Mechanisms of *Staphylococcus aureus* survival of trimethoprim-sulfamethoxazole-induced thymineless death

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Abstract

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Trimethoprim-sulfamethoxazole (SXT) is commonly used to treat diverse *Staphylococcus aureus* infections, including those associated with cystic fibrosis (CF) pulmonary disease. Studies with *Escherichia coli* found that SXT impairs tetrahydrofolate production, leading to DNA damage, stress response induction, and accumulation of reactive oxygen species (ROS) in a process known as thymineless death (TLD). TLD survival can occur through uptake of exogenous thymidine, countering the effects of SXT; however, a growing body of research has implicated central metabolism as another potentially important determinant of bacterial survival of SXT and other antibiotics. Here, we conducted studies to better understand the mechanisms of TLD survival in *S. aureus*. We found that thymidine abundances in CF sputum were insufficient to prevent TLD of *S. aureus*, highlighting the importance of alternative survival mechanisms *in vivo*. In *S. aureus* cultured *in vitro* with SXT and low thymidine, we frequently identified adaptive mutations in genes encoding carbohydrate, nucleotide, and amino acid metabolism, supporting reduced metabolism as a common survival mechanism. Although intracellular ROS levels rose with SXT treatment *in vitro*, survival was not improved in the presence of ROS scavengers, unlike in *E. coli*. SXT challenge induced the SOS response, which was alleviated by added thymidine. Lastly, an inactivating mutation in the phosphotransferase gene *ptsI* conferred both limitation in cellular ATP and improved survival against TLD. Collectively, these results suggest that alterations in core metabolic functions, particularly those that reduce ATP levels, predominantly confer *S. aureus* survival and persistence during SXT treatment, potentially identifying novel targets for co-treatment.

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In loving memory of Gene Podrazik and Usha Pereira, who were unable to see the completion of this journey.

I hope I have made you proud.

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CHAPTER 1: INTRODUCTION

How does Staphylococcus aureus survive antibiotic and nutritional pressures that promote thymineless death?

Staphylococcus aureus biology, pathogenesis, and clinical significance as a human commensal and pathogen

The genus *Staphylococcus* consists of over 30 species (and additional subspecies) of Gram-positive cocci that are both commensals and important pathogens of humans and other mammals[1]. Typically 0.5-1.5μm in diameter, cells of this organism usually occur as individuals or in pairs, short chains, or grape-like clusters. While primarily found in the anterior nares as a facultative anaerobe, because of *S. aureus*' atmospheric adaptability[2], *Staphylococcal* spp. have been cultured from other areas of the human body such as the skin and the mucosal membranes of the throat and intestines[1,3], though evidence suggests its presence these sites may be secondary to its occurrence in the nares[4,5]. Many *Staphylococcus* species are constituents of both the environment and the normal human flora, but several members of this genus can cause infections in humans, such as *Staphylococcus capitis*, *S. warneri, S. lugdunesis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. intermedius*, *S. schleiferi*, and *S. aureus*; the most common and most important pathogenic *Staphylococcus* sp. in humans is *S. aureus*[3,6].

The human nose is the primary reservoir for *S. aureus*[2,4,5]. It's estimated that *S. aureus* asymptomatically and intermittently colonizes at least 60% of the human population, with 20-30% of humans considered persistently colonized. Colonization coincides with an upregulation in the expression of *S. aureus* genes that encode for adhesion, nutrient uptake, and biosynthetic metabolic pathways, and a concurrent downregulation in expression of toxin- and protease-encoding genes[4,7]. The factors that dictate whether an person can be persistently colonized remain relatively unexplored; some studies suggest colonization of certain areas of the body is temporary, with persistent cell types, such as small colony variants (SCVs) and biofilm-capable cells, potentially facilitating colonization[4,7]. Of course, *S. aureus* does not colonize these surfaces in isolation, and the nasal microbiota are thought to play important roles in *S. aureus* colonization. The nasal microbiota consists of diverse organisms that can vary between people, like other microbial communities within the human body[6]. In the nares of healthy people, the dominant phyla are Actinobacteria (*Corynebacterium* spp., *Propionibacterium* spp.) and Firmicutes (*Staphylococcus* spp.), while less abundant community members come from the phyla Proteobacteria, Bacteroides, Fusobacteria, and Deinococcus[6,8]. Community-related determinants of *S. aureus*' presence in the host include potential symbiosis with other community members, competition for adhesion sites, nutrient availability, the presence of antimicrobial molecules, and antagonistic activation of host defenses, highlighting *S. aureus*' inherent need for adaptability in the face of external stressors[6,9].

S. aureus nasal colonization is a risk factor for subsequent *S. aureus* infections, often with same colonizing strain[10]. Carriage of methicillin-resistant *S. aureus* (MRSA), which is resistant to certain broad-spectrum antibiotics, is especially worrisome, though the extent to which MRSA is carried in the population varies widely[4,11–13]. While colonization may be harmless, or at least controlled, in most people, like many major bacterial pathogens, *S. aureus* can be invasive when epithelial barriers or the immune system is compromised. These infections can range from mild

and easily treatable skin and soft tissue infections to systemic infections such as bacteremia, endocarditis, osteomyelitis, and pneumonia, in addition to less common but equally serious infections like meningitis, toxic shock syndrome, foodborne illness, and urinary tract infections[14,15]. Certain human populations are at higher risk of these infections than others, such as those with chronic illnesses, compromised immune systems, cystic fibrosis (CF), traumatic injuries, and individuals undergoing surgical procedures[3,14]. The infections caused by *S. aureus* can vary in severity, highlighting the versatility of *S. aureus* as a pathogen.

This duality—commensal and pathogen—is a contributing factor to *S. aureus*' status as a major global pathogen. *S. aureus* is recognized as one of the most devastating human pathogens. Pre-antibiotics, bacteremia caused by *S. aureus* had an 80% mortality rate, a rate that is substantially lower today[16]. In the United States, the incidence of *S. aureus* bloodstream infections has been declining, with mortality rates at 18%, only representing a portion of the *S. aureus* infections that occur annually[17]. However, *S. aureus* remains a leading pathogen in over 100 countries, surpassing other prominent pathogens like *Escherichia coli*, *Streptococcus pneumoniae*, and *Klebsiella pneumonia*, and is associated with more than one million deaths annually. In 2019, *S. aureus* caused over 500,000 deaths due to lower respiratory and related thorax infections worldwide, second only to *Streptococcus pneumoniae*, while being the leading infectious cause of fatal bloodstream infections, underscoring the continued global burden of *S. aureus*[14,18].

From colonizer to pathogen: The diverse virulence factors of *S. aureus*

S. aureus is a well-equipped pathogen that possesses ample machinery and compounds that allow for efficient colonization, invasion, and self-preservation when the host epithelial barrier is compromised. The cell wall and presence of a capsule both facilitate survival for *S. aureus*. While the cell wall is typically comprised of peptidoglycan and teichoic acid, many *S. aureus* strains also contain protein A (encoded by the *spa* gene), a surface protein that assists immune evasion through the suppression of immune cells[16,19]. The capsule serves as a further protective measure: the extracellular polysaccharides that comprise the capsule mask the bacterial cell during infection and can prevent phagocytosis[20]. In addition to the capsule and cell wall characteristics, *S. aureus* produces a variety of extracellular enzymes and toxins. Cytotoxins produced by *S. aureus*, such as the well-described proinflammatory, pore-forming toxins α-hemolysin and ɣ-hemolysin, cell membrane-damaging toxin β-hemolysin, and enterotoxins (the latter a responsible for toxic shock syndrome and food poisoning) facilitate spread within infected tissues[10,21]; *S. aureus*-produced proteases contribute to cell lysis and inflammation of the epidermis[22]. In some cases, *S. aureus* virulence factors protect bacterial cells rather than target the host. For example, *S. aureus*-produced staphyloxanthin—a pigment responsible for the stereotypical golden hue of *S. aureus* colonies—plays an important role in the scavenging of host-produced reactive oxygen species (ROS), while coagulases aid in coating the cells with cleaved fibrin to allow for immune evasion[22–25].

S. aureus has developed a complex regulatory network consisting of genes that coordinate expression of this array of virulence factors[10,26]. While not essential for growth, these global regulatory genes are important for the niche adaptation *S. aureus* is known for. These regulatory systems include the extensively-studied gene *agr* quorum-sensing system (a master regulator for virulence factors that plays a more specific role in cell-to-cell communication, adhesion, and expression of exotoxin and enzymes), several two-component systems (SaeRS, SrrAB, and ArlRS; responsible for the expression of exoproteins, low-oxygen virulence factors, and extracellular proteolytic activity, respectively), as well as the SarA protein family (global regulators that can modulate *agr*, post-transcriptional functions, and protease and toxin production). The stage of growth of the organism, along with host and environment cues, greatly influence the expression of these proteins. For example, expression of *agr* and its regulated surface proteins largely occurs during the exponential growth phase of the cell populations while secreted proteins under *agr* regulation are expressed during stationary phase. Thus, during initial stages of infection, when immune escape and successful initial colonization are most important, the expression of surface proteins is favored[10]. Environmental cues such as pH and ROS, and host factors like serum components, can also modulate virulence factor expression[26]. The complexity and adaptability of the machinery, compounds, enzymes, and regulatory systems of *S. aureus* makes it an incredibly effective pathogen, even with the extensive antibiotic arsenal at our disposal. In addition to their benefits to pathogens, virulence factors and regulatory systems used by *S. aureus* all serve as potential targets for infection management and eradication. *S. aureus* is nevertheless primed for survival in a range of adverse conditions, including during antimicrobial exposure, making it an especially challenging pathogen in the modern medical era.

The rise and fall of antibiotics: *S. aureus* as a model of antimicrobial resistance

Antibiotics have served as the cornerstone of care for bacterial infections since their discovery. Antimicrobial compounds were approved for clinical use as early as 1910 with the first man-made antibacterial agent, arsphenamine, marketed as a treatment for syphilis. However, discovery of penicillin in 1929 and its public availability in 1945 is often recognized as the beginning of the golden age of antibiotics[27,28]. Regardless, for the two decades following the introduction of penicillin, other antimicrobial compounds were discovered, manufactured, and then shuttled into clinical use, if deemed safe and effective, in rapid succession—for example, sulfonamides, streptomycin, cephalosporin C (the lead compound for modern day cephalosporins), gramicidin S, chlortetracycline (precursor to modern day tetracyclines and tigecycline), chloramphenicol, erythromycin, and vancomycin are only a few of the many antibacterial compounds discovered and used during this period[28,29]. This era also witnessed the rise in synthetic antibacterial agents, giving a renewed life to some of these compounds[30]. Many of the antibiotics discovered since penicillin's introduction remain front-line therapies for treating bacterial infections today. Unfortunately, after the introduction of a few new antibiotics in the 1960s, the pace of discovery substantially slowed; it took several decades before new classes of antibiotics were discovered—reflecting a de-emphasis of antimicrobial discovery, a trend hypothesized by some writers to

reflect the detection of most naturally-occurring antimicrobials[30–32]. Compounding the problem of this slowing pipeline was the increasing frequency of antibiotic resistance to existing compounds.

S. aureus represents a cautionary tale regarding the combined effects of antimicrobial resistance and a shortage of novel antimicrobial compounds. Just as *S. aureus* is naturally susceptible to a wide range of antibiotics, it is also notorious for acquiring resistance to diverse antibiotics, often via horizontal gene transfer and genetic mutations; because of this resistance, several previously indispensable antibiotics are no longer the standard of care for *S. aureus* infections. Historically, the widespread use of many antibiotics typically resulted in resistance among *S. aureus* infections and quickly became a recurring problem. The common use of penicillin in the 1940s was quickly followed by the observation of penicillin-resistant *S. aureus* strains, and the 1960s introduction of methicillin lead to the rise in methicillin-resistant strains a year later, an ancestor of the strains we see today that is responsible for outbreaks in hospitals and communities worldwide[28,33,34]. MRSA was initially largely confined to hospitals and healthcare settings, but spilled into the community (community-associated MRSA) in the 1990s[33]. As resistance became more common, fluroquinolones use increased in the 1980s, followed, predicably, by resistance. The increasing burden of resistant infections ushered in an increasing reliance on the last-line antistaphylococcal vancomycin in the 1990s, an antibiotic that circulating MRSA strains were still susceptible to at the time; like its predecessors, resistance appeared[34]. Today, antimicrobial resistance is considered a major threat to human health—antimicrobial-resistant organisms cause millions of infections worldwide every year and about 1.27 million deaths in 2019[35]. Resistance also poses a significant financial burden, estimated to result in $20 billion per year in additional medical spending in the United States alone[36]. In a world with limited antimicrobial options, individuals undergoing common medical procedures, such as surgery, cancer treatment, and organ transplants, are increasingly at-risk for deadly bacterial infections; antibiotic use in agriculture, horticulture, and veterinary fields may amplify this risk. *S. aureus* (specifically MRSA) is considered one of the top threats to human health by the CDC; worldwide, S. *aureus* is ranked as one of the top six deadly pathogens for deaths associated with antimicrobial resistance, with MRSA causing more than 100,000 deaths per year that are complicated by antimicrobial resistance[35,37].

Trimethoprim-sulfamethoxazole: An effective therapy for *S. aureus* infections that induces thymineless death

While treatment options are more limited now than in the 1940s, there are still antibiotics that are essential to clinical care today, like trimethoprim-sulfamethoxazole (SXT). One of the earlier antimicrobial compounds discovered were sulfonamides. Initially discovered in 1932, the clinical efficacy of sulfonamides were unfortunately limited due to its bacteriostatic nature. However, with the introduction of trimethoprim in the 1960s—a synthetic compound with strong bactericidal activities against both Gram-positive and -negative organisms—and reports of its synergistic effects with sulfamethoxazole within the same year, the pair became and remains, an important clinical option for *S. aureus* infections[29,38,39].

The combination antibiotic SXT exerts its action by inhibiting folate biosynthesis (**Figure 1.1**). The folate biosynthetic pathway in bacteria begins with the conversion of the nucleotide GTP, through several steps, to dihydropteroate pyrophosphate; the modifications of para-aminobenzoic acid (PABA) and dihydropteroate pyrophosphate are catalyzed by dihydropteroate synthase (DHPS) and dihydrofolate synthase, forming 7,8-dihydropteroate and dihydrofolate, respectively. Dihydrofolate production then begins the one-carbon transfer cycle: Dihydrofolate is reduced by dihydrofolate reductase (DHFR) to form tetrahydrofolate, which is converted to 5,10-methylene tetrahydrofolate by serine hydroxymethyltransferase, and then back to dihydrofolate via thymidylate synthase and several co-factor intermediates[39,40]. SXT interrupts two separate steps in this pathway. Sulfamethoxazole (SMX) inhibits DHPS by competitively inhibiting binding of PABA and preventing the downstream formation of 7,8-dihydropteroate and dihydrofolate. Trimethoprim (TMP) inhibits DHFR, halting production of tetrahydrofolate[38–40]. The combination of these two inhibitory activities is synergistically bactericidal for *S. aureus* and other bacteria. Folate biosynthesis is central to the production of one-carbon donors[39,40] that serve as cofactors for synthesis of diverse metabolites, including purines, methionine, histidine, glycine, serine, and the pyrimidine deoxythymidine monophosphate (dTMP). Among these, only the impairment of dTMP synthesis, resulting in thymidine starvation, is lethal; the cell death that occurs as a result is referred to thymineless death (TLD)[41–44].

Despite the continued effectiveness of SXT as an antistaphylococcal, resistance to SXT began to appear in *S. aureus* in the 1980s in both hospital and community settings with expanding use of this drug. The *S. aureus* resistance mechanisms to SXT most often described in the literature involve the folate metabolic pathway, including the known enzymatic target sites. For example, chromosomal mutations in the genes that encode for DHFR and DHPS result in trimethoprim and sulfonamide resistance, respectively; resistance to trimethoprim and sulfamethoxazole through horizontal gene transfer of plasmid-encoded resistant genes have been reported for both Gram-positive and Gram-negative bacteria[39,45]. However, other know, but less frequently discussed, resistance mechanisms involve the metabolite that require folate for their biosynthesis; for example, some bacteria, including *S. aureus*, can survive both agents by taking up exogenous thymidine[46–48], thereby reducing or eliminating the bactericidal activity of SXT. SXT resistance is also exhibited by thymidine-dependent small colony variants (TD-SCVs), a variant subtype of *S. aureus* usually carrying mutations in the thymidylate synthase gene (*thyA*) encoding a critical enzyme for dTMP production[49,50]. TD-SCVs survive TLD by eliminating the primary antagonistic effect of SXT through the loss-of-function of downstream enzyme; survival of these variates generally requires upregulation of a thymidine transporter[50].

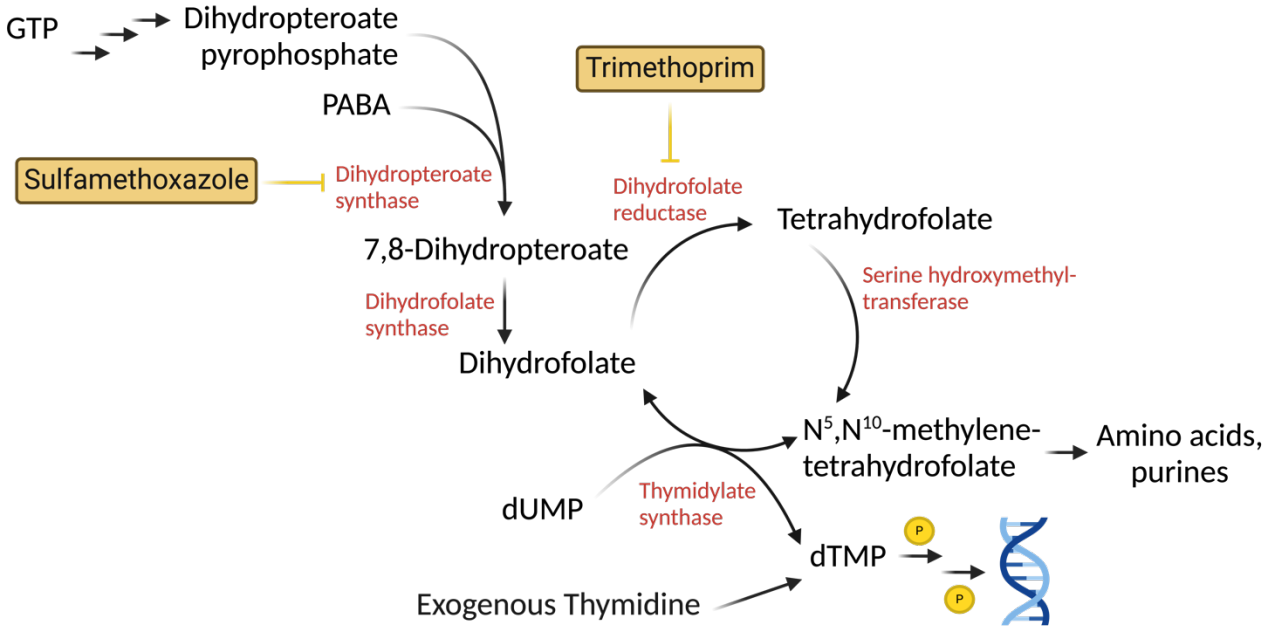

Figure 1.1 Folate biosynthetic pathway Folate biosynthesis from GTP through several intermediates, including one-carbon cycling. Dihydropteroate synthase, important for the formation of dihydrofolate, is inhibited by sulfamethoxazole and dihydrofolate reductase, required for tetrahydrofolate production, is inhibited by trimethoprim. Thymidine uptake and utilization occurs externally to the folate pathway. Enzymes are shown in red. PABA, para-aminobenzoic acid; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate. *Created with BioRender.com.*

Because of the unique features of the lethality of thymidine starvation, and the promise of this pathway as a general antimicrobial target, this process has been extensively studied, most often in the model organism *E. coli*. Early studies focused on *E. coli* without a functional thymidylate synthase (encoded by *thyA*), demonstrating a substantial loss in viability in the absence of thymidine. This outcome differed strikingly from the nongrowing-but-viable quality of cells that was observed when other folate products (i.e., amino acids and purines) were absent. Thymidine's marked impact on bacterial viability has been attributed to its necessity in the synthesis and repair of DNA; it is believed that insufficient thymidine availability results in mis-incorporation of other bases in place of thymidine during DNA replication and cell growth, prompting the initiation of DNA repair mechanisms that ultimately lead to DNA breaks; with repeated but futile damage-repair cycles, cell death occurs[51–54]. Studies have shown that preventing either the initiation of replication or the action of certain DNA repair enzymes can partially alleviate TLD[53,54]. Recent studies in *E. coli* suggested that thymidine depletion increased the production of ROS, resulting in lethal DNA damage[55,56]. However, the involvement of ROS in this and other mechanisms of bacterial killing remains controversial, with several studies suggesting that antibiotic-mediated cell death can occur in the absence of ROS[57–59]. Additionally, it is generally assumed that because the provision of exogenous thymidine during SXT exposure can promote survival, the bactericidal effect of SXT is through TLD and it has been assumed that this mechanism of action applies to other bacterial species[51,53] (**Figure 1.2**). However, relatively little research has examined the details of thymidine starvation and TLD-related mechanisms of SXT action in *S. aureus* compared with *E. coli*. Therefore, there remains, conflicting and unconfirmed hypotheses regarding TLD, SXT, and potential mechanisms of resistance in *E. coli* that has left a knowledge gap in our understanding of TLD and SXT principles in *S. aureus*. This knowledge gap impacts how we understand TLD, exploit it as a bacterial vulnerably, and our ability prevent the development of SXT-resistance in *S. aureus*.

Cystic fibrosis as a paradigm for chronic *S. aureus* infection

Understanding how *S. aureus* can persist under diverse and variable conditions, from both a nutritional and antibiotic point of view, is important for combating chronic infections. *S. aureus* can cause a wide range of infections, both acute and chronic; among these, perhaps the best-studied occurs in the lungs of people with the genetic disease cystic fibrosis (CF). CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR)[60–62], an ion channel that is important for maintaining epithelial homeostasis by controlling sodium, chloride, and bicarbonate flux across epithelial cell membranes. While especially important in the airways, CFTR is expressed in epithelial cells throughout the body, including in sweat ducts, pancreatic ducts, and the gastrointestinal tract. CF has consequences throughout the body, including pancreatic insufficiency, gastrointestinal malabsorption, and abnormalities that result in malnutrition and impaired growth, but progressive lung disease is the primary cause of morbidity and mortality in people with CF (PwCF)[61,63,64].

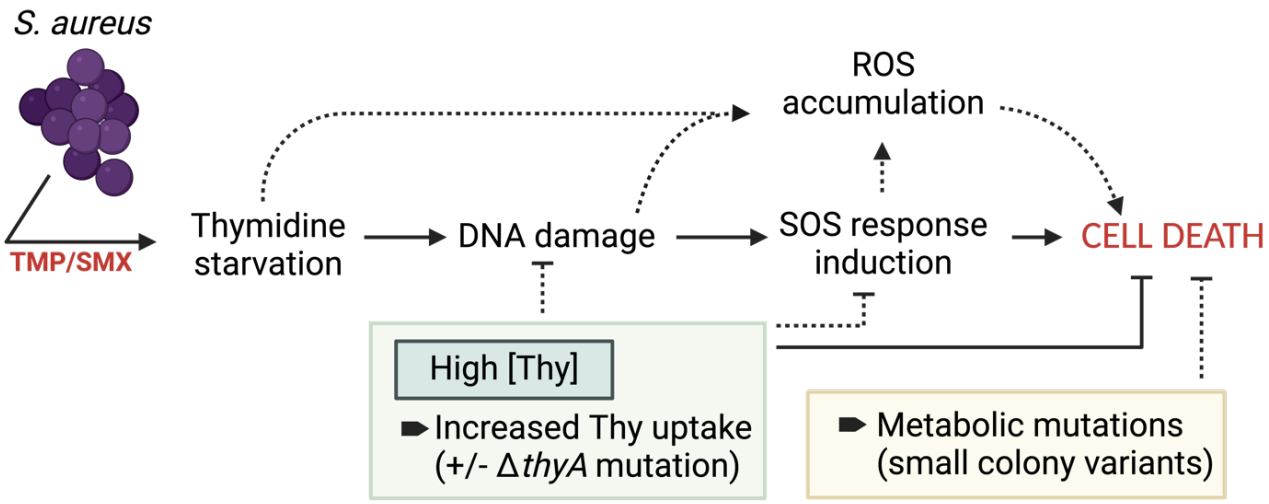

Figure 1.2 Hypothesized model of the proposed mechanism of SXT action in *S. aureus* based on studies of TLD in *E. coli* With SXT exposure, *S. aureus* undergoes thymidine starvation and DNA damage, inducing the SOS response and, ultimately, cell death. Cell death is mitigated with the uptake of environmental thymidine. In contrast, with thymidine depletion, ROS levels increase and contribute to cell death through additional cellular damage. SCV metabolic mutants are associated with SXT use in PwCF and may prevent cell death with SXT exposure. Many of the details of this model remain hypotheses or are unknown (dotted lines) in *S. aureus*, including the points at which ROS accumulation occurs and ROS contribution to cell death, if thymidine supplementation prevents DNA damage itself and/or prevents SOS induction, and the mechanisms behind SCV survival of SXT exposure. *Created with BioRender.com*

In PwCF, mutations in the CFTR channel can severely impact the proper hydration of the airway secretions, resulting in the accumulation of viscous mucus, impaired mucociliary clearance, and the subsequent obstructive pulmonary disease that is characteristic of CF. It has also been hypothesized that mutations in CFTR, and the airways changes that occur as a consequence, impact inflammatory signaling and immune cell recruitment[60,65]. These events create an environment that is susceptible to infection. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* complex, *Achromobacter* spp, *Stenotrophomonas maltophilia*, and non-tuberculous *Mycobacteria* (including *Mycobacterium abscessus* and *Mycobacterium avium-intracellulare*) are all considered canonical CF pathogens[62,66]. Among these, *S. aureus* is often the first pathogen detected in the respiratory secretions of PwCF, especially in younger populations[66,67], and this species is currently the most prevalent standard pathogen infecting the respiratory tracts of PwCF, with the proportion of MRSA among these infections on the rise[66,68–70]. Studies have suggested that *S. aureus* may stimulate lower airway inflammation, contribute to poor nutritional status, and increase the likelihood of antibiotic use and hospitalization[71–73]. *S. aureus* presence in high densities, as MRSA, and as SCVs—slow-growing, antibiotic resistant metabolic mutants—have been found to be associated with worse lung function, increased exacerbations, and increased risk of mortality compared with other pathogen infections, particularly in children[69,73–75]. Unfortunately, it is difficult to discriminate between non-pathogenic *S. aureus* colonization and *S. aureus* infection that results in significant respiratory symptoms. Thus, while an important pathogen in other infection types, the role of *S. aureus* in the pathogenesis of CF remains unclear and understudied.

With the implication of *S. aureus*, particularly that of SCVs, in lung damage and inflammation, effective anti-staphylococcal treatment has become increasingly important[73,76]. Treatment of *S. aureus* infections in PwCF varies among different countries and CF centers, and can generally be categorized a prophylaxis, eradication of existing infection, and treatment with increased symptoms (exacerbations). In the US, prophylaxis treatment is not typically recommended because this therapy is associated with earlier *P. aeruginosa* infections[69]. Eradication strategies for *S. aureus* infections generally involve several weeks of anti-staphylococcal treatment upon detection of *S. aureus* infection, followed by a multi-month course of antibiotics if initial treatment failure occurs. For MRSA, little consensus exists regarding whether to attempt eradication upon detection, and if so, the best eradication strategy[77]. For PwCF with exacerbations who are culture-positive for *S. aureus*, antistaphylococcal therapy is standard of care[78]. In each case, the choice of antibiotics to use depends on certain considerations like the antibiotic susceptibility profile of cultured isolates from the patient, side effects, allergies, or toxicities. Among the several treatment options in common use for *S. aureus* infections in CF, trimethoprim-sulfamethoxazole (SXT) is a first-line, orally available therapy for these infections, especially MRSA[69,79,80]. All antibiotics carry risks; for example, the repeated use of antimicrobials for CF respiratory infections places pressure on bacteria within the lung. While antimicrobials are not the only environmental pressure present in the lungs—oxygen and nutritional levels and the presence of other organisms represent

important influences—antibiotic selective pressure provides an opportunity for adaptation[49]. Adaptive strategies within the lung can present as hypermutation, differential regulation of virulence factors, or a switch to small colony variants (SCVs), the latter a strategy that has been shown to be both common and associated with SXT use in PwCF[74,75,81]. Thus, thoroughly understanding the ways in which adaptation occurs to an important antibiotic like SXT and the clinical impact of these adaptations is not only integral to improving therapies for CF but also has implications beyond CF—these principles may be broadly applicable to other *S. aureus* infection contexts.

Summary and scope of work

Staphylococcus aureus is a ubiquitous organism in people and one of the leading causes of human infections, many of which are difficult to treat due to persistence, antibiotic resistance, or antibiotic tolerance. As our arsenal of effective antibiotics dwindles, the need for improved treatments becomes increasingly urgent, necessitating a better understanding of the precise mechanisms by which pathogens evade our most critical antimicrobial agents. In the following chapters, we report a systematic characterization of the mechanisms of *S. aureus* adaption and persistence to treatment with the first-line antistaphylococcal antibiotic trimethoprim-sulfamethoxazole (SXT) and nutritional pressures that promote TLD. *We hypothesized that: (1) thymidine availability would impact S. aureus survival strategies during exposure to SXT, and that (2) these adaptations would provide insight into the involvement of specific pathways, such as reactive oxygen species (ROS) generation and metabolism, in SXT-mediated cell death in S. aureus.* In **Chapter 2**, we describe the impact of thymidine concentration on the survival of *S. aureus* to SXT, delving further into some of these survival strategies in **Chapter 3**. In **Chapter 4**, we discuss our preliminary work on persistence, specifically focusing on the development of an assay to differentiate persister populations from non-persister and non-viable cell populations. Lastly, in **Chapter 5**, we discuss the implications and future directions of this work. Cumulatively, this work improves our understanding of the mechanisms of action of SXT and subsequent bacterial responses and provides foundational knowledge necessary for developing improved therapies.

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CHAPTER 2: METABOLISM AS A MEDIATOR OF TRIMETHOPRIM SULFAMETHOXAZOLE-INDUCED CELL DEATH *S. AUREUS*—PART 1

Most of the work presented in this chapter was adapted from published work:

Gonsalves, L. J. *et al.* Mechanisms of *Staphylococcus aureus* survival of trimethoprim-sulfamethoxazole-induced thymineless death. *mBio* **15**, e01634-24 (2024).

How does thymidine availability impact S. aureus survival and adaptation to SXT?

Introduction

Among the best-studied chronic *S. aureus* infections are those that occur in the airways of people with cystic fibrosis (PwCF), for whom lung disease is the primary cause of morbidity and mortality[1,2]. *S. aureus* is one of the earliest and most common pathogens isolated from CF secretions[3–6], and the antistaphylococcal agent trimethoprim-sulfamethoxazole (SXT) is a first-line, orally available therapy for these infections, especially for methicillin-resistant *S. aureus* (MRSA)[7,8]. Unfortunately, as is the case for many chronic infections, antibiotics rarely eradicate *S. aureus* from CF airways. Defining the mechanisms of persistence is a key step towards developing more effective antistaphylococcal treatments.

SXT synergistically interrupts two separate steps in folate biosynthesis[9], which is required for producing diverse metabolites. Among these SXT activities, impairing synthesis of deoxythymidine monophosphate (dTMP), a precursor to the triphosphate thymidine nucleotide, is lethal[910–13]. Over the past few decades, research in *E. coli* has led to the general conclusion that SXT impairment of dTMP production results in defective DNA replication and repair in a process known as thymineless death (TLD), with more recent work both suggesting the toxic effects of reactive oxygen species (ROS) play a large role in TLD[14–17]. Thus, while thymidine starvation is hypothesized to be largely responsible for SXT's effectiveness as a bactericidal agent, like other antibiotics, this efficacy is undermined by *S. aureus'* tendency to develop of resistance. Notably, relatively few SXT-specific survival mechanisms have been described for *S. aureus*. Useful clues to the genes and pathways involved in bacterial survival of antibiotics such as SXT have come from characterizing mutants resistant to specific antibiotics. In the case of SXT, target site mutations in dihydrofolate reductase and dihydropteroate synthase cause trimethoprim and sulfonamide resistance, respectively[18]. Some bacteria, including *S. aureus*, can survive both agents by taking up exogenous thymidine[19–21], thereby reducing or eliminating the bactericidal activity of SXT. Alternatively, slow-growing, antibiotic-resistant mutants of *S. aureus* known as small colony variants (SCVs)[22] can carry mutations in the thymidylate synthase gene (*thyA*) encoding a critical enzyme for dTMP production[22,23]; these mutants have been cultured from a variety of both bone and wound infections as well as from respiratory infections after treatment with antibiotics such as SXT.

Thymidine-dependent small colony variants (TD-SCVs) are commonly detected in PwCF after SXT treatment[24,25]. SCVs have been identified in up to 28% of PwCF and are associated with lower lung function and increased exacerbation frequency[24,25]. The thymidine-dependent subtype of SCVs (TD-SCVs), the most common type detected in people with CF, are both selected by and resistant to TMP/SMX, and evidence suggests this SCV subtype accounts for the majority of the adverse clinical associations of SCVs[24,25]. These variants usually carry mutations in the gene encoding thymidylate synthase, which converts deoxyuridine monophosphate (dUMP) to dTMP, a process downstream but dependent on folate biosynthesis. These variants respond to the resulting intracellular thymidine depletion by importing exogenous thymidine sources through the

transporter encoded by *nupC*, simultaneously conferring survival of both TMP/SMX exposure and the resulting TLD[23]. These observations suggest that free thymidine concentrations in CF lung secretions might be sufficient to prevent TLD, supporting *S. aureus* SCVs and, perhaps, allowing a portion of the wild-type population of *S. aureus* cells to survive TMP/SMX[24,25].

However, TD-SCVs are not the only *S. aureus* mutants cultured from CF sputum; often isolates with mutations in genes related to electron transport, such as those encoding menadione and hemin synthesis, are observed among CF patients treated with TMP/SMX[26–28]. These observations suggest that *S. aureus* possesses several, as-yet undefined mechanisms for surviving TMP/SMX and TLD that may involve core metabolic processes. A growing body of evidence links attenuated bacterial metabolic rates to survival of diverse environmental stresses, including not only antibiotics, but also immunity and intracellular defenses[22,29–31]. Therefore, defining the mechanisms of bacterial survival of specific pressures, such as TMP/SMX, can highlight potential targets for preventing and treating diverse bacterial infections.

Here, we explored the relationship between thymidine availability and *S. aureus* survival strategies during exposure to SXT to thoroughly define the mechanisms responsible for TLD. We hypothesized that different cellular processes would be involved in surviving TLD depending on exogenous thymidine levels. We show that *S. aureus* utilizes numerous strategies to survive SXT and the resulting TLD, beyond mutations in folate biosynthetic target sites and *thyA*, particularly under low thymidine conditions.

Results

Thymidine metabolism and uptake play important roles in SXT killing

TD-SCVs escape TLD through uptake of exogenous thymidine and its analogs[23,32]. Because SXT treatment phenocopies TD-SCV-causative mutations by impairing dTMP synthesis, *S. aureus* may also survive SXT treatment in clinical infections (such as those in CF airways) by increasing uptake of available thymidine. However, neither the thymidine concentrations required for *S. aureus* to survive SXT, nor those present in CF sputum, have been defined.

We therefore determined the relationship between thymidine concentration and *S. aureus* survival during *in vitro* culture with and without SXT. Methicillin-susceptible (MSSA) strain Newman, methicillin-resistant (MRSA) strain JE2, and Newman Δ*thyA* (TD-SCV) were cultured over 24h at varying thymidine concentrations (0 μg/mL to 16 μg/mL), with and without clinically relevant SXT concentrations (8- to 16-fold above the parental MICs)[33]. We found the thymidine concentration required to survive SXT *in vitro* differed little among the two non-SCVs (**Figure 2.1A, B**); concentrations ≥0.25 μg/mL supported increasing cell densities over 24h with a decline after 8-10h, while cell densities uniformly declined, but remained above the limit of detection, at thymidine concentrations below 0.25 μg/mL. After a period of rapid cell death following SXT

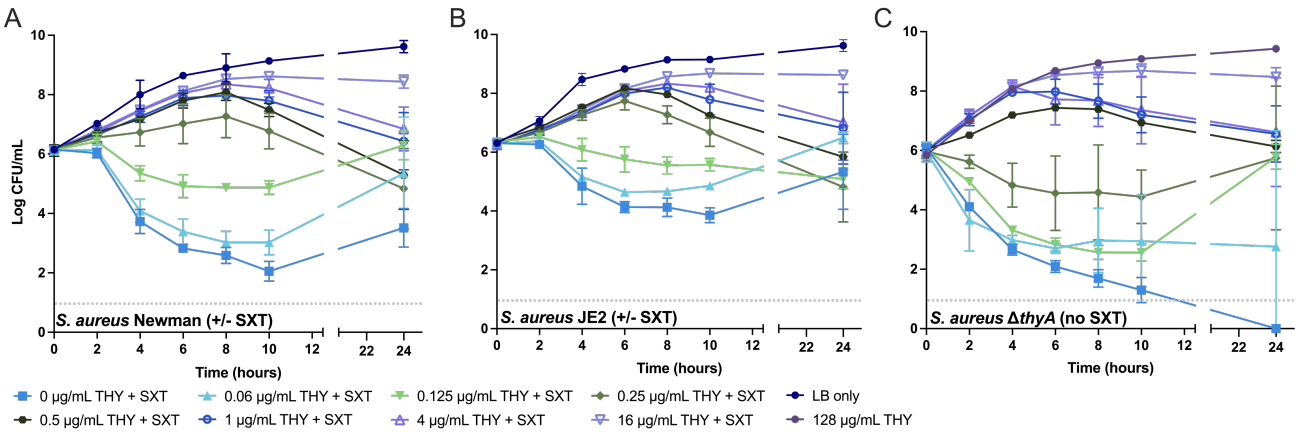

Figure 2.1 Kinetics of survival of two laboratory *S. aureus* strains and a TD-SCV mutant in the presence and absence both of trimethoprim-sulfamethoxazole (SXT) and thymidine supplementation *S. aureus* strains (A) Newman, (B) JE2, and (C) Newman-derived Δ*thyA* cultured in LB over 24h. Each strain was supplemented with the concentrations of thymidine (THY) indicated in the legend (0-16 µg/mL); strains Newman and JE2 were treated with 8 µg/mL TMP and 152 µg/mL SMX; Δ*thyA* was not treated with SXT (indicated in Figure C) due to its natural ability to undergo TLD in the absence of thymidine. A control experiment including supplementation with 128 µg/mL THY to simulate wild-type-like growth was included for Δ*thyA* only (Fig. C). Data represent mean values ± SD (n≥2).

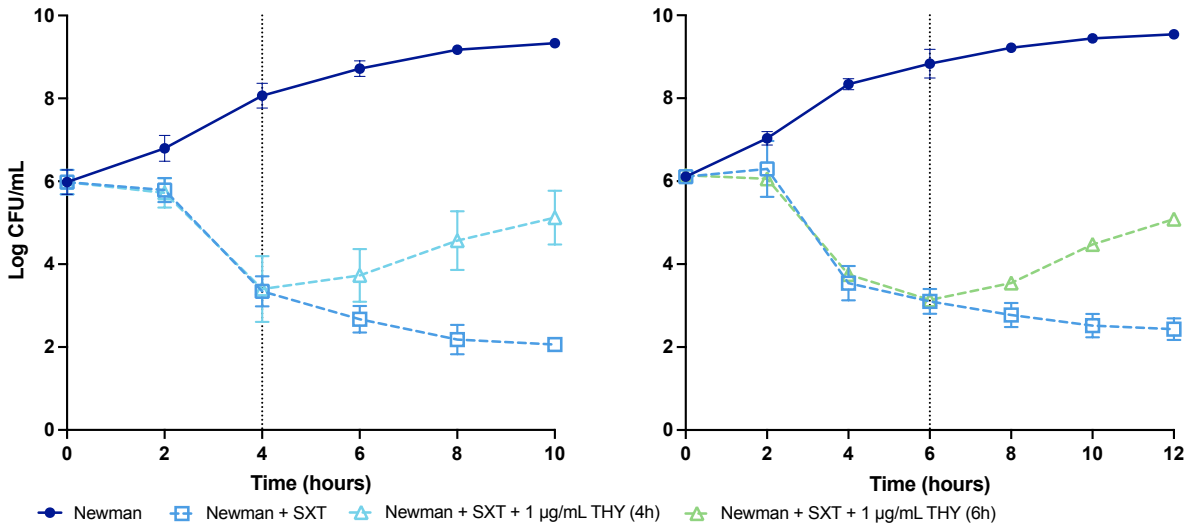

Figure 2.2 Kinetics of survival of *S. aureus* strain Newman in the presence and absence both of trimethoprim-sulfamethoxazole (SXT) and delayed thymidine supplementation *S. aureus* strain Newman was cultured in LB over 10-12h. The culture was supplemented with 1 µg/mL thymidine added at either 4h (dotted vertical line, left) or 6h (dotted vertical line, right) after initiation of SXT treatment. SXT-treated conditions are indicated by a dotted horizontal line; data are mean ± SD (n=3) and treated with 8 µg/mL TMP and 152 µg/mL SMX.

treatment in the absence of exogenous thymidine, culturable cell counts quickly increased with addition of 1 μg/mL of thymidine at 4 or 6h timepoints (**Figure 2.2**), indicating either that damaged cells may be rapidly repaired and replicate, or that a static and uninjured subset of the cell population responds to exogenous thymidine. Despite the two strains having similar MICs to SXT (Newman MIC = 0.5 μg/mL, JE2 MIC = 0.25 μg/mL), Newman was more susceptible to SXT killing than was JE2 (~4 log CFU/mL decrease for Newman versus ~2 log CFU/mL decrease for JE2 at 10h under low thymidine conditions), underscoring a functional distinction between cell death and MICs. Survival patterns of the Δ*thyA* mutant (**Figure 2.1C**) differed slightly from those of non-SCV strains only under low-thymidine conditions. Compared with wild-type parent Newman, which might produce low levels of thymidine due to incomplete folate inhibition with SXT, Δ*thyA* was entirely dependent on exogenous thymidine for viability, ultimately declining to densities below the limit of detection with 0 μg/mL thymidine, but with improved survival at 0.25 μg/mL. These results demonstrated that, under these experimental conditions, 0.25 μg/mL of thymidine was sufficient for all tested *S. aureus* strains to survive SXT challenge or, in the case of Δ*thyA*, TLD.

Based on these results, we reasoned that, if *S. aureus* primarily survives SXT through thymidine uptake during CF respiratory infection, thymidine concentrations in infected secretions should be ≥0.25 μg/mL. Because the concentrations of that nucleoside and its analogs in human tissues are unknown[19,31,32], we analyzed 53 CF sputum samples using mass spectrometry to quantify the nucleoside thymidine and the monophosphate (dTMP), diphosphate (dTDP), and triphosphate (dTTP) nucleotides and cultured for *S. aureus*. dTTP and dTDP were generally undetectable, while measurements ranged from below the limit of detection (0.041 μg/mL) to either 13.55 μg/mL for thymidine or 11.69 μg/mL for dTMP (**Figure 2.3A**). Although dTMP was detected in many sputum samples, this nucleotide cannot support *thyA* mutant growth or antagonize the activity of SXT on its own (**Figure 2.4**). While the median thymidine level for all samples (0.066 μg/mL) was not statistically different than 0.25 μg/mL ($p = 0.173$), only 28% of samples (15/53) contained thymidine levels ≥0.25 μg/mL, with an even smaller proportion (6/34, ~18%) of *S. aureus* culture-positive samples reaching this concentration. Median sputum thymidine concentration was not significantly lower in *S. aureus* culture-positive samples (median = 0.054 μg/mL) compared with culture-negative samples (median = 0.217 μg/mL); median sputum concentration was significantly lower in *S. aureus* SCV-positive samples (median = 0.041 μg/mL), which included TD-SCVs, relative to *S. aureus*-negative samples ($p = 0.0075$) (**Figure 2.3B**), possibly as a result of SCVs consuming the exogenous supply. These data suggest that, while present at detectable levels in some samples, thymidine abundances in CF sputum may frequently be insufficient to confer *S. aureus* survival of SXT.

Mutations selected by SXT challenge vary by thymidine availability

To determine how thymidine availability influences *S. aureus* mutant selection by SXT, strains Newman and JE2 were again cultured in the presence and absence of SXT at clinically-relevant

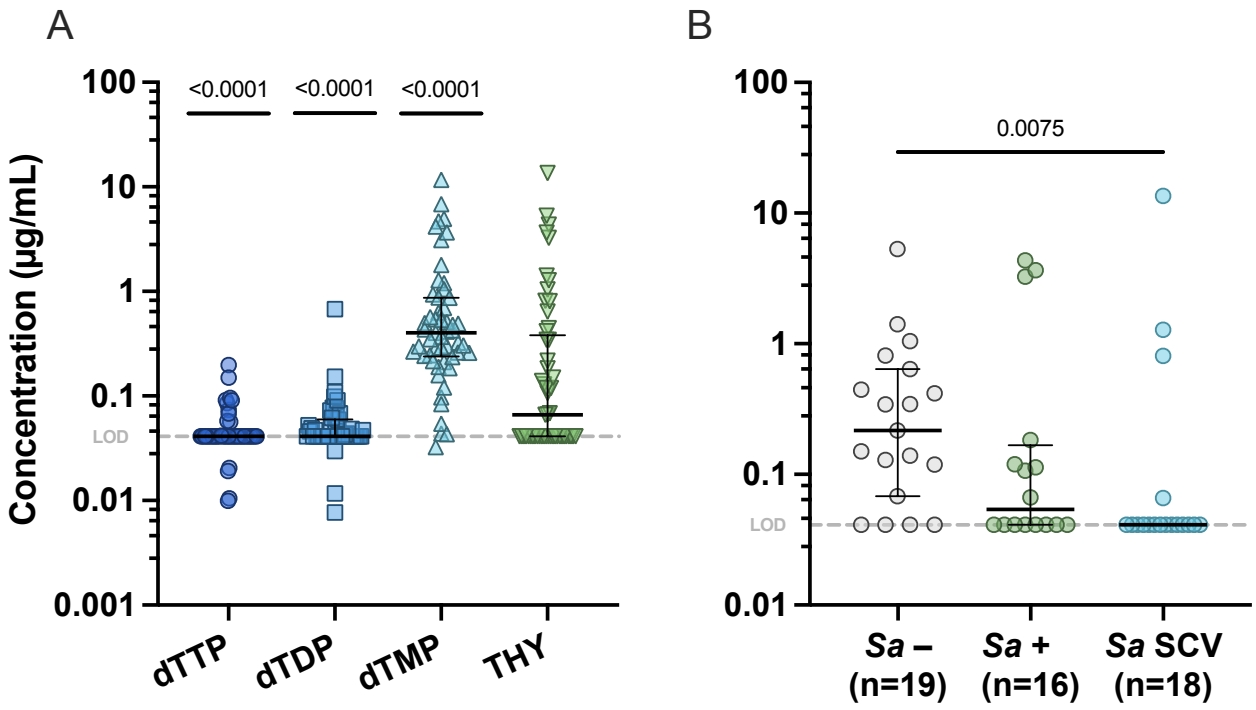

Figure 2.3 Concentrations of free thymidine and its analogs in sputum samples from PwCF (A) LC-MS/MS analysis of free deoxythymidine triphosphate (dTTP), -diphosphate (dTDP), -monophosphate (dTMP), and thymidine in 53 CF sputum samples. Significance was determined using Kruskal-Wallis and Dunn's multiple comparison test; significant p-values reported. **(B)** All analyzed samples were categorized as *S. aureus*-negative, *S. aureus*-positive (non-SCV), or *S. aureus* SCV-positive and the corresponding thymidine concentration as determined by LC-MS/MS; all data reported as median with interquartile range. For A and B, the dotted line indicates the 0.041 µg/mL limit of detection. Significance was determined using either the (A) Wilcoxon signed rank test (theoretical mean of 0.25 µg/mL) or the (B) Kruskal-Wallis test for multiple comparisons (right); only significant p-values reported.

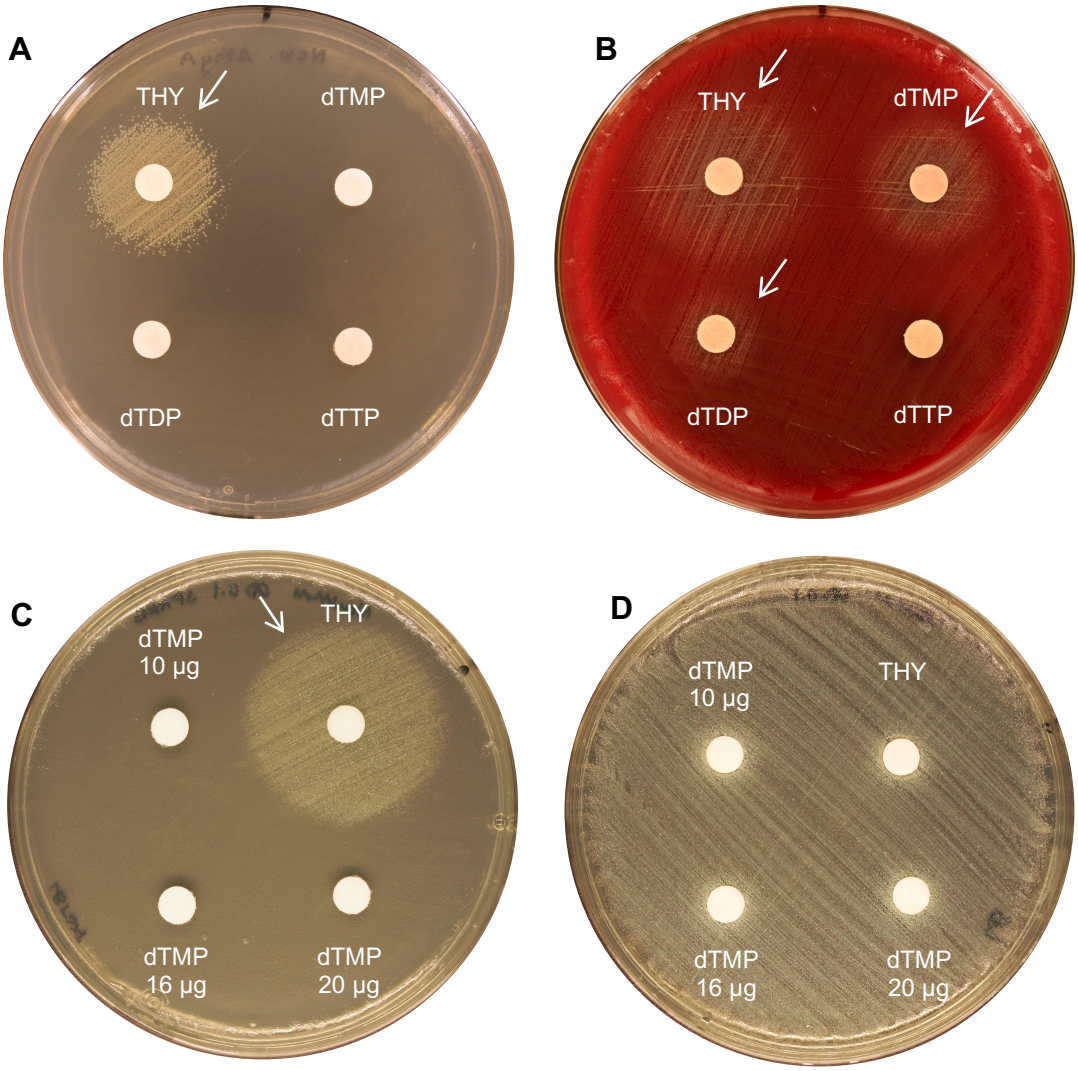

Figure 2.4 Survival of Δ*thyA* and wild-type Newman undergoing TLD in the presence of thymidine and analogs Auxotrophic testing with thymidine (THY) and analogs deoxythymidine-monophosphate, -diphosphate, and -triphosphate (dTMP, dTDP, and dTTP, respectively) for Newman Δ*thyA* grown on **(A)** LB and **(B)** blood agar plates; all disks were saturated with 10 µg of the respective compound. Representative auxotrophic testing of thymidine (10 µg) and dTMP (10-20 µg) for *S. aureus* wild-type strain Newman on **(C)** LB + SXT and **(D)** LB.

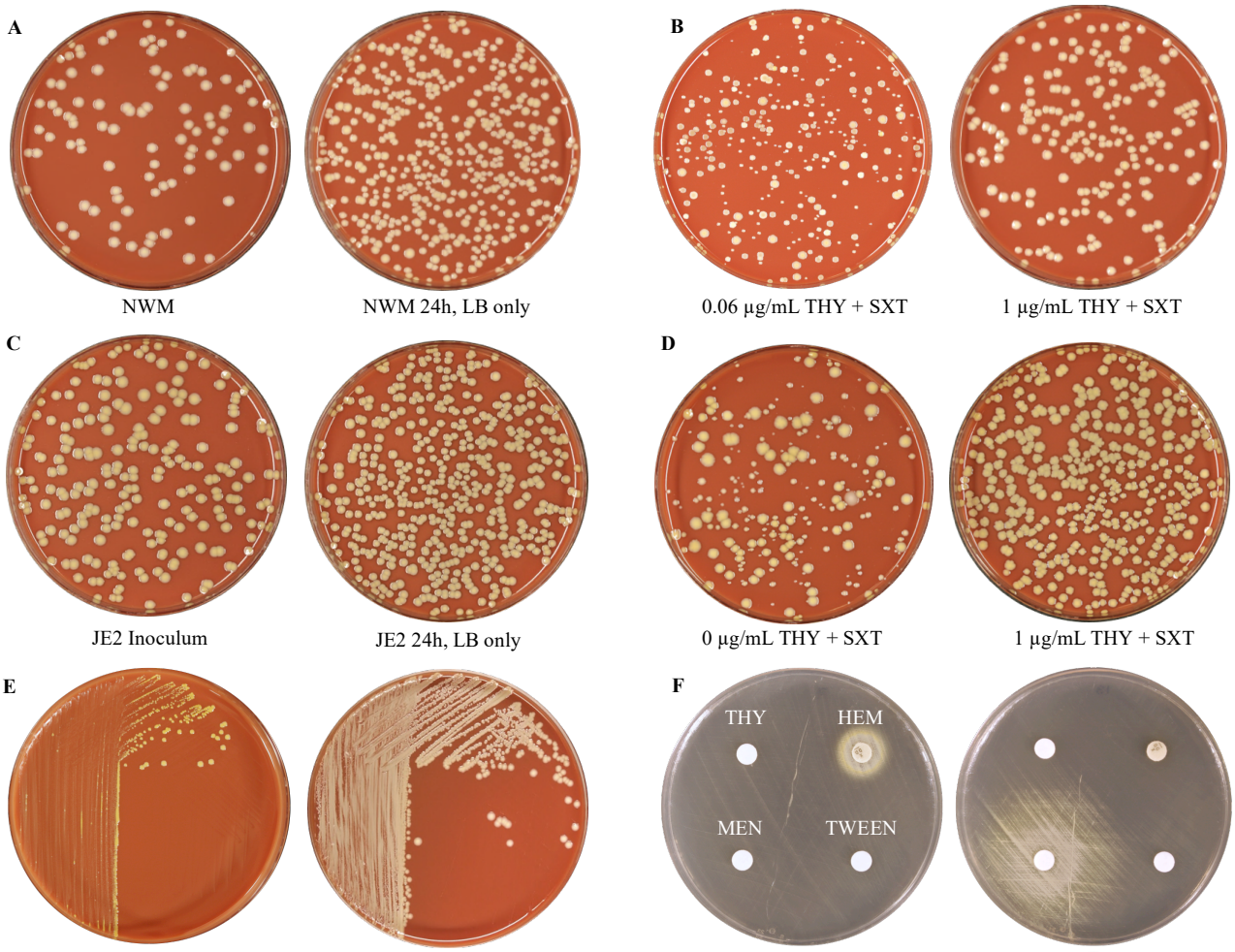

Figure 2.5 Colony phenotypic diversity of *S. aureus* isolates selected by short-term (24h) TMP/SMX exposure (A) Colony morphologies of plated Newman inoculum before (left) and after 24h growth (right) in LB only, revealing homogeneous morphology. **(B)** Representative plates following 24h of SXT treatment of Newman from thymidine conditions 0.06 µg/mL (left) and 1 µg/mL (right) exhibiting diverse colony morphology and normal colony phenotype, respectively. **(C)** Result as in (A) but with JE2 inoculum. **(D)** Representative plates following 24h of SXT treatment of JE2 from thymidine conditions 0 µg/mL (left) and 1 µg/mL (right) exhibiting diverse colony morphology and normal colony phenotype, respectively. **(E)** Plate of representative Newman colonies exhibiting hyperpigmentation (increased staphyloxanthin production; left) following SXT exposure; plate of representative normal colonies for reference (right). **(F)** Representative auxotrophic testing results of select Newman isolates following 24h of treatment with SXT under low thymidine conditions. Plates show growth around discs saturated with hemin and menadione (respectively), indicating auxotrophies complemented with supplementation.

concentrations and 8- to 16-fold above the parental MICs (super-inhibitory) and with various thymidine concentrations. At 24 and 120h, we observed that colony phenotypes from these cultures varied more after selection under low thymidine compared to those from high thymidine, despite originating from a phenotypically homogeneous inoculum (**Figs 2.5A, C**). After selection with higher thymidine concentrations, colonies generally exhibited only normal (**Figure 2.5B and D, right**) or classical TD-SCV morphologies (transparent or fried egg-like colonies) on blood agar. With Newman, we identified *thyA* mutant isolates in 11 of 12 replicate cultures, predominating in cell populations at 120h in most replicates (**Figure 2.6A**). Interestingly, *thyA* mutants were never detected among four separate JE2 cultures when grown with any thymidine concentration (data not shown). At both 24 and 120h, we isolated surviving variants for whole-genome sequencing (WGS). WGS of mutants selected by SXT with high thymidine largely supported these phenotypic observations, revealing mutations in *deoB* (phosphopentomutase, both Newman and JE2), *dfrA* (dihydrofolate reductase, Newman only) and *thyA* (thymidylate synthase, Newman only), encoding genes involved in folate metabolism (*dfrA*)[10] and thymidine uptake and metabolism (*deoB* and *thyA*)[34,35].

By contrast, with low thymidine concentrations, cultures yielded a mixture of hyperpigmented colonies (indicative of staphyloxanthin hyperproduction, **Figure 2.5E**), pinpoint-to-small colonies (many surprisingly hemin- or menadione-dependent SCVs; **Figure 2.5B and D**), and large colonies. This phenotypic variation was reflected genetically by WGS analysis of both Newman and JE2 isolates collected at 24h and 120h (summarized in **Table 2.1**). Mutations were observed in diverse genes involved in nucleotide metabolism (*nrdE/F*), electron transport component synthesis (*hepT*, *hemH/E/Y, memE*, *menF*), translation (*rpoC*), and central energy metabolism (*ptsI*, *thiN, ackA*, *pta*, *aroA/B*, *pdhB*, *pyk*, and *pdhD/lpdA*), in addition to others encoding hypothetical proteins. Genetic alterations in these strains consisted of missense mutations and gene inactivating changes, predicted to result in nonsense mutations and nucleotide insertions/deletions causing frameshifts (see Table S1A and B in Gonsalves *et al.*, 2024).

The culture-based methods we used to select resistant isolates could preferentially identify fixed or stable mutations. To limit this culture-associated bias and better determine relative mutation frequencies, we performed whole genome sequencing directly on the bacterial populations comprising up to four biological replicate cultures each of Newman and JE2 collected at 24h and 120h of SXT treatment with varying thymidine concentrations (summarized in **Table 2.1**; Tables S2-9 in Gonsalves *et al.*, 2024). With high thymidine at 120h, a few genes in these populations were commonly mutated among both Newman and JE2 following SXT challenges, including *deoB* and *mvaK1/mvk* (mevalonate kinase). High proportions of variants in *thyA* and *dfrA* were detected from Newman but not JE2, confirming culture results (**Figure 2.6A**). Additional mutations were observed in genes encoding hypothetical proteins at 120h. With low thymidine at 120h, gene mutations commonly observed in both Newman and JE2 involved central carbon metabolism (*pta*, *ackA*, and *ptsI*), nucleotide synthesis (*nrdE*, *nrdF*), tRNA biosynthesis and transcription (*rpoC*),

Table 2.1 Summary of genes with non-synonymous mutations observed through sequencing of isolates and/or whole cultures after SXT selection with either high or low THY concentrations.

Gene Name	Pathway	Protein	Time[a]	[THY][b]	Strain	Isolate/Whole-Culture
ptsI	Central metabolism	Phosphoenolpyruvate phosphotransferase	Early, Late	Low	Newman, JE2	Isolate and whole-culture
thiN		Thiamine pyrophosphokinase	Early, Late	Low	Newman	Isolate
pdhB		Pyruvate dehydrogenase, beta subunit	Early	Low	JE2	Isolate
ackA		Acetate kinase	Early, Late	Low	Newman, JE2	Isolate and whole-culture
aroA *aroB*		3-phosphoshikimate 1-carboxyvinyltransferase; 3-dehydropuinate synthase	Early	Low, High	Newman, JE2	Isolate and whole-culture
pta		Phosphate acetyltransferase	Early, Late	Low	Newman, JE2	Isolate and whole-culture
pyk		Pyruvate kinase	Early, Late	Low	Newman, JE2	Isolate and whole-culture
pdhD *lpdA*		Dihydrolipoamide dehydrogenase	Early, Late	Low	Newman, JE2	Isolate
heptT	ETC component synthesis	Heptaprenyl diphosphate synthase component II	Early	Low	Newman, JE2	Isolate
hemH *hemE* *hemY*		Ferrochelatase; Uroporphyrinogen decarboxylase; protoporphyrinogen oxidase	Early, Late	Low, High	Newman	Isolate and whole-culture
nrdE *nrdF*	Nucleotide metabolism	Ribonucleotide-diphosphate reductase, alpha and beta subunits	Early, Late	Low	Newman, JE2	Isolate and whole-culture
deoB		Phosphopentomutase	Late	High	Newman, JE2	Isolate and whole-culture
thyA		Thymidylate synthase	Late	High	Newman	Isolate and whole-culture
rpoC	Translation	DNA-directed RNA polymerase, subunit β'	Early, Late	Low	Newman, JE2	Isolate and whole-culture
dfrA	Folate metabolism	Dihydrofolate reductase	Late	High	Newman	Isolate and whole-culture

[a] Time: early and late refer to 24 and 120 h, respectively

[b][THY]: Low includes 0-0.25 µg/mL thymidine conditions; high includes 1-16 µg/mL thymidine conditions

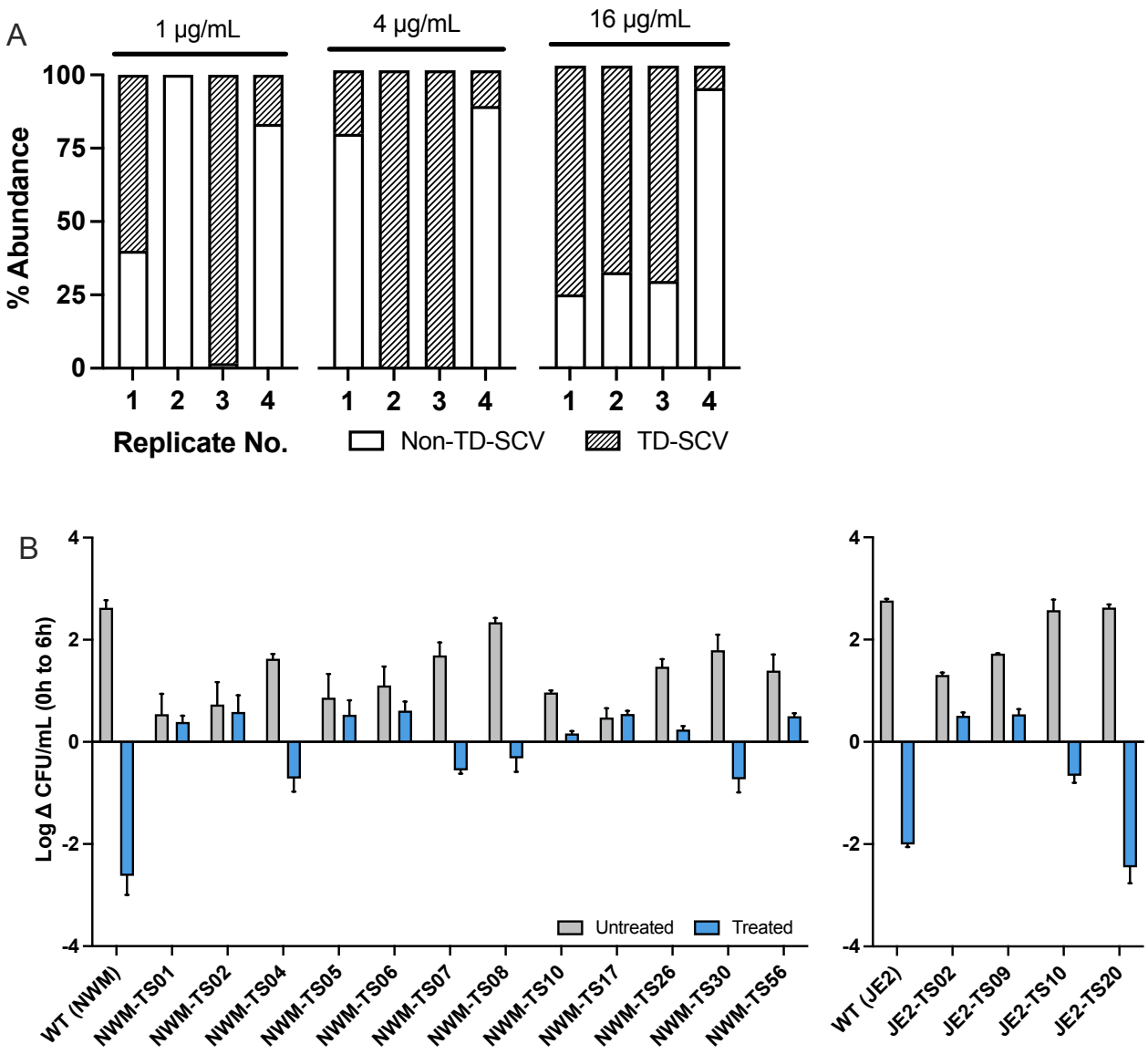

Figure 2.6 Characteristics of adaptive mutants cultured from LB following SXT challenge **(A)** Proportion of colonies exhibiting the TD-SCV morphology following 120 h of SXT exposure of Newman under high thymidine conditions (1, 4, 16 µg/mL). Phenotypes were determined by plating on blood agar, and total bacterial abundances were measured on chocolate agar plates (n=4). **(B)** Survival of a subset of adaptive mutants isolated after 24h from low thymidine concentrations relative to parental counterpart with re-exposure to SXT. Shown is change in Log CFU/mL between 0 and 6h in LB with the addition of SXT and in the absence of supplemental thymidine. Data represent mean ± SD (n≥2). The isolates listed above contain nonsynonymous mutations in the following genes (Table S1): *memE* (NWM-TS01), *menF* (NWM-TS02), *hemE* (NWM-TS04), *aroB* (NWM-TS05), *aroA2* (NWM-TS06), *hemH* (NWM-TS07), *ptsI* (NWM-TS08), *thiN* (NWM-TS10), *ndk* and *hepT* (NWM-TS17), *tkt* (NWM-TS56), *pyk* (JE2-TS02), *pta/eutD* (JE2-TS09), *nrdE* (JE2-TS10), *deoB* (JE2-TS20); no mutations detected in isolates NWM-TS26 and NWM-TS30.

and transcriptional regulation (*ctsR*; CsoR-like sulfurtransferase repressor). Similar to strain-level WGS, population-based sequencing identified various types of mutations, even in the same gene under different conditions, including missense mutations, nonsense mutations, and insertions/deletions. These data further support a model wherein survival of SXT in the presence of ample thymidine involves modulation of either uptake or metabolism of nucleotides, whereas survival under thymidine depletion entails changes in core metabolism, growth, and stress responses.

S. aureus deploys diverse strategies to survive SXT-mediated TLD.

Several of the mutations selected under low-thymidine conditions affected genes involved in generating ATP or metabolites that participate in glycolysis and the TCA cycle, such as pyruvate (*pdhB, pyk, ptsI*)[36–38], acetyl-coA (*ackA, pta*)[39] and thiamine triphosphate (*thiN*)[40], as well as electron transport chain (ETC) components (e.g., *hepT, memE, hemE*)[41,42]. For a subset of selected mutants carrying mutations in these genes, we assessed whether *S. aureus* survival during SXT exposure under thymidine depleted conditions was impacted (**Figure 2.6B**). Most of the SXT-adapted mutants tested exhibited improved survival relative to wild-type upon SXT re-exposure: wild-type viable cell densities decreased ~3 log CFU/mL from 0h to 6h during SXT treatment, while mutant cell densities generally dropped no more than 1-2 log CFU/mL over the same period. Some tested mutants exhibited a slight increase in density at 6h with SXT, despite the absence of thymidine. SXT tolerance was confirmed in Newman menadione- and hemin-dependent SCVs containing single gene knockouts (**Figure 2.7A and B**, respectively) and a hyperpigmented JE2 transposon insertion mutant (*hepT*, NE1920; **Figure 2.7C**)[43]. Supplementation with menadione or hemin re-established death of the respective mutants during SXT challenge, indicating that electron transport impairment can confer SXT tolerance (**Figure 2.7A and B**, respectively).

Relative to wild-type Newman, several tested isolates (*memE, hemE, thiN,* and *hepT* mutants) exhibited growth defects without antibiotic treatment. Slow bacterial growth was previously linked to generally reduced antibiotic efficacy[44,45] and could explain survival against TLD. However, we found that Newman cultured in dilute media, which we used to artificially slow growth rates to those electron transport chain (ETC) mutants in rich media, still resulted in its killing by SXT, albeit at a slower rate relative to undiluted LB (**Figure 2.7D**). In addition, the growth rates of Newman *ptsI* and JE2 *hepT*::Tn mutants were similar to those of their parental strains grown without SXT, and persisted with the addition of SXT. These findings suggest that decreasing the rate of growth improves survival of SXT, perhaps explaining the isolation of some SXT-adapted isolates carrying mutations in metabolic genes; however, factors besides altered growth rate can also protect against TLD.

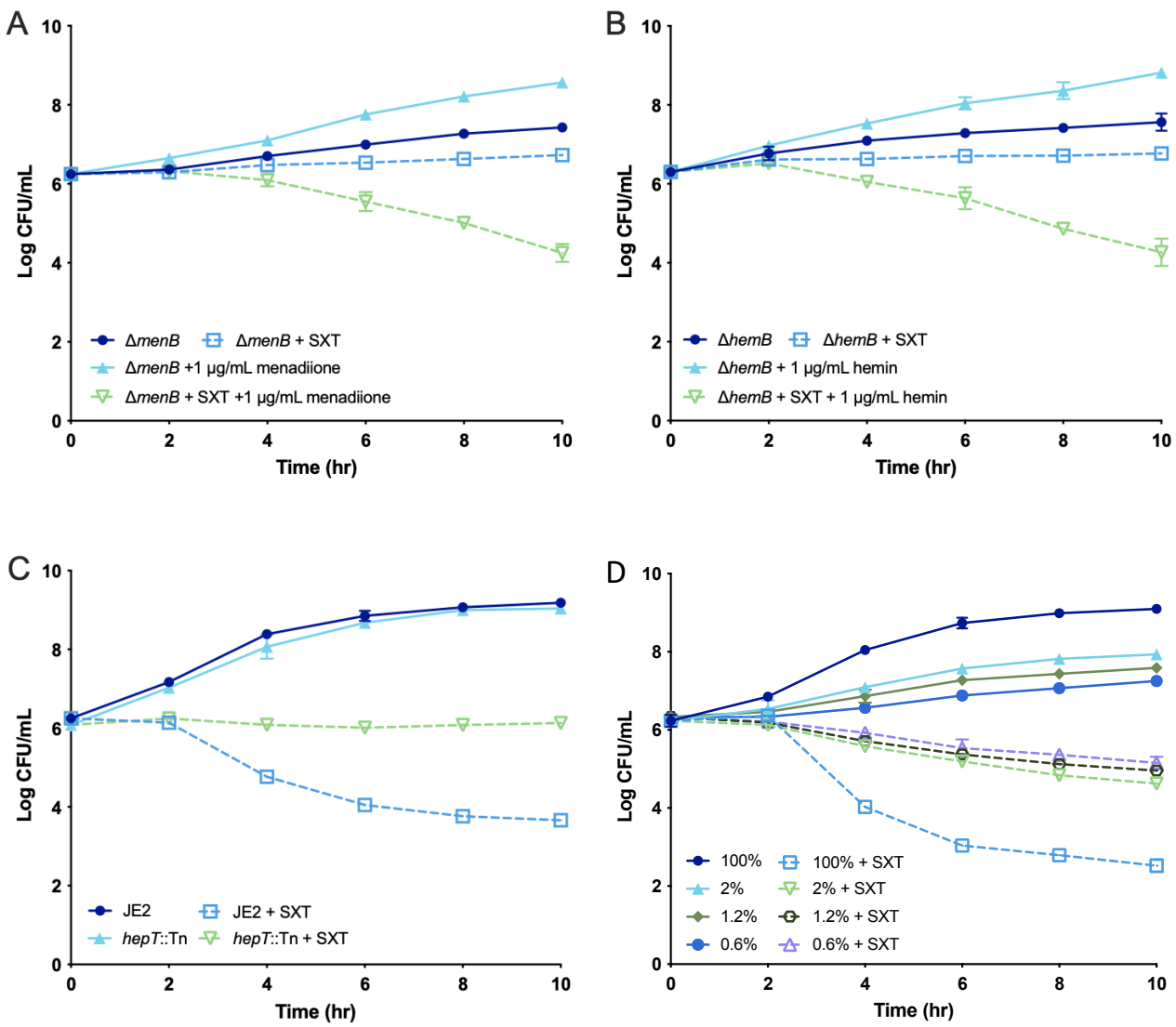

Figure 2.7 Survival kinetics of clean deletion strains or transposon mutants to SXT challenge and *S. aureus* strain Newman during SXT treatment in dilute media Survival kinetics of **(A)** Newman Δ*menB* and **(B)** Newman Δ*hemB*, cultured in LB over 10h in the absence of thymidine with and without SXT and with or without menadione or hemin (1 μg/mL), where indicated. Data are mean ± SD (n≥2). **(C)** Survival kinetics of JE2 *hepT*::Tn cultured in LB over 10h in the absence of additional thymidine and treated with SXT. SXT-treated conditions are indicated by a dotted line and data are mean ± SD (n=3). **(D)** *S. aureus* Newman was cultured in 100% or dilute LB (2%, 1.2%, and 0.6% LB, as indicated) containing 50 mM MOPS (pH 7.0) with and without SXT over 10h; SXT-treated conditions are indicated by a dotted line and shown are mean ± SD (n=3).

Clinical isolate analysis suggests *in vivo* relevance of nucleotide metabolism for *S. aureus* survival to SXT.

We next asked whether the genes that were altered with SXT selection in our *in vitro* assays also exhibited coding changes in clinical isolates of *S. aureus*. We previously performed whole-genome sequencing of ~1,300 isolates collected from 246 children with CF (CwCF)[46]. Using those data, we analyzed the genomic sequences of isolates collected over two years from two populations of children from this isolate collection. The first group, or the exposed group, was comprised of isolates from 103 CwCF who either had reported SXT treatment or were culture-positive for at least one TD-SCV (defined as having at least on isolated with a thymidine auxotrophic phenotype) as an indicator of likely SXT exposure[25]. The second group, or the unexposed group, included 133 children who did not report treatment with SXT nor were TD-SCV-positive. For a set of 44 genes we found to be commonly mutated with *in vitro* selection with SXT in the current study, we compared the frequency of mutations in clinical isolates from the exposed group to those from the unexposed group. Of the candidate genes that we screened, we found that eight of these genes carried coding alterations at a statistically higher rate (uncorrected p-value > 0.05) in isolates from the exposed versus unexposed group: *thyA*, *ropC*, *nrdE*, *lpdA/pdhD*, NWMN_1037, NWMN_1874, NWMN_0013/SAUSA300_0014, and *ptsI*. After Benjamini-Hochberg correction for multiple comparisons, only three of these genes were mutated at a statistically higher rate for this comparison—*thyA*, *rpoC*, and *nrdE* (**Table 2.2**). These findings show that, for three genes of interest, the frequency of mutation in these isolates was greater in the exposed group of CwCF than the unexposed, but the biological relevance for these genes, and other metabolic pathways, remains uncertain, as most genes assessed were not shown to be under positive selection.

Discussion

We have shown that *S. aureus* employs several mechanisms to survive the first-line antibiotic combination trimethoprim-sulfamethoxazole (SXT). The variable concentrations of thymidine that were observed in sputum samples suggested that the route of survival used by *S. aureus* during SXT exposure may differ based on the thymidine concentration. *In vitro*, thymidine concentration was indeed both a major determinant of *S. aureus* viability when treated with SXT as well as a determinant of the types of mutations that were selected by extended SXT exposure, with either folate metabolic or core metabolic mechanisms predominating depending on thymidine levels. Our analysis of clinical isolates underscored the importance of folate and nucleotide metabolism in survival of *S. aureus* to SXT exposure.

When investigating the role of thymidine in survival, a minimum concentration of 0.25 µg/mL of extracellular thymidine promoted *S. aureus* survival *in vitro* under clinically-relevant SXT concentrations (**Figure 2.1**). In Newman, this concentration of thymidine supported the emergence of mutants in the folate metabolic gene *dfrA*, but even higher thymidine concentrations (≥ 1 µg/mL) were required before mutants in another key folate gene, *thyA,* were detected. *thyA* mutants were not detected in JE2, an observation made previously, but that remains unexplained[32], and

Table 2.2 Statistical significance of the frequency of mutations in clinical isolates from the exposed group (n=103) to those from the unexposed group (n=133).

Gene name	Gene ID	Protein	Fisher Estimate	P-value	
				Uncorrected	*Benjamini-Hochberg*
thyA	NWMN_1338 SAUSA300_1320	Thymidylate synthase	56.244	1.04E-18	4.58E-17
rpoC	NWMN_0505 SAUSA300_0528	DNA-directed RNA polymerase subunit beta'	3.978	0.001	0.025
nrdE	NWMN_0700 SAUSA300_0716	Ribonucleotide-diphosphate reductase subunit alpha	Inf	0.003	0.039
pdhD / lpdA	NWMN_0962 SAUSA300_0996	Dihydrolipoamide dehydrogenase	Inf	0.015	0.165
	NWMN_1037	Hypothetical protein	4.631	0.019	0.167
	NWMN_0013 SAUSA300_0014	Hypothetical protein	4.746	0.044	0.249
ptsI	NWMN_0950 SAUSA300_0984	Phosphoenolpyruvate-protein phosphotransferase	8.102	0.045	0.249
	NWMN_1874	Hypothetical protein	Inf	0.035	0.249
	NWMN_0687 SAUSA300_0703	Sulfatase family protein	4.029	0.082	0.300
menF*	NWMN_0912 SAUSA300_0945	Isochorismate synthase	4.029	0.082	0.300
aroA2*	NWMN_1630 SAUSA300_1683	Bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	6.686	0.089	0.300
nrdF	NWMN_0701 SAUSA300_0717	Ribonucleotide-diphosphate reductase subunit beta	Inf	0.082	0.300
rpsB	NWMN_1166 SAUSA300_1149	30S ribosomal protein S2	6.686	0.089	0.300
ndk	NWMN_1378 SAUSA300_1358	Nucleoside diphosphate kinase	5.299	0.171	0.536
pykA / pyk	NWMN_1592 SAUSA300_1644	Pyruvate kinase	3.938	0.321	0.641
memE / menE	NWMN_1686 SAUSA300_1737	O-succinylbenzoic acid-coa ligase	3.938	0.321	0.641
deoB*	NWMN_0083 SAUSA300_0141	Phosphopentomutase	3.938	0.321	0.641
qoxA	NWMN_0930 SAUSA300_0963	Quinol oxidase, subunit II	3.325	0.245	0.641
hepT	NWMN_1379 SAUSA300_1359	Heptaprenyl diphosphate synthase component II	3.938	0.321	0.641
	NWMN_0079 SAUSA300_0137	Predicted GntR family transcriptional regulator	2.203	0.301	0.641
splC	NWMN_1704 SAUSA300_1756	Serine protease SplC	2.203	0.301	0.641

	NWMN_1571 SAUSA300_1624	MutT/nudix family protein	3.938	0.321	0.641
hemY / hemG	NWMN_1723 SAUSA300_1781	Protoporphyrinogen oxidase	2.636	0.408	0.748
hemH	NWMN_1724 SAUSA300_1782	Ferrochelatase	2.636	0.408	0.748
tkt	NWMN_1254 SAUSA300_1239	Transketolase	1.959	0.656	1.000
ftsA	NWMN_1095 SAUSA300_1079	Cell division protein FtsA	0.640	0.699	1.000
	SAUSA300_1408	Phage helicase	1.293	1.000	1.000
eno	NWMN_0745 SAUSA300_0760	Enolase Phosphopyruvate hydratase	1.747	0.702	1.000
ackA	NWMN_1605 SAUSA300_1657	Acetate kinase	0.643	1.000	1.000
	NWMN_2020 SAUSA300_2070	Sua5/YciO/YrdC/YwlC family protein	1.000	1.000	1.000
aroB	NWMN_1376 SAUSA300_1356	3-dehydroquinate synthase	0.859	1.000	1.000
hemE	NWMN_1725 SAUSA300_1783	Uroporphyrinogen decarboxylase	0.426	0.634	1.000
sirC	NWMN_0057 SAUSA300_0115	Iron compound ABC transporter permease SirC	1.000	1.000	1.000
sirB	NWMN_0058 SAUSA300_0116	Siderophore/iron compound ABC transporter permease SirB	1.000	1.000	1.000
pta	NWMN_0551 SAUSA300_0570	Phosphotransacetylase	0.000	1.000	1.000
	NWMN_0303	Hypothetical protein	1.000	1.000	1.000
	NWMN_0590 SAUSA300_0606	Hypothetical protein	1.959	0.656	1.000
mvaK1/ mvk	NWMN_0553 SAUSA300_0572	Mevalonate kinase	2.603	0.582	1.000
	NWMN_0353 SAUSA300_0361	Hypothetical parB-like partition protein	1.296	1.000	1.000
sarU	NWMN_2394 SAUSA300_2438	Accessory regulator U	1.000	1.000	1.000
cmk	NWMN_1386 SAUSA300_1367	Cytidylate kinase	1.000	1.000	1.000
thiN*	NWMN_1133 SAUSA300_1116	Thiamine pyrophosphokinase	1.293	1.000	1.000
rpsG	NWMN_0508 SAUSA300_0531	30S ribosomal protein S7	1.293	1.000	1.000
ctsR	NWMN_0484 SAUSA300_0507	Transcriptional regulator CtsR	1.000	1.000	1.000

*Predicted gene name

suggests physiological differences between strains. The observation that most CF sputum samples contained thymidine levels <0.25 μg/mL suggests that uptake of exogenous thymidine is not the sole mechanism by which *S. aureus* survives SXT *in vivo*. Most gene mutations commonly identified with SXT selection under low-thymidine conditions occurred not in folate metabolism—the target of SXT—but in core metabolic pathways, including glycolysis, the TCA cycle, and electron transport, all of which contribute to ATP production (**Table 2.1;** S1-9 in Gonsalves *et al.*, 2024).

The selection of electron transport mutants (hemin- and menadione-dependent SCVs) by SXT was a surprising result (**Table 2.1;** S1-9 in Gonsalves *et al.*, 2024), as these mutants typically confer aminoglycoside resistance. The slow growth and division of these SCV subtypes confer resistance to penicillin and β-lactams, limiting the active targets available to the antibiotics; decreased membrane potential, another effect of transitioning to a low-oxygen-like state, hampers the uptake of antimicrobials such as aminoglycosides and daptomycin[41,47,48]. Both slow growth and decreased membrane potential of SCVs with ETC mutations may be as effective at protecting cells from SXT as they are for survival to other antibiotics. The unanticipated ability of ETC mutants to survive SXT in the *in vitro* work presented here may explain why these mutants are also observed among CF patients treated with that drug[25,49]. Additional work is needed to understand the SXT-specific occurrence and fitness advantage of SCVs harboring non-thymidine mutations *in vivo*. Our data also showed that slow growth may be beneficial in contexts that do not require direct mutations of ETC components. Our observation that limiting *S. aureus* growth through nutrition slowed the rate of death (**Figure 2.7D**) supports the conclusion that modulation of growth and metabolism impact antibiotic tolerance and resistance, as previously reported in the literature[44,45,50]. Unexpectedly, mutants like the *ptsI* mutant NWM-TS08 and the *nrdE* mutant JE2-TS10, did not show evidence of compromised growth (**Figure 2.6B, 2.7A-C**). Thus, while slow growth can improve *S. aureus* survival (or, at the very least, slow the rate of death) to SXT, survival does not have to come at the expense of growth. Experimental work done in *E. coli* bolsters this conclusion. Zeng *et al*., reported that a *ptsI* mutant identified from several phenol-tolerant isolates exhibited improved survival without an altered growth rate when re-exposed to phenol[51], but additional investigation is needed to understand the underlying mechanism behind the growth-independent tolerance of NWM-TS08 to SXT.

We observed mutations in genes that were associated with nucleotide synthesis, but upstream of *thyA—nrdEF* and *deoB*. Of these, mutations in both *nrdE* (ribonucleotide-diphosphate reductase, alpha subunit) and *nrdF* (ribonucleotide-diphosphate reductase, beta subunit) were observed after selection under low thymidine conditions (**Table 2.1;** S1-9 in Gonsalves *et al.*, 2024). Both genes encode a subunit of the class Ib ribonucleotide reductases (RNRs), an enzyme of the only known *de novo* pathway for nucleotide synthesis in bacteria. However, this class is important for the synthesis of deoxyribonucleotide precursors under aerobic conditions. *S. aureus* also possesses class III RNRs, encoded by *nrdDG*; the expression of these genes has been shown to be upregulated in anaerobic conditions[52,53]. Mutations in *nrdE* and *nrdF* may serve as indicator of a broader metabolic

shift occurring in *S. aureus* during SXT treatment—*S. aureus* may be adapting to environmental pressures in which anaerobic-based metabolic pathways are more advantageous. While our data suggests that coding changes in *nrdE* do promote survival (**Figure 2.6B**), additional investigation of other markers of anaerobic metabolism, such as accumulation of fermentation products or the down regulation of TCA cycle enzymes[54], are necessary to clarify if the transition to anaerobic processes is occurring and staves off the impact of SXT action, or if the alternate routes of deoxyribonucleotide production themselves promote survival. On the other hand, mutations in *deoB* (the gene that encodes phosphopentomutase) in *S. aureus* isolates that survived SXT treatment were observed only at high thymidine levels, particularly in JE2 where *thyA* mutations were not observed. This gene is a part of the pentose phosphate pathway, where *deoB* is important for the conversion of ribose-5-phosphate to ribose-1-phosphate and deoxyribose-5-phosphate to deoxyribose-1-phosophate. Nucleotide production, specifically pyrimidines, is dependent on this pool, as tetrahydrofolate-dependent biosynthesis of dTMP from dUMP is reliant on the intermediate metabolites produced from both ribose-5-phosphate and ribose-1-phosphate, while the folate pathway-independent biosynthesis (pyrimidine salvage pathway) of dTMP from thymidine requires deoxyribose-1-phosophate[34,35,55]. Since both pathways are ultimately important dTMP synthesis, a loss-of-function mutation in *deoB* may play a similar role as a loss-of-function mutation in *thyA* under high thymidine conditions—the cell now becomes reliant on exogenous sources of thymidine to survive SXT. Unfortunately, no literature exists on influence of a *deoB* mutations on thymidine uptake in *S. aureus*, so whether the consequence of mutations in *deoB* mimic those in *thyA* remain to be determined. However, both *nrdEF* and *deoB* mutations highlight the important relationship between nucleotide precursors, nucleotide pools, and metabolism.

While the *in vitro* data presented here is thought-provoking, it is important to understand the *in vivo* relevance. To begin to do so, we leveraged a large clinical isolate collection from CwCF[46], focusing on those children with evidence of SXT treatment and TD-SCV positivity (**Table 2.2**). Coding alterations were observed at a statistically higher rate for *thyA,* confirming again its importance in SXT resistance, an observation that has been made clinically several times[23,25,32]. We also observed mutations at a statistically higher rate for *rpoC* (β'-subunit of RNA polymerase) and *nrdE* (α-subunit of ribonucleotide-diphosphate reductase). Mutations in *rpoB*, the β-subunit of RNA polymerase, are typically associated with resistance to the antibiotic rifampin, but mutations in *rpoC* have been observed both *in vitro* and in clinical isolates and were shown to provide increased tolerance to β-lactams and slower growth in vancomycin-tolerant isolates of *S. aureus*[56–60]. This data and our analysis indicate the potential for mutations in *rpoC* to provide antimicrobial protection for *S. aureus* to various antibiotics, possibly through slowed growth, but whether there is a causal relationship between mutations in *rpoC* in *S. aureus* and tolerance to antimicrobial agents is unclear and requires additional experimental work. The observed coding changes at a higher rate for *nrdE* was particularly interesting. As mentioned, this gene encodes α-subunit (substrate and allosteric binding sites) of class Ib RNRs, enzymes that are oxygen-dependent, unlike their class III counterparts (encoded by *nrdDG*) which are typically upregulated in anaerobic conditions[52,53]. Observing this mutation in isolates of *S. aureus* that survive SXT

treatment *in vitro*, in addition to observing mutations in this gene at a higher rate than the other genes assessed in the SXT-exposed group, suggests its importance to *S. aureus*' adaptation to SXT and potential relevance in the lung of PwCF (**Table 2.2**). Additionally, recent work has shown that the presence of *P. aeruginosa*, another prominent CF pathogen, can result in increased expression of genes that encode class III RNRs in *S. aureus* and an overall switch from aerobic respiration to fermentation[61]; thus, metabolic changes that are mediated by other lung pathogens may impact the effects of antibiotics on *S. aureus*. This underscores the importance of taking the complex and polymicrobial nature the lungs of PwCF into consideration when assessing *S. aureus* adaptation to antibiotics.

The clinical isolate collection from CwCF yielded interesting results; there were unfortunately limits to these results. This analysis lacked a thorough examination of longitudinal isolates that could have clarified how adaptation in *S. aureus* occurred over the course of SXT treatment, specifically, if our genes of interest accrued mutations. Additionally, while we tried to include and exclude children based on their SXT treatment status during the study period, there is a possibility that a portion of them may have been treated with SXT prior to the start of the study, or for whom SXT treatment during the study was not recorded. Moreover, almost all children in the SXT-unexposed group were still exposed to other antibiotics throughout the duration of the study, and while we did not test the effects of other antibiotics in our *in vitro* model, there is convincing evidence from other studies that many antibiotics could select for metabolic mutations similar to those we saw with SXT[62–64], especially because many antibiotics rely on active bacterial metabolism for their efficacy[50,65]. These confounders limited the comparisons we were able to confidently make between our *in vitro* data and clinical isolates from CwCF with evidence of SXT treatment. A proper, matched control group, and an expanded collection that better reflects the *in vivo* diversity of *S. aureus* in PwCF over the course of SXT treatment is ultimately required to better understand mutation accrual in *S. aureus* and their impacts SXT efficacy.

The work described above had several additional important limitations. While we studied several strains/isolates of *S. aureus,* our experiments were largely conducted *in vitro*, and clinical relevance was not always demonstrated. Specifically, we used artificial media to define thymidine requirements for growth, and the conditions we used for mutant selection may not be uniformly physiologically relevant (for example, our monoculture experimental designs do not accurately model the polymicrobial nature of CF respiratory infections[5,66]). In addition, our analysis of thymidine availability *in vivo* was limited to sputum, a complex mixture of mucus, bacterial cells, bacterial byproducts, and other features such as inflammatory cells, carbon sources, ions, and amino acids[67]. Our analysis was unable to the availability of thymidine in different locations in the lung. However, the work presented does highlight the complex, non-linear nature of SXT mechanism of action and *S. aureus* adaptation, emphasizing how auxiliary pathways, or those not directly targeted, can affect survival. These results expand our understanding of the mechanisms behind SXT tolerance.

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CHAPTER 3: METABOLISM AS A MEDIATOR OF TRIMETHOPRIM SULFAMETHOXAZOLE-INDUCED CELL DEATH IN *S. AUREUS*—PART 2

Most of the work presented in this chapter was adapted from published work:

Gonsalves, L. J. *et al.* Mechanisms of *Staphylococcus aureus* survival of trimethoprim-sulfamethoxazole-induced thymineless death. *mBio* **15**, e01634-24 (2024).

What are the ultimate causes of SXT-driven cell death and what can adaptive mutants tell us about survival to SXT?

Introduction

The work presented in **Chapter 2** highlighted two important conclusions: 1) thymidine availability influences survival and the mechanism *S. aureus* relies on to survive SXT-mediated TLD, and 2) metabolism plays an important role in this survival, particularly under low-thymidine conditions. Under thymidine-replete conditions, the predominant mechanisms of survival involved adaptations in both the folate biosynthetic pathway, and in broader nucleotide metabolism were the distinctive mechanisms of survival. Under thymidine deplete conditions, most of the mutations observed suggested a broader mechanism of surviving SXT-induced TLD: Modulation of intracellular ATP levels.

As discussed in **Chapter 1**, thymidine starvation results in defective DNA synthesis and repair in a process known as thymineless death (TLD), which was first identified over 60 years ago[1,2]. Since then, most of the research on thymidine starvation and TLD has been conducted in *Escherichia coli*[1–4], resulting in a model in which thymidine depletion within the cell, coupled with continued attempts at DNA replication, result sequentially in nucleotide mis-incorporation, impaired DNA repair and, ultimately, cell death[1]. Subsequent studies have added to this model, suggesting that increased production of reactive oxygen species (ROS) is responsible for much of this damage and death[5,6]. However, the involvement of ROS in lethality by this and other antibiotics, and among other bacterial species[7], remains controversial[8,9]. Specifically, little work has examined the role of both DNA damage and ROS accumulation in TLD in *S. aureus*. Clarifying the mechanisms of *S. aureus* TLD, including the role of ROS, and the mechanisms with which *S. aureus* survives TLD, represent key steps towards a broader mechanistic understanding of antibiotic-mediated killing.

The data presented in **Chapter 1** showed that thymidine starvation and TLD are not mechanistically independent from other pathways in the cell, but rather intersect with them. For example, we observed mutations in *nrdEF* and *deoB*—two genes that are not canonically associated with core metabolism—in SXT-adapted *S. aureus*; we experimentally validated the relevance of non-canonical adaptations with several mutants containing mutations in the ETC (*menB*, *hemB*) and phosphotransferase system (*ptsI*), showing that they provided protection from SXT-mediated TLD. The primary targets of most antimicrobials are key components or pathways of the bacterial cell, such as cell wall synthesis, chromosomal DNA and its replication, ribosomal function, RNA polymerase function, and, in the case of SXT, tetrahydrofolate synthesis[10,11]. However, it has long been known that cells can also modulate metabolism to circumvent antimicrobial action. For example, in *S. aureus* SCVs, ETC mutations provide resistance to a variety of antibiotics[12–14]. In *E. coli*, mutations that conferred resistance were found in genes unrelated to the antibiotic in question, including tricarboxylic acid (TCA) cycle genes and ROS. Khan and Kuzminov have suggested that central metabolism may play an effective role in alleviating TLD in *E. coli*, a substantial shift in thinking from the conventional understanding of factors that mitigate TLD[15]. Thus, while the mutations described in **Chapter 2** informed the general hypothesis that ATP levels are important for determining *S. aureus* survival to SXT, the

specific dynamics of ATP, and how the levels changed for *S. aureus* exposed to SXT remained unclear, warranting further investigation.

Here, we explored the relevance of ROS, central metabolism, and ATP regulation in TLD in *S. aureus*. We expected ROS to increase in response to SXT treatment, inducing TLD in *S. aureus* and that *S. aureus* survival would be dictated by ATP levels. We showed that that ROS production is not the primary driver of cell death in *S. aureus*, in contrast with prior studies conducted in *E. coli*, but that ATP does play a role in SXT-mediated TLD, and its modulation may determine if *S. aureus* survives SXT exposure.

Results

The effects of functional alterations in *ptsI* indicate the importance of metabolism in surviving TLD

The genetic analyses discussed in **Chapter 2** indicated that modulation of core metabolic activities could confer survival of SXT under thymidine limitation. One of the metabolic gene mutations repeatedly selected among both *S. aureus* strains JE2 and Newman under those conditions occurred in *ptsI* (phosphoenolpyruvate phosphotransferase, **Table 2.1**, S2-9, in Gonsalves *et al.*, 2024). The encoded enzyme catalyzes the first step in the phosphotransferase system (PTS), which regulates sugar import and carbohydrate metabolism[16]. Mutant NWM-TS08 contains a nucleotide change in *ptsI* (T1625A) resulting in a premature stop codon (Leu542*) (Table S1A, in Gonsalves *et al.*, 2024). NWM-TS08 showed little difference in growth relative to wild-type *S. aureus* Newman in the absence of SXT; when treated with SXT, growth was halted in NWM-TS08 over 10h (**Fig. 3.1A**). Complementation of NWM-TS08 with a wild-type copy of *ptsI* (NWM-TS08 + pCN34-*ptsI*) restored SXT susceptibility of NWM-TS08 to that of wild-type (**Fig. 3.1B**), indicating a role for the PTS in mediating susceptibility to SXT. Interestingly, we found no evidence connecting the PTS with folate metabolism from prior published work. However, a recent study reported that mutations in the *E. coli* PTS conferred general antimicrobial tolerance, although this study did not investigate SXT in particular[17]. Because *ptsI* mutants exhibited SXT tolerance but not slow growth, we reasoned that *ptsI* inactivation conferred survival of SXT through as-yet undefined pathways or factors, and we therefore included a *ptsI* mutant (NWM-TS08) in our subsequent experiments to assist with investigating these candidate mechanisms.

ROS are not required for SXT lethality in *S. aureus*

Recent work, largely using *E. coli,* suggested ROS play a dominant role in killing by SXT and perhaps other bactericidal antibiotics[5,6]. Because ROS are produced by partial reduction of oxygen at the ETC and are scavenged by staphyloxanthin[18], the selection of ETC-defective and staphyloxanthin-overproducing mutants by SXT challenge suggested that reducing ROS could be another TLD survival strategy. We first tested this hypothesis by determining the requirement for

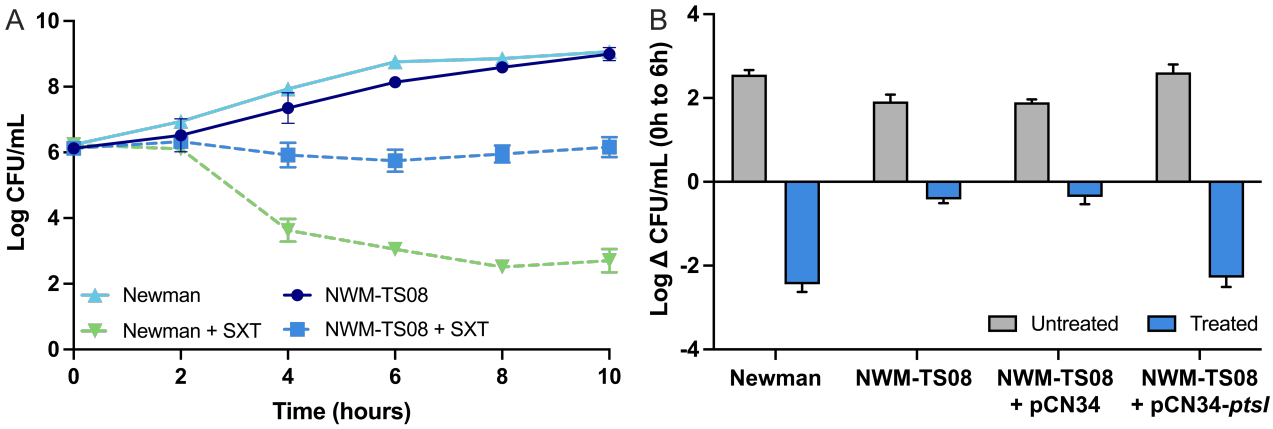

Figure 3.1 Survival of the *S. aureus ptsI* mutant complemented with wildtype *ptsI* during SXT challenge (A) Survival kinetics of *S. aureus* Newman and NWM-TS08 with and without SXT in LB over 10h in the absence of additional thymidine; SXT-treated conditions are indicated by a dotted line and data are mean ± SD, (n=3). **(B)** Values on the y-axis are expressed as change in Log CFU/mL for parental strain Newman, *ptsI* muntNWM-TS08, and NWM-TS08 complemented with wildtype *ptsI* after 6h with or without SXT treatment relative to 0h timepoint. Data represent mean ± SD (n=3).

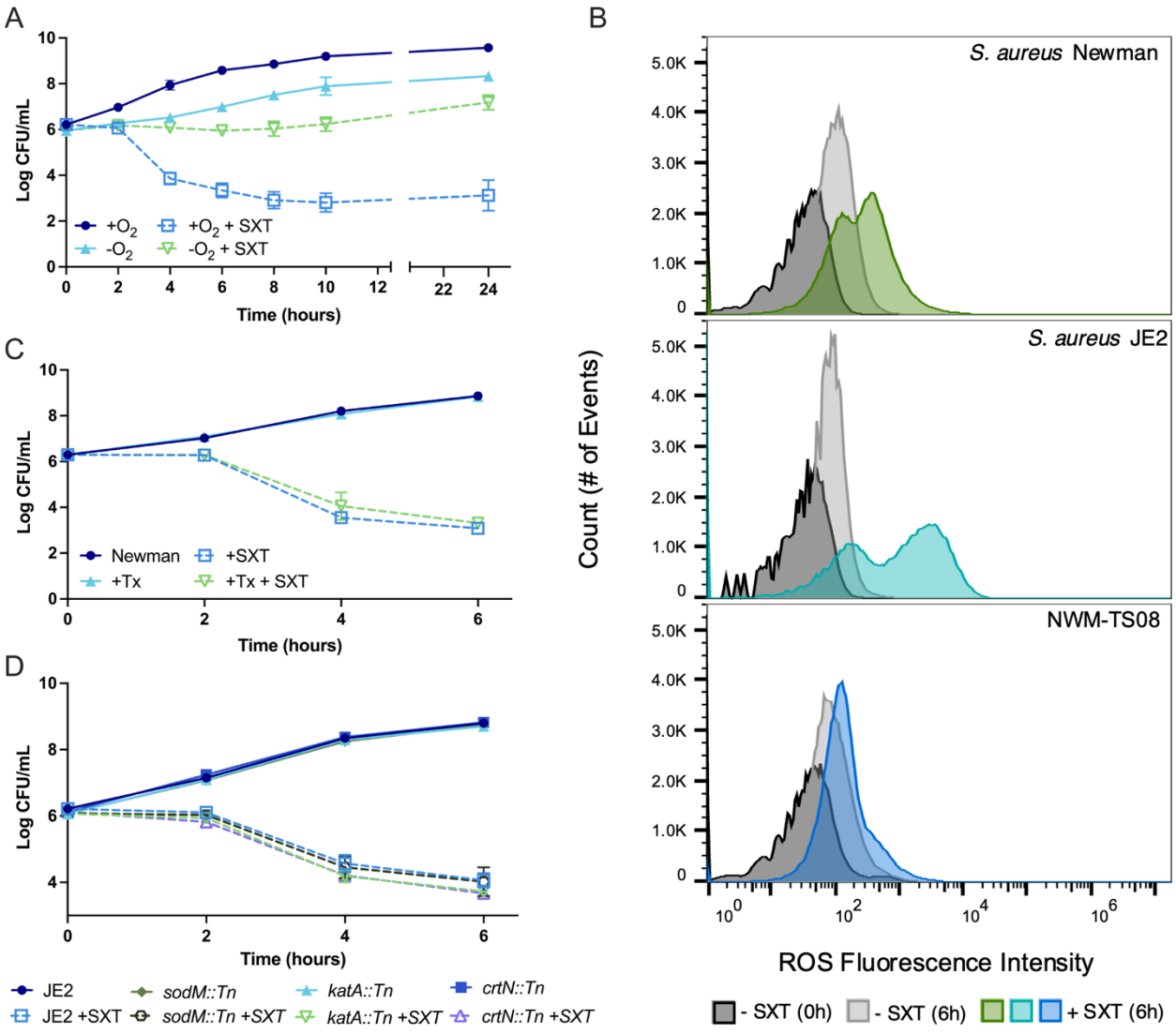

Figure 3.2 Effect of oxygen and reactive oxygen species on *S. aureus* survival of SXT (A) Survival kinetics of *S. aureus* Newman cultured under aerobic versus anaerobic conditions in LB over 10h in the absence of additional thymidine and treated with SXT; data are mean ± SD (n=3). **(B)** Intracellular ROS, measured by flow cytometry and fluorescent indicator CMH_2DCFDA, in *S. aureus* Newman, JE2, and *ptsI* mutant NWM-TS08 with and without SXT at 0h and 6h. 100,000 events were collected for each condition, where possible. Representative data of at least three replicate experiments. **(C)** Survival kinetics of *S. aureus* Newman with and without ROS scavenger Trolox (Tx) and SXT in LB over 6h in the absence of added thymidine; SXT-treated conditions are indicated by a dotted line and data are mean ± SD (n=2). **(D)** Survival kinetics of *S. aureus* JE2 carrying transposons in ROS detoxification genes (*sodM*::Tn, *katA*::Tn, *crtN*::Tn) with and without SXT in LB over 6h in the absence of additional thymidine; SXT-treated conditions are indicated by a dotted line and data are mean ± SD, (n=3).

oxygen in SXT antimicrobial activity. We cultured Newman in the absence and presence of oxygen and SXT for up to 24h (**Fig. 3.2A**). We found that anaerobic growth was slower than aerobic growth, but that the addition of SXT without oxygen had minimal effect on cell viability under these conditions. indicating that either oxygen (and potentially ROS) or maximal growth is required for full SXT lethality and TLD in *S. aureus*.

To determine the relationship between ROS and killing by SXT, we then measured intracellular ROS levels during aerobic growth with and without SXT. Using a flow cytometric assay of the broad-range ROS fluorescent indicator CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate), we found that ROS levels in *S. aureus* were higher in the presence of SXT for both JE2 and Newman (**Fig. 3.2B**), similar to previously published results[7], compatible with a role for ROS in SXT lethality. Mutant NWM-TS08 exhibited only a subtle increase in intracellular ROS with SXT when compared to Newman and JE2 (**Fig. 3.2B**), indicating either that decreased ROS activity supports SXT tolerance or that it is a byproduct of decreased metabolic activity.

We therefore tested whether ROS mitigation improved survival of SXT by treating Newman with the drug in the presence and absence of the membrane-permeable antioxidant Trolox, which scavenges hydroxyl radicals, hydrogen peroxide, and peroxyl radicals. Although Trolox substantially decreased intracellular ROS (**Fig. 3.3**), it exerted no substantial effect on either growth or SXT killing in Newman (**Fig. 3.2C**). We also measured ROS levels and SXT effects for JE2-derived mutants carrying transposon insertions[19] in genes encoding ROS detoxification enzymes, including catalase (*katA,* NE1366), superoxide dismutase (*sodM*, NE1224), and 4,4'-diapophytoene desaturase (*crtN*, an enzyme in the staphyloxanthin biosynthetic pathway, NE382) and found no effect of impairing ROS detoxification on SXT killing (**Fig. 3.2D**). These data indicate that while ROS are increased in the presence of SXT, they are not the primary drivers of SXT-induced cell death in *S. aureus*.

SXT activates the SOS response in wild-type *S. aureus* but not the *ptsI* mutant

DNA damage is considered a sentinel event during TLD in *E. coli*[4,20,21] and during SXT-mediated cell death in *S. aureus*[7]. In thymidine deplete conditions, DNA strand breaks induce the pleiotropic SOS stress response, generating widespread changes in gene expression and corresponding cellular activities. This response is triggered by RecA binding to damaged DNA, activating RecA, which in turn cleaves SOS repressor LexA, resulting in increased expression of SOS-responsive genes such as those encoding DNA repair enzymes[22,23]. In the absence of sufficient nucleotide pools, attempts to repair DNA promotes further DNA damage and cell death. Because the *ptsI* mutant NWM-TS08 exhibited greatly improved survival against SXT relative to wild-type, we compared the SOS response during SXT exposure in these two strains. Using a *recA* promoter-GFP fusion construct, we measured SOS activity at 6h, during active cell death under our experimental conditions, in Newman and NWM-TS08 with and without sub-inhibitory SXT (**Fig. 3.4A**).

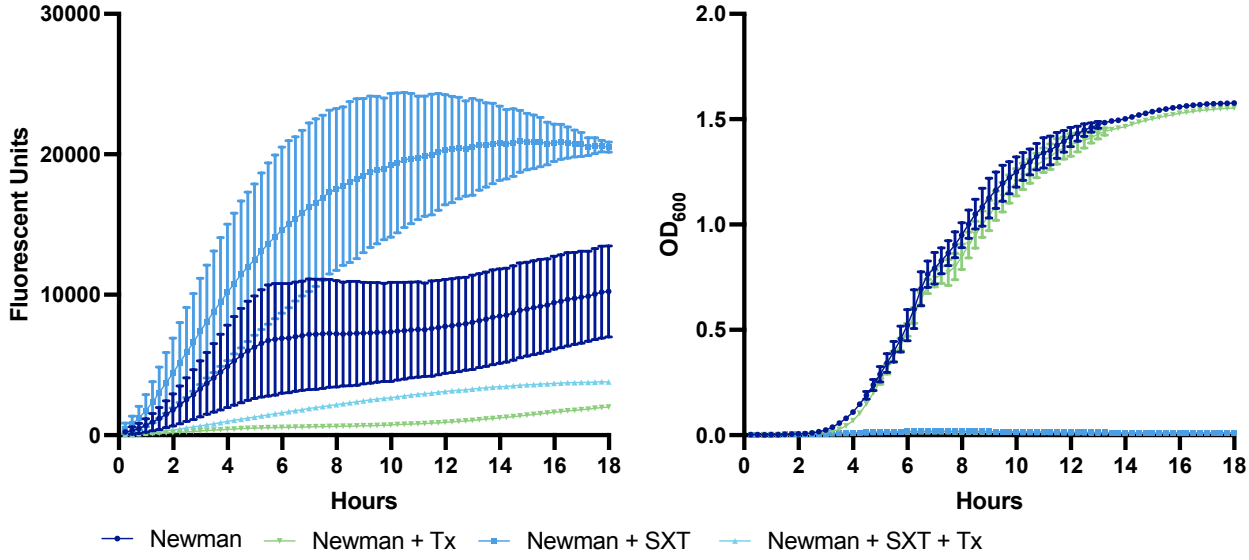

Figure 3.3 Total reactive oxygen species levels present in *S. aureus* in experiments supplemented with the ROS scavenging compound Trolox *S. aureus* Newman treated with SXT, CMH_2DCFDA and Trolox and analyzed via spectrophotometer for ROS levels over 18h. Data presented as fluorescence (left) and OD_{600} (right) over time; data are mean ± SD (n=2).

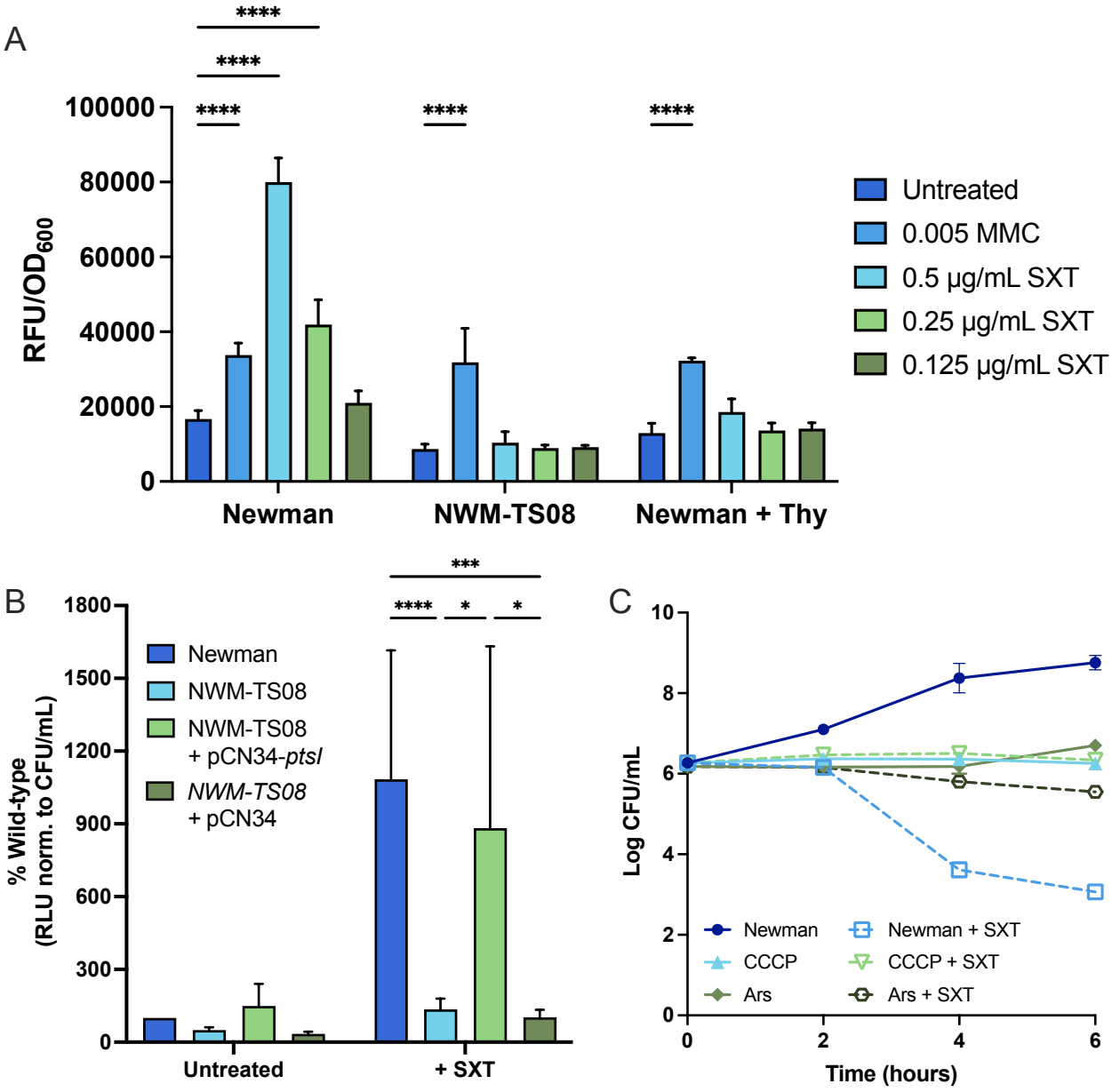

Figure 3.4 SOS induction, ATP levels, and survival during SXT treatment after ATP depletion (A) *recA*-GFP expression in response to sub-inhibitory levels of SXT (1.25-0.5 µg/mL SXT) or mitomycin C (0.005 µg/mL MMC; control) in Newman (with and without 128 µg/mL thymidine) and NWM-TS08 at 6h post-SXT exposure; RFU was normalized to OD_{600} and significance reported as **** when p≤0.05 (two-way ANOVA). Data are mean ± SD (n≥3) **(B)** Relative whole-culture ATP levels at 2h post-SXT exposure (RLU normalized to CFU/mL) presented as percent of wild-type for treated and untreated Newman, NWM-TS08 and NWM-TS08 complemented with wild-type *ptsI* (NWM-TS08 + pCN24-*ptsI*); data are mean ± SD (n≥3) and significance reported as *, ***, or **** where p≤0.05 (two-way ANOVA). **(C)** Survival kinetics of Newman in LB with and without SXT and added CCCP or arsenate (Ars) as indicated; SXT-treated conditions are indicated by a dotted line and data are mean ± SD (n=3)

Experiments with Newman were repeated with excess thymidine (128 μg/mL). As a positive control, strains were also exposed to mitomycin C (0.005 μg/mL), a well-characterized SOS response-inducing agent[24]. SOS induction dynamics were indistinguishable among all strains treated with mitomycin C, indicating that all strain and conditions had similar intrinsic SOS response capacities. In contrast, SOS was induced in Newman, but not NWM-TS08, during treatment with SXT. Exogenous thymidine suppressed SOS induction by SXT in Newman, signifying a close relationship between SXT lethality and the SOS response. These data indicate that SOS induction plays a role in SXT lethality and suggest that mitigation of this response could contribute to cell survival in wild-type and the *ptsI* mutant.

Preliminary work suggests DNA fragmentation occurs with TLD

The above results demonstrate that SXT activates the SOS response in *S. aureus*. Because elements of the SOS response, including ROS production, are known to contribute to DNA damage in *E. coli*[3,5,6,25,26], we aimed to determine whether SXT treatment leads to DNA damage in *S. aureus* using pulsed-filed gel electrophoresis (PFGE) to qualitatively assess chromosomal fragmentation, as PFGE allowed the visualization of larger fragments that may occur separate from the intact chromosomal DNA[26,27]. We assessed the fragmentation profiles of genomic DNA extracted from cultures of Newman at 0h and 3h with and without SXT treatment and Δ*thyA* at 0h, 0.5h, 1h and 3h without SXT treatment (**Figure 3.5**). Where possible, we used the restriction enzymes *SmaI* or *SbfI* on Newman without SXT as a positive control with known fragmentation patterns. Initially, we observed (**Figure 3.5**, **left panel**) an extended DNA fragment band (presenting as a smear along the length of the gel) at the top portion of the gel for all conditions tested, similar to the gels presented by Khan and Kuzminov in their work with *E. coli*[26]. We observed that the intensity of the fragment band was less for Δ*thyA* at 0.5h and 3h compared to Δ*thyA* at 0h and 1h, potentially due to the stage of TLD occurring within the cells. Surprisingly, we observed that a fragment band was present for the Newman untreated conditions but not in the SXT-treated conditions.

We hypothesized that the absence of DNA in the SXT-treated lane for wild-type Newman could be due to the presence of DNA fragments that were too small to be retained in the gel. With shorter electrophoresis times, we observed that there were indeed two fragment band (**Figure 3.5**, **middle panel**). Fragmentation bands in group "A" (bands present at the top of the gel) corresponded to bands present in original PFGE experiment (**Figure 3.5**, **left panel**). For the Newman treated and untreated conditions, the fragmentation bands band in group "B" (bands present at the bottom of the gel) looked similar to each other, but the group "B" bands of the Δ*thyA* showed increasing intensity over time. To better understand the bands in group "B", which are presumably made up of smaller fragments, we conducted a similar experiment, with and without SXT, using conventional gel electrophoresis and extended the timeframe to 8h to better assess the presence of smaller DNA fragments. The presence of bands was much clearer, indicating small fragments present in cell lysates (**Figure 3.5**, **right panel**); however, the relationship between traditional gel band profiles and the PFGE profiles remains unknown. Together, these data

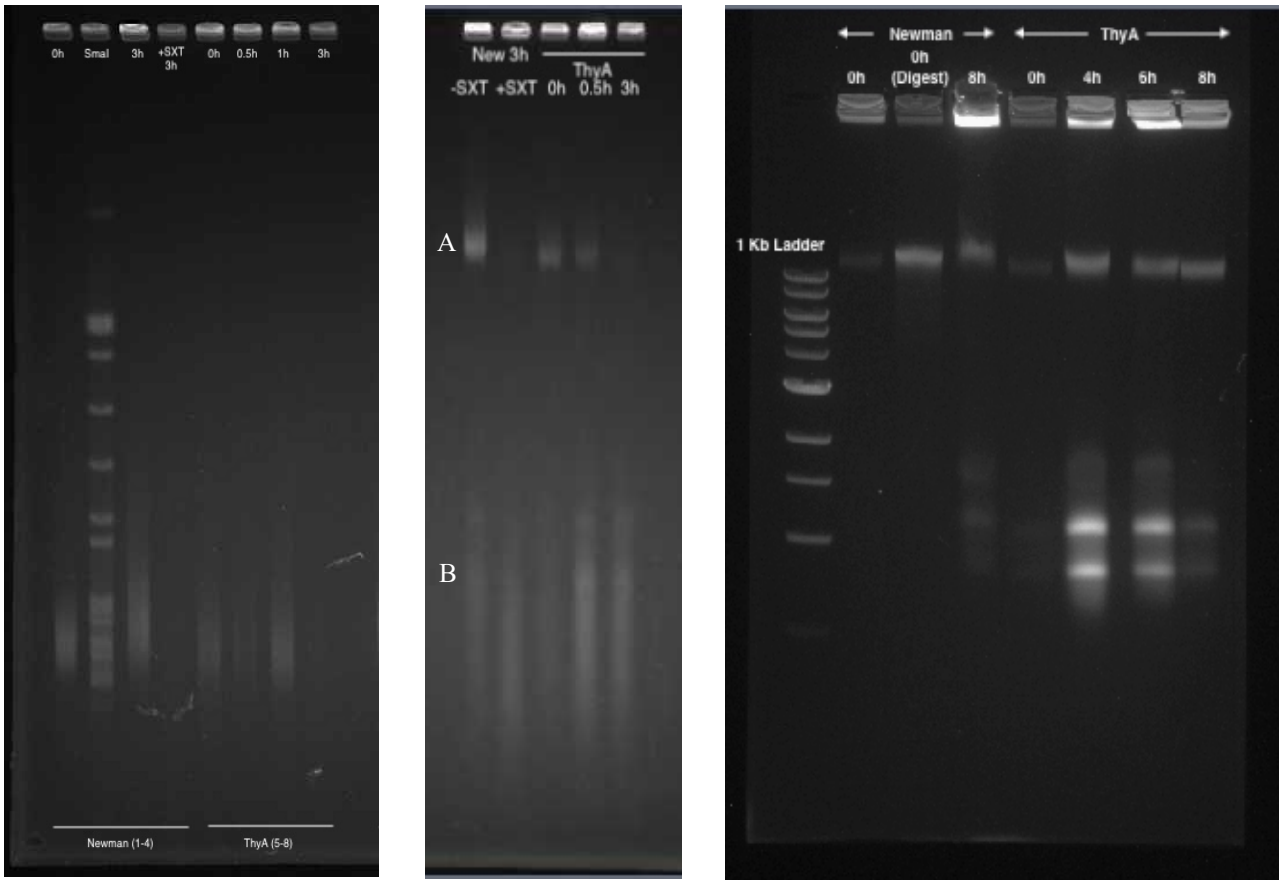

Figure 3.5 Pulsed-field and conventional gel electrophoresis of SXT-treated *S. aureus* strain Newman and a Δ*thyA* mutant (untreated) (Left) Ethidium bromide-stained pulsed-field gel of DNA extracted from Newman cells at 0h (untreated) and 3h (SXT-treated and untreated) and untreated ΔthyA cells over 0h-3h; a *SmaI* digest was included as a fragmented control. Electrophoresis was conducted for 23h, split into two periods comprising of 10h (initial switch: 5s, final switch: 10s) and 13h (initial switch: 15s, final switch: 60s), at 6 V/cm and 120°. **(Middle)** Ethidium bromide-stained pulsed-field gel of Newman cells at 3h (treated and untreated) and ΔthyA cells over 0h-3h; electrophoresis was conducted for 8h, split into two blocks of 3h (initial switch: 5s, final switch: 10s) and 5h (initial switch: 15s, final switch: 60s), at 6 V/cm and 120°. **(Right)** Conventional gel of Newman cells at 0 and 8h and ΔthyA cells over 0h-8h run alongside a restriction digest control (*SbfI*); a 1 kb ladder was included for fragment size estimation.

suggest that thymidine starvation (including SXT-mediated TLD) may result in chromosomal fragmentation in *S. aureus*, but our data did not definitively demonstrate this fragmentation; additional work is needed to truly determine what portion of fragmentation occurs naturally versus because of thymidine starvation.

A central role for ATP production in lethality and tolerance of SXT in *S. aureus*

Several SXT-selected mutations we identified by sequencing, and results from anaerobic SXT challenge (**Fig. 3.2A**), suggested that ATP generation may be a critical component of TLD. ATP is an important cofactor for RecA and the SOS response[22,23]. ATP plays important roles in stress responses and their consequences, including the formation of multidrug-resistant dormant cells known as persisters[28–30]. Therefore, we investigated the role of ATP in TLD and whether diminished ATP levels might confer survival of SXT-induced TLD in *S. aureus.* We took advantage of the temporal lag in TLD onset—evident in the growth curves in **Figs. 2.1A-B** where no decrease in cell density was apparent until after 2 hours of SXT exposure—to compare the relative dynamics of intracellular ATP and cell density. Using a luciferase-based assay, we measured ATP in wild-type Newman, NWM-TS08, NWM-TS08 + pCN24-*ptsI*, and NWM-TS08 + pCN24 over 2h of SXT exposure. In the absence of SXT treatment, amounts of ATP were similar between all strains with no significant differences observed during the experiment (**Fig. 3.4B**). SXT treatment increased ATP levels in strains with a wild-type *ptsI* gene (Newman and NWM-TS08 + pCN24-*ptsI*) 10.8- and 5.4-fold higher, respectively, compared to the same strains without treatment. ATP levels were significantly higher in SXT-treated, *ptsI* wild-type containing strains (Newman and NWM-TS08-pCN24-*ptsI*) compared to *ptsI* mutant NWM-TS08 with and without an empty vector (**Fig. 3.4B**). Based on these results, we hypothesized that limiting ATP levels could improve survival against SXT.

To test this hypothesis, we first determined the effect of impairing ATP production on SXT survival in Newman by disrupting electron transport with the proton gradient-uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The addition of 10μm CCCP simultaneously halted growth and mitigated SXT-induced cell death compared to no CCCP conditions (**Fig. 3.4C**). To test the effects of limiting ATP levels on SXT survival more directly, we repeated the experiment using arsenate, which irreversibly inactivates ATP[31]. The effects of 1mM arsenate on both cell density (**Fig. 3.4C**) and ATP production (**Fig. 3.6**) in the presence and absence of SXT resembled those of CCCP, providing further evidence that limiting ATP production improves *S. aureus* survival with SXT treatment.

Discussion

We have shown that under thymidine-deplete conditions, as many of the metabolic mutations observed in **Chapter 2** suggested, survival was influenced by ATP and metabolic rates but not by ROS, in contrast with findings from studies in *E. coli*. We found that beyond thymidine,

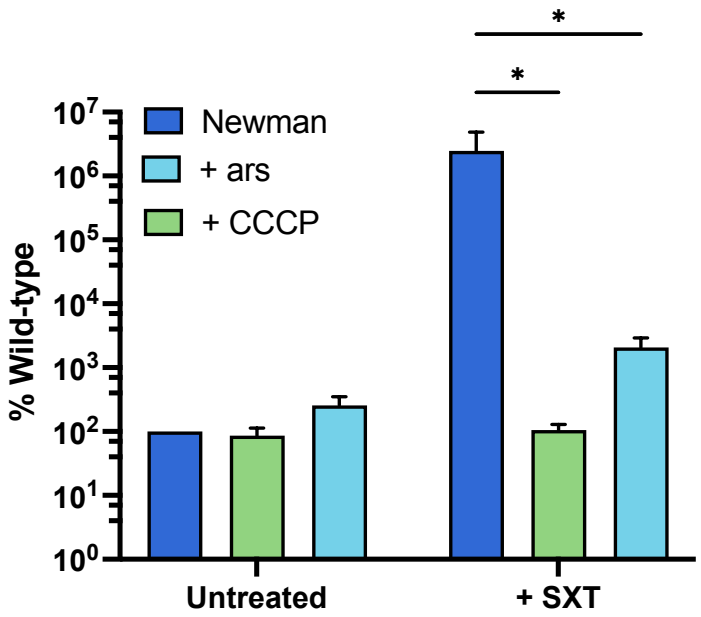

Figure 3.6 Whole-culture ATP levels with SXT treatment and either arsenate (ars) or CCCP Relative ATP levels at 6h post-SXT exposure (RLU normalized to CFU/mL) presented as percent of wild-type for treated and untreated Newman and isolate 029. The ratio of treated to untreated condition for Newman only, Newman with ars, and Newman with CCCP is 24,765.47, 1.23, and 8.03, respectively; data are mean ± SD (n=3) and significance (*) reported where p-value ≤ 0.05, as determined via a two-way ANOVA.

manipulating growth (oxygen and nutrient limitation) and ATP metabolism (CCCP and arsenate treatment) conferred survival of SXT in predictable patterns, indicating that even reversible alterations in respiratory activity limit killing by this antibiotic. Chemically neutralizing the ROS generated during SXT exposure did not improve survival; instead, the work presented here demonstrated a strong relationship between SXT exposure and SOS activation. Whether this activation is due to DNA damage within the cells remains unclear.

The work presented in this chapter demonstrated the importance of ATP in *S. aureus* survival to SXT. While we initially hypothesized that improved survival in an anaerobic environment (**Fig. 3.2A**) was due to a reduction in ROS accumulation, further investigation revealed a dramatic increase in ATP levels following SXT exposure in *S. aureus* Newman, indicating that perhaps survival to SXT was dependent on metabolic activity of the cell. The observed increase in ATP occurred after just 2h of SXT exposure, a timepoint at which treated cells exhibited very little loss in viability (**Fig. 2.1A, B**), may be a marker of thymidine starvation, before TLD has occurred. This possibility was supported by the improved survival of the *ptsI* mutant NWM-TS08 (**Figure 2.6B**), a mutant that exhibited drastically reduced ATP levels following SXT exposure relative to wild-type under the same conditions. Moreover, the chemical-based reduction of ATP also promoted survival of TLD (**Figure 3.4C**), collectively strongly implicating ATP fluctuations in *S. aureus* responses to SXT exposure. Considering the mutants selected by SXT *in vitro* described in **Chapter 2**, it was not surprising that we observed the involvement of ATP in SXT tolerance. Similarly, recent studies from several groups demonstrated that decreasing core metabolic activity can confer broad antibiotic tolerance in diverse species. For example, in *Pseudomonas aeruginosa*, the disruption of tolerance-promoting genes resulted in increased cellular ATP and decreased persistence[32]. In *E. coli*, growth arrest through nutrient starvation or exposure to certain bacteriostatic antimicrobials (including trimethoprim) reduced ATP levels and conferred protection during subsequent antibiotic exposure[33]. In both *E. coli* and *S. aureus*, ATP depletion was linked to both drug tolerance and persistence[30,34]. While cellular energetics and the role of the environment are at the center of the work described here, the importance of complex factors such as the type and level of stress and the metabolic states of the cell itself in antimicrobial lethality remain incompletely defined.

DNA damage and the SOS response have been reported to play important roles in TLD,[7,35] and thymidine starvation likely increases lethality by impairing DNA repair[21,25]. Evidence supporting a role for DNA repair in TLD include the observation that related enzymes, such as *dnaA* or *mutM* and *mutY* in *E. coli,*[4] and rexAB and *xerC* in *S. aureus*[7,35]*,* were required for survival against antifolates. Our observation of a close relationship between SOS induction, presumably induced by DNA breaks that are difficult to experimentally demonstrate, and SXT lethality suggests a role for DNA damage in SXT action, at least in *S. aureus*. Given the demonstrated importance of ATP for both RecA function and overall SOS responses in other species[22,23] and the parallels we observed in *S. aureus* survival (observed decreases in SOS induction and ATP in NWM-TS08 with

SXT, relative to SXT-treated wild-type), ATP may play a relatively complex role in regulating survival of TLD in a mechanism coupled to DNA repair; whether SOS induction stimulates higher rates of ATP production or if excess ATP is a prerequisite for SOS induction, and the precise impact, if any, of single- and double-stranded DNA breaks, remains unexplored in *S. aureus*. To complement our work on the role of SOS in SXT-mediated TLD, we investigated the occurrence of DNA damage in *S. aureus* as a potential consequence of thymidine starvation. While our data is highly suggestive and requires substantial additional work to confirm and quantify any DNA damage that may be occurring during thymidine starvation, thymidine starvation-driven DNA damage has been widely reported in the literature. For example, several studies reported evidence of DNA single-stranded breaks during thymidine starvation, demonstrating that thymidine starvation does impact the integrity of the DNA itself[1,5,21]. Khan and Kuzminov studied DNA damage on a larger scale, reporting the presence of chromosomal fragmentation during TLD, but their findings suggests that chromosomal fragmentation is ultimately not required for TLD. The explicit impact DNA damage has on downstream processes has largely studied in *E. coli* and future studies are required to clarify the role, and types (single- or double-strand breaks), of DNA damage in lethality in other species, including *S. aureus*.

It has long been proposed that ROS are directly responsible for cell death in an *E. coli* model of TLD,[5,6,17] although other work with that species contradicts that view[8,9,36]. For example, Rao and Kuzminov showed that neither blocking ROS accumulation in *E. coli* through chelation nor amplifying oxidative damage influenced TLD[36]; similarly, the work done by both Keren et al.[9] and Liu and Imlay[8] suggested that, in general, antibiotic cell death can occur without the involvement of ROS. Our results also indicated no direct role for ROS in SXT lethality in *S. aureus.* In agreement with observations by Clarke et al.,[7] we observed elevated intracellular ROS with SXT exposure in *S. aureus*. However, we found that chemically inactivating ROS did not improve survival in *S. aureus*, suggesting that, in general, cell death does not require changes in ROS levels during antibiotic treatment. Recent studies have explored whether, rather than contributing to cell death, ROS may instead play a role in survival. Observations made by Rowe et al. suggest that ROS may (either through exogenous exposure or during macrophage internalization) prime *S. aureus* cells for increased tolerance to subsequent antibiotic exposure by disrupting core metabolic enzymes (often those containing iron-sulfur cluster and thus prone to oxidation)[37,38]. Peyrusson et al. drew similar conclusions, but instead suggested that ROS promote tolerance through the induction of dormancy, driven by translation and ATP production deficits[29,39]. Taken together, these studies suggest that ROS may reflect the induction of a stress response that benefits the cell, but that the nature of ROS involvement in TLD and TLD-like cell death is complex, species-specific, and potentially dependent on both internal and external factors that requires further study.

The work discussed in this chapter has expanded our understanding of the mechanisms of SXT-mediated TLD but is not without limitations. Our study focused on bacterial determinants of SXT lethality and *S. aureus* survival, while other effects may predominate in limiting the *in vivo* efficacy

of antibiotics such as SXT, such as drug penetration in infected tissues or other nutrients/pressures not accounted for in our model system, limiting generalizability to other infections and species. Our understanding of the role of DNA damage in TLD is currently limited to SOS activation—our investigation of DNA damage remains preliminarily and was not able to discriminate among different types of DNA damage or determine whether any DNA damage is essential for TLD. Additionally, our understanding of the role of ROS in SXT-mediated TLD in *S. aureus* is still incomplete; ROS are difficult to quantify via indicator dyes and the impact of ROS on the cell (death versus growth inhibition) is difficult to decern[40].

The work presented here and in **Chapter 2** are parts of a whole. **Chapter 2** describes the many potential avenues of survival for *S. aureus* to SXT-mediated TLD. This **Chapter** (**3**) further defines these routes of survival by distinguishing mechanisms of TLD in *S. aureus* from those found in *E. coli*, while in the process laying a foundation for a better understanding of what truly drives SXT-mediated TLD in *S. aureus* and thus, survival. These two chapters suggest a model of *S. aureus* survival during SXT exposure (**Fig. 3.7**) in which thymidine starvation causes DNA damage that induces stress responses, ATP production[23], and ROS generation (perhaps as by-products of increased aerobic respiration) either through independent or interconnected pathways. While elevated ROS abundance is not a major contributor to lethality in *S. aureus*, we propose that increased ATP availability mediates cell death that can be prevented through exogenous thymidine uptake and utilization, reduced SOS response, or mutations or treatments that promote decreased electron transport, core metabolism and/or ATP availability. In support of this model, we found that manipulating availability of thymidine or ATP, or dampening growth, each conferred survival of *S. aureus* to SXT in predictable patterns, indicating that even reversible alterations in respiratory activity limit killing by that antibiotic. This work supports a broader, context-dependent paradigm for the mechanism of TLD in bacteria in which environmental conditions, duration of stress exposure, and even the species determine the dominant pathways of bacterial death. These findings emphasize the importance of considering the diversity of host tissue conditions (such as nutrient and thymidine concentrations) in designing improved, broad-spectrum antimicrobials targeting pathways related to TLD.

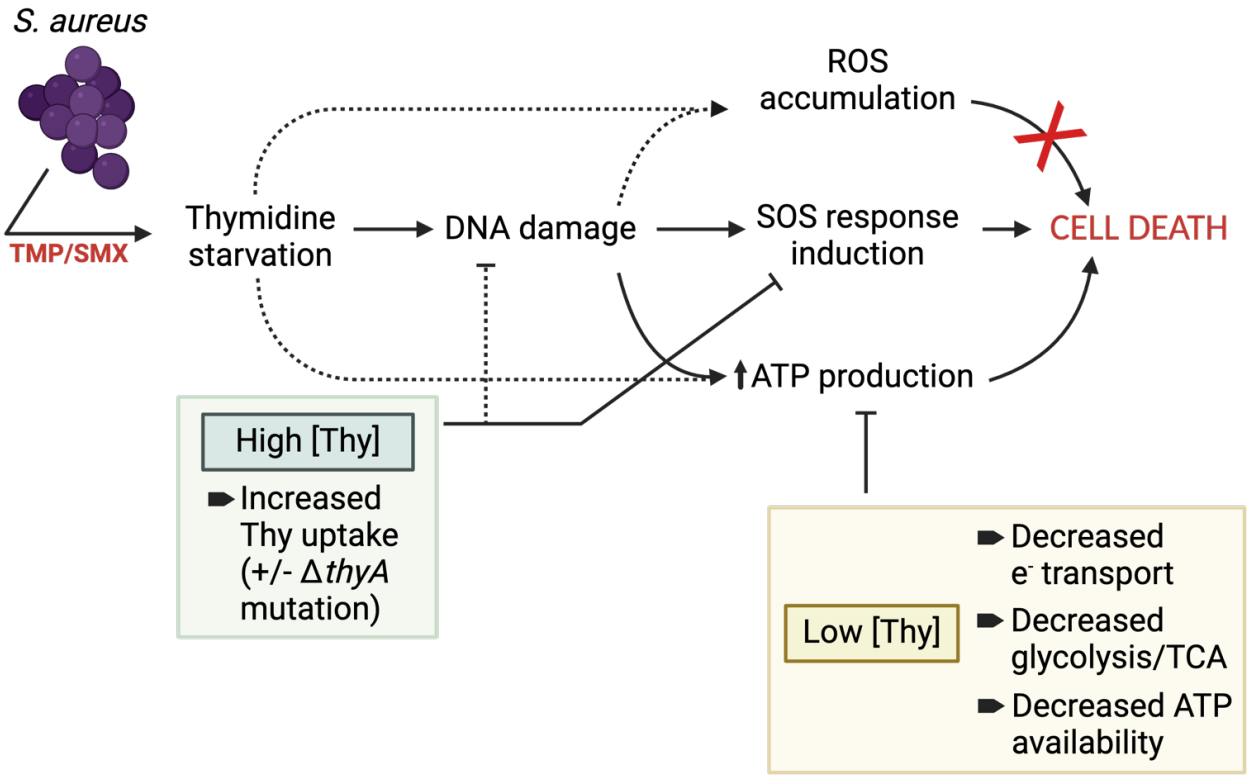

Figure 3.7 Model of the proposed mechanism of SXT action in *S. aureus* Upon SXT exposure, *S. aureus* undergoes thymidine starvation and DNA damage, inducing the SOS response and, ultimately, cell death. SOS induction can be mitigated, and survival conferred, by environmental thymidine levels above a threshold of 0.25 µg/mL. In contrast, with continued thymidine depletion, both ROS and ATP levels increase through undefined mechanisms. Of these, only ATP is strongly associated with lethality. Multiple conditions that decrease ATP levels, including decreased electron transport, decreased substrate phosphorylation, or decreased ATP production (either through slow growth or by impairing metabolism or ATP production) improve survival with SXT. Several details of this model remain unknown (dotted lines), including the points at which ROS accumulation and increased ATP production occur, whether thymidine supplementation prevents DNA damage itself, and the mechanism(s) through which ATP levels correlate with lethality. *Created with BioRender (L. Gonsalves, 2024, BioRender.com/f49f324).*

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CHAPTER 4: PRELIMINARY EXAMINATION OF THE EFFECTS OF SXT ON PERSISTER CELL FORMATION IN *S. AUREUS*

Can we identify the presence/absence of S. aureus persister subpopulations during SXT exposure?

Introduction

In **Chapters 2** and **Chapter 3**, we discussed the role of thymidine in *S. aureus* survival to SXT, the specific mutations that were selected by SXT, many of which involved core metabolism and slow or halted growth. Some of the observed mutations allowed *S. aureus* to grow in the presence of the antibiotic; other mutations limited cell death instead but did not quite permit growth with SXT exposure, instead allowing allow *S. aureus* to persist (**Figure 2.6B, 2.7A-C**). Modulation of metabolic pathways, either mutation-based or through changes in the nutritional environment (**Figure 2.7D**) suggests that *S. aureus* may not need to use traditional resistance mechanism to survive SXT-exposure. Our observations are reminiscent of the subset of bacterial cells that been observed to survive antibiotics without the need to acquire resistance, cell types that have been described in the literature as early as 1944. These alternative survival strategies, or states, have been referred to as tolerance, persistence, dormancy, and viable but nonculturable (VBNC)[1].

The term "resistance" is typically used to describe an inherited ability to grow in lethal concentrations of an antibiotic. Tolerance, on the other hand, is a more general term, used to describe an organism's ability to survive temporary exposure to otherwise lethal concentrations of an antibiotic (generally through slowing down growth, a concept that is difficult to distinguish from dormancy, which is considered an exaggerated form of slow growth) and can occur through mutation or can be triggered by environmental factors[1,2]. In contrast to these states, persistence describes a subpopulation of cells that survive antibiotics that have killed most other cells in the population and that exist in a non-dividing but viable state for extended periods of time[1,3]. This state is sometimes equated to the VBNC state, and both are considered by some to represent subtypes or different stages of dormancy[4]. Bacterial cells are considered "resistant" to an antibiotic when they exhibit a minimum inhibitory concentration (MIC) that is substantially higher than that of a susceptible population. In addition, resistant cells demonstrate growth in the presence of an antibiotic[1,3]. In contrast, the MIC of tolerant cells is considered to reflect susceptibility in standard tests, but the time it takes an antimicrobial to kill the bacterial population is longer than for wild-type cells, usually as a result of slower growth rates. Persistence reflects a state intermediate between these other two; while the MICs of persister cell populations resemble those of susceptible cell populations, persisters represent the cells that remain after an initial, rapid loss of viability of the majority of the bacterial cells in a bacterial population (e.g., the susceptible cells). The persisters then exhibit stable cell counts, or at most a slow decline (e.g., the tolerant cells)[1,5]. This biphasic curve has been regarded as a defining characteristic of a persister subpopulation[2].

The environmental cues that lead to persister formation, and the mechanisms underlying their phenotypes, represent an active field of study. Certain mutations, including those in the *hip* operon, as well as the nonmutational, stress-mediated activation of cellular pathways such as toxin-antitoxin systems, the SOS response, or the stringent response have been associated with increases in the size of the persister population[6–9]. Toxin-antitoxin modules, specifically, have been hypothesized to be key in persister formation in Gram-negative bacteria[10]. These modules typically

encode a stable toxin, that can inhibit a variety of metabolic targets, usually paired with a stable antitoxin that can defuse toxin activity but is degraded under stress, leaving the unopposed toxin to modify metabolic processes (translation, DNA metabolism inhibition, altered proton motive force function). However, the evidence for an important role for these modules in persistence formation among Gram-positive bacteria is weaker[11,12]; instead, external stressors that lead to halted protein synthesis or low metabolic activity[11,13,14] may be as important in the initiation of persistence as toxin-antitoxin modules, in both Gram-positive and -negative organisms. In most cases, slow or arrested growth is at the core of persister formation[5,15]. Despite these general principles the complex nature of persistence, and the differences in the details of persister formation among and within different taxa, highlight the need for additional research.

Many of the findings described in **Chapters 2 and 3**—for example, the identification of SXT-selected metabolic mutations, and the biphasic appearance of death curves of Newman and JE2 with SXT treatment (**Fig. 2.1A and B**) that resemble the characteristics typically attributed to persisters—raise the possibility that persister formation is a common mechanism of SXT survival for *S. aureus.* We therefore hypothesized that *S. aureus* survival of SXT involves a persistent state. To ideally test this hypothesis would require separating this non-growing cell population from both non-viable cells and actively growing cells. Thus, we adapted a published method[16] that allowed us to begin to distinguish between these populations and interrogate the impact of SXT on persister formation in *S. aureus*.

Results

eBioscience™ Cell Proliferation eFluor™ 670 provides a promising method for differentiating non-growing and growing cell populations

We first sought a method for discriminating persister cells (i.e., those that are viable but nonproliferating) from those that were either actively growing or nonviable. Methods used to study bacterial persistence and cell viability have relied on either single-cell transcriptional studies of non-lysed cells following antibiotic exposure or viability stains coupled with time-kill assays to differentiate between viable but non growing cells[2,3,17]. Here, we adapted the method of Flannagan and Heinrichs[16] (a fluorescence-based assay originally devised for identifying replicating *S. aureus* within host cells, repurposed here for free-living bacterial cells). We used the proliferation dye eFluor™670 (Thermo Fisher) as a general stain to mark all cells, with the viability dye BactoView™ Dead 500/515 (Biotium) as a counterstain. eFluor™670 is a fluorescent dye that non-specifically binds to primary amines of any protein within a cell; BactoView™ Dead 500/515 is a cell membrane-impermeant dye that selectively stains cell with a compromised cell membrane (dead cells) and fluoresces only when bound to DNA. This approach allowed us to differentiate *S. aureus* cell subpopulations: actively replicating cells were expected to have decreasing eFluor™670 staining over time, while non-growing cells—persisters—would retain this proliferation dye; in both cases, if cells were live and functioning, the viability dye signal would not be present in either.

Our initial goals were to 1) ensure the general (proliferation) dye would label *S. aureus* cells in a way that allows us to differentiate them from unstained cells and 2) assess proliferation dye retention in both growing and non-growing populations. To achieve these goals, we measured the fluorescence of a dyed, starting population of *S. aureus* cells grown in LB via flow cytometer after 0h, 2h and 4h of culture; the distribution of events/cells proliferation dye fluorescence for each sample is reported in **Figure 4.1A**. As expected, the fluorescence measurements of the dyed (+dye) populations and undyed (-dye) populations were distinct from each other at the initial timepoint (0h). At 4h, while the fluorescence of the undyed population did not change discernibly, we observed a reduction in fluorescence in the dyed population, corresponding with an increase in cell density (**Figure 4.1A**); importantly, the presence of the proliferation dye did not impact the cell viability of *S. aureus* when compared to undyed conditions (**Figure 4.2**). There was a distinct difference between the fluorescence profiles 4h timepoint with that of the 0h timepoint. Additionally, we measured the baseline fluorescence of a 1:1 mix of dyed and undyed cells (at 0h only), demonstrating distinct peaks for the two cell populations. The low-intensity peak overlapping with undyed cells, reflecting the background fluorescence of *S. aureus* cells, while the high-intensity peak had similar fluorescence profile to the dye-positive cells at 0h, further emphasizing that the fluorescence profiles between starting dyed and undyed populations are distinguishable from each other. These data both demonstrates the dilution of this dye over time in growing cells and that dyed cells have a distinct profile relative to undyed cells.

To ensure that this proliferation dye is retained over time in non-growing cells, we utilized the proton gradient-uncoupling agent CCCP (carbonyl cyanide m-chlorophenyl hydrazone), which disrupts cell metabolism without killing most cells, to simulate persistence; as reported in **Chapter 3** (**Figure 3.4C**) and here (**Figure 4.2, left**), we demonstrated that this compound halted growth in *S. aureus* over an extended period. Repeating our initial tests over 6h, we measured the fluorescence of dyed and undyed populations of cells relative to dyed and undyed cells that were treated with 10 μM CCCP. As observed previously, the undyed populations maintained a distinct and low baseline fluorescence, with or without CCCP. In contrast and consistent our previous observations, we observed a gradual loss in fluorescence among the untreated, dyed populations, showing that the proliferation dye is diluted out in growing cell populations. At 4h and 6h, the fluorescence profiles of the CCCP-treated populations closely resembled the 0h fluorescence profile (**Figure 4.1B**) and corresponded to the halt in growth observed when cultured (**Figure 4.2, left**). These data establish that the dye can be retained in non-growing cells over at least 6h, but loss of the proliferation dye does require actively replicating cells.

SXT exposure results in a population of cells with a distinct proliferation and viability fluorescence profile relative to an untreated *S. aureus* population

Persistent organisms play important roles in chronic and recurrent infections, but our understanding of how and why they arise in bacterial cell populations is still understudied. In the

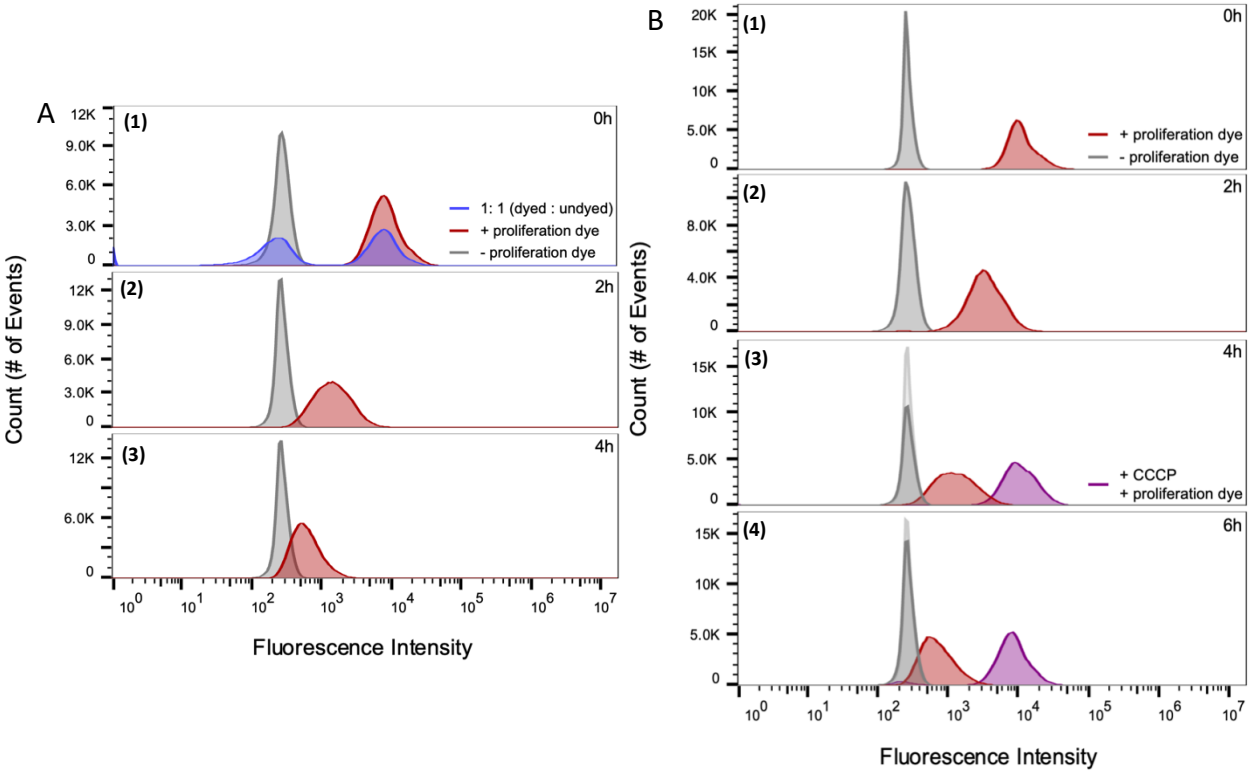

Figure 4.1 Change in eFluor™670 fluorescence over time in growing and non-growing *S. aureus* cells (A) Intracellular levels of proliferation dye, eFluor™670, was measured via flow cytometry in *S. aureus* Newman grown in LB at 0h (A1), 2h (A2), and 4h (A3). **(B)** Intracellular levels of proliferation dye in Newman measured with flow cytometry in *S. aureus* Newman grown in LB at 0h (B1), 2h (B2), 4h (B3), and 6h (B4). CCCP-treated (10 µM) Newman was used as a control for non-growing cells to determine dye retention time at 4h and 6h. Undyed *S. aureus* conditions were included in the analysis to show background fluorescence levels. Proliferation dye-negative conditions are indicated by gray curves, proliferation dye-positive are indicated by red curves, a 1:1 mix of dyed and undyed cells is indicated by a blue curve (at 0h/baseline only), and proliferation dye positive conditions treated with CCCP are indicated by magenta curves. Measured by flow cytometry; 100,000 events collected for each condition.

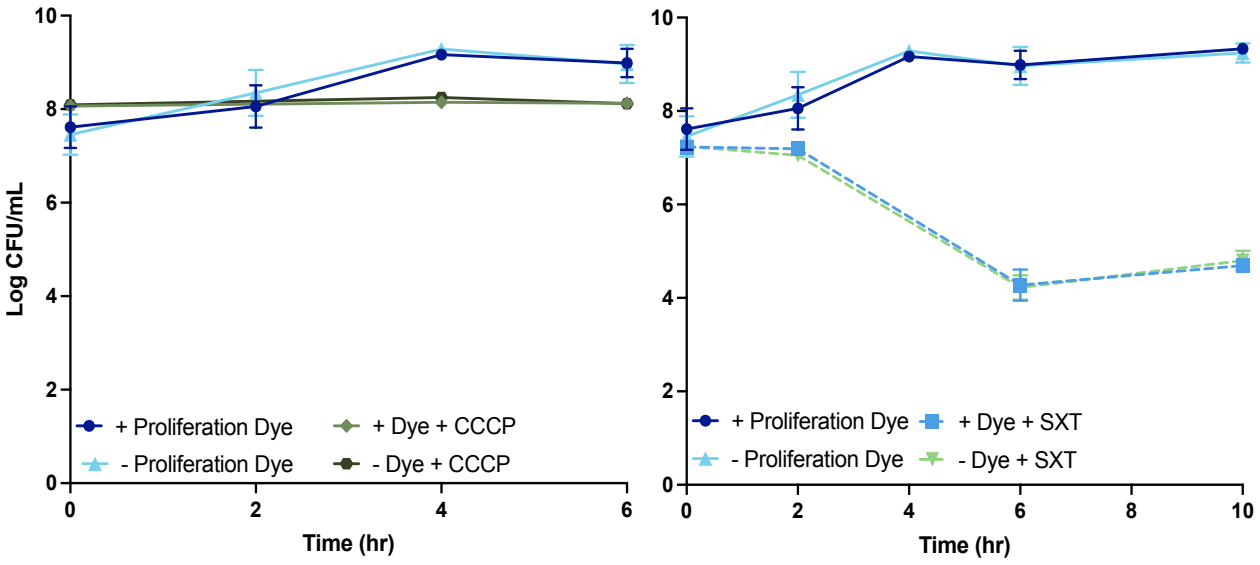

Figure 4.2 Survival curves of *S. aureus* Newman dyed with eFluor™670 relative to un-dyed cells (Left) *S. aureus* Newman cultured in LB, dyed or undyed, over 6h. Conditions in which CCCP was used are indicated. Total bacterial abundances were measured on chocolate agar plates; data are mean ± SD (n≥3; for CCCP conditions, n=1). **(Right)** Survival kinetics of Newman cultured in LB, dyed or undyed, and with and without SXT over 10h; SXT-treated conditions are indicated by a dotted line, total bacterial abundances were measured on chocolate agar plates, and data are mean ± SD (n≥2).

case of SXT-exposed *S. aureus*, the adoption of persister cell states is a plausible survival strategy, but the nature and size of the persister population, including the specific metabolic characteristics of this *S. aureus* cell population with SXT or other antibiotics, have not been defined. To facilitate investigations into this bacterial state, we incorporated SXT exposure in the method described above. An initial population of dyed cells was exposed to SXT over a period of 6h, as for the survival assays described in **Chapters 2 and 3**. The fluorescence profiles of treated and untreated *S. aureus* populations were then analyzed via flow cytometry at 0h and 6h (**Figure 4.3A**). Undyed populations all showed only background fluorescence levels regardless of treatment status. Untreated, dyed cells demonstrated a decrease in fluorescence at 6h relative to 0h. In the case of the SXT treated, dyed population, a slight decrease in fluorescence was observed at 6h when compared to the untreated, dyed conditions at 0h; this small loss in fluorescence indicated that some growth may be occurring in the *S. aureus* cell population during 0h-6h of SXT-treatment. The 6h SXT-treated, dyed cells sampled were gated—selected and grouped based on having a fluorescence measurement that fell within the fluorescence peak of the starting population (0h). Approximately 95% of the sampled cells from the 6h SXT-treated *S. aureus* population remained within this range. These data show that SXT treatment prevents the loss of proliferation dye in *S. aureus*, suggesting the presence of either a non-growing or slow-growing population of cells.

As shown in **Chapter 2**, SXT treatment results in variability in survival between SXT-treated cultures (**Figure 2.5B and D**). We wanted to ensure that the fluorescence profiles of both the treated and untreated conditions were consistent across replicates. To assess replicability of the fluorescence profiles we observed, we compared the fluorescence profiles two different biological replicates, as shown in **Figure 4.3B** (these data are separated by time and dye status). We observed similar fluorescence profiles when replicates were compared for both undyed and dyed cell populations at 0h and 6h, regardless of SXT-treatment status. While we observed slight differences in the mean fluorescence of each population between replicates (data not shown), those subtle differences were largely found in the treated, dyed populations, rather than the untreated conditions, indicating that cell labeling with eFluor^{TM}670 is relatively consistent across replicates.

Most of the SXT-treated cells sampled retained a proliferation dye fluorescence level resembling that of the starting population (0h), but we observed a small shift in fluorescence at 6h relative to 0h (**Figure 4.3A**). This shift could indicate several possibilities: a subpopulation of cells is actively growing and resistant to SXT; resumption of growth for the entire population after a period of halted growth; or the entire population is slowly growing over the course of 6h. The work described above is not able to differentiate between these possibilities. However, whether this fluorescence shift occurred early and was maintained was readily testable. As shown in **Figure 2.1A**, cell densities uniformly declined for *S. aureus* strain Newman, but remained above the limit of detection, at thymidine concentrations below 0.25 µg/mL. We observed, however, no change in cell viability between 0h and 2h, possibly indicative of a delay in TLD and attempted cell division.

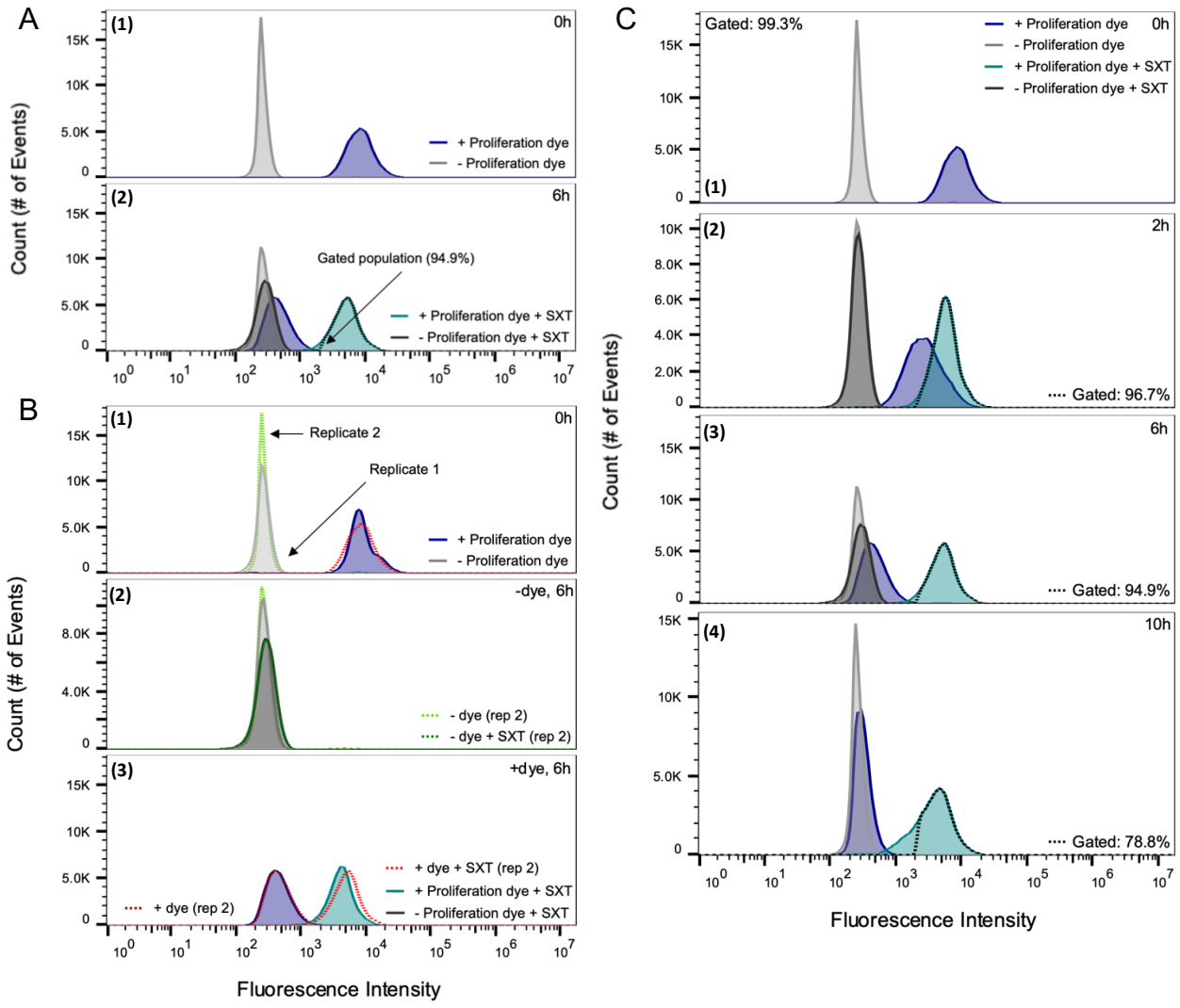

Figure 4.3 Assessment of eFluor™670-stained *S. aureus* over time with and without SXT treatment (A) Intracellular levels of proliferation dye, eFluor™670, was measured via flow cytometry in *S. aureus* Newman grown in LB with and without SXT at 0h and 6h. **(B)** Proliferation dye levels in Newman grown in LB with and without SXT at 0h (B1) and 6h from two biological replicates (B2 and B3). Data is separated by condition and timepoint—starting populations at 0h (B1), undyed cells with and without SXT (B2), and dyed cells with and without SXT (B3). Replicate 1 is indicated by solid lined peaks while replicated 2 is indicated by dotted lines. **(C)** Proliferation dye fluorescence in *S. aureus* Newman grown in LB, with or without SXT at 0h (C1), 2h (C2), 6h (C3), and 10h (C4). For (A) and (C), the fluorescence profile of the dyed inoculum (0h) was used as a gate to group the fraction of the sampled population at (2h, 6h, or 10h) that fall within the fluorescence peak at 0h—reported as percent of total cells sampled for dyed cells treated with SXT. Undyed *S. aureus* conditions were included in the analysis to show background fluorescence levels. Measured by flow cytometry; 100,000 events collected for each condition. Representative data of at least two replicated experiments.

With this lag period in mind, the above experiment was repeated but with the inclusion of an early and late timepoint (2h and 10h), in addition to the 6h timepoint (**Figure 4.3C**). As expected, there was a steady loss in fluorescence for the untreated, dyed condition. When assessing the dyed and SXT-treated conditions, a small loss in fluorescence was again observed, but this shift was observed at 2h and was largely maintained at 6h. Interestingly, there was a more noticeable loss in fluorescence at 10h, that corresponds to the observed rebound in *S. aureus* population's viability (**Figure 4.2, right**). These data suggest that there may be subtle changes in cell proliferation within an SXT-treated population that depends on the duration of exposure to SXT.

Unfortunately, the proliferation dye eFluor™670 alone was not able to distinguish whether the cells sampled via flow cytometry were truly viable, as opposed to intact but dead cells. To address this deficiency, we incorporated BactoView™ Dead 500/515, a DNA-binding viability dye selective for dead cells. Proliferation-dyed *S. aureus* was treated with the viability stain at specific timepoints, and the fluorescence of both stains assessed via flow cytometry. Proliferation dye fluorescence profiles of both treated and untreated *S. aureus* were similar to previous experiments—dyed, untreated conditions showed a decrease in fluorescence over 10h while dyed, SXT-treated showed only a small decrease in fluorescence at 2h and 6h, with a larger decrease at 10h, relative to 0h (**Figure 4.4, A**). Similar to the proliferation dye results, the viability dye fluorescence profiles of treated and untreated conditions differed. The viability fluorescence profile of *S. aureus* not treated with SXT at 6h and 10h was similar to the background fluorescence of the starting population (no viability dye). The viability fluorescence profiles of SXT-treated *S. aureus*, however, demonstrated increased fluorescence relative to treated cells, indicating an increased population of non-viable cells (cells retaining the BactoView™ Dead 500/515) (**Figure 4.4, B**). This data shows there are different viability profiles with SXT-treatment compared to untreated *S. aureus* cells, and a portion of cells within the sampled population may be non-viable cells. Comparison to a non-viable control is required to determine a proper fluorescence threshold for viability and for specifically identifying and quantifying the cells sampled that are both proliferation dye-positive and viability dye negative, reflecting persister cells.

Discussion

In this chapter, we developed a fluorescence staining scheme that can be used to identify and quantify viable, nongrowing (persister) cells from other cells. Specifically, we showed that eFluor™670-dyed and undyed populations have distinct fluorescence profiles. In a proliferation dye-positive population of cells, a reduction in fluorescence is observed over time, as growth occurs. This loss in fluorescence was not observed for *S. aureus* treated with 10uM CCCP, demonstrating that the dye is retained in non-growing cells more than a growing culture. When *S. aureus* was treated with SXT, proliferation dye fluorescence was maintained, similar to CCCP-treated *S. aureus*, indicative of a non-growing population after SXT treatment. When we instead measured cell viability under these conditions using BactoView™ Dead 500/515, we observed

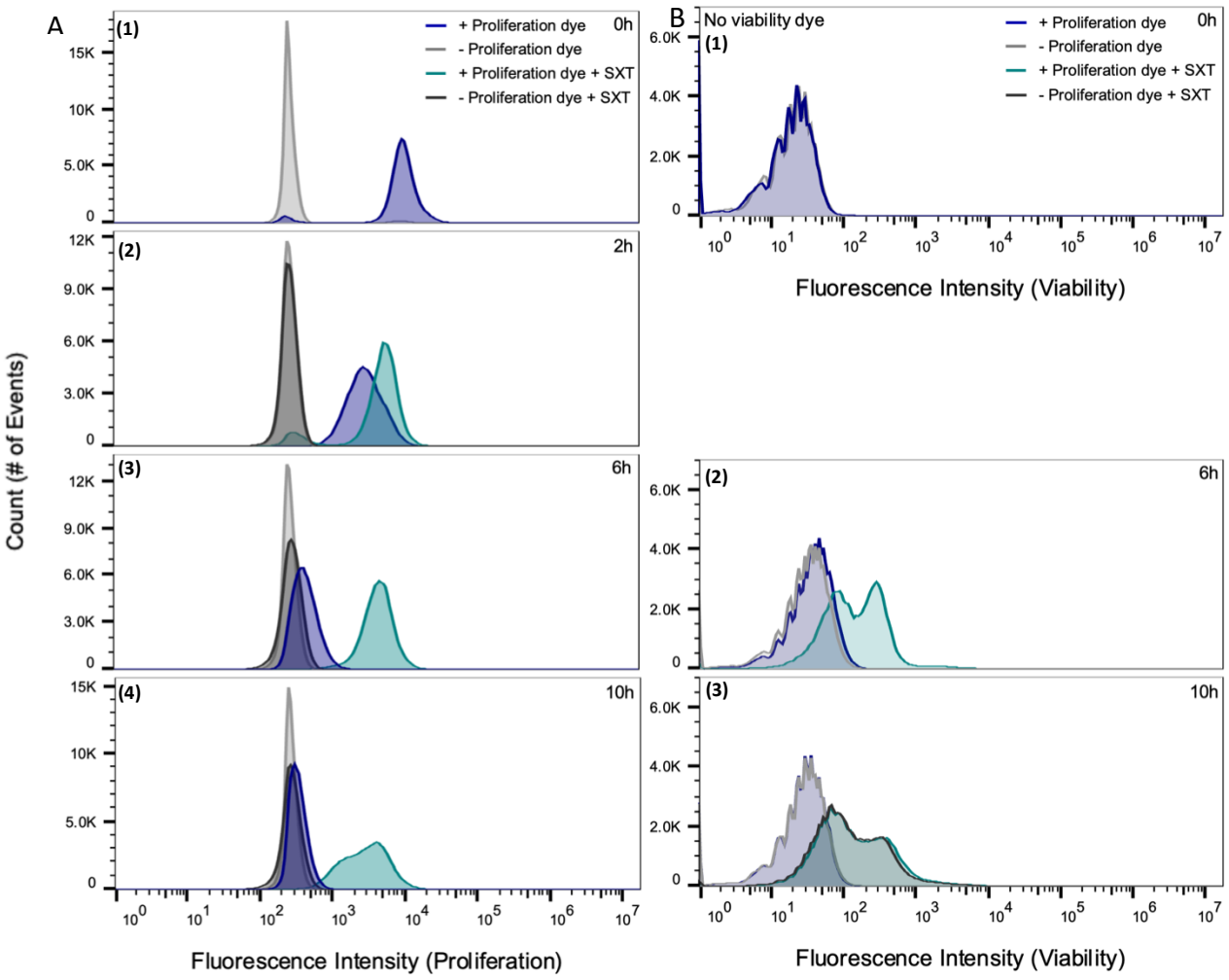

Figure 4.4 Assessment of eFluor™670 and BactoView™ Dead 500/515 fluorescence profiles in *S. aureus* over time with and without SXT treatment (A) Proliferation dye presence in *S. aureus* Newman grown in LB, with or without SXT at 0h (A1), 2h (A2), 6h (A3), and 10h (A4). Fluorescence measured via flow cytometry. **(B)** BactoView™ Dead 500/515, viability dye, was assessed via flow cytometry in proliferation-dyed and undyed Newman with and without SXT treatment at 0h (B1), 6h (B2), 10h (B3). Undyed *S. aureus* conditions were included in all analyses to show background fluorescence levels; baseline fluorescence of sampled cells without viability staining is presented in the B1. Measured by flow cytometry; 100,000 events collected for each condition. Representative data of at least two replicated experiments.

different fluorescence profiles among SXT-treated compared with untreaded *S. aureus* cultures. While this work demonstrates that a portion of the SXT-treated population is potentially non-viable, further work is needed to specifically quantify viable, nongrowing cells, indicating persister cells, and differentiate them from nonviable cells and viable, proliferating cells. Do so would enable the isolation (i.e., through cell sorting) and analysis of persister cells though, for example, transcriptional profiling.

This work underscores the technically and conceptually complex nature of studying persistence in bacteria. Persisters exist in small numbers in any bacterial cell population, reflecting a transitory state without known associated genetic markers (like mutations). Additionally, the characteristics that differentiate persistence, tolerance, and viable-but-nonculturable cells may overlap, thwarting efforts to study a single state[4,18]. Properly studying persistence requires the development of methods that are simple, specific, and modular. The work presented in this chapter was aimed at developing a method that meets these qualifications. A defining characteristic of the method presented here is the use of the proliferation dye eFluor™670. We demonstrated this dye circumvented the complications of genetic engineering without compromising the growth of the organism, was simple-to-use and readily available, and could be used to interrogate *S. aureus* under different conditions—normal growth, chemically-induced persistence, and antibiotic treatment. Additionally, this method has already been shown to be a powerful tool for tracking the proliferation status *S. aureus in vivo*, demonstrating this method's potential for physiologically-relevant downstream applications[16]. One limitation of the original method, as presented by Flannagan and Heinrichs, was the use of GFP expression vectors as a general tag for all cells in the population[16]—this effort required genetic engineering and did not indicate viability of the cells, complicating both experimental design and the subsequent conclusions. We have demonstrated that the method used here does not require such a tagging system, because the proliferation dye used is compatible with a counterstain like the viability dye BactoView™ Dead 500/515. The work presented in this chapter provides a foundation for further experimentation.

Although the methods described here are in the early stages of development, we made several interesting observations on which to build a new project. We observed a marked difference in fluorescence profiles between actively growing and SXT-treated cells, for both the proliferation dye and the viability dye. We have previously described the absence in a change in viability over the first 2h following SXT-exposure in both *S. aureus* Newman and JE2 (**Figure 2.1**). When we assessed the proliferation profiles of SXT-treated Newman, we observed a subtle loss in fluorescence intensity of the proliferation dye within 2h of SXT-exposure, indicative of growth within the *S. aureus* population during this lag period. This 2h-period may therefore correspond to an otherwise similar (but shorter) pre-TLD lag period—or "resistance phase"—observed by others in *E. coli*, in which the cell may try to function normally (initiating replication and cell division) under thymidine starvation conditions, depleting non-replenishing intracellular thymidine reserves before undergoing TLD[19,20]. We also observed that the stability in proliferation fluorescence levels

through 6h was temporary; an additional loss in fluorescence intensity was observed at 10h. This peak change indicated a possible transition between states over the course of SXT exposure, one that was not unexpected based on the observations we made previously. In **Chapter 2**, we observed a loss in viability when *S. aureus* was exposed to SXT under thymidine deplete conditions (**Figure 2.1A**); despite this viability loss, cell densities remained above the limit of detection, transitioning from a steep decline from 2-6h (3 log CFU/mL loss) to a slower decline at 6-10h (1 log CFU/mL loss). Taken together, the proliferation data alongside the survival curves suggest that *S. aureus* cell populations treated with SXT may be transitioning to an antibiotic-tolerant, persistent state that promotes *S. aureus* survival and sets the stage for subsequent cell density rebound at 10-24h. Because persistence is considered a transient state that protects bacterial cell populations during stress, relief of this stress (such as a removal of the antibiotic pressure) allows bacterial cells to transition to a growing, and possibly resistant, states[6,15,21]; this may be the case for *S. aureus* during later stages of SXT exposure. Not only would such a switch be important for survival in a stressful environment, but it would also play an important role in intercellular survival in the host[3,22]. Targeting such a "defensive" mechanism could greatly enhance the efficacy of antimicrobials such as SXT. While these are intriguing observations, further work will be required to further characterize these cellular states with exposure to SXT, as well as other clinically-useful antibiotics.

While novel, the method described here is not without limitations. We were able to differentiate between actively growing cells and non-growing cells, given the right timeframe, but this method was not able to distinguish persisters from cells in other dormancy states. With SXT treatment, some loss in viability is expected. We demonstrated that the viability dye was compatible with the proliferation dye, but we were unable to precisely determine the proportion of cell populations that reflected viable versus non-viable cells following SXT treatment, an important distinction necessary for the downstream analysis of specific cell states that arise following exposure. For these limitations, additional optimization will be required alongside the inclusion of relevant controls. Nevertheless, the approach presented in this chapter has the capacity, with continued refinement, to aid in the differentiation of persister cells under a variety of conditions for a variety of infection types. Understanding persistence and its development during infections is critical in revitalizing the effectiveness of our antibiotics, and the method described here has to potential to provide valuable insights into how bacterial persistence may lead to treatment failure.

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CHAPTER 5: BRINGING IT ALL TOGETHER—A DISCUSSION OF FUTURE DIRECTIONS

Where do we go from here?

Overview

In the work described in this thesis, we used traditional microbiological methods, population level sequencing, clinical isolate data, and molecular techniques and assays to begin to better define how *S. aureus* survives treatment with trimethoprim-sulfamethoxazole (SXT), an antibiotic used particularly often for people with CF (PwCF)[1,2]. SXT is a folate inhibitor[3], effectively promoting cell death (thymineless death; TLD) through thymidine starvation[4–7]. While numerous studies have explored the details of TLD and SXT action, there remains a lack of sufficient mechanistic clarity, particularly in organisms other than the model organism *E. coli*[8–10]. Improving our understanding of one of our most important antimicrobial compounds is critical for not only improving current therapies but preventing the loss of yet another antimicrobial compound in an age of dwindling treatment options for infections. Together, the data presented here show that thymidine availability plays an important role in *S. aureus'* response to SXT, a response that is complex and associated with core metabolism. This work extends our understanding of TLD and the contribution (or, as our results suggest, lack thereof) of reactive oxygen species (ROS) to bactericidal action of SXT in *S. aureus*. Despite the thorough analysis presented here, ongoing work is still necessary to fully understand TLD, mechanisms of SXT action, the role of metabolism in antimicrobial resistance and persistence, and the *in vivo* relevance of *S. aureus* adaptation to SXT.

Chapter 2: Metabolism as a mediator of trimethoprim-sulfamethoxazole-induced cell death in S. aureus – Part 1

We began our assessment of *S. aureus* survival strategies to SXT in Chapter 2. The importance of exogenous thymidine in *S. aureus* mitigation of SXT action has been well documented clinically—thymidine-dependent SCVs are slow-growing mutants that are selected by SXT and, accordingly, intrinsically resistant to SXT, but also highly reliant on the uptake of exogenous thymidine[11]. Thymidine-dependent small colony variants (TD-SCVs) have been recognized as an important adaptation to SXT *in vivo* [12,13], but the recent examination of other SCV subtypes, such as those with mutations in the electron transport chain (ETC)[14–16], has prompted the reevaluation of the canonical modes of resistance to SXT, and in particularly to answer the question: Does *S. aureus* utilize survival strategies other than reliance on thymidine to circumvent the bactericidal effects of SXT? Because of the common occurrence of TD-SCVs, we hypothesized that thymidine is readily available at sites of infection, which would improve the growth of these variants. However, little work had specifically investigated thymidine requirements in *S. aureus*. To answer this question and address these gaps, we analyzed the effect of thymidine concentration on *S. aureus* survival to SXT *in vitro*, observing a positive relationship between thymidine concentration and *S. aureus* survival under conditions of TLD. However, analysis of sputum samples from PwCF showed varied, and often low, levels of thymidine in those samples, suggesting *S. aureus* may use other survival methods *in vivo*. To understand how thymidine concentration influenced *S. aureus* survival, and thus the mutants that SXT selected for, we analyzed sequencing data from both SXT-selected isolates and SXT-treated whole cultures of *S. aureus* populations for mutations relative to parent strains. This analysis revealed that mechanisms of survival were thymidine concentration

dependent, with higher thymidine conditions promoting mutations in genes associated with nucleotide and folate biosynthetic pathways, while low thymidine conditions seemed to promote mutations in genes related to more diverse, and core metabolic pathways. While these *in vitro* data provided a valuable foundation for further investigation, it prompted the question: are the pathways selected by SXT and observed to confer survival *in vitro* modified in clinical isolates of PwCF treated with SXT? We interrogated the mutational frequency of select genes in isolates from children with CF (CwCF) that were either exposed or unexposed to SXT; among the 44 genes we screened, we observed evidence of *in vivo* selection in only three genes in the SXT-exposed group, but this may have been a result of limited statistical power.

An interesting observation discussed in this chapter was the differential survival and adaptive capabilities of *S. aureus* strain Newman relative to JE2. Despite similar MICs, Newman showed a larger decline in viability with SXT exposure under thymidine-deplete conditions compared to JE2 (**Figure 2.1A**). Additionally, isolates with mutations in *thyA* were never recovered from JE2 exposed to SXT *in vitro* (an observation that has been previously described[17] but not further investigated), suggesting strain-specific difference in survival. When Newman and JE2 adaptive mutants were compared, we also observed variation in the types of mutations present following SXT exposure at both the *S. aureus* isolate and cell population level. While there was some overlap (*deoB*, *mvaK1/mvk*, *ackA*, *nrdEF*, *pta ptsI*), there was a substantial number of genes with mutations that were unique to one strain or the other, prompting the question: what strain-specific differences exist that might account for the different responses between JE2 and Newman to SXT and the mutants the arise in the cell population following this exposure? Single cell and whole-culture transcriptomic analysis focusing on metabolic pathways may allow us to better understand the origin of these differences. Global transcriptomic analysis would provide the most comprehensive assessment of cellular changes occurring over the course of SXT treatment, and how that might be different between strains; genes identified through WGS and population-level sequencing can serve as good candidates for a targeted transcriptomic analysis to understand changes in mRNA levels of specific genes in response to SXT. Since there may be differences in the transcriptional responses among individual cells after SXT exposure, single-cell analysis could provide the granularity necessary to understand this heterogeneity in *S. aureus*' response to SXT. These approaches would allow us to define the transcriptional response(s) that may be occurring at the single cell level, the population-level and the strain-level, when *S. aureus* is exposed to SXT. These methods would be particularly useful in understanding the changes in mRNA that occur within the first 2 hours of SXT exposure, before significant cell death occurs, compared to the period of active cell death (4-6 hours following SXT exposure), and which pathways may explain the ATP changes observed during SXT challenge.

The goal of the work presented in this thesis was to provide a foundational understanding of adaptive mechanisms of *S. aureus* to SXT that may help identify novel targets for treatment; understanding *in vivo* relevance is important to achieve goal. The question of whether the genes

and pathways altered in *S. aureus* mutants and cell populations that survived SXT *in vitro* were also altered in clinical isolates, remained unanswered. To address this uncertainty, we analyzed the genomic sequences of isolates from children with CF (CwCF) that were either exposed to SXT or unexposed[18] for coding changes in a set of genes that we found to be commonly mutated *in vitro.* (**Table 2.2**). Unfortunately, our analysis was limited in that there was inadequate statistical power to determine whether these genes of interest were under selection *in vivo*—the absence of a proper unexposed group, the short duration of the study period, and the limited number of participating children all contributed to this limitation. A more rigorous study to understand whether genes/pathways altered in SXT-exposed *S. aureus in vitro* are also altered *in vivo* could include a control isolate collection that is ultimately derived from individuals who were not routinely exposed to antibiotics in the same way as PwCF, such as isolates collected from either healthy individuals carrying *S. aureus* or individuals admitted to the hospital prior to antibiotic treatment. The addition of a proper control isolate collection can provide improved statistical power to address our question(s) regarding the relevance of certain altered genes *in vivo*. Alternatively, a study that follows a cohort of people with CF before and over the course of SXT treatment could provide the evolutionary granularity that the analysis presented in Chapter 2 was unable achieve—this would require extensive and frequent collection of *S. aureus* isolates to ensure the diversity of the bacterial population is captured, allowing for *in vitro* and *in vivo* comparisons of evolutionary adaptation to an antimicrobial stressor. Our analysis raised an additional question: can the mutations selected for *in vitro* also be selected for by other antibiotics? The cohort of CwCF included our original analysis (Chapter 2) were likely exposed to multiple antibiotics over the course of the study. Thus, the overlap that we observed between mutations detected in SXT-exposed *S. aureus in vitro* and among clinical isolates could be attributable to SXT, other antibiotics, and/or other selective pressures of the lung environment or other aspects of treatment. As the analysis described in Chapter 2 was unable to distinguish between these, the inclusion of a proper control isolate collection could aid in parsing out the contributions of different antibiotics to positive selection for certain genes; a more extensive isolate collection from individuals exposed to SXT is required.

Chapter 3: Metabolism as a mediator of trimethoprim-sulfamethoxazole-induced cell death in S. aureus – Part 2

In Chapter 3, we expanded our observations from Chapter 2. Considering the genes and pathways altered in *S. aureus* isolates from SXT selection *in vitro*, we noted that many of those mutations were predicted to result in altered intracellular ATP levels. While the accumulation of reactive oxygen species (ROS) has largely been assumed to be the primary cause of TLD[19–21], after several studies demonstrated elevated ROS in Δ*thyA* mutants of *E. coli* compared with wild-type strains, these species were thought to mediate cell death with SXT, and other antibiotics as well[22,23]. Because the processes that generate ATP also yield ROS, our *in vitro*-selected mutants could be indicators of the importance of ROS in SXT killing, or, alternatively, ATP metabolism itself. While we observed an increase in ROS accumulation in SXT-treated strains of *S. aureus*, neither

ROS scavenging nor mutations in ROS detoxifying enzymes altered SXT lethality, suggesting ROS was not a primary driver of SXT-mediated TLD in *S. aureus*. We hypothesized that ATP production, or ATP levels themselves, and the role of this molecules in essential cell processes like DNA damage repair, were the primary drivers of cell death in SXT-exposed *S. aureus*, rather than ROS. We found that with SXT treatment, both the SOS response and ATP levels were higher relative to untreated controls. Using an SXT-adapted mutant (NWM-TS08), we demonstrated that SXT re-exposure resulted in lower levels of SOS activation and ATP compared with wild-type *S. aureus*, indicating that a cell's ability to dampen certain stress responses and mitigate ATP spikes may be the key to surviving conditions that induce TLD. In support of this hypothesis, we found that chemically inhibiting the production of ATP during SXT exposure with arsenate or CCCP improved *S. aureus* survival.

The results presented in Chapter 3 highlighted several important directions for further investigation. Because SOS system activation, and trimethoprim exposure itself, (**Figure 3.4A**) is known to activate the replication of prophages and the production of bacteriophage components and particles, such activation could have occurred as a result of SXT exposure, potentially contributing to lethality[8,24]. Phage have been implicated previously in TLD and SXT action[25], but whether SXT-induced phage contributes to cell death has not been thoroughly explored. To begin to determine the role of phage induction in SXT lethality, we propose experiments comparing the survival dynamics of *S. aureus* with genetic knockouts in both individual and combination prophage genetic elements to that of wild-type *S. aureus*. Plaque assay-based quantification of phage particle production over the course of SXT exposure of *S. aureus* relative to unexposed *S. aureus* provide the opportunity to determine if SXT can induce phage replication and production as well as how changes in the phage particle population in these experiments may coincide with changes in *S. aureus* viability over the course of exposure. This experiment would allow us to better understand which phage, if any, contribute to SXT lethality. Additionally, these experiments would be complimented with a population-level sequencing approach like that described in Chapter 2—analyzing the dynamics and ratio of sequencing reads generated for prophage-specific genetic elements relative to all bacterial sequences over the course of SXT exposure *in vitro* could clarify whether phage induction is occurring during SXT exposure of *S. aureus*, especially if low or no phage particle production is detected using plaque assays during SXT challenge. These experiments may help determine if phage induction occurs with SXT exposure and if prophage replication and particle production (or lack of) alters *S. aureus* viability during SXT-mediated TLD *in vitro*.

One of our SXT-adapted isolates, NWM-TS08 (*ptsI* mutant), exhibited characteristics that made it an informative comparator for wild-type Newman in studying the effects of SXT. In the absence of SXT, the *ptsI* mutant exhibited wild-type growth, but tolerance to SXT (**Figure 2.6B**), a unique and useful combination of characteristics for studying the relationships ROS, the SOS response, and ATP levels with SXT separate from growth. While this mutant was repeatedly selected for in

both JE2 and Newman, we don't fully understand the mechanism behind its improved tolerance to SXT, prompting the question: What underlying characteristics might contribute to the *ptsI* mutant's tolerance to SXT? Using transcriptional analysis or monitoring the concentrations of certain metabolic intermediate of glycolysis, the TCA cycle, and the ETC in the *ptsI* mutant relative to wild-type *S. aureus* during SXT exposure could be used to answer this question, but this is an untargeted approach that may not yield a specific answer. However, more defined studies such as quantifying viability changes of a *ptsI* mutant in different conditions may provide a more specific answer. For example, all *S. aureus* strains described in this work were grown in LB, a media rich in peptides but not glucose; thus *S. aureus* may not have needed to rely on *ptsI* and the phosphotransferase system (PTS) for survival. Either altering the media composition, such as the available carbon sources within the media, both with and without challenging the *ptsI* mutant to SXT, could begin to narrow down the pathways that the *ptsI* mutant can use for growth and survival and whether the mutant retains its tolerance to SXT, or shows an increase in sensitivity. Additionally, quantifying changes in *S. aureus* cell viability with and without SXT challenges in mutants containing deletion of genes encoding different components of the PTS downstream of *ptsI* will highlight which components of the PTS pathway are important for SXT lethality. *PtsI* mutants were previously shown to exhibit reduced susceptibility for a range of antimicrobials[21], demonstrating a potential role for the PTS in mediating susceptibility to SXT in *S. aureus.* An additional question that was raised was whether a *ptsI* mutation in *S. aureus* could provide cross-resistance to other antimicrobials, as it did in *E. coli*, and if other SXT-generated metabolic mutations that arose in *S. aureus* could do the same? A simple approach to begin to answer this question would be to expose the stable, SXT-adapted isolates to a panel of antimicrobials from different classes, assessing the MIC and survival curves of these isolates. Leveraging strains available in the Nebraska Transposon Mutant Library[28] would provide an additional opportunity to understand whether mutations selected in Newman provide the same protection in the context of JE2 and corroborate findings from isolate data using these transposon insertion mutants rather than rely on antibiotic-generated mutations. These experiments would provide an opportunity to investigate relationships between MIC and viability as well as whether certain mutations provided broader protection to diverse antibiotics than others.

While the *ptsI* mutants was an intriguing finding, many mutations selected under low thymidine conditions with SXT exposure affected genes that are involved in carbohydrate metabolism—which of these cellular pathways are important for *S. aureus* susceptibility to SXT? Assessing how the mutation landscape changes when *S. aureus* is cultured in conditions that vary in the carbon source or oxygen availability over both short- and long-term SXT exposure (e.g., 24h and 120h), like the experiments presented in Chapter 2 could begin to answer this question. Comparing the mutations that arise in each of these conditions may provide insight into which genes and pathways are indispensable for survival to SXT. Additionally, quantifying the severity of TLD that occurs following SXT exposure in mutants unable to access certain pathways or adapt to SXT exposure relative to wild-type may help tease apart which pathways are potentially important for decreasing

S. aureus susceptibility to SXT; genes such as *pckA* or *fbp* (both enzymes important in gluconeogenesis), *ackA* (involved in pyruvate-to-acetate conversion and ATP production), *pdhD* (important for the conversion of pyruvate to acetyl-CoA), *pyk* (the conversion of phosphoenolpyruvate to pyruvate) may be ideal candidates for this approach[26,27]. Lastly, tracking both SOS system activation and ATP levels, as done in Chapter 3, during these experiments may offer more information on how central metabolism and ATP production affect DNA repair in the context of SXT.

Chapter 4: Preliminary examination of the effects of SXT on persister cell formation in S. aureus

The aim of Chapter 4 was to develop a method to study the cell subpopulations that survive antimicrobial treatment; SXT-exposed *S. aureus* served as an effective test case for this method. Based on the data presented in Chapters 2, both the identification of metabolic mutants and the biphasic appearance of the death curves of SXT-exposed Newman and JE2, we hypothesized that persisters were present in SXT-treated *S. aureus* cell populations (**Fig. 2.1A and B, Table 2.1**). The proliferation dye eFluor™ 670 was previously demonstrated to be effective in signifying *S. aureus* proliferation[29], providing a potentially effective method for studying persistence *in vitro* in the context of antibiotic exposure. This dye reproducibly stained *S. aureus* cultures and dyed and undyed cells had distinct fluorescence profiles. This method also allowed us to gauge dye retention under normal growing conditions and conditions that stalled proliferation (CCCP treatment); fluorescence levels remained unchanged in CCCP-treated cells (non-growing cells) over at least 6 hours indicating that those cells retained the dye, while growing cells lost fluorescence signal, indicating the dye was diluted out during growth. This method also provided the opportunity to assess how SXT changes the proliferation fluorescence profile of *S. aureus*. We have not thoroughly tested the viability dye BactoView™ Dead 500/515 for its ability to discriminate dead cells from live, non-proliferating cells (persisters), an issue that prevents the immediate application of this method to properly test our hypotheses.

The work in this chapter remains a work in progress. The assay described in Chapter 4 did not yet clearly discriminate between live and dead cells. Propidium iodide (PI) is typically used for viability assessment, but studies have shown PI can result in either the over- or underestimation of the viability of a population of cells, depending on the conditions and cell state[30,31], indicating that PI is suboptimal for assessing viability. While found the viability dye BactoView™ Dead 500/515 to be fluorescently compatible with the proliferation dye, unfortunately there is no precedence in the literature for the successful use of this dye, despite manufacture claims that this dye is more specific than PI, especially for Gram-positive bacteria. While our data are promising, the use of this viability dye requires extensive testing. The use of cells known to be dead to set a fluorescence threshold for what qualifies as a truly dead cell is especially important. For example, UV-treated, heat-killed, antibiotic-treated, or fixed cells may be viable options for such a non-viable control, but the method used to create this control cell population must maintain the integrity of the cellular

structure to allow for analysis via flow cytometry. Additional testing may be required to determine whether SXT-induced cellular stress permeabilizes the cell and allows for entry of the dye even in viable cells; this may underestimate the viable proportion of a cell population.

Once properly validated, this method has a variety of exciting potential applications. In particular, we hope to use this assay to identify environmental and therapeutic triggers that contribute to persister formation. Testing a variety of antibiotics and experimenting with environmental conditions such as oxygen availability and carbons sources, and the addition of certain compounds, would allow us to study the effects of certain exposures and the environment on persister formation; for example, growing *S. aureus* in an anaerobic environment may yield a larger persister population with a specific exposure (**Figure 3.2**), while the addition of menadione or hemin may prevent the formation of persisters, sensitizing cells to SXT lethality (**Figure 2.7**). Each of these proposed experiments could include transcriptomic analysis of identified persister cells compared to non-persisters (isolated by cell sorting) to identify transcriptional mechanism of persister formation. However, because persisters may comprise only a small proportion of a cell population, preparation of a RNAseq library could require methods that are specifically tailored for small populations, low input, and, ideally, affordable. Wang *et al*., developed a method that meets these qualifications and has proven useful for assessing the transcriptome of non-growing (persistent) *E. coli*[32], reflecting an appropriate place to start. The successful development, refinement, and validation of this method permits the exploration of unanswered questions that have plagued the field for decades—for example, whether persisters play a role in all chronic infections, how to initiate "resuscitation" of persister cell populations to re-sensitize them to antibiotics, how to test for and identify persisters in clinical infections (and whether there are specific genes that designate persistence that allow for molecular testing), and if (and which) adjuvants may improve our current therapies to better target this subpopulation.

Final Remarks

Antibiotic resistance remains a growing and urgent concern, especially for vulnerable and chronically-infected human populations. It has become increasingly important to understand the mechanisms of action of, and resistance to, our most essential antibiotics. This work describes a detailed investigation into the microbial adaptations that confer survival of SXT in the human pathogen *S. aureus*, providing new insight into the mechanism of action with which SXT kills this bacterium and revealing the central role of metabolic adaptations in survival. This work paves the way for new studies of the real-world implications of the of these adaptations. While we did not definitively define all modes of *S. aureus* adaptation to this or other antibiotics, environments, or other stresses nor are these results generalizable to other bacterial species, the knowledge presented here provides a critical step towards identifying novel targets for improved antibiotic efficacy for a multitude of *S. aureus* infections, beyond just those associated with CF.

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CHAPTER 6: MATERIALS AND METHODS

Bacterial strains, growth conditions, reagents, and antibiotics

Strains used in this study are listed in the **Appendix, Table 6.1**. *S. aureus* was cultured with Lennox LB agar or broth (BD Difco) at 35°C. Mass spectrometry analysis (as described in methods below) of two different lots of LB found thymidine amounts were at or below the level of quantitation (0.041 µg/mL) and insufficient to support *thyA* growth or antagonize SXT activity. Δ*thyA* was cultured on LB agar supplemented with a final concentration of 1.5 µg/mL thymidine. Liquid cultures were grown with agitation (225 rpm). Where indicated, media were supplemented with a final concentration of 0-16 µg/mL thymidine, 10 µM CCCP or 1 mM sodium arsenate dibasic heptahydrate (all Sigma Aldrich) for survival assays. Antibiotics used in survival assays and cloning included trimethoprim-sulfamethoxazole 8/152 µg/mL, 10 µg/mL erythromycin (transposon maintenance), 50 µg/mL ampicillin or 50 µg/mL kanamycin. For enumeration, blood and chocolate agar plates were used (ThermoFisher).

SXT survival assays

Overnight cultures of *S. aureus* were diluted in fresh LB broth to a final density of $1x10^6$ CFU/mL. Cultures were incubated with SXT and supplements as indicated. Cultures were serially diluted in 1X PBS pH 7.4 (Gibco) and plated for viable counts at specific timepoints. For anaerobic experiments, initial starting cultures and all reagents and media were deoxygenated overnight where possible, and all work was conducted in a Coy anaerobic chamber.

Enrichment of adaptive mutants

Experiments to select for adaptive mutants were performed as survival assays except that cultures were backdiluted in LB with fresh thymidine supplementation and SXT addition every 24h, at which point aliquots of culture were removed for DNA extraction. Representative colonies of diverse morphologies on LB, chocolate, and blood agar were selected for phenotyping and sequencing.

DNA extraction, whole genome and population-level sequencing, and analysis

DNA was extracted from cultures using the Qiagen DNeasy UltraClean Microbial Kit (Qiagen) as recommended by the manufacturer except that lysostaphin was added to the resuspended pellet at a final concentration of 0.14 mg/mL and incubated at 37°C for 30 min; the volume of solution SL added was then adjusted to account for the additional volume, followed by light vortexing and incubation at 70°C for 10 min.

Sequencing libraries were prepared as previously described[1], followed by sequencing on the NextSeq500 (Illumina) with 300 cycle chemistries. Sequencing depth for isolates was at least 43x coverage. Reads were analyzed as previously described[2], except using BWA-MEM (v0.7.12)[3] to align sequences to appropriate reference genomes (Newman, GenBank accession AP009351.1; JE2, GenBank accession CP000255.1). SAMtools (v1.1) was used for variant calling[4]. Mutations

were annotated with sequence features using SnpEff[5]. Average sequencing depth for population sequencing was 8.1 million reads per specimen (range 2.1 to 41.2 million reads), which were mapped as described above, followed by SNP variant calling using LoFreq2 "call" algorithm[6] and SnpEff for mutation annotation. Upstream and synonymous variants were filtered out and the additive variant allele frequency for each gene was calculated (aVAF; the total proportion of unique, nonsynonymous mutations). Genes with mutations detected in control experiments were excluded from further analysis, and the average aVAF for each experimental condition by timepoint (high thymidine, 1, 4, and 1 µg/mL; low thymidine, 0, 0.06, and 0.25 µg/mL) across available replicates was determined.

Analysis of clinical isolates from PwCF

Isolates (n=1382) collected from 246 children were previously sequenced and analyzed as described[7]. The current analysis was restricted to two populations of children from the isolate collection described in Long et al.[7]: 1) children with CF (CwCF) who either reported treatment with SXT or were TD-SCV-positive (the exposed group) and 2) CwCF who did not report treatment with SXT nor were TD-SCV-positive (unexposed group). *De novo* mutation analysis of the clonal groups that exist within these populations was previously determined and described[7]. This analysis was then used to determine the differences in mutational frequency between the exposed and unexposed groups for a set of 44 genes of interest that were found to be commonly mutated with *in vitro* selection with SXT. For each gene of interest for both exposed and unexposed groups, the number of clonal groups containing non-synonymous mutations was calculated (individuals with mutations); the number of clonal groups without mutations was then determined by subtracting the number of individuals with mutations from the total population size (total number of clonal groups). A contingency table containing the counts for individuals with mutations and without mutation for both the exposed and unexposed groups was generated. A Fisher's exact test was then preformed to determine the presence of a significant association between the mutation frequency and exposure status. The generated p-values were then adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

Auxotrophic assays

Colony phenotypes and auxotrophy were determined as previously described[8].

Liquid chromatography-tandem mass spectrometry of sputum samples

CF sputum samples were collected with informed consent and after approval from the University of Washington Institutional Review Board (STUDY00006773). Fifty microliters of sputolysin-treated (Calbiochem) CF sputum samples were analyzed for amounts of thymidine and its analogs by mass spectrometry using standard curves generated for thymidine (Thy; Fisher), deoxythymidine mono- (dTMP; Sigma), di- (dTDP; Fisher), or triphosphate (dTTP; Sigma). Ten microliters of C13 MTP internal standard (Sigma; 100ng/ml) was added followed by 150 µL of acetonitrile. Samples were centrifuged at 16.1 rcf for 5 min. Samples were reconstituted with 50

µl of mobile phase/solvent A, 0.1M ammonia acetate in water, pH 9.5 (Sigma Aldrich). Twenty microliter sample injections were analyzed on a Water's Xevo TQ-s triple quadrupole mass spectrometer, coupled to a Water's Acquity I-Class Ultra high-pressure liquid chromatography system (UPLC) in negative ionization mode (Neg-ESI) with the following transitions for each analyte: Thymidine - 241.2 to 125.1 and 151.2, dTMP – 321.2 to 125.2 and 195.2, dTDP – 401.2 to 159.0 and 275.2, dTTP -481.0 to 159.0 and 383.1. IS C13 MTP exhibited a transition of 333.2 to 182.0 and 200.0. Separation was achieved using a Thermo Hypercarb 2.1x50mm, 3u column with a 0.2u frit as a guard and a flow rate of 0.3 mL/min with gradients listed in Table **Appendix, Table 6.2**.

Strain construction

PtsI complementation of mutant NWM-TS08 and *RecA*-GFP reporter strains were constructed essentially as described[9]. For the *ptsI* complementation mutant, the target gene, preceded by a *sarA* promoter and *sod* RBS[10], was constructed by spliced overlap extension (SOE) PCR and the primers SbfI-*sarA* promoter forward, *sarA* promoter-*sod* RBS internal reverse, *sod* RBS-*ptsI* internal forward and *ptsI*-KpnI reverse (**Appendix, Table 6.1**). For construction of the RecA reporter strain, a gblock™ *recA*-GFP insert with restriction enzyme sites was designed as described[11,12] and constructed by IDT. The insert fragments were cloned into restriction sites *KpnI* and *SbfI* of pCN34 (BEI resources, strain NR-46121), transformed into NEB® 10-beta competent *E. coli* cells, followed by RN4220, and finally transformed into *S. aureus* strain Newman and mutant isolate NWM-TS08 by electroporation.

Measurement of intracellular ROS and ROS mitigation assays

CM-H2DCFDA (ThermoFisher, excitation ~492-495 nm and emission 517-527 nm) was reconstituted to 1mM in DMSO and used to quantify ROS in cultures as recommended by the manufacturer with modifications. Briefly, reconstituted dye was added to $1x10^7$ CFU/mL-containing culture at 5 µM alongside other supplements and SXT and incubated for 20 minutes. Following initial incubation, 1-2 mL of culture was removed to establish intracellular ROS baseline and remaining cultures were incubated. At specific timepoints, 1-3 mL aliquots were removed from culture, centrifuged at 6500 x g for 5 min, pellets washed with 1X PBS, and resuspended in 1X PBS. Samples were analyzed on a BD Accuri C6 Plus with a flow speed of 14 µL/min and a core size of 10 micron was used. Fluorescence was detected using a 533/30 standard optical filter (FL1), and data was analyzed using FlowJo software (version 10.8.1). ROS mitigation assays were prepared similarly to survival assays except with the addition of Trolox (Cayman Chemical) to cultures at a final concentration of 0.2 mM. Intracellular ROS was analyzed via BioTek Synergy H1 Hybrid reader.

Measurement of relative ATP

ATP was quantified in cultures using the BacTiter-Glo™ Microbial Cell Viability Kit (Promega), according to the manufacturer's instructions.

SOS induction assay

Overnight cultures of strain Newman harboring the pCN34-RecA-GFP plasmid were diluted to ~1x10^6 CFU/mL in LB with kanamycin for plasmid maintenance and treated with either 0.005 µg/mL mitomycin C, 128 µg/mL of thymidine, and/or varying concentrations of subinhibitory levels of SXT (0.125-0.5 µg/mL) and incubated at 35°C with constant agitation. Fluorescent data and OD_{600} were measured with a BioTek Synergy H1 Hybrid reader.

DNA Fragmentation Assay

Pulsed-field gel electrophoresis (PFGE) and conventional gel electrophoresis was used to assess DNA fragmentation. PFGE was conducted as previously described[13]. Briefly, agarose plugs were prepared prior to electrophoresis: 2% SeaKem® Gold agarose in EC buffer (Fisher Scientific) was prepared and kept molten in boiling water. Cells from SXT challenges or Δ*thyA* grown in the absence or minimal thymidine were concentrated (13,000 rpm for 1 min) and resuspended in 200 uL of EC buffer and placed in a water bath (55C); 40 uL of 0.5mg/mL lysostaphin was added to the cell suspension, followed by 200 uL of molten 2% agarose. The mixture was gently mixed and cast into an agarose plug mold (Bio-Rad). Plugs were solidified at room temperature for 15 minutes before removed from the mold and incubated in 5 mL EC buffer at 37°C for 1h to lyse cells. Following cells lysis, the EC buffer was removed, replaced with 5 mL of 1x TE buffer (10mM Tris, 1 mM EDTA; pH 8.0) and incubated at 55°C for 1h. Fresh 1xTE buffer (5mL) was added and the plugs were stored in solution at 4°C until ready for downstream use. In cases where plugs underwent restriction digest, the plug was first transferred to a 1.5 mL microcentrifuge tube and 150 uL of the restriction enzyme mix was added (per plug: 15uL 10x restriction buffer, 10 U/uL restriction enzyme, 130 uL sterile molecular grade water); plugs were incubated at 25°C for 2-4h, then directly used for PFGE. For PFGE, a 0.8% SeaKem® gold agarose gel was prepared using 0.5X TBE; a 1% MegaBase Agarose (Bio-Rad) in 1x TAE was prepared for traditional gel electrophoresis. Plug slices were placed onto the teeth of a gel comb, trimmed if necessary, and extra buffer was removed with a Kimwipe. Plugs were airdried for 10-15 min and then sealed to comb with ~5-10uL of agarose. The comb with the plugs was then positioned into the gel mold, molten agarose poured in, and was left to solidify for at least 30 min. *For PFGE:* While gel was solidifying, 2 L of 0.5X TBE was added to the electrophoresis chamber (CHEF DR-III) and circulated to cool the buffer to 14°C using a variable speed pump (1 L/min, set to 70) for 30 min. The gel was placed in the chamber and electrophoresis was run using the following settings unless otherwise stated: 6 V/cm, 120-degree angle; block 1 = initial switch 5s, final switch 15s for 10hrs; block 2 = initial switch 15s, final switch 60s for 13hrs). *For traditional gel electrophoresis:* the gel was placed in the chamber; 1x TAE was added to the fill line. 1kb ladder was added as a size reference. The following settings were used: 70 V for 1.5-2h. Gels were imaged with the Bio-Rad Molecular Imager® Gel Doc™ XR System.

Cell Proliferation Dye and Viability Assay

S. aureus cells were stained with eBioscience™ Cell Proliferation eFluor™ 670 (excitation 647 nm and emission 670 nm) as previously described with modifications[14].The Cell Proliferation Dye eFluor™ 670 was reconstituted according to manufacturer's instructions; a 10 µM working stock was prepared immediately prior to use. An overnight culture of *S. aureus* strain Newman was diluted to a final OD_{600} of ~1.0, from which 1 mL was removed and centrifuged at 6500 x g for 3 minutes. The cell pellet was then washed in 1 mL of PBS and centrifuged. The resulting pellet was resuspended in 1 mL PBS contain 1.25 µM of the working solution of Cell Proliferation Dye eFluor™ 670 and incubated in the dark at room temperature for 5 minutes. Following labeling, the cells were pelleted and resuspended in 1 mL of LB broth to quench unreacted dye; the cell suspension was incubated in the dark at room temperature for 2 min. Following incubation and centrifugation, the cell pellet was washed once with 1 mL PBS, and resuspended in 200 uL of LB. This bacterial suspension was then used to generate cultures with a final density of $1x10^7$ CFU/mL. SXT-specific proliferation assays were prepared similarly to survival assays. Cultures for all proliferation assays were prepared for flow cytometry at specific timepoints as described above. When testing for viability, the BactoView™ Dead Stain (Biotium, excitation 497 nm and emission 515 nm) was added according to manufacturer's instructions. Fluorescence was detected using a 533/30 standard optical filter (FL1) for viability and 660/20 standard optical filter (FL4) for cell proliferation; data was analyzed using FlowJo software (version 10.8.1).

Data analysis and availability

Data analysis and figure construction was performed with GraphPad Prism 9, version 10.1.1. Sequencing data from this study are publicly available through NCBI Sequence Read Archive under the BioProject ID PRJNA1083920.

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CHAPTER 7: APPENDIX

Table 7.1 Strains, primers, and sequences created or used in this study.

Strain or Resource	Source
Bacterial Strains	
Staphylococcus aureus Newman	Laboratory Stock
Staphylococcus aureus JE2	Laboratory Stock
Staphylococcus aureus RN4220	Laboratory Stock
Escherichia coli DC10β	Laboratory Stock
NE1920 (polyprenyl synthetase, *hepT*)	Fey, P. D. et al. mBio 2013
NE1224 (superoxide dismutase, *sodM*)	Fey, P. D. et al. mBio 2013
NE382 (squalene synthase, *crtN*)	Fey, P. D. et al. mBio 2013
NE1366 (catalase, *katA*)	Fey, P. D. et al. mBio 2013
S. aureus Newman Δ*thyA*	Tang, Q. et al. Cell 2022
S. aureus Newman Δ*hemB*	Tang, Q. et al. Cell 2022
S. aureus Newman Δ*menB*	Hammer, N., et al. mBio 2013
S. aureus Newman + pCN34	This paper
S. aureus Newman + pCN34 recA-gfp	This paper
S. aureus Newman ptsI (NWM-TS08)	This paper
NWM-TS08 + pCN34	This paper
NWM-TS08 + pCN34-ptsI	This paper
NWM-TS08 ptsI + pCN34-recA-gfp	This paper
Primers and plasmids	Sequence (5' to 3')
Construction of recA-gfp reporter	
IDT gblock ™ recA-gfp w/ restriction enzyme *sbfI* and *kpnI* sites	GTCCGCCTGCAGGAAACCTATTGAGCAGCAATTAAAA GATGCAGTGCAATTTGTTAATAAATTGTTTAATGTGT CATCAGCAATTATTCTATTAGAGTATGATGGTGTAGT TCATATAGGCTATGATAATAACTTTGAATTTAAAACT GAGCAATTTAAAATGTCTAAATCTAGAAATTTATTAA AGAACAGAAGTCAAAATTATGCGCTCATAAGATTATT AAATTGGCTTAGAACAACAAATTAATTGTATTATCGA TAAAAATATAAGCACGTTTGTTCGTTTTTGCGTTTTGA TTTCAAGATTTTATACGAACAAATATTCGCAAAACAC TTGTATTTTATTTTGAATCCTTGTATAGTATTGGTAAG ATAATTAAAAGATAGCAATTTCAATTAGGAGGTCTCG CTTTGGATAACGATCGTCAAAAAGCTTTAGATACAGT AATTAAAAATATGGAGAAATCTTTCGGTAAAGGTGCC GTAATGAAGTTGGGTGACAATATAGGTCGCATGTCAA AAGGCGAAGAGTTGTTCACGGGTGTTGTGCCGATCTT AGTAGAATTGGATGGTGACGTTAACGGCCATAAATTT TCTGTGCGAGGTGAAGGAGAGGGCGACGCGACTAAC

	GGAAAATTGACATTAAAGTTCATTTGTACGACAGGTA AGTTGCCTGTTCCATGGCCTACTTTAGTGACGACGTT AACATACGGAGTCCAATGCTTCAGTCGTTATCCAGAC CACATGAAGAGACACGACTTTTTCAAAAGTGCTATGC CGGAAGGTTATGTACAAGAACGTACAATTTCATTCAA AGATGACGGTACTTACAAAACAAGAGCTGAAGTGAA ATTCGAGGGCGACACTTTGGTGAATAGAATTGAGTTA AAAGGTATTGATTTCAAAGAGGATGGCAATATCTTAG GACATAAGTTAGAATACAATTTCAACTCACATAATGT GTACATCACTGCTGACAAACAGAAGAATGGTATCAA AGCAAATTTTAAGATAAGACATAACGTAGAAGATGG AAGTGTTCAGTTAGCAGACCACTACCAGCAGAATACG CCAATCGGTGACGGACCTGTGTTGTTGCCGGATAATC ACTATTTATCTACACAGTCAGTATTATCTAAGGACCC GAACGAGAAACGTGACCACATGGTTTTATTGGAGTTT GTAACAGCAGCTGGCATCACGCACGGCATGGACGAG TTGTACAAATAATGATAATGGTACCCCGTC
Construction of *ptsI* complementation plasmid	
sbfI-sarA promoter Fwd	CCGCCTGCAGG AAACACTTTTTTGTTTACTTC
sarA promoter-sod RBS internal Rev	CATCCTCCTAATTTGATGCATG
sod RBS-*ptsI* internal Fwd	TTAGGAGGATGATTATTTATGTCTAAATTAATTAAAG GTATTGCC
ptsI-kpnI Rev	TCCGGTACCTTATTTTACGTAGTTGTTAACTAATTC
Plasmid-specific primers (for sequencing)	
pCN34-MCS Flanking Fwd	GTATTACCGCCTTTGAGTGAG
pCN34-MCS Flanking Rev	CATTTAGTTTTGGTTCATCTTCTG

Table 7.2 Gradient used for LC/MS-MS analysis of thymidine analogs within sputum samples.

Time (min)	%A	%B
0	95	5
1	95	5
6	70	30
8.1	5	95
8.2	95	5
10	95	5