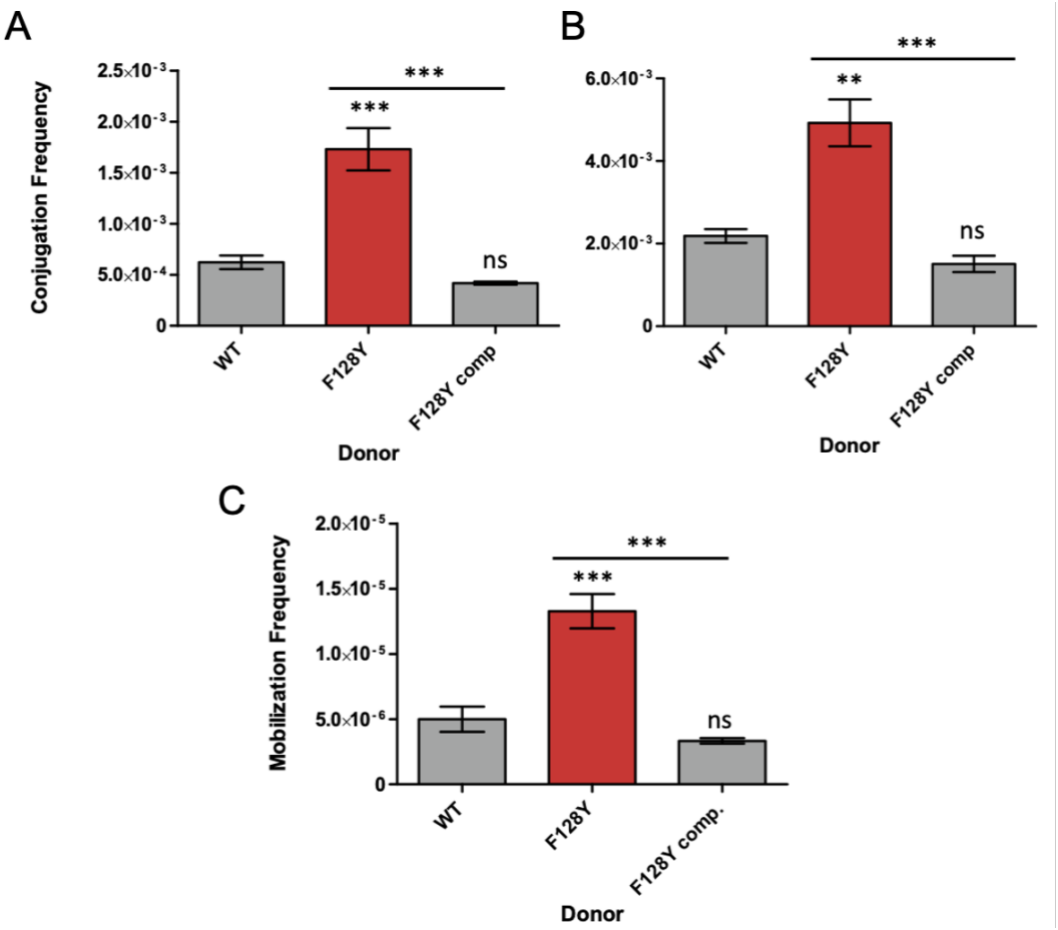

Figure 6. SR activation in the donor strain increases conjugal transfer of plasmids from all three families of conjugative staphylococcal plasmid, and plasmid mobilization. (A) pWBG707[6]. (B) pWBG749e[7]. (C) pC221 co-resident with pWBG749e. SH1000 was used as the recipient strain for all matings. Conjugation/mobilization frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Asterisks indicate statistical significance as stated in the legend for Fig. 5.

Although SR activation resulted in a significant increase in the donation of these plasmids, it was not as drastic as the increase observed with pGO1. It is important to note that pGO1 is 54 kb in size, whereas pWBG707 is ~33.9 kb and pWBG749e is ~43.3 kb. Carriage of large conjugative plasmids such as pGO1 is associated with significant fitness costs[166]. Furthermore, the process of conjugation is energetically expensive and needs to be tightly regulated to reduce the burden on the host cell[167]. Given the substantial energetic cost associated with pGO1 donation, conjugal transfer occurs at a low frequency. Compared to the frequency of pWBG707 donation, the frequency of pGO1 donation is four orders of magnitude lower. The low baseline donation frequency of pGO1 may make it easier to observe changes

in conjugation frequency and therefore, provide an explanation for the ~10-fold increase in pGO1 donation from the F128Y mutant.

3.2.3 Elevated cellular (p)ppGpp levels increase conjugal transfer

The F128Y mutant differs from wildtype Newman by its elevated cellular (p)ppGpp levels[88]. To further investigate the effects of elevated (p)ppGpp levels on plasmid donation, filter matings were performed using *S. aureus* Newman Rel L152F. This SR-activated Rel mutant exhibits elevated (p)ppGpp levels, similar to that of the F128Y mutant[88]. Like the F128Y mutant, SR activation in the L152F mutant resulted in increased donation of pGO1 (Figure 7A). Notably, the L152F mutant exhibited a ~5-fold increase in conjugal transfer, while the F128Y mutant exhibited a ~10-fold increase. Previous work has shown that the F128Y mutant has slightly higher (p)ppGpp levels compared to the L152F mutant, but the difference is not statistically significant[88]. Furthermore, the F128Y mutant exhibits more severe growth defects compared to L152F and has a more prominent tolerance phenotype as a result[88].

To further support the link between increased plasmid donation and elevated (p)ppGpp levels, the SR was chemically-induced in wildtype Newman with subinhibitory concentrations of mupirocin. Wildtype Newman treated with mupirocin exhibited a small colony phenotype consistent with SR activation and donated pGO1 at a significantly higher rate compared to untreated wildtype Newman (Figure 7B). There is a narrow range of mupirocin concentrations that are sufficient for SR induction while remaining subinhibitory; therefore, it was not possible to evaluate a linear relationship between mupirocin concentration and conjugation frequency. To determine whether lowering the cellular (p)ppGpp concentration would have the opposite effect (and whether (p)ppGpp is critical for conjugation), filter matings were performed using Rel mutants with reduced levels of (p)ppGpp. The Δrel_{syn} mutant carries three mutations that render the Rel synthetase domain non-functional while leaving the hydrolase domain unaltered. This mutant still contains a low level of (p)ppGpp due to the presence of the small alarmone synthetases, RelP and RelQ[168]. The Δrel_{syn}Δ*relP*Δ*relQ* mutant lacks (p)ppGpp entirely due to abolished Rel synthestase activity and deletion of *relP* and *relQ*[129]. Decreased cellular (p)ppGpp levels resulted in decreased plasmid donation by both mutants (Figure 7C). Deletion

of Rel synthetase activity resulted in a ~20% decrease in conjugal transfer of pGO1. Interestingly, the Δ*rel*$_{syn}$Δ*relP*Δ*relQ* mutant exhibited a ~40% decrease in the donation of pGO1. Although these two mutants demonstrated an apparent correlation between (p)ppGpp levels and plasmid donation rate, the differences in conjugation frequency were not statistically significant. In all filter matings, donor and recipient strains were combined at a 1:1 ratio for optimal conjugation. Both deletion mutants exhibit decreased fitness due to reduced (p)ppGpp levels, making it difficult to reach a 1:1 ratio of donor to recipient cells during mating[169]. Deviation from the 1:1 ratio produced variability in conjugations frequencies, contributing to the high standard error of the mean and hindering statistical significance. To further examine the effects of reduced cellular (p)ppGpp levels, filter matings were repeated with Δ*rel*$_{syn}$ and Δ*rel*$_{syn}$Δ*relP*Δ*relQ* mutants bearing pWBG749e. Interestingly, both the Δ*rel*$_{syn}$ and Δ*rel*$_{syn}$Δ*relP*Δ*relQ* mutants demonstrated a ~60% decrease in conjugation frequency, suggesting that the small alarmone synthetases do not play a significant role in regulating pWBG749e transfer rates under these conditions (Figure 7D). Previous work has shown that *relP* and *relQ* are transcriptionally induced upon cell wall stress and only synthesize small amounts of (p)ppGpp under normal conditions[129]. Rel is responsible for the majority of (p)ppGpp synthesis; therefore, it is expected that deletion of Rel synthetase activity has the greatest impact in the absence of cell wall stress. Notably, a complete lack of (p)ppGpp in the Δ*rel*$_{syn}$Δ*relP*Δ*relQ* mutant did not abolish conjugal transfer of pGO1 or pWBG749e. This suggests that conjugation is not fully dependent on (p)ppGpp; however, (p)ppGpp levels appear to correlate with the frequency of conjugation.

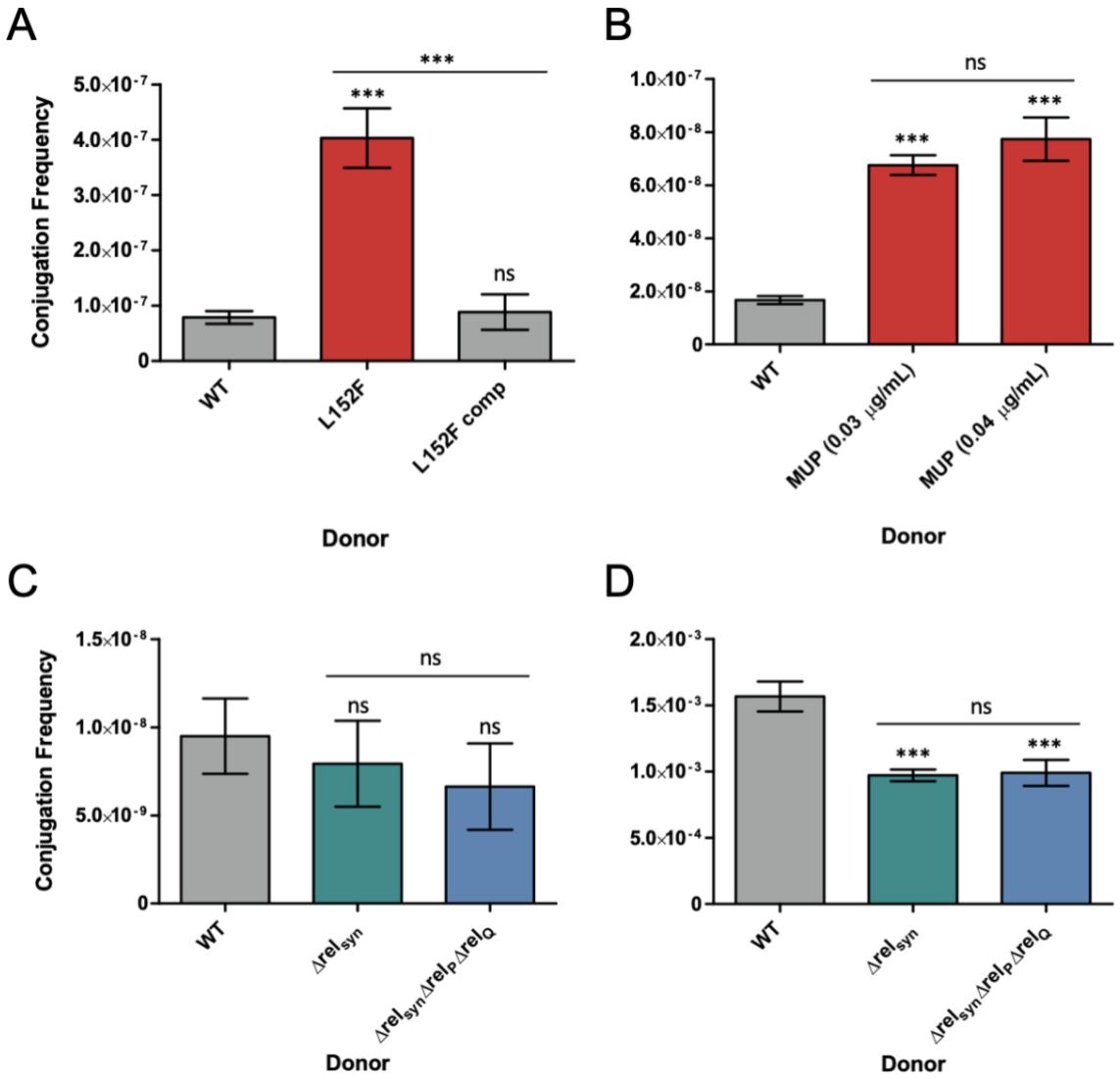

Figure 7. Cellular (p)ppGpp levels correlate with the frequency of conjugal transfer. (A). SR activation in the L152F mutant increases the donation of pGO1. (B) Chemical induction of the SR with a subinhibitory concentration of mupirocin increases the donation of pGO1. (C) Deleting Rel synthetase activity and the small alarmone synthetases decreases the frequency of donation of pGO1, but not significantly. (D) Deleting Rel synthetase activity and the small alarmone synthetases has a small, but significant negative impact on conjugal transfer of pWBG749e. SH1000 was used as the recipient strain for all matings. Conjugation frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Asterisks indicate statistical significance as stated in the legend for Fig. 5.

To determine whether RelP and RelQ are important for conjugal transfer under cell wall stress, filter matings were performed using wildtype Newman treated with a subinhibitory concentration of vancomycin prior to mating. A range of vancomycin concentrations were tested to determine the highest concentration that induced cell wall stress while still allowing

growth. Wildtype Newman treated with vancomycin at 1.0 μg/mL exhibited a small colony phenotype consistent with cell wall stress. Induction of *relP* and *relQ* with cell wall stress did not affect the rate of pWBG749e donation, further suggesting that Rel is the predominant contributor to (p)ppGpp-mediated changes in conjugation frequency (Figure 8). Given these results, the roles of RelP and RelQ in conjugation were not investigated further.

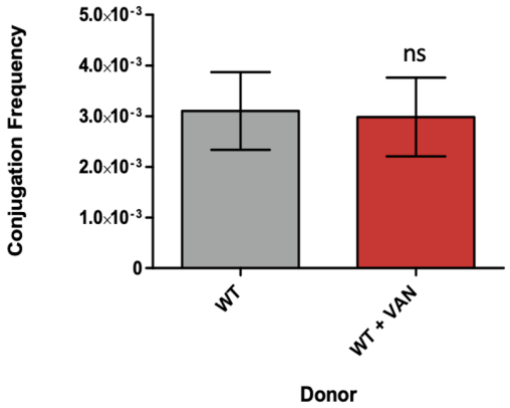

Figure 8. Rel is the predominant contributor to (p)ppGpp-mediated changes in conjugation frequency. Cell wall stress was induced with vancomycin treatment at 1.0 μg/mL. SH1000 was used as the recipient strain for all matings. Conjugation frequency is expressed per donor. Data shown are the mean of three replicates; error bars represent the SEM. Statistical significance was determined with an unpaired t-test (ns: not significant).

3.2.4 Mechanism(s) underlying increased conjugal transfer

It is clear from the data presented that SR activation and elevated cellular (p)ppGpp levels promote the donation of diverse plasmids in *S. aureus*. In order to investigate the potential molecular mechanism(s) behind this relationship, it is important to consider the downstream effects of SR activation. Transcriptional profiling in *E. coli* revealed that SR induction resulted in upregulated expression of six genes involved in the SOS response[170]. Furthermore, SR activation has been shown to promote the dissemination of resistance genes *via* induction of the SOS response[143]. Induction of the SOS response resulted in increased integron-integrase expression in *E. coli* biofilms. The SOS response is an inducible pathway triggered by DNA damage that is regulated by RecA and LexA[171]. Upon DNA damage, RecA binds to single-stranded DNA and is activated (RecA*). RecA* stimulates self-cleavage of the repressor LexA, leading to de-repression of SOS genes. Mutating the catalytic serine to alanine in LexA prevents autoproteolysis and induction of the SOS response[172]. To determine whether SR activation increases conjugal transfer *via* induction of the SOS response, the effect of

mupirocin exposure on pGO1 donation by a LexA S130A mutant was tested. Interestingly, SR induction with mupirocin treatment resulted in a ~6.7-fold increase in pGO1 donation by the LexA mutant (Figure 9), which is similar to the increase seen upon mupirocin treatment of wildtype Newman (Figure 7B). This suggests that SR activation does not increase conjugal transfer *via* induction of the SOS response in *S. aureus*.

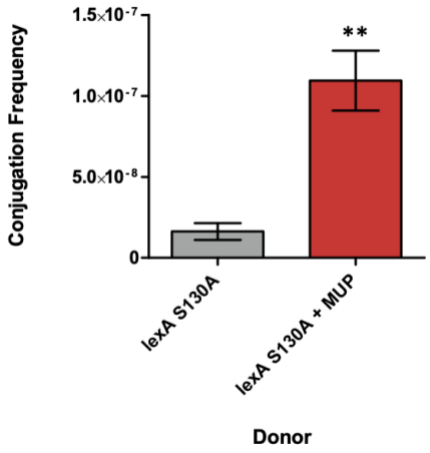

Figure 9. SR activation does not increase conjugal transfer *via* induction of the SOS response. Chemical induction of the SR was achieved with mupirocin treatment at 0.04 μg/mL. SH1000 was used as the recipient strain for all matings. Conjugation frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Statistical significance was determined with an unpaired t-test. Asterisks indicate statistical significance as stated in the legend for Fig. 5.

Next, I considered the potential role of CodY in the regulation of plasmid conjugation in *S. aureus*. CodY is a global transcriptional repressor that requires GTP in order to bind to DNA. Upon SR induction, GTP levels decrease and transcriptional repression of > 140 genes by CodY is alleviated[173]. The CodY regulon consists of a number of virulence factor genes, as well as genes associated with metabolism[132]. To determine whether CodY regulation affects the frequency of conjugal transfer, filter matings were performed with a Δ*codY* mutant bearing pGO1. Compared to wildtype Newman, the frequency of plasmid donation by the Δ*codY* mutant was slightly increased by ~1.5-fold; however, the increase was not statistically significant (Figure 10A). Additionally, bioinformatic analysis revealed that the CodY consensus binding sequence is not present in any of the three conjugative plasmids used in this study. This suggests that CodY does not regulate genes that influence the rate of conjugal transfer.

Conjugal transfer requires cell-to-cell contact between donor and recipient cells. The high cell density in biofilms promotes cell-to-cell contact and has been shown increase the frequency of HGT[174]. The mating method used in this study allows biofilm formation to occur on nylon filters, thus promoting conjugation. Interestingly, SR induction has previously been linked to biofilm formation[124,129]. To investigate whether the F128Y mutant exhibited increased conjugal transfer as a result of increased biofilm formation, the densities of biofilms formed by wildtype Newman and the F128Y mutant in polypropylene wells were quantified using crystal violet staining. The densities of biofilms formed by the two strains were not significantly different (Figure 10B). This suggests that SR induction does not increase conjugal transfer *via* increased biofilm formation.

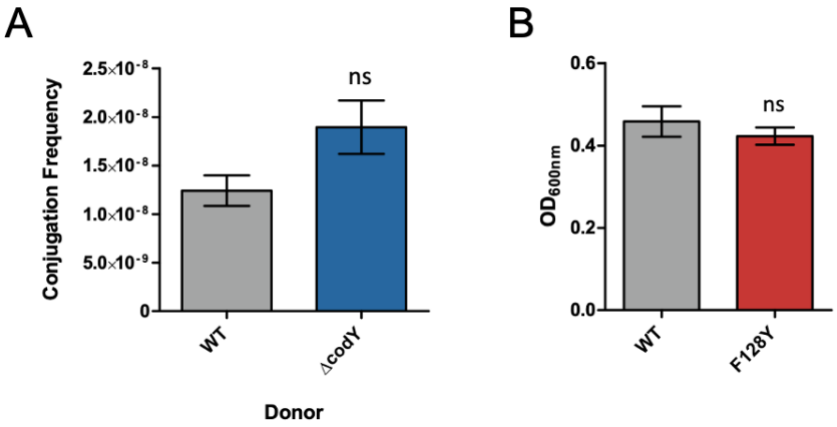

Figure 10. SR activation does not increase conjugal transfer *via* CodY regulation or increased biofilm formation. (A) CodY does not regulate genes involved in conjugal transfer. SH1000 was used as the recipient strain for all matings. Conjugation frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Statistical significance was determined with an unpaired t-test (ns: not significant). (B) SR-activated F128Y mutant does not exhibit increased biofilm formation. Biofilm formation in TSB + 1% glucose after incubation at 37°C for 24 hours. Biofilm was stained with crystal violet and quantified by OD_{590} measurement. Data shown are the mean of 12 replicates; error bars represent the SEM. Statistical significance was determined with an unpaired t-test (ns: not significant).

Investigation into the downstream effects of SR activation suggested that increased plasmid donation does not occur *via* induction of the SOS response, CodY regulation or increased biofilm formation. Given that (p)ppGpp accumulation has a diverse range of binding partners and downstream consequences in the cell, there are a number of mechanisms *via* which SR activation could increase the donation of plasmids. While the data presented ruled out three main pathways in the host cell, it is possible that SR activation could increase plasmid donation

by modulating plasmid-encoded factors. The data presented here suggest that (p)ppGpp increases conjugation by acting on a gene or protein that is shared by plasmids in different families. Previous studies have demonstrated that the frequency of conjugal transfer can be controlled by plasmid copy number and/or the expression of conjugative machinery[175]. Of the staphylococcal conjugative plasmids, pGO1 is the most well-characterized; therefore, pGO1 was used to investigate the effects of SR activation on plasmid-encoded factors. Studies of pGO1 have identified two distinct plasmid regions that are necessary for conjugation: the conjugative transfer region (*trs*) and the origin of transfer (*oriT*)[80]. The *trs* gene cluster consists of 14 open reading frames (ORFs) annotated as *trsA* to *trsN*. Previous work has shown that purified TrsN binds to DNA fragments containing promoters for *trsA*, *trsL*, and *trsN*[81]. Provision of excess TrsN decreased β-galactosidase activity from a *trsL-lacZ* transcriptional reporter and decreased pGO1 conjugation frequency. Conversely, excess *trsL* increased transcription and the frequency of conjugation with pGO1. This study demonstrated that regulation of conjugative machinery expression affects pGO1 donation. To determine whether SR activation increases the expression of conjugative machinery, the expression of *nes*, *trsA* and *trsL* was compared between wildtype Newman and the F128Y mutant carrying pGO1 using *lacZ* reporter constructs. The relaxase-encoding gene, *nes*, was used as control as it is not regulated by TrsN[176]. Interestingly, expression of all three genes was lower in the F128Y mutant (Figure 11). F128Y expressed *trsA* and *trsL* at a lower level than wildtype Newman, suggesting that (p)ppGpp does not alleviate repression of the *trs* genes. Although *nes* is not included in the *trs* operon and is not regulated by TrsN, it was also expressed at a lower level in the SR-activated mutant. These findings are consistent with previous work that has shown SR induction results in downregulated expression of genes that are not involved in stress adaptation[170].

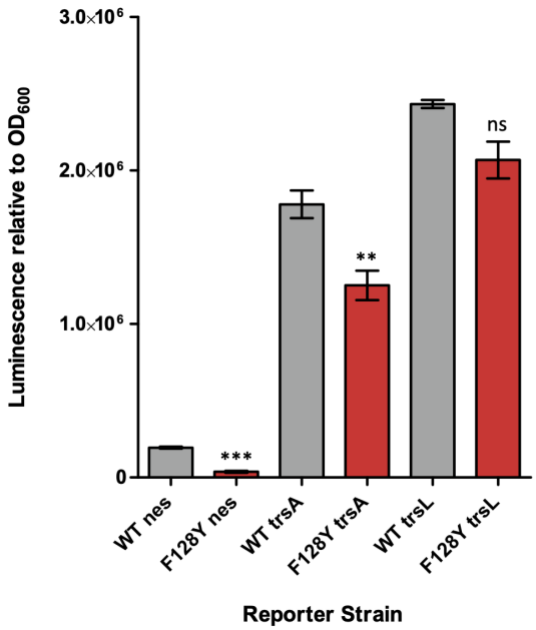

Figure 11. SR activation does not increase expression of pGO1 *trs* genes. In strains bearing pGO1, β-galactosidase activity driven by the *nes*, *trsA* or *trsL* promoter was measured by luminescence. Luminescence is expressed relative to OD_{600}. Data shown are the mean of 4 replicates; error bars represent the SEM. Asterisks indicate a statistically significant difference between the wildtype and F128Y mutant for each reporter construct as determined by an unpaired t-test. Asterisks indicate statistical significance as stated in the legend for Fig. 5.

In an attempt to further narrow down the molecular mechanism of (p)ppGpp-mediated control of plasmid donation, I assessed the impact of SR activation on ICE transmission. Like conjugative plasmids, ICEs encode all of the genes necessary for DNA transfer and mating pore formation[74]. If SR activation also increases conjugal transfer of an ICE, it suggests that (p)ppGpp is acting on a gene or protein that is shared by ICEs and conjugative plasmids. Filter matings were performed using wildtype Newman and the F128Y mutant bearing ICE*6013*. ICE*6013* represents one of two families of staphylococcal ICE and the variant of it used in these experiments encodes for erythromycin resistance to allow selection[154]. SR activation in the F128Y mutant resulted in a ~2.0-fold increase in the frequency of conjugal donation of ICE*6013* compared to wildtype Newman; however, the increase was not statistically significant (Figure 12A). To further confirm that SR activation does not increase the transfer of ICE*6013*, the SR was chemically induced in wildtype Newman with mupirocin treatment. The frequency of conjugal transfer did not change significantly with SR activation in the

wildtype strain, further suggesting that the mechanism of (p)pGpp-mediated conjugal control is unique to plasmids (Figure 12B).

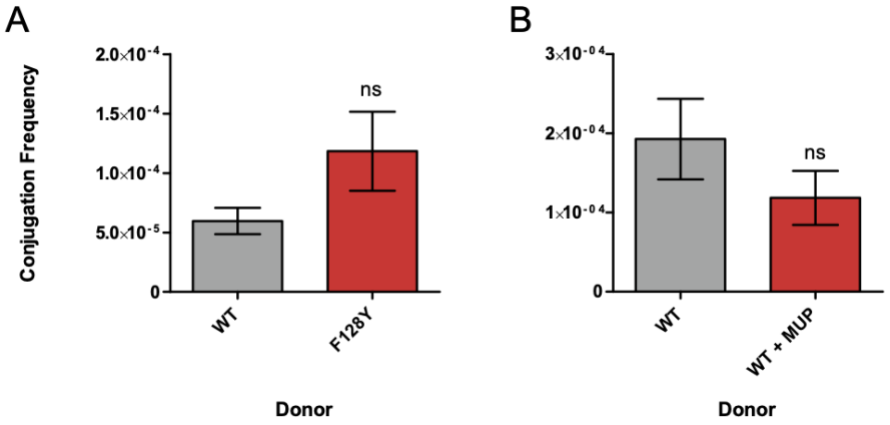

Figure 12. SR activation does not increase conjugal transfer of ICE*6013*. (A) SR activation in the F128Y mutant does not increase conjugal transfer of ICE*6013*. (B) Chemical induction of the SR in wildtype Newman with a subinhibitory concentration of mupirocin does not increase the donation of ICE*6013*. SH1000 was used as the recipient strain for all matings. Conjugation frequency is expressed per donor. Data shown are the mean of six replicates; error bars represent the SEM. Statistical significance was determined with an unpaired t-test (ns: not significant).

Studies in *E. coli* have shown that increased plasmid copy number results in increased conjugal transfer[175]. To examine whether SR activation in *S. aureus* results in increased plasmid copy number, the relative copy number of pGO1 was determined in wildtype Newman and the F128Y mutant by quantitative PCR (Figure 13). A single-copy chromosomal reference gene was used to calculate the relative copy number of a plasmid-encoded gene. Interestingly, the average relative copy number of pGO1 was 2.46 in the F128Y mutant, while it was 1.61 in wildtype Newman. This moderate 1.53-fold change was deemed to be statistically significant, suggesting that SR activation results in an elevation in pGO1 copy number. Notably, among four biological replicates the relative copy number did not exceed 2.0 in wildtype Newman. In contrast, the minimum relative copy number was 2.2 among four replicates of the F128Y mutant. Although the 1.53-fold change in relative copy number was not drastic, it is important to note that carriage of pGO1 is associated with significant metabolic costs. At 60kb, the conjugative plasmid RP4 is similar in size to pGO1[177]. Previous work has estimated that the copy number of RP4 is less than three in *Pseudomonas* spp[178]. While the absolute copy number of pGO1 remains unknown, it is likely similar to that of RP4 given their comparable sizes.

With an average relative copy number of 2.46, the F128Y mutant may be carrying pGO1 at the maximum copy number that is energetically feasible for the cell. Future work could involve determination of the relative copy number of a smaller conjugative/mobilizable plasmid in wildtype Newman and the F128Y mutant. Compared to large multidrug resistance plasmids, smaller plasmids present lower energetic costs to the host cell and can be carried at a higher copy number. In staphylococci, plasmids less than ~8kb are maintained at 10-60 copies per cell[74]. The low metabolic costs of small plasmids may allow for a larger deviation in copy number to occur with SR activation compared to the 1.53-fold change observed with pGO1.

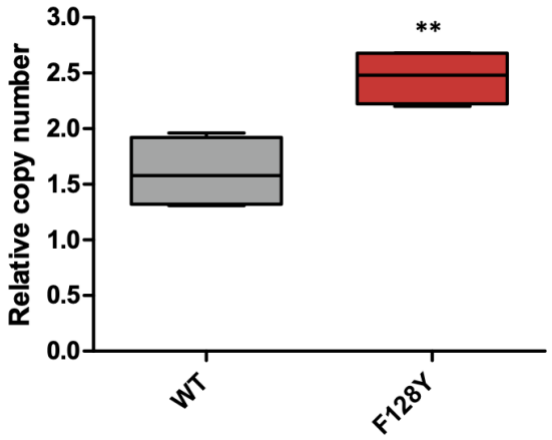

Figure 13. SR activation increases the relative copy number of pGO1. Data shown are the mean of four replicates; whiskers represent the minimum and maximum values Statistical significance was determined with an unpaired t-test. Asterisks indicate statistical significance as stated in the legend for Fig. 5.

3.2.5 Potential mechanistic basis for increased plasmid copy number with SR activation

The data presented here suggest that SR activation increases the donation of conjugative and mobilizable plasmids from diverse families by increasing plasmid copy number. It appears that this increase in conjugal transfer is directly related to elevated cellular levels of (p)ppGpp. This raises the question of how (p)ppGpp mediates an increase in plasmid copy number. Plasmid copy number is controlled by the frequency of plasmid replication[179]. All three of the conjugative plasmids used in this study employ theta-mode replication[74]. In theta-type plasmids, binding of the replication initiation protein to the origin of replication leads to the formation of a nucleoprotein complex that opens the DNA duplex. Duplex melting is necessary for assembly of the replisome complex that coordinates DNA replication. pGO1 and pWBG749e encode a replication initiation protein referred to as RepA[180]. The conserved N-

terminal RepA_N domain binds to repeat DNA sequences located within the *rep* gene, while the C-terminal domain interacts with DnaG primase[181,182]. Translation of RepA is thought to be negatively regulated by an antisense RNA that binds to the leader region of *rep* mRNA[179]. It has been proposed that antisense RNA binding to the mRNA leader induces formation of a stem loop in the *rep* translation initiation region that sequesters the ribosome binding site[181]. pWBG707 encodes a replication initiation protein containing a conserved PriCT_1 domain[180]. It has been predicted that replication of pWBG707 is also negatively regulated by an antisense RNA in addition to a small repressor protein[180]. While the mechanism of antisense RNA regulation in pWBG707 is poorly understood, it has been proposed that binding of antisense RNA to the leader mRNA induces formation of a terminator-like stem loop that results in transcriptional termination[180]. The smaller non-conjugative plasmid pC221 uses rolling circle replication and is predicted to employ a similar mechanism of copy number regulation with an antisense RNA[74]. One possible explanation for a (p)ppGpp-mediated increase in plasmid copy number is that (p)ppGpp could bind to the mRNA leader sequence and prevent interaction with antisense RNA. Interestingly, the replication initiation proteins of pGO1 and pWBG749e share 55% amino acid sequence identity, whereas the replication proteins of pGO1 and pWBG707 only share 11% sequence identity. Although low sequence identity suggests that the replication initiation proteins of pGO1 and pWBG707 are not homologous, a consensus binding site for (p)ppGpp has not been identified; therefore, (p)ppGpp could be capable of binding to the leader mRNA in both plasmids. Furthermore, many RNAs are conserved at the structural level, but they lack sequence conservation[183]. One limitation with this hypothesis is that perhaps binding of (p)ppGpp to the leader mRNA could hinder ribosome binding, and therefore, translation of the *rep* transcript. Another possibility is that (p)ppGpp could bind to antisense RNA and prevent interaction with the mRNA leader sequence. The predicted secondary structure of the antisense RNA for pGO1/pSK41 includes three stem-loop structures[179]. Intriguingly, this secondary structure resembles the predicted secondary structure model for (p)ppGpp riboswitches[184]. Given the previous work on riboswitches, it is plausible that (p)ppGpp could bind to similar stem-loop structures in antisense RNA. Additionally, antisense RNAs are transcribed from a constitutive promoter to ensure that they are in excess of the target mRNA[181]. The abundance of antisense RNA is thought to prevent significant deviations in plasmid copy number that could impose a metabolic burden on the host cell. Perhaps binding

of (p)ppGpp to antisense RNA moderately reduces the amount of functional antisense RNA present in the cell; however, the effects of (p)ppGpp are limited by the abundance of antisense RNA. This could explain why SR activation resulted in a moderate increase in pGO1 copy number, as opposed to a dramatic increase. More work is needed to determine the predicted structures for antisense RNA in pGO1, pWBG749e and pWBG707, and investigate whether these structures possess conserved motifs that could be bound by (p)ppGpp. This hypothesis could be directly tested by generating reporter constructs with the replication control region translationally fused to *lacZ*. Translation of the replication initiation mRNAs could be measured with and without (p)ppGpp using a β-galactosidase assay. Increased translation in the presence of (p)ppGpp could suggest that (p)ppGpp is protecting the mRNA from interactions with antisense RNA.

3.2.6 Implications and future directions

Elevated plasmid copy number is coupled with an increase in gene dosage[185]. Previous work has suggested that elevated copy number increases the transfer of conjugative and mobilizable plasmids due to increased *oriT* dosage[175]. For conjugative plasmids, it has been suggested that increased dosage of the conjugative transfer genes may further increase conjugal transfer[175]. Interestingly, increased expression of the *trs* genes with elevated plasmid copy number was not observed in this study. In the method used here, OD_{600} measurements were used to correct for bacterial numbers. Given the downregulation of translation during SR activation, correcting for total protein concentration may provide a more accurate measurement of *trs* gene expression in the F128Y mutant. This method of standardization may result in comparable levels of *trs* gene expression between wildtype Newman and the F128Y mutant, or even a slight increase in expression of the *trs* genes in the F128Y mutant that corresponds to the 1.53-fold increase in plasmid copy number. Increased gene dosage affects both the conjugative transfer region and the expression of other phenotypes encoded by the plasmid. Previous studies have correlated high levels of resistance with increased gene dosage *via* plasmid copy number[175,186,187]. This suggests that increased pGO1 copy number in the F128Y mutant is coupled with high-level gentamycin and trimethoprim resistance. To further support an increase in pGO1 copy number, the levels of resistance in wildtype Newman and the F128Y mutant bearing pGO1 could be examined with MIC determinations. From a clinical

perspective, while increased gene dosage of resistance genes is unlikely to impact the treatment of infections with antibiotics, plasmid copy number has a general effect on the dosage of all plasmid-encoded genes, including those associated with virulence[175]. Increased gene dosage has been linked to increased virulence and improved ability to grow on limited nutrients[188,189]. Ultimately, increased dosage of plasmid-encoded genes as a result of SR activation may contribute to the evolution of highly resistant *S. aureus* strains with enhanced pathogenicity.

In addition to increased conjugal transfer, elevated plasmid copy number has implications for phage transduction. Many strains of *S. aureus* harbor one to four bacteriophages that contribute to a significant proportion of HGT[73]. These bacteriophages are integrated into the genome as prophages that can excise spontaneously or due to induction of the SOS response. During generalized transduction, bacterial DNA is packaged into transducing particles instead of phage DNA. Approximately 45kb of chromosomal or plasmid DNA can be transferred, leading to the dissemination of resistance genes[190]. As one fifth of staphylococcal plasmids are less than 45kb in size, it is possible to transfer an entire plasmid by generalized transduction[75,76]. Transduction is a stochastic process; therefore, it is predicted that elevated plasmid copy number increases the likelihood of transduction with plasmid DNA. Previous work has shown that plasmid transduction efficiency is indeed related to plasmid copy number[191]. Given the role transduction plays in the dissemination of resistance genes, the impact of SR-mediated increases in plasmid copy number should be further investigated.

Overall, the data presented in this chapter uncovers a novel role of the SR in bacterial physiology that has important implications for human health. SR activation resulted in increased transfer of both conjugative and mobilizable plasmids. A recent study suggested that nearly all non-conjugative plasmids are potentially mobilizable; therefore, this increase in transfer likely has significant consequences for multidrug resistance[76]. Furthermore, it has been suggested that conjugation is the preferred mechanism of HGT when DNA is transferred long genetic distances between species or even genera[73]. Conjugal transfer of resistance plasmids has been observed between *S. aureus* and *S. epidermidis*[192,193]. Although *S. epidermidis* is typically less virulent compared to *S. aureus*, increased plasmid donation could result in more pathogenic strains of *S. epidermidis*[194]. In the clinical setting, increased conjugal transfer is of

particular concern for infections with high bacterial cell densities such as wound infections or indwelling medical device infections. The high cell density increases cell-to-cell contact and promotes conjugation[195]. These infections are already difficult to eradicate, and the dissemination of high-level resistance could further complicate antibiotic treatment. Antibiotic exposure has been shown to select for tolerance-conferring mutations in Rel[111]. Tolerance mutations present a significant challenge to antibiotic therapy in their own right and Rel mutations have the coincidental consequence of promoting the dissemination of multidrug resistance plasmids. Without routine tolerance detection in the clinical setting, Rel mutations will not be identified. Although previous work has focused on the development of conjugation inhibitors, SR inhibitors may provide protection against both resistance and tolerance. Ultimately, more research is needed to assess the clinical consequences of SR activation and guide the development of therapeutic strategies for effective treatment.

CHAPTER 4

Detection of antibiotic tolerance among clinical isolates of *S. aureus* from persistent infections

4.1 Introduction

SR-activating Rel mutations have important consequences in relation to antibiotic therapy. In addition to playing a role in the dissemination of resistance genes, clinical Rel mutations have been linked to antibiotic tolerance. In 2010, the first clinical isolate bearing a Rel mutation was reported from a case of persistent *S. aureus* bacteremia[136]. The isolate exhibited a reduced growth rate and elevated cellular (p)ppGpp levels as a result of a F128Y mutation in the hydrolase domain of Rel[88]. In 2017, a second clinical Rel mutation was reported in an isolate of *Enterococcus faecium* associated with persistent bacteremia[135]. A L152F mutation in the linker region of Rel conferred elevated (p)ppGpp levels[135]. Bryson *et al.* recently introduced the F128Y and L152F mutations into a laboratory strain of *S. aureus*, Newman, and demonstrated that they confer tolerance to multiple classes of antibiotic as a result of SR activation[88]. Recently, additional Rel mutations have been identified in clinical isolates associated with relapsing endocarditis and persistent bacteremia[110,111]. Interestingly, it has been shown that isolates from persistent bacteremia exhibit significantly higher levels of (p)ppGpp compared to isolates from resolving bacteremia[196]. These reports suggest that elevated (p)ppGpp levels and Rel mutations are prevalent among clinical isolates of *S. aureus* from persistent infections. SR activation is a clinically-relevant mechanism of tolerance; therefore, Rel mutant strains provide a much-needed model system for studying tolerance in *S. aureus*. In small-scale studies, tolerance has been identified in up to 60% of clinical isolates[92]. Furthermore, tolerance has been linked to higher rates of treatment failure and mortality compared to resolving infections[96,97]. The prevalence and clinical significance of tolerance is only just beginning to emerge in part due to the lack of routine tolerance detection in clinical laboratories. Time-kill assays are the current gold standard for tolerance detection; however, the labour-intensive protocol is not feasible in the clinical setting. In this thesis chapter, the Rel mutants generated by Bryson *et al.* will be used to develop a simple screen to detect genotypic tolerance. The screen will be used to conduct a pilot study on the prevalence of tolerance among *S. aureus* isolates from CF lung infections.

4.2 Results and Discussion

4.2.1 Detecting genotypic tolerance with cellular ATP content

ATP is a crucial component of cellular metabolism that is essential for the growth and proliferation of bacteria. As such, previous studies have demonstrated a correlation between cellular ATP content and viability[197,198]. Following cell death, the ability to synthesize ATP is lost and any remaining ATP is rapidly degraded by ATPases[199]. Therefore, cellular ATP content has been used as a proxy for viability in time-kill assays, to eliminate the need for laborious viable count determinations[197,198]. In order to quantify cellular ATP, it must be released from cells and protected from degradation. DMSO has been shown to instantly disrupt cell walls and denature proteins responsible for ATP hydrolysis[198,200]. As such, DMSO treatment releases and stabilizes intracellular ATP in a single step[198]. To test the stability of ATP in DMSO, the ATP content of culture samples was measured immediately after and 90 minutes after cell lysis. The samples being measured 90 minutes after cell lysis were stored at 4°C to minimize degradation, as recommended by previous studies[198]. Luminescence measurement of samples 90 minutes after lysis showed minimal degradation of ATP (< 5%). This suggests that, with DMSO treatment, ATP is stable enough for use as proxy of cellular viability in a high-throughput assay.

Previous studies have demonstrated that cellular ATP levels are linked to antibiotic killing rates[201,202]. Furthermore, low cellular ATP levels have been associated with tolerance[203]. To determine whether cellular ATP content can be used to detect antibiotic tolerance, the standard time-kill protocol was adapted to a luminescence assay where luminescence readout is proportional to ATP concentration. In developing the protocol, it was important to ensure that the assay could detect the two main forms of tolerance: tolerance by extended lag and tolerance by slow growth. Therefore, two well-characterized tolerant strains of *S. aureus* were used as model strains. The *S. aureus* Newman Rel F128Y mutant exhibits tolerance due to a reduced growth rate[88] and *S. aureus* COL exhibits tolerance due to an extended lag phase (Claire Stevens, unpublished data). In the traditional time-kill assay, the F128Y mutant and COL are killed at a significantly slower rate compared to wildtype Newman, indicating tolerance (Figure 14A). The time-kill assay also shows varying levels of the tolerance phenotype, with COL exhibiting a higher degree of tolerance than the F128Y mutant. In the

ATP-based assay, vancomycin was used to examine tolerance as the rate of resistance is low at 2.4%. The concentration of vancomycin used in the ATP-based assay is 10 × the MIC at the susceptibility breakpoint[56]. This high concentration accounts for differences in MICs among clinical isolates and prevents the need for MIC determinations to occur prior to the assay. In the ATP-based assay, both the F128Y mutant and COL were killed by vancomycin at a slower rate compared to wildtype Newman (Figure 14B). Consistent with previous studies, the F128Y mutant and COL both exhibited lower raw luminescence values compared to wildtype Newman (data not shown)[203]. However, the F128Y mutant and COL exhibited relative luminescence values (relative to time zero) > 1.0 throughout the entire duration of the assay. Although the relative luminescence values remained above 1.0 for both tolerant strains, ATP content slowly decreased following 40 minutes of vancomycin exposure. This steady decrease in ATP content is indicative of tolerance rather than resistance. In agreement with the time-kill assay, COL appeared to be more tolerant than the F128Y mutant in the ATP-based assay. In contrast to the tolerant strains, wildtype Newman exhibited relative luminescence values less than 1.0 after 60 minutes of vancomycin exposure. These results indicate that cellular ATP content can be used to detect varying levels of genotypic tolerance conferred by extended lag and slow growth.

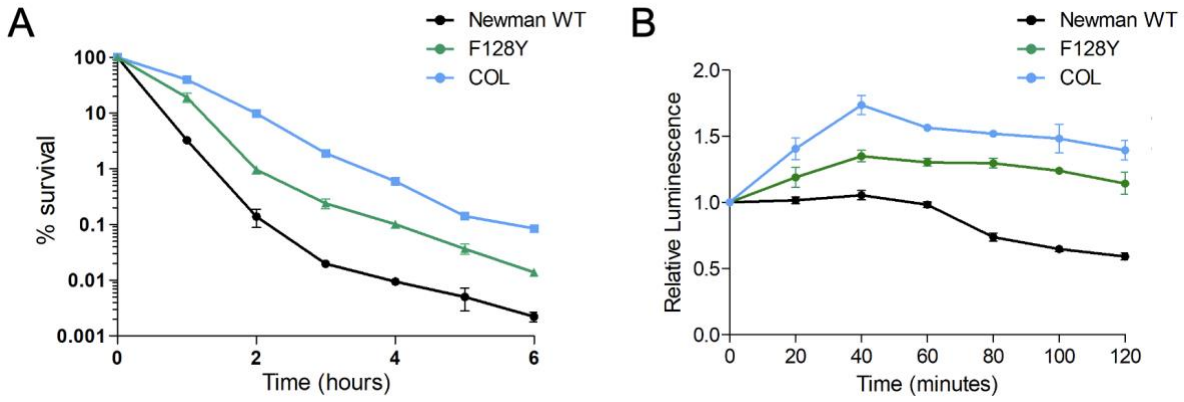

Figure 14. Antibiotic tolerance detected in a time-kill assay and an ATP-based luminescence assay. (A) Wildtype Newman, Newman Rel F128Y and COL were exposed to daptomycin at 4 × MIC in the traditional time-kill assay. Data provided by Claire Stevens. (B) Wildtype Newman, Newman Rel F128Y and COL were exposed to vancomycin at 20 μg/mL (~10 × MIC) in an ATP-based tolerance detection assay. Data shown are relative to time zero and the mean of three replicates (error bars represent the SEM).

To further validate the assay, a panel of known tolerant and non-tolerant strains were tested. Like the F128Y mutation, the Rel L152F mutation has been shown to confer tolerance as a result of (p)ppGpp-mediated growth defects. In the ATP-based assay, the L152F mutant closely resembled the F128Y mutant (Figure 15A). The relative luminescence value peaked at 60 minutes and remained above 1.0 throughout the assay, indicating that the tolerance phenotype of the L152F mutant was detected. N315 and MW2 are common strains of community-associated MRSA that are non-tolerant in time-kill assays (Claire Stevens, unpublished data). Interestingly, both of these strains were nearly identical to wildtype Newman based on relative luminescence values (Figure 15A). In all three non-tolerant strains, ATP levels steadily decreased over time and produced relative luminescence values below 1.0. To further support the assay, a wider panel of known tolerant and non-tolerant *S. aureus* Newman mutants were examined. The PrsE112K mutant has been shown to exhibit a tolerance phenotype in time-kill assays and the basis of tolerance in this mutant is not related to the SR (Claire Stevens, unpublished data). The tolerant Prs E112K mutant was comparable to the F128Y mutant in the ATP-based assay, whereas the non-tolerant IleS Y723H and Rel E169G mutants closely resembled wildtype Newman (Figure 15B). Taken together, data for all of these strains revealed that tolerant strains exhibit a distinct profile in the assay compared to non-tolerant strains. Tolerant strains consistently demonstrated relative luminescence values greater than 1.0, whereas non-tolerant strains exhibited relative luminescence values well below 1.0. Previous studies have indicated that bactericidal antibiotics accelerate respiration, leading to the formation of reactive oxygen species that contribute to lethality[202]. Accelerated initial respiration is evident in all strains, but most drastically in the tolerant strains. However, tolerant strains are able to survive the initial burst of respiration, whereas susceptible strains are quickly killed. The difference between tolerant and non-tolerant strains was the most pronounced around the 90-minute time point; therefore, the following criteria were established: tolerance is defined as relative luminescence > 1.0 after 90 minutes of vancomycin exposure, and non-tolerance is defined as relative luminescence ≤ 1.0 after 90 minutes of vancomycin exposure. Based on this clear threshold for tolerance, the duration of the assay was reduced to 90 minutes and fewer time points were included for increased throughput.

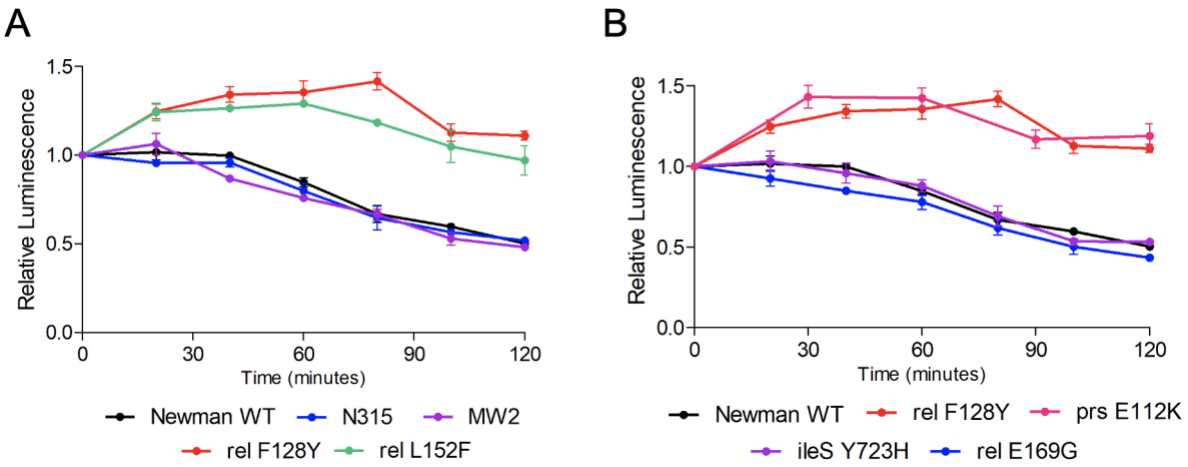

Figure 15. Validation of the ATP-based tolerance detection assay using a panel of known tolerant and non-tolerant strains. (A) Non-tolerant CA-MRSA strains N315 and MW2 are quickly killed by vancomycin at ~10 × MIC, whereas the Rel F128Y and Rel L152F mutants are tolerant. (B) Tolerant strains are distinguished from non-tolerant strains based on their distinct profiles in the assay. Data shown are the mean of three replicates and error bars represent the SEM.

4.2.2 Pilot study with *S. aureus* isolates derived from CF lung infections

The purpose for developing the ATP-based assay was to be able to quickly screen clinical isolates of *S. aureus* for tolerance. Therefore, the next step after validating the assay against a panel of known tolerant and non-tolerant strains was to test it against a library of clinical isolates. *S. aureus* infections frequently occur in the respiratory tract of CF patients and are often persistent despite antibiotic therapy. As such, the screen was applied to a library of 39 *S. aureus* isolates derived from CF lung infections at RJH. Given the comparative nature of tolerance, a non-tolerant counterpart is a necessary control; however, directly related "wildtype" non-tolerant strains are not available for comparison in the clinical setting. To account for the diversity observed among clinical isolates from different backgrounds, five common non-tolerant model strains were used as benchmarks. These strains represent a range of different lineages, clonal complexes, and sequence types of *S. aureus*. In agreement with the criteria defined above, Newman WT, MW2, N315, USA300, and LAC exhibited relative luminescence values below 1.0 after 90 minutes (Figure 16). Data for three known tolerant strains are also shown for reference.

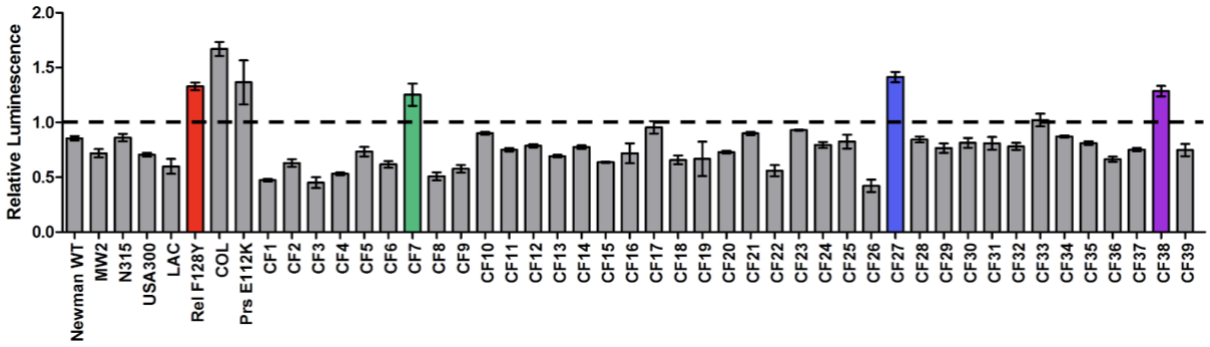

Figure 16. Prevalence of tolerance among *S. aureus* isolates from CF lung infections at RJH. CF7, CF27 and CF38 exhibit a tolerance phenotype with relative luminescence values greater than 1.0 after 90 minutes of vancomycin exposure. Newman WT, MW2, N315, USA300 and LAC are non-tolerant strains. Rel F128Y, COL and Prs E112K are tolerant strains. Data shown are the mean of three replicates and error bars represent the SEM.

Three clinical isolates (CF7, CF27, and CF38) were identified as tolerant, with relative luminescence values greater than 1.0 after 90 minutes (Figure 16). An additional three isolates (CF10, CF17, and CF33) appeared to be on the borderline with relative luminescence values nearing 1.0 after 90 minutes. To further investigate the phenotype of these six isolates, MIC determinations were performed. Multiple antibiotics were used to assess their general susceptibility profiles. All six isolates exhibited a low flucloxacillin MIC, suggesting that they are strains of MSSA (Table 8). Five of the isolates exhibited ciprofloxacin MICs below or equal to the susceptibility breakpoint of 1.0 μg/mL, suggesting that they are susceptible. CF33 exhibited an MIC above the resistance breakpoint, suggesting that it is resistant to ciprofloxacin. To further assess susceptibility in CF7, CF27, and CF38, MIC determinations were performed with daptomycin and vancomycin. All three isolates exhibited daptomycin MICs below the susceptibility breakpoint of 1.0 μg/mL, suggesting that they are susceptible to daptomycin. All three isolates also exhibited vancomycin MICs below or equal to the susceptibility breakpoint of 2.0 μg/mL, suggesting that they are susceptible to vancomycin.

Table 8. Broth microdilution MIC values for CF7, CF10, CF17, CF27, CF33 and CF38

Strain	Flucloxacillin MIC (μg/mL)	Ciprofloxacin MIC (μg/mL)	Daptomycin MIC (μg/mL)	Vancomycin MIC (μg/mL)
Newman WT	0.125	0.5	1.0	2.0
CF7	0.25	0.25	0.25	0.25
CF10	0.25	0.5	nd	nd
CF17	0.25	1.0	nd	nd
CF27	0.5	0.5	0.25	1.0
CF33	0.25	2.0	nd	nd
CF38	0.25	1.0	0.5	2.0

Abbreviations: Not determined (nd)

To validate the tolerance phenotypes exhibited by these isolates in the ATP-based assay, time-kill assays were performed. Given the widespread susceptibility to flucloxacillin demonstrated by MIC determinations, flucloxacillin was used for initial investigation. CF7 and CF27 demonstrated tolerance to flucloxacillin with a 1.4-fold increase in MDK_{99} compared to wildtype Newman (Figure 17A and B). These two CF isolates exhibited a lower degree of tolerance compared to the F128Y mutant, suggesting that the ATP-based tolerance assay is capable of detecting low-level tolerance. Interestingly, CF38 was not tolerant to flucloxacillin, with no change in MDK_{99} compared to wildtype Newman (Figure 17A and B). This was unexpected as CF38 exhibited a relative luminescence value well above the threshold for tolerance in the ATP-based assay. Flucloxacillin exerted full bactericidal activity against CF10, CF17, and CF33 as all three isolates exhibited a similar or reduced MDK_{99} compared to wildtype Newman (Figure 17C and D). In the ATP-based assay, these three non-tolerant isolates exhibited relative luminescence values slightly less than or equal to 1.0 after 90 minutes of vancomycin exposure, suggesting that the threshold established in the ATP-based assay is a reliable indicator of tolerance.

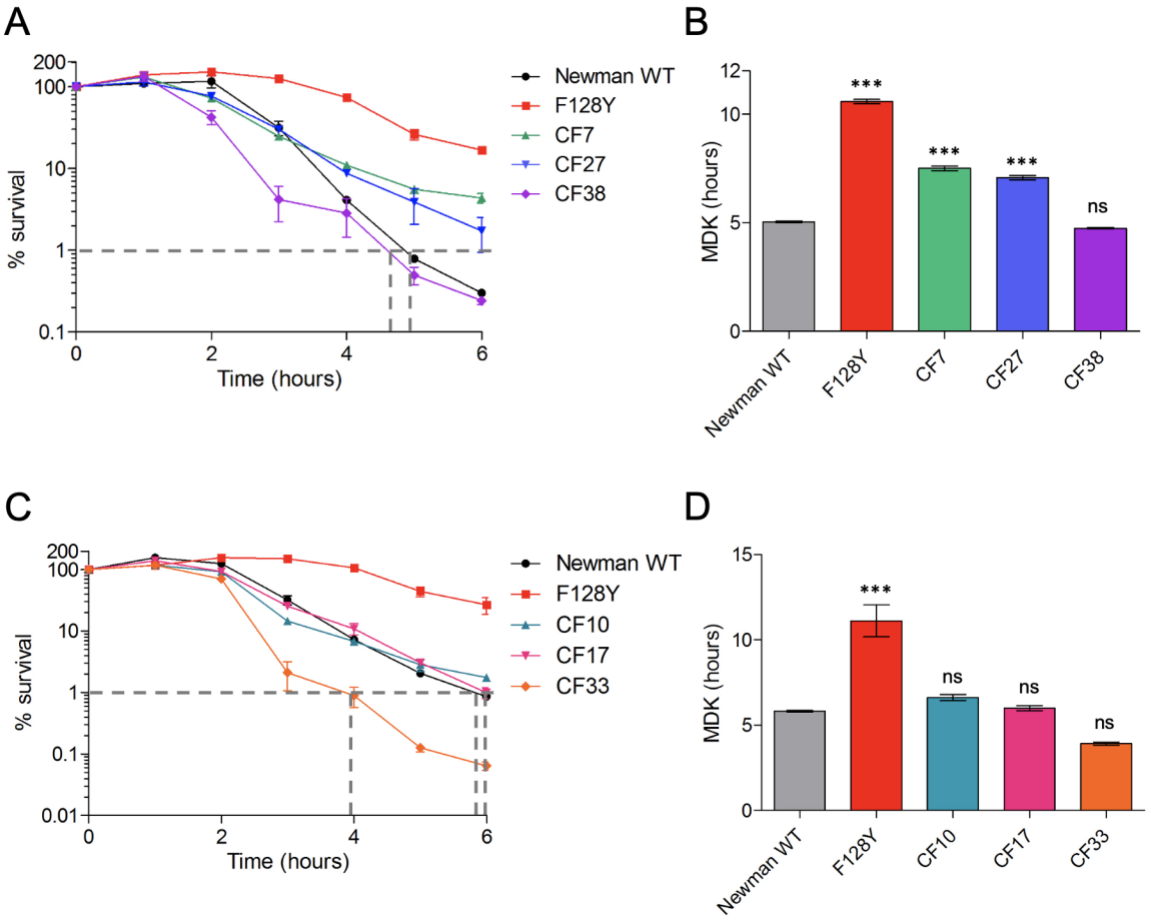

Figure 17. CF isolates exhibit tolerance to flucloxacillin. (A and C) Wildtype Newman, Newman Rel F128Y, CF7, CF10, CF17, CF27, CF33, and CF38 were exposed to flucloxacillin at 4 × MIC and viable counts were determined at intervals. Dashed gray lines indicate the approximate minimum duration for killing (MDK) 99% of the population for each strain. (B and D) MDK values interpolated from the time-kill data. Data shown are the mean of three replicates and error bars represent the SEM. Asterisks indicate a statistically significant difference from the wildtype strain as determined by one-way ANOVA with Tukey's multiple comparisons test (*, ** and *** indicate p = 0.01 to 0.05, 0.001 to 0.01 and < 0.001, respectively; ns, not significant).

Previous work suggests that tolerance provides protection against multiple classes of antibiotic[88]. To investigate multidrug tolerance in CF7, CF27, and CF38, time-kill assays were performed with daptomycin. CF7 exhibited high-level daptomycin tolerance with a 2.1-fold increase in $MDK_{99.9}$ compared to wildtype Newman (Figure 18A and B). Notably, CF7 also demonstrated a slightly higher degree of tolerance compared with the F128Y mutant. Although CF27 exhibited tolerance to flucloxacillin, no statistically significant difference in $MDK_{99.9}$ values was observed between CF27 and wildtype Newman for daptomycin (Figure 18A

and B). Interestingly, CF38 was not tolerant to flucloxacillin; however, high-level daptomycin tolerance was exhibited with a 2.0-fold increase in $MDK_{99.9}$ compared to wildtype Newman (Figure 18A and B).

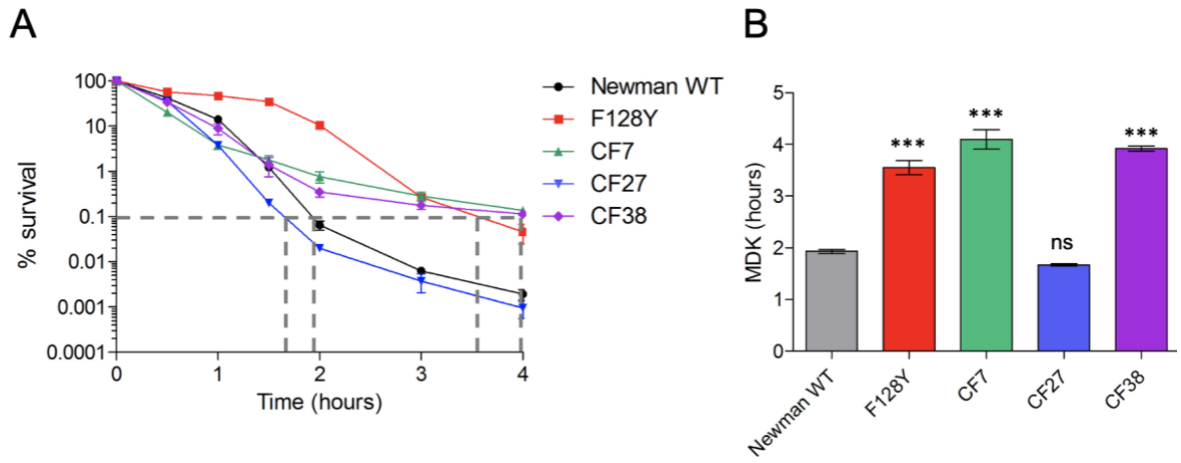

Figure 18. CF isolates exhibit tolerance to daptomycin. (A) Wildtype Newman, Newman Rel F128Y, CF7, CF27, and CF38 were exposed to daptomycin at 4 × MIC. Viable counts were determined at intervals. Dashed gray lines indicate the approximate minimum duration for killing (MDK) 99.9% of the population for each strain. (B) MDK values interpolated from the time-kill data. Data shown are the mean of three replicates and error bars represent the SEM. Asterisks indicate a statistically significant difference from the wildtype strain as stated in Figure 17.

The ATP-based screen suggests that CF7, CF27, and CF38 are tolerant to vancomycin. To further investigate vancomycin tolerance, time-kill assays were performed with the same concentration of vancomycin used in the ATP-based assay. Vancomycin is a concentration-independent antibiotic (also referred to as time-dependent); therefore, increasing the concentration above the MIC will not increase the rate of bactericidal activity[204]. Surprisingly, vancomycin remained fully effective against all three clinical isolates, with no increase in MDK_{90} (Figure 19A and B). The F128Y mutant exhibited tolerance to vancomycin with a 1.9-fold increase in MDK_{90} compared to wildtype Newman, suggesting that the time-kill assay is capable of detecting vancomycin tolerance.

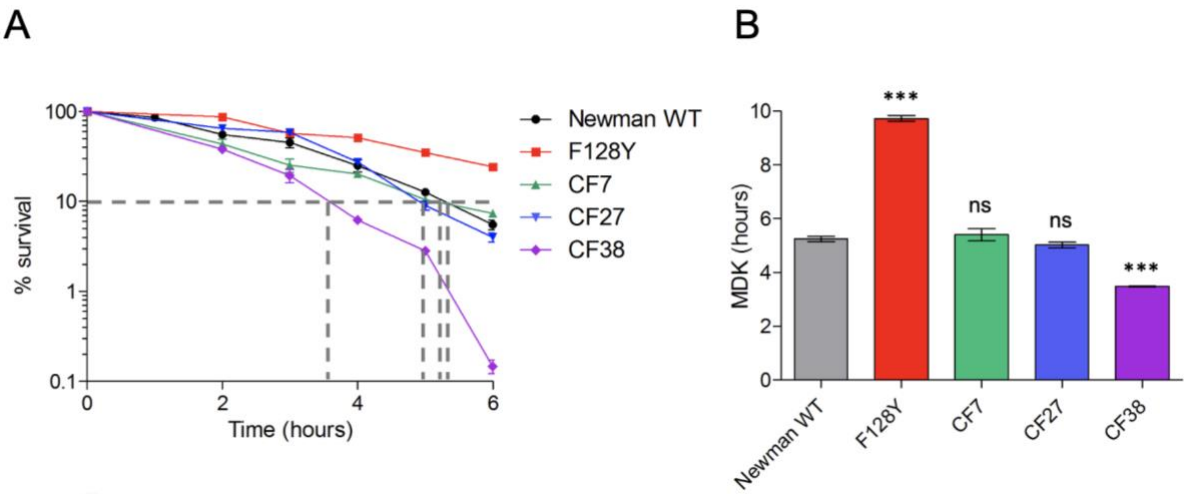

Figure 19. CF isolates do not exhibit tolerance to vancomycin in a time-kill assay. (A) Wildtype Newman, Newman Rel F128Y, CF7, CF27, and CF38 were exposed to vancomycin at 20 μg/mL (~10 × MIC). Viable counts were determined at intervals. Dashed gray lines indicate the approximate minimum duration for killing (MDK) 90% of the population for each strain. (B) MDK values interpolated from the time-kill data. Data shown are the mean of three replicates and error bars represent the SEM. Asterisks indicate a statistically significant difference from the wildtype strain as stated in Figure 17.

Taken together, the flucloxacillin and daptomycin time-kill assays demonstrate that the clinical isolates identified by the ATP-based screen are each tolerant to at least one antibiotic. In the ATP-based assay, CF7, CF27, and CF38 exhibited a reduced rate of killing by vancomycin that is indicative of tolerance. However, none of these isolates appeared tolerant to the same concentration of vancomycin in a time-kill assay. While these contradictory results could suggest a fault in the ATP-based assay, time-kill assays with flucloxacillin and daptomycin suggest that these three isolates are indeed tolerant. Furthermore, the relationship between cellular ATP content and viability is well-established[197,198]. In the traditional time-kill assay, viability is defined as the ability of a cell to form a detectable colony on agar. Although culture samples are removed and immediately plated onto agar following dilution, visible colonies are detected ~16 hours later. Therefore, it is possible that a cell was viable when the culture sample was removed, but the cell did not survive on agar to form a visible colony. Bound vancomycin molecules that were retained following dilution could be carried onto the agar, resulting in cell death. In contrast, the ATP-based assay provides a real-time measurement of viability. ATP content serves as an indicator of metabolic activity that enables precise timing of cell death. Real-time measurements of ATP content are more sensitive than viable count

determinations; therefore, the ATP-based assay can likely detect low-level tolerance that is overlooked by agar-based detection methods. Interestingly, the F128Y mutant exhibited vancomycin tolerance in a time-kill assay. Among the four tolerant isolates, the F128Y mutant is the only strain with SR activation due to a Rel mutation. SR-mediated tolerance provides protection against a broad range of antibiotics, whereas the mechanisms of tolerance in CF7, CF27, and CF38 appear to be more specific. Time-kill assays may be sufficient for the detection of robust tolerance phenotypes; however, ATP measurements are more sensitive to subtle metabolic changes that occur with antibiotic exposure.

4.2.3 Preliminary investigation into the tolerance phenotypes of *S. aureus* isolates derived from CF lung infections

SR activation has been shown to confer tolerance and Rel mutations appear to be common among clinical isolates. To determine whether CF7, CF27, and CF38 carry Rel mutations, the full length *rel* gene was sequenced from each isolate. Interestingly, sequencing did not reveal any nonsynonymous mutations in CF7, CF27 or CF38. Tolerance is often associated with severe growth defects; therefore, growth phenotypes of the three isolates were characterized in a rich medium. CF7 exhibited a significant growth defect compared to wildtype Newman, with a ~1.5-fold increase in doubling time and a ~1.9-fold increase in lag time (Figure 20). Compared to wildtype Newman, CF27 and CF38 did not exhibit a significant difference in doubling time or lag time (Figure 20). It is important to note that growth phenotypes were compared to a single wildtype strain and Newman may not be the most appropriate non-tolerant reference for the CF isolates. Whole genome sequencing of the CF isolates is required to determine which non-tolerant model strain has the closest genetic background. Nonetheless, it is notable that CF7 is the only isolate of the three that exhibited a growth defect, and it is the only isolate that demonstrated tolerance toward flucloxacillin and daptomycin in time-kill assays.

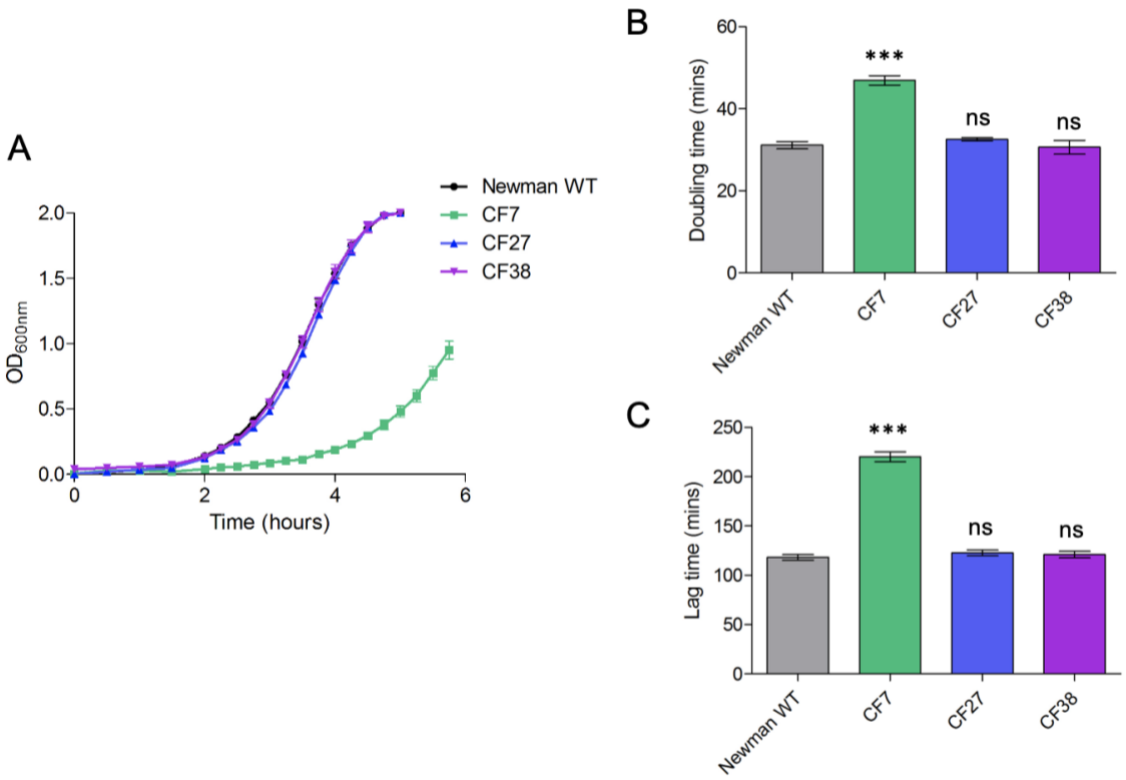

Figure 20. Growth phenotypes of tolerant *S. aureus* isolates derived from CF lung infections. (A) Growth curves of wildtype Newman, CF7, CF27, and CF38 in TSB. (B and C) Doubling times (B) and lag times (C) derived from the growth curves in panel A. Data shown are the mean of four replicates and error bars represent the SEM. Asterisks indicate a statistically significant difference from the wildtype strain as stated in Figure 17.

4.2.4 Cellular pathways that are potentially involved in the tolerance phenotypes of *S. aureus* isolates derived from CF lung infections

Several different cellular pathways have been implicated in tolerance (Table 1). Depending on the mechanism, tolerance can provide protection against multiple classes of antibiotic, or be specific to one antibiotic. A recent study demonstrated that nutrient deprivation induced the SR, leading to multidrug tolerance[205]. In contrast, impaired electron transport conferred tolerance to aminoglycosides as they require proton motive force for internalization, but not to antibiotics that act extracellularly[205]. In this pilot study presented here, each of the three tolerant isolates exhibited a different phenotype, suggesting that the mechanism of tolerance is different in each isolate (Table 9).

Table 9. Summary of tolerant *S. aureus* isolates derived from CF lung infections

Phenotype	CF7	CF27	CF38
Tolerant to FLC	Yes	Yes	No
Tolerant to DAP	Yes	No	Yes
Growth defects present	Yes	No	No

Previous studies have demonstrated that tolerance can reduce the efficacy of multiple classes of antibiotic[88]. In time-kills assays, CF7 exhibited high-level tolerance to flucloxacillin and daptomycin. CF7 also demonstrated tolerance to vancomycin in the ATP-based assay. Evidence suggests that multidrug tolerance occurs as a result of slow growth and reduced metabolism. As most antibiotics target active metabolic processes, slowing down these processes can confer protection to a broad range of antibiotics. Several different pathways have been associated with multidrug tolerance, including the SR, tricarboxylic acid cycle, purine biosynthesis, transcription, translation, and ETC[88,111,113]. SR-activating Rel mutations have been shown to confer multidrug tolerance as a result of (p)ppGpp-mediated growth defects[88]. The severe growth defects in CF7 suggest that a metabolic pathway is implicated in tolerance. Although CF7 did not carry Rel mutations, mutations in the wider SR network could be involved in tolerance. There are a number of different mechanisms through which indirect SR induction could occur. Reduced tRNA synthetase activity can lead to the presence of uncharged tRNAs and SR activation. Nonsynonymous mutations in the *ileS* gene encoding isoleucyl-tRNA synthetase have been shown to confer tolerance to vancomycin, daptomycin and a β-lactam[120]. Like CF7, *ileS* mutants exhibited slow growth. Downstream of Rel, GTPases are critical in facilitating the cellular effects of SR activation. Upon SR activation, (p)ppGpp binds to the GTPase RsgA, thereby inhibiting its activity and disrupting ribosome assembly. Deletion of *rsgA* or inactivation of its GTPase activity has been shown to mimic the effects of (p)ppGpp in *S. aureus*[115]. Both *rsgA* mutants demonstrated growth defects that conferred tolerance to β-lactams, vancomycin, and ciprofloxacin. In addition to SR activation, defects in the TCA cycle and ETC have been associated with multidrug tolerance[111,113]. Among clinical isolates derived from MRSA persistent bacteremia, a significant enrichment of nonsynonymous mutations was found in genes involved in the TCA cycle (*citZ* and *odhA*)[111]. Interestingly, tolerant *citZ* mutants do not exhibit overt growth defects[111]. In contrast, defects in the ETC through

mutations in *hemB* and *menD* are associated with an SCV phenotype that confers multidrug tolerance[113]. While these examples highlight pathways that are frequently implicated in tolerance, it is important to note that a vast number of genes could be responsible for the tolerance phenotype of CF7. Given the number of genes that have been linked to multidrug tolerance, whole genome sequencing is needed to identify putative mutation(s) conferring the tolerance phenotype in CF7.

In time-kill assays, CF27 demonstrated tolerance toward flucloxacillin, but not to daptomycin. The ATP-based assay suggested that CF27 is also tolerant to vancomycin. Although tolerance generally provides protection against killing by nearly all classes of antibiotic, recent work suggests that antibiotic efficacy is dependent on the mechanism of tolerance[205]. Interestingly, CF27 did not exhibit significant changes in doubling time and lag time compared to wildtype Newman. The absence of growth defects suggests that the mechanism of tolerance in CF27 could be specific to one cellular pathway that is targeted by antibiotics. Flucloxacillin and vancomycin both target cell wall synthesis, whereas daptomycin targets the cytoplasmic membrane. Flucloxacillin interferes with cell wall synthesis by binding to and inactivating PBPs. This prevents cross-linking of peptidoglycan, leading to weakening of the cell wall and eventually lysis. Vancomycin has similar effects as a result of binding to the terminal residues on nascent peptidoglycan chains. In contrast, daptomycin complexes with calcium and forms micelles that are inserted in the membrane. Aggregates of daptomycin form pores in the membrane, resulting in ion leakage and subsequent membrane depolarization, as well as interfering with membrane-bound enzymes and processes. Tolerance to β-lactams and vancomycin, but not to daptomycin, has been reported in a Δ*gdpP* mutant of *S. aureus*[206]. GdpP is a phosphodiesterase that helps maintain intracellular levels of the second messenger cyclic di-AMP (CDA)[207]. CDA is essential for a variety of processes, including peptidoglycan metabolism and osmoregulation[208]. In a Δ*gdpP* mutant, increased CDA levels resulted in increased peptidoglycan cross-linking and a concurrent decrease in the amount of monomeric muropeptides, leading to tolerance to cell-wall targeting antibiotics[207]. Increased peptidoglycan cross-linking likely contributes to tolerance by reducing the number of monomeric targets for vancomycin. Furthermore, increased cross-linking strengthens the cell wall and could make PBPs less accessible to flucloxacillin.

Recent clinical surveillance studies have identified a number of inactivating truncation mutations in *gdpP*[209,210]. The β-lactam/glycopeptide tolerance phenotype of Δ*gdpP* mutants is strikingly similar to the tolerance phenotype of CF27. However, unlike CF27, Δ*gdpP* mutants have been shown to exhibit significant growth defects[211]. Interestingly, both reduced and elevated CDA levels have been reported to inhibit growth, but only the latter is associated with tolerance[208]. This suggests that the mechanism of tolerance is related to increased CDA levels, not slow growth. Elevated levels of CDA are one possible explanation for β-lactam/glycopeptide tolerance in CF27. The number of *S. aureus* proteins involved in CDA production and hydrolysis remains unknown; therefore, whole genome sequencing will be critical to uncovering the underlying mechanism of tolerance in CF27.

In contrast to CF27, CF38 demonstrated tolerance to daptomycin, but not to flucloxacillin in time-kill assays. CF38 also appeared tolerant to vancomycin in the ATP-based assay. Compared to wildtype Newman, CF38 did not exhibit any significant changes in doubling time or lag time. Taken together, this suggests that the mechanism of tolerance in CF38 could provide protection against antibiotics that target a specific cellular process (or processes). Although tolerance is frequently associated with slow growth and reduced metabolism, daptomycin remains effective against non-replicating bacteria[212]. This suggests that the mechanism of tolerance towards daptomycin is quite complex. Previous studies have shown that activation of the GraRS two-component signaling system is partially responsible for daptomycin tolerance[213]. GraRS activation resulted in increased accumulation of peptidoglycan due to a reduction in cell wall degradation. Furthermore, a GraRS-independent increase in cardiolipin in the cell membrane contributed to tolerance. Notably, increased peptidoglycan and cardiolipin content conferred tolerance to daptomycin and vancomycin, as observed with CF38. Increased abundance of peptidoglycan may hinder access to the membrane targets of daptomycin. Furthermore, increased cardiolipin content in the membrane could reduce the bactericidal activity of daptomycin that did gain access by preventing translocation and pore formation[214]. Interestingly, thickened cell walls are a common feature of VISA strains that frequently exhibit decreased susceptibility to daptomycin[215]. Given the large size of vancomycin, the thickened cell wall traps vancomycin at the periphery of the cell and blocks access[216]. Although vancomycin and flucloxacillin have similar cellular targets,

flucloxacillin is significantly smaller in size. Previous studies have shown that β-lactams remain effective against VISA strains with thickened cell walls; therefore, it is possible that flucloxacillin molecules are small enough to access their targets[217]. A mutation in the GraRS system was identified in a clinical isolate of *S. aureus* derived from persistent bacteremia that did not resolve with vancomycin treatment[218]. It is possible that CF38 carries one or more mutations in the GraRS signaling pathway leading to daptomycin and glycopeptide tolerance. However, a number of genes are involved in cell wall and cell membrane regulation, thus emphasizing the need for whole genome sequencing of tolerant clinical isolates.

4.2.4 Conclusions and future directions

Tolerance is an underappreciated contributor to treatment failures as it is not routinely detected in the clinical setting. Following validation with time-kill assays, tolerance was identified in 3/39 (~7.7%) *S. aureus* isolates derived from CF lung infections at RJH. It is possible that clinical isolates in the library may have been derived from the same patient. While the total number of source patients is unknown, chart reviews revealed that CF7, CF27, and CF38 were each linked to a different patient. This suggests that the prevalence of tolerance among cases of *S. aureus* CF lung infections is quite significant. Furthermore, this library of isolates represents all available *S. aureus* isolates from CF lung infections at Royal Jubilee Hospital for a given time period. The prevalence of tolerance would likely increase with criteria such as recurrence or persistence (*i.e.* positive culture after five days of appropriate antibiotic therapy). In addition to *S. aureus*, tolerance has been identified in other pathogenic bacteria, including *E. coli*, enterococci, and streptococci. Future work will involve modifying the ATP-based assay to detect tolerance in other organisms. Furthermore, screening clinical isolates from other types of persistent infections such as bacteremia and infective endocarditis will provide a wider assessment of the prevalence of tolerance.

Real-time detection of tolerance in the ATP-based assay is a tremendous advantage compared to the traditional time-kill assay and the recently developed TDtest. The ATP-based assay provides same-day results, whereas agar-based detection methods require an additional ~18 hours. Rapid detection of tolerance is necessary in cases of life-threatening infections that require immediate antibiotic administration. The ATP-based assay could easily be miniaturized

for high-throughput and could be adapted for use in the clinical setting. Currently, optimization of the assay protocol is limited by the small number of known tolerant mutants available. While a number of tolerance-conferring mutations have been reported in the literature, many of these mutations are in essential genes. Genetic manipulation of essential genes is a challenging and time-consuming process. Future work will involve both genetically engineering and/or sourcing more known tolerant strains based on the literature available, and screening larger libraries of clinical isolates from additional types of infections such as persistent/relapsing bacteremia. Both of these approaches will inform further optimization of the assay protocol. Tolerant isolates identified with the assay will also be used for further investigation into the underlying mechanisms of tolerance. Based on the pilot study data, the prevalence of tolerance among clinical isolates of *S. aureus* is likely quite significant. The ATP-based tolerance screen is a powerful tool that could be used to provide the first real assessment of the prevalence of tolerance in the clinical setting. The first step in addressing the threat posed by tolerance is understanding the magnitude of the problem. Additionally, previous work has demonstrated that some antibiotics remain effective against tolerant bacteria in time-kill assays. However, without detection of tolerance, it is unlikely that these antibiotics would be prescribed as they are not considered to be first-line treatment. Detection of tolerance will inform antibiotic prescribing practices and improve the treatment of persistent infections. Investigation of the molecular basis of tolerance in clinical isolates could also lead to the development of novel inhibitors to mitigate the effects of tolerance. Ultimately, the work presented in this thesis chapter will improve the diagnosis and treatment of persistent *S. aureus* infections.

CHAPTER 5

Conclusions and future directions

Antibiotic evasion strategies such as resistance and tolerance play a major role in the global burden of bacterial infections. Both of these antibiotic escape strategies are associated with higher healthcare costs, longer hospital stays, and higher mortality rates[19,20,38,219]. Given the lack of new antibiotics coming into use, it is critical to address these threats to antibiotic efficacy and preserve the use of existing antibiotics. The SR has recently emerged as a clinically-relevant mechanism implicated in both resistance and tolerance[88,137,142,143]. Rel mutations promote the dissemination of multidrug resistance plasmids and represent an important mechanism of tolerance. As recent studies have suggested that nearly all staphylococcal plasmids can potentially be mobilized, SR activation has significant consequences for HGT[76]. In addition to carrying resistance genes, plasmids often carry virulence factors that will also be mobilized at a higher rate. Increased plasmid copy number could also increase the dissemination of resistance genes *via* phage transduction; therefore, SR activation likely contributes to increased pathogenicity through both of the main mechanisms of HGT in *S. aureus*. Previous work has demonstrated that clinical Rel mutations also lead to a higher level of β-lactam resistance and a more homogenous resistance expression profile[137]. In the clinical setting, Rel mutations could lead to the evolution of highly virulent strains of *S. aureus* that are resistant to multiple classes of antibiotic. Multidrug resistance limits treatment options and often requires the use of last-resort antibiotics such as vancomycin. While this study focused on Rel mutations, other mutations that indirectly activate the SR may be expected to have similar effects on plasmid donation. SR-activating mutations in tRNA synthetases have been associated with elevated cellular (p)ppGpp levels and could also increase the frequency of plasmid donation.

In addition to resistance, the SR-activating mutations have been linked to multidrug tolerance. In this study, the Rel mutant strains generated by Bryson *et al.* were used to develop a screen to detect genotypic tolerance based on cellular ATP content. Compared to alternative agar-based detection methods, this screen enables rapid detection of tolerance without a labour-intensive protocol. Although validation with additional strains is needed, preliminary results suggest that detection of cellular ATP content could be a more sensitive measure of viability

compared to plating for colony-forming units. Detection of ATP content measures viability in real time, whereas plating for colony-forming units measures the ability of a cell to form a detectable colony on agar after ~16 hours. The high sensitivity of the ATP-based assay could enable identification of low-level tolerance that would be undetected in the traditional time-kill assay. Pilot study data indicated that tolerance is relatively common among isolates of *S. aureus* derived from CF lung infections. Assessing the prevalence of tolerance among additional types of persistent infections, such as bacteremia, will highlight the magnitude of the problem and identify tolerant isolates for further investigation. SR-activating mutations represent an important mechanism of tolerance with coincidental consequences for resistance; however, the SR is not the only mechanism of tolerance. The effects of tolerance appear to be dependent on the mechanism; therefore, more work is needed to understand the potential implications associated with a wide range of tolerance-conferring mutations.

Implementation of the ATP-based assay in the clinical setting would provide physicians with a powerful tool to inform antibiotic prescribing practices when tolerance is suspected. Time-kill assays have shown that some antibiotics retain full bactericidal activity against tolerant bacteria. Furthermore, extending the course of antibiotic treatment may be an effective strategy against tolerant isolates. Recent coculture experiments demonstrated that in the absence of antibiotic *in vitro*, tolerant Rel mutants are outcompeted by the wildtype strain due to their growth defects[137]. However, tolerant Rel mutants have a significant fitness advantage in the presence of antibiotic. During an infection, cyclic antibiotic exposure would be expected to temporally favour the growth of the tolerant or wildtype strain with supra-MICs favouring the tolerant mutant and sub-MICs favouring the wildtype strain. Although these experiments provided insight into the complex implications of clinical Rel mutations, they do not accurately model the clinical situation. Additionally, Rel mutations only represent one mechanism of tolerance. The fitness relationship between wildtype and tolerant strains is likely dependent on the mechanism of tolerance and the host environment. An important step in assessing the clinical consequences of tolerance will be examination of tolerant mutants in *in vivo* models of infection. Future work will compare the ability of tolerant mutants and wildtype strains to cause infections, and the subsequent response to antibiotic treatment. Antibiotic regimens will be given in-line with current clinical recommendations in order to determine which antibiotics are

the most/least effective against tolerant strains. Studies using *in vivo* models of infection will also determine the extent of antibiotic exposure necessary to achieve the same level of killing of tolerant strains compared to wildtype strains. This will provide physicians with valuable information on how to effectively treat tolerant bacteria with existing antibiotics.

The data presented in this thesis demonstrates the broad consequences of SR activation and shows that the SR is a clinically-relevant model system for studying antibiotic evasion strategies. Given the apparent prevalence of SR activation among clinical isolates of *S. aureus*, it is critical to better understand the role of the SR in resistance and tolerance. Despite increased investments to stimulate the research and development pipeline globally, few novel antibiotics have been approved for clinical use. The World Health Organization reported that between 2017 and 2021, only 12 new antibiotics were approved for clinical development[220]. Of the 12 antibiotics, 10 belong to existing classes of antibiotic for which there are well-established mechanisms of resistance. This provides a rationale for the development of SR inhibitors that could limit the dissemination of resistance and reverse the effects of tolerance. Ultimately, this study demonstrates the multifaceted implications of clinical Rel mutations and highlights the SR as a useful model system for studying threats to antibiotic efficacy.

CHAPTER 6

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