EUKARYOTIC-LIKE SERINE/THREONINE KINASE SIGNALING IN STAPHYLOCOCCUS AUREUS

DISSERTATION

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Signal transduction in prokaryotes is normally attributed to two component regulatory systems in which a membrane-bound sensor kinase facilitates phosphotransfer to a cytosolic response regulator, which can then appropriately modulate transcription. However, one-component regulatory systems have emerged as critical signaling molecules in prokaryotes as well. Members of this group include eukaryotic-like serine/threonine kinases (ESTKs) and phosphatases (ESTPs), named due to similarities to their eukaryotic counterparts. These enzymes are implicated in nearly all steps of bacterial pathogenesis, however their role in *Staphylococcus aureus* (*S. aureus*) unknown. This resilient organism is one of the most common causes of hospital-acquired and community-associated infections, affecting immunocompromised and immunocompetent hosts alike. We characterized a major functional ESTK (STK) and ESTP (STP) in *S. aureus* and found them to be critical modulators of the bacterial cell wall. Both enzymes are functional in vitro. Using gene knockout strategies, we created *S. aureus* N315 mutant strains lacking *stp* and/or *stk*. The strain lacking both *stp* and *stk* displayed notable cell division defects including multiple and incomplete septa and bulging, as observed by transmission electron microscopy. Mutant strains lacking *stp* alone displayed thickened cell walls and increased resistance to the peptidoglycan-
targeting glycylglycine endopeptidase lysostaphin, as compared to wild-type. Despite these observations, the core structure in peptidoglycan isolated from each strain was found to be the same, as determined by LC/MS/MS. Penicillin-binding proteins (PBPs), the enzymes responsible for peptidoglycan assembly in bacteria, were differentially expressed in mutant strains. Transcripts of all pbps (A, 2, 2x, 3, and 4) were up-regulated in the absence of stp, while pbps A and 2 were down-regulated in the absence of stk or stp and stk. Additionally, mutant strains lacking stk or both stk and stp displayed increased sensitivity (up to 50-fold in the case of N315ΔSTP/STK and ertapenem) to cell wall-acting β-lactam antibiotics. Finally, both STK and STP reversibly phosphorylate the response regulator WalR in vitro. This protein is a key regulator of cell division and cell wall gene transcription. Together, these results indicate that S. aureus STK- and STP-mediated reversible phosphorylation plays a critical role in peptidoglycan synthesis by modulating pbp expression.
To Steve.

We made it!
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CHAPTER 1

INTRODUCTION

*Staphylococcus aureus* (*S. aureus*), a robust, nonmotile, spherical bacterium, has emerged over the past century as a major public health threat, causing infection outbreaks worldwide. Currently it is estimated that approximately one-third of the United States population is colonized with *S. aureus* [1]. This pathogen is capable of infecting nearly every organ and tissue in the body [2], in part due to its extensive arsenal of toxins, host binding proteins, and other virulence factors. The emergence of antibiotic-resistant strains of *S. aureus* has only compounded the problem.

**Antibiotic Resistance**

Alexander Fleming’s 1929 discovery of penicillin [3] prompted the start of the antibiotic era, and the development of new antimicrobials. However, bacteria are constantly evolving mechanisms of resistance to survive, and *S. aureus* is no exception. By the 1940s, a mere decade after this Nobel prize-winning discovery, nearly half of all staphylococcal clinical isolates in the United States were reported to be resistant to penicillin [4, 5]. Not surprisingly, these resistant isolates appeared to be localized to
hospitals—areas of high antibiotic use. Thus *S. aureus*’ reputation for being “hospital-associated” began. Penicillin resistance was combated in the 1960s by the development of methicillin, a penicillin derivative resistant to the effects of β-lactamase, a serine protease produced by staphylococci which destroys the β-lactam ring common in penicillins [6]. Unfortunately, less than a year after its initial administration, methicillin-resistant *S. aureus* (MRSA) bacteria were isolated in England [7], which was followed shortly by the appearance of similar isolates worldwide. In 2003, 59.5% of all *S. aureus* isolates from intensive care units were resistant to methicillin, an increase of nearly 11% from a decade earlier [8].

MRSA bacteria are able to resist the effects of methicillin by their acquisition of the *staphylococcal cassette chromosome mec* (SCCmec). This 21-67kB fragment integrates into the staphylococcal genome near the origin of replication [9]. Genetic recombination is believed to occur at very low frequency within the *Staphylococcus*, evidenced in part by its resistance to genetic manipulations in the laboratory [10] and immediate digestion of foreign DNA. Based on this, the acquisition of the SCCmec element, which cannot be explained by simple nucleotide substitutions, is thought to be phage-mediated [10]. Notably, this element has not been found in any bacterial genera outside of *Staphylococcus*. Of the nine *S. aureus* genomes sequenced to date (clinical isolates MW2, Mu50, N315, RF122, COL, USA300, MRSA252, and MSSA476 and laboratory strain NCTC8325), five different variations (I-V) of the SCCmec element have been identified in the MRSA strains [10]. These variations are based on the combination of genes on each element. All SCCmec elements contain *mecA*, a penicillin-binding
protein (PBP) with reduced affinity for β-lactam antibiotics (which are designed to bind to and disable the endogenous PBPs) and one or more recombinases (ccrA, ccrB, and ccrC) that enable insertion of the cassette into the genome. Some variants (SCCmec II and III) also contain genes encoding resistance to non-β-lactam antibiotics or heavy metals [9]. Importantly, subtle changes in these components have been identified in all SCCmec elements except for SCCmec type V, resulting in other designations (SCCmec types IA, IIA-E, IIIA, and IVa-c, E, F) [9]. The multitude of SCCmec elements identified to date may, in some part, explain the persistence of MRSA within hospitals, and how it has been able to spread to the community in recent years.

Community-associated MRSA

Community-associated MRSA (CA-MRSA) emerged in the 1980s and was originally thought to be limited to high-risk individuals (institutionalized, intravenous drug-using, or those with indwelling medical devices) [11, 12]. However, current research suggests that MRSA can persist asymptomatically, primarily in the nares [2, 13], which likely facilitated the original spread of MRSA from the hospital to the community. Interestingly, SCCmec elements in CA-MRSA appear to be limited to types IV and V [14], confirming that these isolates are in fact distinct from their hospital-associated (HA-MRSA) counterparts, although ~20% of CA-MRSA genetic material is believed to be acquired horizontally [15]. Since each strain utilizes different virulence factors, the patterns of infection and symptoms they cause differ. CA-MRSA tends to infect young, healthy individuals, and normally remains localized in skin and soft tissue infections [12,
HA-MRSA infections often follow catheter placement, surgery, or immunotherapy, resulting in more severe and even fatal sepsis, toxic shock syndrome, pneumonia, endocarditis or osteomyelitis [16, 17]. CA-MRSA is actually thought to be more virulent than HA-MRSA, causing more significant disease in the immunocompromised, and sporadically in healthy individuals [18]. Thus, the incidence of severe CA-MRSA infection has significantly risen in recent years, allowing these strains to make their way back in to the hospital setting [16].

Invasive MRSA infections, regardless of origin, result in death in approximately 6% of cases [16]. This translates to an estimated 18,650 United States deaths in 2005—2,000 more than HIV and AIDS [16]. Current treatment for MRSA infections often includes vancomycin, however *S. aureus* strains have been isolated with resistance to this drug as well. This is mediated by *S. aureus*’ acquisition of the vancomycin-resistance gene cluster from *Enterococcus* species [19]. Enterococci are able to resist vancomycin-mediated inhibition of cell wall synthesis by expressing alternative peptide sequences within their peptidoglycan, specifically sequences that terminate in D-Ala-D-Ser or D-Ala-D-Lac, rather than the vancomycin substrate D-Ala-D-Ala [19]. *S. aureus* with intermediate vancomycin utilize VanA from *Enterococcus* species and also express D-Ala-D-Lac in lieu of D-Ala-D-Ala [19]. Interestingly, these bacteria also appear to simply increase the production of glycopeptides substrates to outcompete the antibiotic, resulting in significantly thickened cell walls [20]. Although current levels of vancomycin-resistant *S. aureus* (VRSA) infections are low, treatment options are extremely limited, and consequently these infections are associated with a mortality rate of more than 60% [21].
Virulence Factors

To cause such diverse diseases, *S. aureus* bacteria utilize a myriad of virulence factors. As a Gram-positive organism, the single cell membrane of *S. aureus* is coated with a thick peptidoglycan layer, a meshwork of sugars (glycans) and protein (peptides). This layer gives the bacterium its shape and protects it. Staphylococci have an exceptionally thick and highly crosslinked layer of peptidoglycan, particularly as compared to that of Gram-negative bacteria. *Escherichia coli* (*E. coli*) peptidoglycan for example is normally 1.5-15nm thick, whereas that of *S. aureus* can reach 30nm in thickness, nearly all of which is crosslinked in a tight lattice [22, 23]. Such a thick peptidoglycan layer imparts halotolerance to the staphylococcus, which can grow even in relatively high salt concentrations (2.6M NaCl), such as those found in salty foods [24]. All together, peptidoglycan makes up approximately 20% of the dry weight of the cell [25]. Anchored in the cell membrane and covalently linked to peptidoglycan are surface proteins, many of which help the *Staphylococcus* colonize its host, the first step in pathogenesis. During exponential phase, staphylococci express surface proteins (Fig. 1) which specifically bind to glycoproteins within the host’s extracellular matrix (ECM) and are as such termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). The best characterized MSCRAMMs include fibronectin-binding proteins (FnbA, FnbB), and clumping factors (ClfA and ClfB), which can bind to integrins on the surface of host cells via fibrinogen or fibronectin bridges [26] (Fig. 2). Another surface protein, Cna, serves as a collagen adhesin and is believed to facilitate invasive infection, particularly osteomyelitis [27]. These proteins also allow
**Fig.1: Growth phase-associated virulence factor expression.** A typical growth curve for *S. aureus* is indicated by the solid black line, including lag, exponential, and stationary phases of growth. Surface protein (fibronectin/fibrinogen binding proteins, protein A, collagen adhesin, etc.) expression, indicated in green, peaks at early-mid exponential phase. Secreted (hemolysins, toxins, etc.) and major cytoplasmic protein expression is activated at late exponential phase, and production continues through stationary phase (indicated in yellow). Peak Agr expression (indicated by dashes) falls between mid and late exponential phases.

Staphylococci to adhere to implanted medical devices covered in serum protein deposits, as well as intact epithelium. *S. aureus*. Extracellular fibrinogen-binding protein (Efb) can bind free complement factor C3b in addition to fibrinogen [29], again sequestering this host immune molecule.

In addition to ECM binding proteins, several surface and secreted proteins on *S. aureus* are designed to combat the host’s immune system through enzymatic activities and by binding specific components. Protein A (SpA) binds constant (Fc) domains on host immunoglobulin G (IgG) molecules (Fig. 2) [28]. This sequesters the antibodies and
**Fig. 2: Major staphylococcal virulence factors.** Schematic indicates major host virulence factors, although they are not all expressed simultaneously (see Fig. 2). Fibronectin binding proteins (FnbA, FnbB) can bind to host cells through a similar bridge mechanism to that of clumping factors (ClfA, ClfB) using fibronectin instead of fibrinogen. Note not all strains produce capsule. Host factors are indicated in purple.
inhibits their ability to properly opsonize the bacteria, thus it is a key virulence factor of Chemotaxis inhibitory proteins (CHIPs), secreted by about 60% of *S. aureus* strains, bind complement factor C5a and formyl peptide receptors on neutrophils inhibiting their activation and propagation of the complement pathway [28]. Cysteine and serine proteases, metalloproteases, and lipases secreted by *S. aureus* are all thought to provide small molecular weight nutrients for the bacterium through their individual enzymatic activities, but they may also play a role in pathogenesis by cleaving host proteins and membranes [17]. V8 protease (SspA), a serine protease, is commonly found in most *S. aureus* strains and can cleave the heavy chains of host immunoglobulins, but more commonly cleaves its own bacterial surface proteins to facilitate planktonic growth and spread [30]. Staphylokinase (Sak) is only found in lysogenic *S. aureus* strains carrying specific phages and is a potent activator of plasminogen, the precursor of plasmin, a fibrin protease [31]. In this way, Sak is activated to facilitate growth and tissue invasion. It has also been proposed as a therapeutic thrombolytic agent [17, 31]. Sak can also neutralize the effects of antimicrobial peptides such as α-defensin [31]. All *S. aureus* strains also produce coagulase (Coa), which is a key difference between *S. aureus* and the commensal *Staphylococcus epidermis* (*S. epidermis*), and this property is often used to distinguish the two species [17]. Coagulase is tightly associated with the bacterial cell wall by an unclear mechanism, and binds soluble prothrombin in the serum, forming a coagulant complex termed ‘staphylothrombin’ [32]. Staphylothrombin converts
fibrinogen to fibrin, clotting the blood and coating the bacteria in a layer of host protein, helping *S. aureus* resist phagocytosis by host immune cells [17].

If the bacteria are subjected to phagocytosis, one of the primary defense mechanisms of the innate immune system, they are equipped with catalase (KatA), thioredoxin (TrxA), superoxide dismutase (SodA and SodM) and glutathione peroxidase (GpxA), among others, to combat any reactive oxygen species they may encounter [33]. Several *S. aureus* strains also produce a thin polysaccharide capsule *in vivo* to resist phagocytosis. Eleven capsule types have been identified, the most common being serotypes 5 (CP5) and 8 (CP8), each encoded by 16 gene operons on the bacterial chromosome [34, 35]. Types 5 and 8 share similar sugar structures, and are composed of O-acetyl-β-D-N-acetylmannosaminuronic acid and N-acetylfucosamine [35]. Staphylococci expressing capsule are more virulent and persistent in animal infection models [28].

**Staphylococcal Toxins**

Toxins produced by *S. aureus* during late exponential phase and stationary phase (Fig. 1) are major players in its ability to invade host tissues and cause disease. Alpha, beta, and gamma toxins are hemolysins that all interfere with host cell membranes, causing lysis, release of cytokines and internalized staphylococci, and inflammation. Alpha toxin (Hla), the most potent of the staphylococcal hemolysins, oligomerizes to form hexa- and heptameric pores, primarily in erythrocytes but also in epithelial and
endothelial cells, monocytes and platelets [36, 37]. Cellular contents leak through these pores causing rapid cell death. Similarly, beta toxin (Hlb) also causes membrane damage, albeit through a different mechanism. Beta toxin is a sphingomyelinase which cleaves sphingomyelin into phosphorylcholine and ceramide. The buildup of ceramide causes the membranes to collapse, appearing as invaginations on the cell surface [37]. Beta toxin is secreted by approximately 88% of animal S. aureus isolates, but is far less commonly produced by human isolates, whereas alpha and delta toxin can be detected in nearly all S. aureus isolates [36, 37]. The mechanism of delta toxin-mediated cell lysis is not clear [37].

Another pore-forming toxin, Panton-Valentine leukocidin (PVL) is encoded on prophages and thus is not present in all S. aureus strains. It has been epidemiologically linked to CA-MRSA, but it remains controversial how critical PVL is to CA-MRSA pathogenesis, as strains lacking PVL are still capable of causing significant disease [18, 38]. PVL is a bi-component toxin encoded by the lukPV operon in ΦSLT, ΦPVL, and ΦSA2MW phages [15]. How the pores are formed in host cells is not entirely clear, but PVL is specific to leukocytes [15]. Despite its association with CA-MRSA strains responsible for causing disease, PVL has been shown to be dispensable in fatal sepsis, abscess formation, and even in vitro neutrophil lysis [18]. This may be due in part to assay techniques, as the virulent effects of PVL in vivo are thought to be transient and specific to the early stages of infection [39]. Direct inoculation of mice with purified PVL causes significant mortality in a concentration-dependent manner [40], indicating that PVL itself is a virulence factor of S. aureus. It is perhaps for this reason that antibodies
against PVL are protective [41]. There is also some evidence that PVL may regulate
expression of other virulence genes in *S. aureus* [40]. Increased expression of protein A
(Spa) and other cell wall-anchored proteins is directly correlated with PVL expression
levels [40]. Thus, although it is clear that PVL plays a role in CA-MRSA pathogenesis,
its necessity and place as the “primary” virulence factor associated with such isolates
remains contentious.

In addition to the aforementioned virulence factors, *S. aureus* also produces 20
different enterotoxins, or “superantigens,” named as such for their ability to cause
extreme, non-specific T-cell activation [42]. They are most often encoded on mobile
genetic elements, such as prophages, pathogenicity islands, or transposons, thus are not
equally distributed across strains [43]. All superantigens bind non-specifically to T-cell
receptors and major histocompatibility complex (MHC) class II molecules on the
surfaces of antigen-presenting cells. Superantigen binding to MHC molecules is
extremely stable, and can last up to 40 hours as the antigen “waits” for a T-cell receptor
to engage [36]. The binding to both molecules occurs outside of the peptide groove.
Engagement of both receptors results in extremely high levels of T-cell activation and
proliferation, and a consequent enormous efflux of inflammatory cytokines, causing
shock [37]. Because superantigens are highly resistant to heat and pepsin digestion and
are secreted [36], they can cause disease in the absence of the bacterium. For this reason,
staphylococcal superantigens, specifically enterotoxin subtypes A, B, C, D, E, and G, are
believed to be the leading cause of food poisoning (and its resulting emesis) in the United
States [36]. Ingestion of <1µg of enterotoxin is sufficient to cause disease in humans [36].

A separate subset of enterotoxins includes toxic shock syndrome toxin-1 (TSST-1), encoded by _tstH_ on staphylococcal pathogenicity island 1 [36]. Interestingly, this toxin does not cause emesis, despite its stability and ability to cross mucosal surfaces [36]. It is the primary toxin associated with menstrual toxic shock syndrome (TSS), although the incidence of such disease has decreased significantly since the 1980s due to public education initiatives regarding tampon use [44]. During TSST-1-mediated toxic shock syndrome, up to 1 in 5 T-cells may be activated systemically, as compared to 1 in 10,000 during normal antigen presentation [36, 37]. Amazingly, TSST-1 and staphylococcal enterotoxin B, but not others, can also serve as transcription factors, repressing exoprotein production and their own synthesis [45].

**TCRSs and the Agr System**

The various virulence factors of _S. aureus_ (and those of prokaryotes in general) are controlled by complex regulatory networks which both overlap and work in concert. These often include two component regulatory systems (TCRSs). TCRSs involve the use of membrane-bound, extracellular sensors (histidine kinases) and intracellular transcription factors (response regulators). Upon sensing an extracellular stimulus, the histidine kinase autophosphorylates on a conserved intracellular histidine residue and transfers this phosphoryl group to a conserved aspartic acid residue on the response regulator. The response regulator can then bind directly to DNA to mediate appropriate
transcription [46]. In *S. aureus*, there are 16 known TCRSs [47], including the Agr system, which has been established as a global regulator [48]. The Agr system is self-activating, in that the histidine kinase AgrC acts on the response regulator AgrA to modulate divergent transcription of the *agr* locus [48]. The sequence of the *agr* locus is conserved across staphylococci [48], and expression of Agr peaks during late exponential phase (Fig. 1). In addition to the AgrC/A TCRS, the *agr* locus also encodes an autoinducing peptide (AgrD, processed and secreted by AgrB), which serves as the extracellular stimuli for AgrC, and RNAIII, which modulates intracellular gene transcription [48]. In general, RNAIII down-regulates transcription of surface proteins while up-regulating transcription of extracellular proteins (e.g. secreted toxins), implicating it and the Agr system in virulence control [48]. In fact, δ-hemolysin is encoded on the RNAIII transcript [46], and RNAIII can modulate α-hemolysin both transcriptionally and translationally [49]. The Agr system is considered a quorum sensing system, whereby the autoinducing peptide is secreted and can be sensed by neighboring staphylococci, in turn activating their own *agr* regulons (including RNAIII). Thus, transcription of virulence factors is density-dependent [46]. This quorum sensing is strain-specific. Autoinducing peptides, which are just 7-9 amino acids in length [48], from one *S. aureus* strain can inhibit the *agr* system of another [49].

RNAIII modulates transcription of another major TCRS in *S. aureus*, the *S. aureus* exoprotein TCRS, saeRS. Orthologs of this system are seen in *Bacillus subtilis* (*B. subtilis*) and *Enterococcus faecalis* (*E. faecalis*) [49]. This system is essential for hemolysin production [49], and acts independently of the Agr system. SaeR/S is activated
by hydrogen peroxide and α-defensin, therefore it is believed to play a role in *S. aureus* survival within phagocytes [50]. Other major TCRs in *S. aureus* include SrrA/B (controls staphylococcal respiratory response), ArlS/R (autolysin regulating locus), and LytR/S (responsible for autolysis).

**Staphylococcal Accessory Regulators**

Another major contributor to *S. aureus* gene regulation is the staphylococcal accessory regulator, or Sar, protein family, which includes the small cytoplasmic regulator SarA and its various paralogs (SarR, SarS, SarT, SarU, SarV, SarX, SarY, SarZ, MgrA, and Rot), all of which serve as DNA-binding proteins [47]. Transcription of *sarA* is driven by three independent promoters, and it produces three overlapping transcripts: *sarA*, *sarB*, and *sarC* [51]. The levels of *sarA* are generally consistent with those of *agr* transcripts [51]. SarA, expressed during all growth phases [49], can bind to AT-rich regions termed “Sar boxes” [49] and modulate transcription. It has been shown to bind to P2 and P3 promoters of the *agr* locus [52, 53], and is also implicated (perhaps as a result of *agr* regulation) in the transcription of both extracellular and cell wall-associated proteins, including PVL, various toxins, protein A, and fibronectin-binding proteins, among others [50, 54]. Although most Sar protein family members serve as transcriptional activators, some can serve as repressors in specific cases. Among these, SarR serves as a SarA repressor [49], SarX (which is self-activating) represses *agr* transcription [54], and Rot negatively regulates toxin expression (although it up-regulates cell wall protein synthesis) [54]. All of the Sar protein family members play major roles
in staphylococcal gene regulation, as evidenced by transcriptional profiling whereby MgrA alone was found to regulate 355 genes, approximately equally split between up- and down-regulation [55]. Thus, Sar proteins, while not members of TCRSs, provides another mechanism of regulation within *S. aureus*.

**Alternative Sigma Factors**

*S. aureus* also possesses a single alternative sigma factor, σ^B^ [48]. Sigma factors are key components of the RNA holoenzyme, providing promoter recognition specificity and contributing to DNA unwinding [56]. In prokaryotes, RNA holoenzyme normally associates with the same sigma factor for transcription of housekeeping genes and utilizes dissociable alternative sigma factors to transcriptionally regulate more environment-dependent genes, such as virulence factors [46]. In *S. aureus*, σ^B^ regulates carotenoid expression responsible for the characteristic golden color of colonies [48], which confers resistance to oxidative damage [57]. However, its precise role in global regulatory networks is enigmatic. It is regulated post-translationally, normally bound by an anti-sigma factor, RsbW, which is dephosphorylated and released under conditions of environmental stress [48]. There is a great deal of overlap between the 251 genes regulated by σ^B^ in *S. aureus* and the 127 genes regulated by σ^B^ of *B. subtilis* [56, 58], including genes associated with cell wall metabolism, membrane transport, virulence, and environmental stress responses [46, 58]. Most importantly, *S. aureus* σ^B^ plays a key role in sarA transcription [58], and appears to be antagonistic to the Agr system [48].
One Component Regulatory Systems

Although conventional dogma dictates that TCRSs (and to a lesser extent alternative sigma factors) are the primary method of signal transduction in prokaryotes, a recent report suggests that one component regulatory systems are in fact the most abundant signaling systems in prokaryotes [59]. One component regulatory systems include eukaryotic-like serine/threonine kinases (ESTKs) and phosphatases (ESTPs), and have never before been described in S. aureus. Serine/threonine kinases and phosphatases are well known for their involvement in eukaryotic signaling cascades [60, 61], thus ESTKs and ESTPs are termed “eukaryotic-like” due to their high sequence homology to their eukaryotic counterparts [62, 63]. This suggests a eukaryotic origin [64], however in reality ESTKs and ESTPs have also been found in Archaea [64-66], making the term “eukaryotic-like” a misnomer. The first ESTK was identified in Myxococcus xanthus (M. xanthus) in 1991 [67], and since then similar ESTKs and ESTPs have been identified and characterized in both Gram-negative and -positive pathogens, including Salmonella enterica serovar Typhi (S. Typhi) [68], Pseudomonas aeruginosa (P. aeruginosa) [69, 70], Streptococcus agalactiae (S. agalactiae) [71], Streptococcus pneumoniae (S. pneumoniae) [72], Streptococcus pyogenes (S. pyogenes) [73], E. faecalis [74], B. subtilis [75, 76] and Streptococcus mutans (S. mutans) [77], as well as Mycobacterium tuberculosis (M. tuberculosis) (reviewed in [78]). In Gram-positive genomes, such as those of B. subtilis [75], S. pneumoniae [72], S. pyogenes [73], S. agalactiae (5963), and E. faecalis [74], genes encoding ESTPs and ESTKs often overlap, a pattern that is not
generally seen in Gram-negative genomes. Notably, perhaps due to their abundance and primary signaling roles, ESTKs have been studied more extensively than ESTPs.

**ESTK/ESTP Structure**

Structurally, ESTKs vary across organisms depending on their function(s), but Gram-positive forms are usually membrane-bound with an extracellular sensing, or PASTA (penicillin binding protein and serine/threonine kinase associated) domain, a variable number of transmembrane domains (often just one), and a catalytic, intracellular kinase domain (Fig. 3). In this way, a bacterium can transmit a signal across its cell membrane. Notably, ESTKs have been shown to act as dimers in vivo \[79, 80\]. The kinase, or catalytic, domain is highly conserved and sequence homology studies have shown that this region consists of approximately 280 amino acids \[81, 82\]. Multiple studies indicate autophosphorylation occurs at one or more specific serine and/or threonine residues within the catalytic domain of ESTKs to stimulate their phosphotransfer activity, and site-directed mutagenesis of these residues inhibits the catalytic reaction \[69, 83-86\]. It is in this catalytic region that the phosphodonor (usually an ATP or GTP molecule bound to a divalent cation) and substrate are thought to bind, and the phosphoryl group is transferred \[81\]. As a result of the sequence being so highly conserved, the three dimensional structure of the catalytic domain across species is believed to be quite similar, as is how the phosphodonor and recipient bind.
Fig. 3: Schematic of a typical Gram-positive ESTK. Gram-negative ESTKs are not normally membrane bound. Autophosphorylation (indicated in green) occurs on one or more intracellular serine and/or threonine residues. Intracellular N-terminus and extracellular C-terminus are indicated by N and C respectively. PASTA indicates penicillin binding protein and serine/threonine kinase associated domain.

Nevertheless, there are exceptions to these characteristics. *M. xanthus* contains 102 ESTKs, including several uncharacterized cytoplasmic forms [87]. Gram-negative ESTKs are often soluble, such as an ESTK in *Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) which is not only soluble, but secreted and translocated to membranes of host cells [88].

Additionally, sequences of ESTKs are not always conserved. For example, a mycobacterial ESTK (PknB) has a structure that is highly similar to ESTKs, despite
exhibiting less than one third sequence homology [89]. In fact, inactive PknB most closely resembles activated mouse cyclic AMP-dependent protein kinase [89]. Identification of the catalytic region of PknB by searching for projected phosphoryl binding sites indicates that its location is such that if phosphorylated, the phosphoryl groups may actually inhibit proper folding [89]. Consistent with this idea, three phosphorylation sites outside of the catalytic domain have been identified for PknB [83]. However, overall, in most ESTK catalytic domains the phosphoryl donor is believed to bind and phosphorylate the smaller N-terminal portion of the catalytic domain (Fig. 3), while the recipient binds to the larger C-terminal portion. In this way, the catalytic domain of ESTKs is bi-lobed, with an N-terminal portion (primarily comprised of β-sheets) and a C-terminal portion (primarily α-helical) [81, 82, 89]. This catalytic region is further divided into 12 individually conserved subdomains (termed “Hanks’ domains”), with subdomains II-VII and IX being required for catalysis, and I, X, and XI taking on more structural roles [81, 82]. These domains are also seen in eukaryotic serine/threonine kinases [81].

The extracellular sensing domains of ESTKs are comprised of a variable number of PASTA repeats (Fig.4). These repeats bind unlinked peptidoglycan [90], and are essential for growth and development [91]. It is perhaps due to each organism’s unique peptidoglycan structure that there is minimal sequence homology across PASTA repeats [91, 92]. Linking the extracellular domain to the cytosolic domain in ESTKs is normally a single hydrophobic transmembrane domain. This region is thought to serve as an anchor
Fig. 4: PASTA-containing Gram-positive ESTKs. The number of PASTA repeats (shown in green) in ESTKs is variable, typically 3-5 in Gram-positive varieties. The serine/threonine kinase domain is conserved.

for the kinase [86], and have little catalytic function. The sequence of the transmembrane domain is often, but not always, conserved. In *M. xanthus*, Inouye et al. reported at least one conserved transmembrane domain in all but two of 13 characterized serine/threonine kinase sequences [84]. This conservation makes it hard to discount function for this transmembrane region, although ESTKs lacking this domain in *Bacillus* and *Streptococcus* species maintain their catalytic activity [73, 85]. Among the 11 reported ESTKs in *M. tuberculosis*, nine are believed to be transmembrane signaling molecules passing through the plasma membrane via a single helix [78, 80]. It is unknown whether non-mycobacterial ESTKs consistently exhibit this single helix transmembrane domain.
or not, although this is the most common structure predicted from sequence analyses and hydrophobicity plots.

ESTPs serve to reverse the reactions of ESTKs [93]. Despite this, these molecules are not always present in equal numbers in a given prokaryotic genome. For instance, the *M. tuberculosis* genome encodes 11 ESTKs [78], but only one ESTP [94]. On the other hand, the genomes of *Caulobacter crescentus* and *Xylella fastidiosa* each contain multiple putative ESTPs, but not a single ESTK [93]. The catalytic domains of ESTPs vary, and are spread across the four eukaryotic protein phosphatase families PP1, PP2A, PP2B, and PP2C [82, 93], which are identified by their specific conserved motifs [82, 95, 96]. ESTPs appear to be quite versatile, capable of dephosphorylating multiple substrates on both serine and threonine residues [93]. To date, all ESTPs identified are soluble and primarily cytoplasmic, although in the case of *S. pyogenes* this enzyme is also secreted [73].

**ESTKs and Pathogenesis**

Both ESTKs and ESTPs have been implicated in various steps of bacterial pathogenesis. To identify their specific roles, knock-out and over-expression studies have been the method of choice. Altered expression of ESTKs and/or ESTPs in a variety of bacterial species has had widespread effects on crucial bacterial functions, including growth, adherence, cell division, environmental sensing, metabolism, and virulence. For this reason, ESTKs in particular are considered global regulators of gene expression and work is underway to identify similar roles for ESTPs [59, 97].
Specifically, throughout the literature ESTKs have been implicated as key players in bacterial morphology, and septation in particular. In *S. pyogenes*, deletion of its sole ESTK causes extreme bulging and a loss of chain formation [73]. Bacteria appear to divide inefficiently, resulting in multiple septa in a single oversized (1.17μm as opposed to 0.94μm diameters in the wild type) coccoid-like cell [73]. In *S. agalactiae*, the ESTK Stk1 is also implicated in chain formation, however deletion of *stk1* results in formation of extremely long chains, as does deletion of both *stk1* and its cognate ESTP, *stp1* [71]. *E. faecalis* cells are slightly bulged and collapsed upon deletion of the ESTK *prkC* [74].

In organisms with multiple ESTKs, less severe phenotypes are seen upon modulation of a single ESTK. Depletion of PknA or PknB in the nonpathogenic Gram-positive actinomycete *Corynebacterium glutamicum* (*C. glutamicum*) results in multiple incomplete septa, whereas over-expression of the same proteins converts the normally rod-shaped organism to a coccoid shape [98]. Deletion of the other two ESTKs in *C. glutamicum*, *pknG* or *pknL*, does not affect morphology [98]. Over-expression of two ESTKs (PknA or PknB) in mycobacterial species also affects cellular morphology, causing bulging, branching, and slowed growth as compared to wild type [99]. Major effects of ESTP deletions on cellular morphology remain to be determined, as *prpC* mutants in *B. subtilis* do not have difficulty reaching exponential phase [100], and *S. agalactiae* cells lacking both *stk1* and *stp1* exhibit similar growth patterns to cells lacking just *stk1* [71].

Disruption of ESTK production in group A streptococcal cells has also been shown to have deleterious effects on bacterial function, specifically adherence. Normally
this bacterium has a thick, fibrous, electron-dense layer attached to its cell wall. Removal of either the kinase’s outer membrane domain or the intracellular catalytic domain results in an absence of this layer [73]. Furthermore, the resulting inability of the bacteria to efficiently adhere to mammalian pharyngeal cells suggests a place for ESTKs in bacterial adhesion [73]. In *S. mutans*, ESTK knockout strains are unable to form a cohesive biofilm, even after prolonged incubation. Rather, the biofilm is thin and gapped [77]. Similar results are seen in *Mycobacterium smegmatis* (*M. smegmatis*) bacteria lacking the ESTK PknF [101]. Inefficient biofilm formation could be due to slowed growth of ESTK mutant bacteria as described above, reinforced by the fact that nearly all published studies investigating ESTK mutants report a reduced growth rate, even after media supplementation [71, 77]. These few studies suggest that ESTKs may indirectly modulate bacterial adherence to host cells and substrata, although further investigation is needed in this area to confirm this.

It is perhaps due to these morphological and growth defects that many ESTK mutant bacterial strains have demonstrated reduced virulence as well [71, 102, Jin *et al.* unpublished data]. Genetic manipulations of ESTKs have also been shown to severely inhibit virulence in the pathogenic fungus *Aspergillus fumigatus* [103] and Gram-negative *Y. pseudotuberculosis* [104]. Gram-positive *E. faecalis* strains lacking the ESTK PrkC are 6-fold less abundant than wild type after a 16 hour intestinal persistence assay, although the effect of this persistence on morbidity/mortality has not been investigated [74]. Direct evidence for ESTK involvement in virulence has been seen in *S. pneumoniae*, whereby the ESTK StkP is required for lung and bloodstream infection in
mice by inducing competence via the *comCDE* operon [105]. Elimination of the PASTA domain-containing C-terminus of SP-STK in *S. pyogenes* increases susceptibility of the bacteria to phagocytosis [73]. However, in *P. aeruginosa*, deletion of the ESTK *ppkA* (which is preferentially expressed during infection) results in no change in virulence in animal models [70], emphasizing the importance of further research in this area to elucidate common mechanisms for these observations.

In relation to environmental sensing, the ESTK Pkn22 from the cyanobacteria *Anabaena* is activated under iron-depleted conditions [106]. Additionally, insertion inactivation of *pkn22* results in growth arrest under oxidative stress conditions [106]. The ESTK PrkC in *E. faecalis* is required for resistance to, and growth in the presence of, multiple antibiotics, particularly those that act on the cell wall [74]. ESTK mutants in *S. agalactiae* and the dental pathogen *S. mutans* have been shown to be sensitive to low pH, growing more slowly in agar with pH 5 as well as in the presence of hydrogen peroxide, as compared to wild type [77, 107]. *Synechocystis* cyanobacteria lacking the ESTK SpkD are unable to utilize carbon dioxide or ammonium as carbon and nitrogen sources, respectively [108], suggesting involvement of this ESTK gene in extracellular nutrient uptake and metabolism. SpkA, another ESTK in *Synechocystis* species, regulates pilus production [109]. While pili are primarily associated with cellular morphology and used for adhesion, they should not be discounted as an important method used by bacteria to sense their environments. Thus ESTKs play a key role in transmitting signals from the extracellular milieu to the cytoplasm, although the cofactors and other stimuli involved in this process remain to be elucidated.
ESTK Substrates

A handful of proteins have been identified in the literature as *in vitro or in vivo* substrates for prokaryotic ESTKs (Table 1). Not surprisingly (based on the peptidoglycan-binding PASTA domains present on ESTKs), many substrates are involved in cell division processes. Several mycobacterial ESTKs directly phosphorylate cell division-related proteins, including FtsZ [110], MurD [111], Wag31 [112], and penicillin-binding protein A (PBPA) [113]. Interestingly, Wag31 is phosphorylated to a much greater degree when two ESTKs (PknA and PknB) are added simultaneously to *in vitro* kinase assays [99], suggesting ESTKs may in fact work together to phosphorylate their substrates and transmit their signals. ESTKs also phosphorylate proteins in other pathways, such as OdhI in *C. glutamicum*, a 2-oxoglutarate dehydrogenase inhibitor protein in the TCA cycle [114], and RshA and alternative sigma factor SigH in *M. tuberculosis*, which are involved in the cellular stress response [115]. PrkC of *B. subtilis* can also phosphorylate elongation factor G (EF-G), which is required for mRNA and tRNA translocation [100]. The *M. tuberculosis* ESTKs PknA, PknB, and PknH are all able to phosphorylate EmbR, a transcriptional activator for arabinosyltransferases [116]. This phosphorylation ultimately alters the lipoarabinomannan/lipmann (LAM/LM) ratio, virulence, and antibiotic resistance [116]. In *S. pyogenes*, the ESTK SP-STK has been shown to phosphorylate multiple (unidentified) substrates, although it preferentially phosphorylates P10 (histone-like-protein, HLP) [73], the function of which is unknown. This observed promiscuity of ESTKs and their substrates suggests complex signaling cascades are at work in these systems. Forkhead-associated domains, conserved
<table>
<thead>
<tr>
<th>Organism</th>
<th>ESTK</th>
<th>Substrate Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tr>
<td><em>C. glutamicum</em></td>
<td>PknA</td>
<td>PknG</td>
<td>ESTK</td>
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<td></td>
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<td>MurC</td>
<td>UDP-N-acetylmuramate:L-alanine ligase</td>
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<td>PknG</td>
<td>OdhI</td>
<td>2-oxoglutarate dehydrogenase inhibitor</td>
<td>[114]</td>
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<td><em>M. tuberculosis</em></td>
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<td>FtsZ</td>
<td>GTP-binding tubulin-like cell division protein</td>
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<td></td>
<td></td>
<td>Wag31</td>
<td>Essential cell division protein</td>
<td>[112]</td>
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<td></td>
<td></td>
<td>EmbR</td>
<td>transcriptional regulatory protein</td>
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<tr>
<td></td>
<td>PknH</td>
<td>EmbR</td>
<td>transcriptional regulatory protein</td>
<td>[116]</td>
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<td>[121]</td>
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<td>EF-G</td>
<td>Elongation factor</td>
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<td>TCRS Response regulator</td>
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<td>Histone-like protein</td>
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<td>AsfR</td>
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<td></td>
<td>PkaG</td>
<td>AsfR</td>
<td>Transcriptional activator</td>
<td>[124]</td>
</tr>
</tbody>
</table>

Table 1: Known ESTK Substrates.
sequences of approximately 75 amino acids that recognize phosphoproteins [125], are also implicated in ESTK-mediated processes. Forkhead-associated domain-containing proteins (Rv0020c and Rv1747) in *M. tuberculosis* have been shown to associate with four serine/threonine kinases (PknB, D, E, and F) [126]. These associations may facilitate binding of specific substrates (such as the aforementioned) or serve to inhibit kinase activity by blocking catalytic binding sites [127]. Notably, these domains are not always present in ESTK substrates.

**Convergence of Signaling Pathways**

It is important to consider each signaling system in *S. aureus*, or any organism for that matter, not as an individual linear pathway, but as part of a larger consortium that integrates to produce a given phenotype. That is, TCRSs and ESTK-mediated signaling are not mutually exclusive. In fact, response regulators of TCRSs, such as CovR in group A and B streptococcal species, are not only phosphorylated on aspartic acid residues by their cognate histidine kinases, but also on threonine residues by ESTKs, impacting the binding of the response regulator to DNA in different ways [107, 122, Jin, *et al.* unpublished data]. Genome-wide analysis using microarray data in *S. pneumoniae* corroborates convergence of these two types of signaling. Deletion of the ESTK *stkP* in this organism directly affected expression levels of a response regulator and histidine kinase involved in competence (*comD/E*), as well as genes known to be controlled by the TCRS VicR/K, suggesting “direct cross talk between two different signaling systems exists” [97]. Fruiting body formation in *M. xanthus* is also known to be regulated by
ESTKs (of which *M. xanthus* has 102) and TCRSs, although this regulation appears to occur in the absence of direct cross-phosphorylation [87]. The involvement of ESTK and/or ESTP signaling in alternative sigma factor regulation remains to be clarified, although the mycobacterial ESTK PknD has been shown to phosphorylate an anti-anti-sigma factor *in vivo*, eliminating its ability to bind to another sigma factor regulator [128].

Work on these interesting eukaryotic-like signaling molecules has only begun, particularly in regards to ESTPs. However, from the limited number of studies directly addressing the role of ESTKs and ESTPs in bacteria, it has become apparent that these one component systems truly are “global regulators.” Since these enzymes are found in all phyla (e.g. Bacteria, Archaea, and Eukaryota), further research into this area will not only provide insight into the roles of these proteins in prokaryotes, but may also help better characterize the relationships between these hierarchies. It is clear that serine/threonine phosphotransfer is critical in determining bacterial morphology, as well as a bacterium’s ability to divide, adhere, remain virulent, and sense its environment. Each of these processes is key to bacterial pathogenesis. Yet, despite the significance of these enzymes in bacterial pathogenesis, there has been no work done to investigate the role of ESTK- and ESTP-mediated signaling in one of the most well-armed and antibiotic resistant pathogens known—*S. aureus*. The following studies explore the roles of such enzymes in the *Staphylococcus*. 
CHAPTER 2

CHARACTERISTICS OF ESTKS AND ESTPS IN S. AUREUS

Although ESTKs and ESTPs have been implicated in nearly all steps of bacterial pathogenesis (see Introduction), they have never before been investigated in S. aureus, thus the role of these enzymes, if any, in S. aureus is unknown. To gain a better understanding of the function of eukaryotic-like serine/threonine kinase-mediated signaling in S. aureus, we first investigated the presence and characteristics of ESTK- and ESTP-specific gene operons in the S. aureus N315 genome.

We employed the Microbial Signal Transduction Database (MiST) to identify relevant estk/estp genes within the available genomes of S. aureus strains. This database combines sequence and domain profile analysis to specifically identify proteins involved in phosphotransfer [129]. The number of ESTKs and ESTPs within a single genome varies significantly [64, 82, 93, 129] and it is imperative to first identify any potential redundancy or variations across all putative ESTK/ESTP proteins. Additionally, since these enzymes are highly conserved, we compared amino acid sequences of the primary S. aureus ESTK to those of previously characterized ESTKs from Gram-
negative/positive bacteria and mycobacteria, aligning them using specific bioinformatic freeware (ClustalW) [130]. We predicted that these enzymes may have similar functions proportionate to their sequence similarities. We also employed MegAlign software, which quantifies similarities and also provides visual representation of relationships between proteins by way of phylogenetic trees.

Finally, it is critical to be sure that the genes of interest do in fact encode functional proteins. Serine/threonine kinases generally autophosphorylate and then phosphorylate their substrates, and both reactions can be reversed by cognate phosphatases [81, 82]. Thus, we performed *in vitro* kinase assays using radiolabelled $[^{32}\text{P}]$ATP to test whether this held true for staphylococcal recombinant proteins. We tested both magnesium and manganese as cofactors in our assays as ESTKs and their cognate ESTPs are often annotated in the literature as manganese-and/or magnesium-dependent [69, 71, 131]. We also performed thin-layer chromatography (TLC) to identify residues phosphorylated by a major *S. aureus* ESTK, as it is predicted that ESTKs target serine and/or threonine for phosphotransfer. Phosphorylation on either of these residues (or tyrosine) is acid stable, thus acid hydrolysis of phosphorylated, recombinant proteins during TLC sample preparation allows separation of phosphorylated serine, threonine, or tyrosine residues specifically [71-73]. These characterization studies are the critical first step to understanding ESTK-mediated signaling in *S. aureus*.

**Materials and Methods**

**Bioinformatic analyses.** The *S. aureus* N315 genome was searched for the presence of
ESTKs and ESTPs using MiST, a signal transduction database [129]. Characteristics of conserved domains within proteins were identified using the Conserved Domain Database [132] available from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and TIGRFAM and Pfam analyses from J. Craig Venter Institute’s Comprehensive Microbial Resource (CMR, http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). Alignments were performed using ClustalW software available at http://www.ebi.ac.uk/Tools/clustalw2/index.html [130]. Pylogenetic analyses and identity/divergence values were obtained from MegAlign software (DNASTAR, Inc.). Hydophobicity was determined by transmembrane HMM analysis (CMR).

Sequences of individual ESTKs were obtained from UniProtKB (http://www.uniprot.org), NCBI, and CMR using the indicated loci and the following accession numbers where applicable: SA0077: Q7A8A0, SA1063: Q7A5Z8, PrkC: Y13937, SPy1625: Q99YN0, PknB: P71584, TGFBRI: P36897, TGFBRII: P37173, Stk1 (P. aeruginosa): Q9I354, Stk1 (S. agalactiae): Q8VQA0, PknA: P65727, AfsK: P54741, StkP: Q8KY50, and YpkA: Q05608.

Cotranscription assay. This, and subsequent assays in this Chapter are also described previously [133]. Total RNA was isolated from mid-log phase (OD600nm=0.6) cultures of N315 grown in tryptic soy broth (TSB, BD Diagnostic Systems) using Qiagen’s RNeasy Mini Kit. During the isolation process, RNA was subjected to on-column DNA digestion using Qiagen’s RNase-free DNase Set. 2.5µg of isolated RNA (quantified using Invitrogen’s Qubit Quantitation Platform) was used to create cDNA using Superscript RTII (Invitrogen) as per the manufacturer’s instructions. An identical reaction was
performed without reverse transcriptase as a negative control. cDNA with or without reverse transcriptase and genomic DNA (gDNA) were used as templates in PCRs using primer sets specific for the overlapping (#108/109) and outermost regions of stp and stk (#44/107 and #110/46).

**Preparation of chemically competent cells and transformation**

Overnight cultures of *E. coli* BL-21(DE3) pLysS and XL-1-Blue cells grown overnight in Luria-Bertani (LB) broth were seeded into 100mL fresh LB and grown at 37°C with shaking to an OD$_{600nm}$≈0.6-0.7. Cultures were then spun down at 4°C and resuspended and washed sequentially in 50, 25, and 15mL 100mM CaCl$_2$. Pelleted cells were then resuspended in ~500μL (depending on pellet size) 10% glycerol, divided into 50μL aliquots, and stored at -80°C. During transformations, a single thawed aliquot was incubated with plasmid DNA on ice for at least 30 minutes. Cells were then shocked at 42°C for 45 seconds and allowed to recover for 5-15 minutes on ice. One milliliter LB broth or SOC media (Invitrogen) was then added and cells were incubated for one hour at 37°C before plating.

**Production of recombinant STP/STK.** Primer sets STP-F/STP-R and STK-F/STK-R were used to create 744bp and 1995bp fragments respectively encoding SA1062 (stp) and SA1063 (stk) from *S. aureus* N315 genomic DNA. These fragments were inserted separately into the multiple cloning site of the His-tag expression vector pET14B (Novagen), to create pET14B-STP and pET14B-STK using Epicentre Biotechnologies’ Fast-Link™ DNA Ligation Kit and transformed into *E. coli* XL-1-Blue cells with selection on LB agar containing 100μg/mL ampicillin. The next day, colonies were
chosen, reinoculated, and grown overnight in LB broth containing 100μg/mL ampicillin.

Plasmids were reisolated and checked for the appropriate inserts by restriction digest analysis before being transformed into *E. coli* BL-21(DE3) pLysS cells and selected again on LB agar containing 100μg/mL ampicillin. A single clone for each construct was selected and grown in LB containing 100μg/mL ampicillin to an OD₆₀₀nm≈0.6-0.7, at which point expression of recombinant His-tagged STP and STK was induced for 4 hours by the addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). rSTK and rSTP were purified from the respective cell lysates by denaturing (8M urea) and non-denaturing methods respectively, using Ni-NTA column chromatography as recommended by the manufacturer’s instructions (Qiagen, [134]). In both cases, buffers were identical with the exception of 8M urea addition to each (lysis buffer: 50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH 8; wash buffer: 50mM NaH₂PO₄, 300mM NaCl, 20mM imidazole, pH 8; elution buffer: 50mM NaH₂PO₄, 300mM NaCl, 250mM imidazole, pH 8). rSTK was further purified by FPLC (Amersham ÄKTA™ purifier) using a Superdex 200 HR 10/30 column. Eluted proteins were dialyzed overnight against 10mM Tris/HCl buffer, pH 8 before being divided into aliquots and stored at -80°C.

*In vitro* kinase assays. Briefly, 0.5μg rSTK or rSA0077 and/or 1μg myelin basic protein (MBP) were incubated separately (control reactions) and in the indicated combinations with 1μCi [γ⁻³²P]ATP (specific activity 4500Ci/mmol) in 40μl of kinase buffer (50mM Tris/HCl, pH 7.5, 1mM dithiothreitol, 10mM MgCl₂ or MnCl₂) in the absence or presence of 1μg rSTP for up to 30 minutes at room temperature. Reactions were stopped by the addition of 4x SDS-PAGE loading buffer (8% β-mercaptoethanol, 4% SDS, 40%
glycerol, 250mM Tris/HCl (pH 6.8), 0.1% bromphenol blue) and the entire reaction mixtures were resolved by 12% SDS-PAGE. Separated proteins were transferred to PVDF membranes (Biorad) at 25V for 1 hour, and phosphorylated proteins were identified by autoradiography.

**Thin-layer chromatography.** Autophosphorylated STK and MBP phosphorylated by STK were separated on 12% SDS-PAGE, excised from the gel, and subjected to 6M HCl hydrolysis at 110ºC for 6 hours in screw-cap Reacti vials. The samples (~50ng) were spotted on cellulose sheets (EMD Chemicals) along with 0.3pg each phosphoserine, phosphothreonine, and phosphotyrosine standards (Sigma) and separated by two-dimensional thin layer chromatography. A 5:3 (v/v) mixture of isobutyric acid and 0.5M NH₄OH was used as solvent for the first 12 hour dimension run, and a 7:1.5:1.5 (v/v/v) mixture of 2-propanol, HCl, and H₂O was used for the second 8 hour dimension run. Phosphoamino standards were visualized by ninhydrin spray and aligned with radioactive spots of the hydrolysates (visualized by autoradiography) to identify phosphorylated residues in STK and MBP.

**Results**

**Bioinformatic analyses.**

Analysis of the *S. aureus* N315 genome revealed the presence of two putative ESTKs (SA0077 and SA1063) and four putative ESTPs (SA0140, SA1062, SA1662, and SA2225). These proteins were identified as such by the presence of ESTK-specific kinase
Fig. 5: Domains of *S. aureus* ESTPs/ESTKs. Highly conserved cotranscribing SA1062 and SA1063 are bolded. PP: protein phosphatase, T: transmembrane, PASTA: penicillin binding protein and serine/threonine kinase associated.

or ESTP-specific protein phosphatase conserved domains (Fig. 5). Homologs of SA0077 and SA2225 are seen in other *S. aureus* genomes (Mu50, MRSA252 and Mu50, MW2, RF122, MRSA252, MSSA476, respectively). However, SA1062 and SA1063 are 100% conserved across strains we examined (Mu50, MW2, RF122, COL, MRSA252, MSSA476, USA300, NCTC8325, and N315), and thus were the focus of the following studies.

SA1062 (744bp/247aa) and SA1063 (1995bp/664aa) are designated STP and STK, respectively. Based on hydrophobicity plots, STK is predicted to be a transmembrane protein, crossing the membrane a single time, with an intracellular N-terminus and extracellular C-terminus (Fig. 5). The N-terminus of STK contains a single
### Table 2: Locations of conserved features in SA1063

Conserved domains are underlined. Red: catalytic loop; blue: activation loop; bold: ATP binding pocket; italics: substrate binding pocket. Sites were identified using NCBI’s Conserved Domain Database [132].

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serine/threonine protein kinase catalytic domain, including an ATP binding pocket, substrate binding pocket, catalytic loop, and activation loop (Table 2). The C-terminus contains three similar but not identical PASTA repeats (Fig. 5). The single PP2C domain in STP (SA1062) contains 8 specific active sites (Table 3) and spans nearly the entire
Table 3: Locations of conserved features in SA1062. PP2C domain is underlined. Active sites are indicated in blue. Sites were identified using NCBI’s Conserved Domain Database [132].

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</table>

Table 3: Locations of conserved features in SA1062. PP2C domain is underlined. Active sites are indicated in blue. Sites were identified using NCBI’s Conserved Domain Database [132].

protein (Fig. 5). STP does not possess a hydrophobic domain, thus is predicted to be in the cytoplasm.

We performed alignments of STK and STP with other ESTKs and ESTPs to identify conserved features [130]. As seen in Fig. 6, STK (and SA0077) contains the 11 (I-XI) conserved Hanks’ motifs [81] seen in other ESTKs, including PrkC from B. subtilis [75], SPy1625 from S. pyogenes [73], PknB from M. tuberculosis [78], and TGFβ receptor II, a transmembrane serine/threonine kinase from H. sapiens (analyzed in [75]). Similarly, SA1062 contains 11 conserved protein phosphatase 2C (PP2C) motifs [135] (Fig. 7) common in other ESTKs such as PrpC and Spy1626 (the cognate ESTPs for PrkC [100] and SPy1625 [73] respectively), Mstp from M. tuberculosis [136], and PP2C from H. sapiens. The presence of these specific motifs strongly suggests that STP and STK are in fact a eukaryotic-like serine/threonine phosphatase and kinase respectively.
Fig. 6: Location of conserved motifs in ESTKs. Shown is the alignment of serine/threonine kinases from *S. aureus* (SA0077, SA1063), *B. subtilis* (PrkC), *S. pyogenes* (Spy1625), *M. tuberculosis* (PknB), and *H. sapiens* (TGFBRII) using ClustalW [130]. Conserved Hanks’ kinase motifs [81] are boxed.
Fig. 7: Location of conserved motifs in ESTPs. Shown is the alignment of PP2C domain-containing serine/threonine phosphatases from *S. aureus* (SA1062), *B. subtilis* (PrpC), *S. pyogenes* (Spy1626), *M. tuberculosis* (Mstp), and *H. sapiens* (Pp2C) using ClustalW [130]. Conserved PP2C motifs [82] are boxed.
Fig. 8: Phylogenetic analysis of ESTKs. (A) Phylogenetic tree produced upon alignment of the indicated proteins. (B) Percent identity of proteins and arbitrary divergence scores based on (A). Accession numbers used: SA0077: Q7A8A0, SA1063: Q7AZ58, PrkC: Y13937, Spy1625: Q99YN0, PknB: P71584, TGFβRI: P36897, TGFβRII: P37173, Stk1 (P. aeruginosa): Q9J534, Stk1 (S. agalactiae): Q8VQA0, PknA: P65727, AfsK: P54741, StkP: Q8KY50, YpkA: Q05608. Tree produced using MegAlign software.
After phylogenetic analysis of 15 ESTK sequences (Fig. 8A), STK (SA1063) clustered most closely with *B. subtilis’* PrkC. This is likely due to the fact that these two proteins were the most identical of those included in the analysis (34.6% identical; Fig. 8B). Not surprisingly, eukaryotic ESTKs (TGFβRI and II) clustered together as did ESTKs from Gram-negative *S. Typhi* and all of the streptococcal ESTKs (SPy1625, Stk1, StkP). Interestingly, SA0077 from *S. aureus* clustered with Gram-negative ESTKs YpkA (from *Yersinia pestis*) and Stk1 (from *Pseudomonas aeruginosa*) rather than SA1063, suggesting that these two ESTKs may play separate roles in *S. aureus*.

**stp and stk are cotranscribed.** Four nucleotides within *stp* (SA1062) and *stk* (SA1063) overlap (ATGA) at the junction of their 3’ and 5’ ends respectively, with the putative *stp* preceding the *stk* (see schematic in Fig. 9A), and as such these genes are predicted to cotranscribe. To investigate this, we performed RT-PCR assays using cDNA or gDNA (gDNA) templates and *stp-stk* overlapping region-(#108/109) and flanking region-specific (#44/107 and #110/46) primer sets, as shown by schematic in Fig. 9A. Flanking region-specific primers only produced amplicons when gDNA (Fig. 9B, lanes 4 and 6) but not cDNA (Fig. 9B, lanes 1 and 3), was used as a template, while overlapping region-specific primers produced amplicons when both cDNA and gDNA was used as a template (Fig. 9B, lanes 2 and 5). This, together with the absence of PCR products in reactions lacking reverse transcriptase and cDNA as a template (Fig. 9B lanes 7-9) confirmed that *SA1062* and *SA1063* are encoded on a common RNA transcript.

**STK and STP are enzymatically active proteins.** To determine biochemical and biological functions of *S. aureus* STP and STK, we cloned *SA1062* and *SA1063*,
**Fig. 9: stp and stk are cotranscribed.** (A) Schematic of overlapping pattern of SA1062 (stp) and SA1063 (stk) and primer annealing sites. Four nucleotide overlap is indicated in bold. Stop codon for SA1062 is underlined. Primer binding sites are indicated by arrows. (B) Results of PCRs using cDNA, gDNA, or cDNA reactions mixtures lacking reverse transcriptase (-RT) as templates. Lanes 1, 4, and 7 were performed using primer set 44/107, lanes 2, 5, and 8 utilized 108/109, and lanes 3, 6, and 9 utilized 110/46, annealing on flanking or overlapping regions of stp/stk as indicated in (A). Figure reproduced [133].

produced purified, recombinant proteins (rSTP and rSTK), and examined their roles in *in vitro* phosphorylation reactions.

As seen in Fig. 10A, rSTK autophosphorylated and catalyzed phosphotransfer to a non-specific model substrate myelin basic protein (MBP). Addition of rSTP into the kinase reaction resulted in dephosphorylation of both autophosphorylated STK and STK-phosphorylated MBP, indicating that both rSTK and rSTP are functionally active. STK autophosphorylation occurred in time- and dose-dependent manners (Fig. 10B) and
Fig. 10: Autoradiographs of *in vitro* kinase reactions. (A) Purified, recombinant STK (rSTK) was incubated in kinase buffer with [γ-32P]ATP and Mn\(^{2+}\) alone and in the presence of myelin basic protein (MBP) and recombinant STP (rSTP) as indicated. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Experiments were repeated at least three times and shown are representative autoradiographs. (B) Increasing amounts of purified rSTK was added to separate kinase reactions to observe the dose-dependent effect. 1μg rSTK was used for the time course and cation-dependency experiments, where minutes and cations are shown above the autoradiographs. (C) Thin layer chromatography results showing phosphoamino standards stained with ninhydrin (left) and autophosphorylated rSTK hydrolysis products revealed by autoradiography (right). 1st and 2nd dimensions are indicated by arrows. Figure reproduced [133].
occurred most efficiently when Mg$^{2+}$ was replaced with Mn$^{2+}$ as a sole cation, suggesting that *S. aureus* STK-mediated phosphorylation favors manganese as a cofactor. To further determine specificity of the phosphorylation, we performed two-dimensional thin-layer chromatography using acid hydrolysates of autophosphorylated rSTK and phosphorylated MBP. Alignment of radioactive-phosphorylated amino acid spots, migrated from the hydrolysates of autophosphorylated STK (Fig. 10C) or phosphorylated MBP (data not shown), with the ninhydrin-stained phosphothreonine standards revealed that *S. aureus* STK autophosphorylates on threonine residues and specifically targets threonine residues in its substrate(s).

**Discussion**

*S. aureus* STK and STP are considered an ESTK and ESTP respectively due to the presence of specific Hanks’ motifs in STK and PP2C motifs in STP. Protein kinases in eukaryotes and prokaryotes catalyze the transfer of phosphoryl groups from donors (often ATP or GTP) to acceptors (substrates) [81]. This mechanism is highly conserved, thus the catalytic domains of all protein kinases contain conserved residues specific for binding ATP or GTP, binding the acceptor molecule, and transferring the phosphoryl group. These conserved residues have been collectively termed “Hanks’ motifs” after Steven Hanks, who first identified them in eukaryotic protein kinases [81]. These motifs are specific to serine, threonine, and tyrosine kinases and are not seen in histidine kinases (such as those comprising two component regulatory systems in prokaryotes) [81]. 11 conserved Hanks’ motifs are seen in the amino acid sequences of both SA0077 and
SA1063 (Fig. 6) indicating that these proteins are in fact eukaryotic-like serine/threonine kinases.

Four different types of protein phosphatases have been identified in eukaryotes, termed PP1, PP2A, PP2B and PP2C [95]. In *S. aureus* ESTPs, two types of serine/threonine phosphatase domains were seen: PP2Ac and PP2C. PP2A, seen in *S. aureus* ESTPs SA0140, SA1662, and SA2225, alone is a tumor suppressor in mammalian cells, and is identified by the presence of 15 tandem “HEAT” (Huntingtin elongation A subunit TOR-like) motifs [137]. PP2C domains are identified by the presence of 11 conserved motifs [135], all of which are seen in *S. aureus* ESTP SA1062 (STP) (Fig. 7). Catalytic activity of a given PP2A or PP2C domain is dependent on manganese or magnesium binding [95, 138]. STP is a soluble protein, in contrast to some ESTPs, such as PstP of *M. tuberculosis*, which has an intracellular PP2C domain followed by a transmembrane domain and extracellular region [94]. PP2C domain-containing proteins are common in ESTPs, and are often encoded in operons with cognate ESTKs [69, 71, 94, 133]. This pattern was also observed in *S. aureus* (see schematic in Fig. 9). Notably, a single ESTP is capable of dephosphorylating multiple substrates, specifically autophosphorylated ESTKs [83], thus the presence of other ESTPs in the *S. aureus* genome may indicate overlapping mechanisms.

STK also contains a three PASTA repeat domain at its C-terminus end. PASTA repeats have been found in nearly all prokaryotic ESTKs (primarily Gram-positive) and 82 penicillin-binding proteins, with repeats in ESTKs ranging from a single copy to up to 5 [74, 129, 132]. PASTA repeats have limited overall sequence similarity (10-25%) [91,
and bind unlinked peptidoglycan [90]. The combination of extracellular PASTA domains and intracellular serine/threonine kinase domains allow ESTKs to serve as extracellular sensors, capable of mediating intracellular signaling cascades in response to environmental signals though autophosphorylation and phosphorylation of specific substrates [92].

The overlapping pattern seen in stp and stk is also found in a number of other Gram-positive stp/stk pairs, including those of B. subtilis [75], S. pneumoniae [72], S. pyogenes [73], and S. agalactiae [71] (see also supplemental material in [73]). The highly conserved arrangement of stp and stk in S. aureus suggests that this pair comprises the primary ESTK and ESTP in staphylococci, whereas the other “non-cotranscribing” ESTK and ESTPs, which are not highly conserved, may play secondary roles.

STK was observed to autophosphorylate and phosphorylate the non-specific substrate MBP on one or more threonine residues (Fig. 10). Phosphorylation on threonine residues is most common among ESTKs [73, 83, 100] although there are exceptions, including M. tuberculosis ESTK PknB [83] and Stk1 of P. aeruginosa [69] which can autophosphorylate on both serine and threonine residues (albeit more weakly on serine residues), and Stk1 of S. agalactiae, which autophosphorylates on serine residues, but phosphorylates threonine residues within its substrates [71]. Certain ESTKs have also been shown to phosphorylate each other [98, 99] and share substrates [116], and this may very well be the case in S. aureus based on the presence of multiple ESTKs in the genome.
Upon confirmation that $stk$ and $stp$ indeed encode functional proteins, we next created $S. aureus$ N315 strains lacking each of these genes and identified multiple phenotypic changes, specifically in regards to morphogenesis.
CHAPTER 3

THE ROLE OF STK AND STP IN *S. AUREUS* MORPHOLOGY

Based on the observed *in vitro* functionality of STK and STP and similarities between their corresponding sequences and other characterized ESTKs and ESTPs, we hypothesized that STK and STP are signaling molecules within *S. aureus*. To identify cellular processes that they may regulate, we created three mutant strains lacking *stp*, *stk*, or both and compared characteristics of these mutant bacteria to those of the isogenic parent strain.

Restriction enzymes within *S. aureus* immediately digest foreign DNA. To circumvent this and create each mutant strain, we utilized a *S. aureus* laboratory strain (RN4220) to serve as an intermediary between *E. coli* and *S. aureus* N315. RN4220 is restriction deficient and easily transformed with plasmids propagated in *E. coli* [139]. Methylated plasmids can then be isolated from this deficient strain and transformed into N315 without concern about plasmid stability. N315ΔSTK was created by homologous recombination using plasmid pMADΔSTKchl, which facilitated insertion of the chloramphenical resistance cassette (*cat*) in place of *stk*. pMAD is a temperature-sensitive vector which offers blue/white colony selection along with an erythromycin resistance
marker [140]. To create N315ΔSTP and N315ΔSTP/STK, we used pKOR1ΔSTP and pKOR1ΔSTP/STK. This pKOR1-based markerless vector system contains a chloramphenicol resistance gene to serve as a positive selection marker and an antisense secY cassette which, upon induction, inhibits staphylococcal growth and serves as a negative selection marker [141]. For complementation, we employed a modified version of pCN40 [142]. S. aureus N315 is inherently resistant to erythromycin, therefore we replaced the erythromycin resistance cassette from pCN40 with a tetracycline version taken from pCN36 [142] to create pCN40tet. This plasmid includes an endogenous staphylococcal promoter (PblaZ) directly upstream of its multiple cloning site, providing a simple way to reintroduce stp and/or stk expression into the appropriate deletion strains.

We analyzed in vitro growth of each strain by turbidity assay and visual assessment of colony morphology. ESTK mutants in other bacteria often display unique growth characteristics in complex solid and liquid media, particularly growth defects [71, 73, 77]. Detailed analysis of cellular appearance was performed by high power scanning (SEM) and transmission (TEM) electron microscopy. A number of studies have utilized similar techniques to implicate ESTKs from other organisms in regulation of cellular morphology [73, 74, 99]. SEM and TEM at high resolution, respectively, provide detailed outer views of the bacterial surface and inner views of the cell wall, septum, and division patterns. At resolutions of 100,000x or more, even slight structural differentiations between the wild type and mutant strains can be identified. These studies provided key physical analysis of each mutant strain to begin to identify cellular processes in which they are involved.
Materials and Methods

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used are shown in Table 4. Wild type *S. aureus* N315 and derived mutant strains were grown in TSB or tryptic soy agar (TSA) (BD Diagnostic Systems). The media were supplemented with 10μg/mL chloramphenicol, 12μg/mL tetracycline, 5μg/mL erythromycin, 250μg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and/or 1.5μg/mL anhydrotetracycline (Clontech) where indicated. *E. coli* strains DH5α and XL-1-Blue were grown in LB broth or agar (BD Diagnostic Systems) supplemented with 50μg/mL carbenicillin or 100μg/mL ampicillin where indicated. All bacterial strains were grown at 37°C unless otherwise stated.

**Preparation of *S. aureus* electrocompetent cells and electroporation**

Overnight cultures of *S. aureus* N315, RN4220, and the appropriate *S. aureus* mutant strains were grown overnight in B2 medium (10g casamino acids, 25g yeast extract, 25g NaCl, 1g K2HPO4 and 5mL 20% glycerol in 1L; pH 7.5; filter sterilized [145]) and subcultured into 100mL fresh B2 the next day. These cultures were allowed to grow at 37°C with shaking to an OD600nm≈0.6. Cells were then spun down and washed sequentially in 50, 25, 15, and 7.5mL sterile water followed by a final wash in 25mL 10% glycerol. Final washed pellets were resuspended in ~1mL 10% glycerol, separated into 60μL aliquots, and stored at -80°C until use. For electroporation, desired plasmid DNA was added to a single thawed aliquot of electrocompetent cells (on ice) and the mixture was transferred to a chilled 0.2cm electroporation cuvette (Biorad). Cells were pulsed at
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<td>pCN40tet</td>
<td>pCN40 with <em>ermC</em> gene replaced by tet(M) from pCN36</td>
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<tr>
<td>pCN40tet-STP</td>
<td>pCN40tet containing entire SA1062 gene and putative promoter upstream</td>
<td>This study</td>
</tr>
<tr>
<td>pCN40tet-STK</td>
<td>pCN40tet containing entire SA1063 gene and putative promoter upstream of SA1062</td>
<td>This study</td>
</tr>
<tr>
<td>pCN40tet-STPSTK</td>
<td>pCN40tet containing overlapping SA1062-SA1063 genes and putative promoter upstream of SA1062</td>
<td>This study</td>
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### E. coli strains

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<th>Mutant alleles:</th>
<th>Source</th>
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<td>XL-1-Blue</td>
<td>*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F' proAB lacF'ZAM15 Tn10 (Tetr)</td>
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<td>DH5α</td>
<td><em>Subcloning efficiency</em> F&lt;sup&gt;+&lt;/sup&gt; Δ*lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(r&lt;sup&gt;K&lt;/sup&gt;&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;−&lt;/sup&gt;) phoA supE44 λ- thi-1 gyrA96 relA1</td>
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<tr>
<td>BL-21 (pLysS DE3)</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; *ompT hsdS&lt;sub&gt;B&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) gal dcm λ(DE3) pLysS (Cam&lt;sup&gt;R&lt;/sup&gt;)</td>
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Continued on next page
### Table 4: Strains and plasmids used in the current study.

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<td>RN4220</td>
<td>Restriction-deficient derivative of NCTC 8325-4</td>
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<tr>
<td>N315</td>
<td>Methicillin-resistant <em>S. aureus</em> parent strain</td>
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<tr>
<td>N315ΔSTP</td>
<td>N315 lacking <em>SA1062</em></td>
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<tr>
<td>N315ΔSTK</td>
<td>N315 lacking <em>SA1063</em></td>
<td>This study</td>
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<tr>
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<tr>
<td>N315pCN40tet</td>
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<td>This study</td>
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<tr>
<td>N315ΔSTPΩSTP</td>
<td>N315ΔSTP complemented strain containing pCN40tet-STP</td>
<td>This study</td>
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<td>N315ΔSTK complemented strain containing pCN40tet-STK</td>
<td>This study</td>
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<tr>
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<td><strong>Recombinant proteins</strong></td>
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<td><strong>Deletion strains</strong></td>
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**Complementation**

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**Real-time PCR**

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Continued on next page
Table 5: Primers used in the current study.

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<tr>
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2.5kV/100Ω/25μF (Biorad Gene Pulser® II) followed by the immediate addition of 1mL B2 medium and recovery for 1 hour at 37°C with shaking before plating on the appropriate selection media.

**Construction of N315ΔSTK.** Primer sets AF/AR and BF/BR were used to amplify approximately 600bp upstream and 750bp downstream of *SA1063* (*stk*). These primers were designed to leave the co-transcribing *SA1062* intact. Similarly, primer set catF/catR was used to amplify the 743bp chloramphenicol acetyl transferase (*cat*) gene and its promoter from pDC123 [143]. These fragments were purified (Qiagen Qiaquick® PCR Purification Kit), digested with the appropriate enzymes (see Table 5) and sequentially inserted into the multiple cloning site of pMAD [140] to create pMADΔSTKchl such that the chloramphenicol acetyl transferase gene was flanked by upstream and downstream regions of *SA1063*. This plasmid was then used to create *S. aureus* strain N315ΔSA1063 essentially as described [140]. Specifically, the new pMAD construct (propagated in *E. coli* XL-1-Blue) was electroporated into *S. aureus* strain RN4220 and recovered cells were plated on TSA supplemented with chloramphenicol, erythromycin, and X-gal and allowed to grow at 30°C for two days. A single blue colony was selected and grown in the broth equivalent of the same media overnight at 30°C, and the pMAD construct was isolated and verified via PCR (primer set AF/BR) and sequencing. Isolation of plasmid DNA from *S. aureus* was performed using the QIAGEN® Plasmid Mini Kit as per the manufacturer’s instructions, with the exception of a 30 minute incubation at 37°C after addition of Buffer P1 containing 3-10μg lysostaphin. pMADΔSTKchl isolated from
RN4220 was then electroporated into *S. aureus* strain N315. After growth on TSA supplemented with chloramphenicol, erythromycin, and X-gal, many blue colonies were present. A single blue colony was selected and grown in TSB with shaking overnight at 30°C. The culture was then streaked on TSA containing chloramphenicol and X-gal and grown for two days at 43°C. From a mixture of blue, white, and light blue colonies a single white colony was selected and restreaked on the same media and grown overnight at 43°C. This process was repeated until only white colonies remained (approximately 8 generations), at which point a single (white) colony was inoculated into TSB supplemented with chloramphenicol and incubated at 37°C overnight. Genomic DNA was then isolated from both this strain and the wild type (N315), and the mutant was confirmed via PCR and sequencing.

**Construction of N315ΔSTP and N315ΔSTP/STK.** Both mutant strains were created using plasmid pKOR1 [141]. Primer sets #33/34, #58/59, and #35/36 were used to amplify approximately 1kB upstream of SA1062, 1kB downstream of SA1062, and 1kB downstream of SA1063, respectively. The resulting gene products were subjected to SacII (New England Biolabs) digestion and ligated (Epicentre Biotechnologies Fast-Link™ DNA Ligation Kit) such that two fragments containing ligated up- and downstream regions of *SA1062* and *SA1062-SA1063* were created. The ligation products were used as templates in two PCRs using primer sets #33/59 and #33/36. Respective PCR products were then inserted separately into the temperature-sensitive plasmid pKOR1 using BP Clonase (Invitrogen Gateway® Clonase System). These plasmids were then transformed into DH5α *E. coli* with selection on LB containing chloramphenicol and
verified by DNA sequencing. After being passed through *S. aureus* strain RN4220, the plasmids were ultimately electroporated separately into *S. aureus* N315 for allelic replacement. Initial selection of N315 colonies was made on TSA supplemented with chloramphenicol incubated overnight at 30°C. For each strain, a single colony was selected and inoculated into TSB supplemented with chloramphenicol and grown at 43°C overnight with vigorous shaking to initiate plasmid integration. The strain was then streaked on TSA supplemented with chloramphenicol and grown overnight at 43°C. A single, well-isolated colony was chosen and inoculated into TSB (no antibiotic) and grown at 30°C overnight with shaking to promote plasmid excision. This culture was then diluted 1:10,000, spread on TSA containing anhydrotetracycline, and grown overnight at 30°C. 30 colonies per strain were patched on TSA and TSA with chloramphenicol and grown overnight at 37°C. A single chloramphenicol-sensitive transformant was identified and restreaked on TSA for approximately 8 generations. Finally, mutant strains with the desired deletions were confirmed by PCR, DNA sequencing, and Western blotting.

**Complementation of mutants with SA1062 and/or SA1063.** To enable selection in the erythromycin-resistant *S. aureus* strain N315, the erythromycin resistance cassette (ermC) from pCN40 was replaced with the tetracycline resistance cassette (tet(M)) from pCN36 [142] to create pCN40tet. The sequence and location of the endogenous promoter which facilitates *stp* and *stk* transcription in *S. aureus* is not known. pCN40 contains the endogenous *S. aureus* β-lactamase promoter module immediately upstream of its multiple cloning site [142]. In addition to this, a 53bp DNA fragment (nucleotides 1201589-1201642, located directly upstream of the cotranscribing SA1062/SA1063 (stp/stk)) was
included in frame and upstream of *stp* and/or *stk* to include the endogenous ribosomal binding site in all complementation constructs. To create the appropriate constructs, primer set #73/65 was used to amplify *SA1062* including the upstream region to create pCN40tet-STP. In the same manner, primer set #73/66 was used to create pCN40tetSTP/STK, again including the upstream region. Similarly primer sets #73/87 and #88/66 were used to amplify *SA1063* and the 53bp upstream region. These fragments were ligated together after digestion with *Nde*I and used as template in a PCR with primers #73/66, the product of which was inserted into the complementation vector to create pCN40tet-STK. All complementation plasmids were passed through *S. aureus* strain RN4220 before being inserted into their respective mutants, and verified by sequencing and Western blotting.

**Western blotting.** Overnight cultures of N315, N315ΔSTP, N315ΔSTK, N315ΔSTP/STK and their corresponding complemented strains were subcultured into fresh TSB (supplemented with tetracycline for complemented strains) and allowed to grow to an OD_{600nm}=1.0. 1mL of each culture was then spun down and resuspended in 75μL 25mM Tris/HCl (pH 7.5) containing 5μg lysostaphin (Sigma). Cells were lysed in a 37°C water bath for ~40 minutes until samples appeared clear, at which point 25μL 4x SDS-PAGE buffer was added. Samples were then boiled for 5 minutes and insoluble material pelleted by centrifugation. 15μL of supernatant from each sample was resolved by 12% SDS-PAGE and transferred to PVDF membranes (Biorad) at 25V for 1 hour. Antisera against rSTP and rSTK were custom made by Lampire Biologicals using a 50 day express-line rabbit protocol involving a series of three 100μg rSTK or rSTP (mixed
with Freund’s adjuvant) injections. Antisera obtained were used to reveal the presence and absence of STK and STP in the appropriate lysates. As a control, N315 total lysates were also probed with pre-bleed serum obtained prior to immunization of the rabbits with rSTP or rSTK. For Western blotting, PVDF membranes were blocked overnight in TBST buffer (50mM Tris/HCl, pH 8; 150mM NaCl; 0.005% Tween-20) containing 5% BLOT-QuickBlocker™ (Geno Technology, Inc.). Membranes were further incubated for 1 hour at room temperature (with gentle rocking) in TBST containing 1% BLOT-QuickBlocker™ and primary antisera (diluted 1:1000). After three 10-minute washes in TBST, membranes were further incubated with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:10,000 in TBST containing 1% BLOT-QuickBlocker™. Membranes were then washed thoroughly three times in TBST (~10 minutes/wash), followed by a wash in 50mM Tris/HCl (pH 10)/10mM MgCl₂ for at least 15 minutes. Reactive protein bands were revealed by the addition of 10mL developing solution (50mM Tris/HCl, pH 10; 10mM MgCl₂; 50μg/mL nitro blue tetrazolium salt (Denville Scientific); 50μg/mL 5-bromo-4-chloro-3-indolylphosphate (Denville Scientific)).

**Growth curve analysis:** 100μL of OD₆₀₀=1.0 cultures of *S. aureus* N315, N315ΔSTP, N315ΔSTK, N315ΔSTP/STK, and their associated complemented strains were subcultured into 10mL TSB (supplemented with tetracycline for complemented strains) and grown at 37°C with shaking for 6 hours. The OD₆₀₀ of each strain was measured every 30 minutes (Thermospectronic Spectronic 20+).

**Microscopy.** Stationary phase cultures of *S. aureus* N315 (control) and each mutant strain grown in TSB were fixed overnight at 4°C in 0.1M Ca-codylate buffer (pH 7.4)
containing 2.5% gluteraldehyde and 4% paraformaldehyde. For TEM, fixed cells were washed and embedded in 2% low temperature gelling agarose, cut into blocks, and post-fixed in 1% osmium tetroxide. Blocks were then rinsed in water and stained in 1% uranyl acetate before being subjected to multi-wash dehydration using increasing (50%-100%) concentrations of ethanol. Samples were further rinsed sequentially in propylene oxide, 1:1 propylene oxide/Eponate 12 resin, 1:2 propylene oxide/resin, and 100% resin. Blocks were then sectioned, stained in 2% uranyl acetate and Reynold's lead citrate, and viewed with an FEI Tecnai G2 Spirit. In the case of SEM preparation, fixed samples were dehydrated using an ascending series of ethanol washes (ending in 100%) and further dried using a critical point drier (Pelco). They were then mounted on aluminum stubs with silver paint, and sputter coated with gold/palladium before viewing with an FEI NOVA nanoSEM. All procedures subsequent to fixing were performed at the Campus Microscopy and Imaging Facility (CMIF) at The Ohio State University.

**Results**

**Creation and confirmation of N315ΔSTP, N315ΔSTK and N315ΔSTP/STK.** To study the contributions of STK-mediated kinase and STP-mediated phosphatase activities in staphylococcal biology, we created three mutant strains lacking *stp* (N315ΔSTP), *stk* (N315ΔSTK) or both *stp* and *stk* (N315ΔSTP/STK). We also validated their functional properties using corresponding complemented strains (N315pCN40tet, N315ΔSTPΩSTP, N315ΔSTKΩSTK, and N315ΔSTP/STKΩSTP/STK). As seen in Fig. 11A, PCR analysis using primers specific for *stp* (STP-F/STP-R) and *stk* (STK-F/STK-R) confirmed the
Fig.11: Confirmation of deletion strains. (A) Representative gel from PCR analysis of genomic DNA isolated from the indicated strains using primers specific for stp and stk. (B) Representative Western blots of total cell lysates probed separately with (top) anti-rSTP and (bottom) anti-rSTK production sera (right panels) and corresponding pre-bleed (PB, left panels) sera. Lane 1: N315; 2: N315ΔSTP; 3: N315ΔSTK; 4: N315ΔSTP/STK; 5: N315pCN40tet; 6: N315ΔSTPΩSTP; 7: N315ΔSTKΩSTK; 8: N315ΔSTP/STKΩSTP/STK. Arrows indicate the location of STK and STP within the lysates. Figure reproduced from [133].
absence of the appropriate genes in each deletion strain. Additionally, Western blotting analysis of total bacterial lysates from each strain probed with anti-rSTP (Fig. 11B, top panels) or anti-rSTK serum (Fig. 11B, bottom panels) revealed distinct antibody-reacting bands corresponding to STP (~32kDa) and STK (~77kDa) in the wild-type, all complemented strains, and the appropriate mutants. To express the missing gene products in the mutant strains, pCN40tet vectors containing a *S. aureus* β-lactamase promoter (P\_blaZ) and the appropriate wild-type gene were used. Importantly, P\_blaZ-mediated expression may not be optimally activated in the absence of β-lactam antibiotics. Thus, the inconsistent expression levels of STP and/or STK in the corresponding complemented strains as compared to wild type are likely due to the use of P\_blaZ instead of the native promoter, which so far has not been identified. However, the amount of STK and/or STP expressed in the complemented strains seemed to be sufficient to regain functionality of the wild-type strain. Importantly, the presence of an STP-reacting band in N315ΔSTK (Fig. 11B, lane 3) and STK-reacting band in N315ΔSTP (Fig. 11B, lane 2), indicated that the employed deletion strategies left the stp transcript stable in the absence of stk and had no polar effects on the stk transcript in the absence of stp.

To determine the function of STK and STP in *S. aureus* growth, the isogenic mutant strains were grown on TSA containing 5% sheep’s blood (Hemostat Laboratories) and their colony morphology and hemolytic patterns were compared to those of the parent N315 strain. All strains displayed consistent β-hemolysis, with no obvious
Fig. 12: Growth curves analysis. Growth curves were created simultaneously for (A) all wild type and mutant strains and (B) their corresponding complement strains. Results are separated for ease of reading. Assay was repeated three times. Data shown are from a single representative experiment performed in triplicate. Error bars indicate standard deviations. *p<0.005 as compared to wild type (N315) by student’s unpaired t test.
differences between the four strains (data not shown). This suggests, albeit indirectly, that production of β-hemolysin is not significantly affected upon deletion of *stp* and/or *stk*. General colony appearance also remained consistent across deletion strains as compared to wild type. All strains produced round, light yellow colonies with smooth edges and a somewhat shiny appearance. Additionally, growth curves were constructed simultaneously for all mutants and their complemented strains using a turbidity assay. The results of this are displayed in Fig. 12. N315ΔSTP and N315ΔSTK displayed growth characteristics similar to those of the wild-type strain (N315) in TSB, reaching an OD$_{600}$=1.0 by approximately 5 hours. N315ΔSTP/STK growth was significantly ($p$<0.005) slower than wild type during exponential phase (3-4 hours), only reaching an OD$_{600}$=0.85±0.05 by 5 hours (Fig. 12A). Growth of complemented strains in TSB (supplemented with tetracycline) was relatively consistent with that of wild type, with the exception of N315ΔSTP (Fig. 12B), which did not reach an OD$_{600nm}$=1.0 by 5 hours. These alterations are likely due to the presence of tetracycline in the growth media, and the varying expression levels of STP and STK under β-lactamase, rather than endogenous, promoter control.

**Role of STP/STK in *S. aureus* cell wall morphology.** To more closely examine any morphological changes between mutants, we subjected stationary phase cultures of the three deletion strains (N315ΔSTP, N315ΔSTK, and N315ΔSTP/STK) and the wild-type strain (N315) to scanning and/or transmission electron microscopy (SEM and/or TEM). Although their general surface morphologies looked similar under SEM, N315ΔSTP/STK cells appeared much larger than wild type cells at the same
Fig. 13: Analysis of cell morphology by scanning electron microscopy (SEM); 30,000x. Shown are representative fields of TSB-grown, stationary-phase *S. aureus* (A) wild-type (control) and (B) N315ΔSTP/STK. Insets show 2μm magnification (30,000x, Fig. 13). Measurement of cell diameters in these images revealed that N315ΔSTP/STK cells are larger than wild type, with an average diameter of 1.0μm (Fig. 13B) as compared to 0.78μm in the wild type (Fig. 13A). In TEM images (11,200x magnification) N315ΔSTP and N315ΔSTK cells did not appear significantly different than wild type N315 cells in terms of cell size and shape (Fig. 14). However, consistent with SEM findings, N315ΔSTP/STK cells appeared bulged and irregularly shaped (Fig. 14, circles). At higher magnification (30,500x; Fig. 15), cell division defects in N315ΔSTP/STK cells became more apparent. Multiple and incomplete septa (Fig. 15, circle), asymmetry, and again irregularity in cell size all suggest dysregulation of cell division machinery in the absence of STP and STK (see also Fig. 17). In particular, these cells displayed formation of new septa parallel, rather than perpendicular, to established
Fig. 14: Analysis of cell morphology by transmission electron microscopy (TEM); 11,200x. Shown are representative fields of TSB-grown, stationary-phase *S. aureus* wild-type (control) and mutant strains as indicated. Circles indicate bulging and irregular shape. Insets show 2µm.
Fig. 15: Analysis of cell morphology by transmission electron microscopy (TEM); 30,500x. Shown are representative fields of TSB-grown, stationary-phase *S. aureus* wild-type (control) and mutant strains as indicated. Circle indicates bulging and irregular septa. Arrows indicate improper septa placement. Insets show 500nm.
Fig. 16: Analysis of cell morphology by transmission electron microscopy (TEM); 198,000x. Shown are representative fields of TSB-grown, stationary-phase *S. aureus* wild-type (control) and mutant strains as indicated. Insets show 100nm.
Fig. 17: Analysis of N315ΔSTP/STK by transmission electron microscopy (TEM). Shown are images of N315ΔSTP/STK cells at 49,500x. Enlarged images of individual cells were taken at 80,900x. Insets show 100 and 500nm.
septa (Fig. 15, arrows). Septa in N315, N315ΔSTP, and N315ΔSTK generally all appear centered and normal (Fig. 15). However, at extremely high magnification (198,000x; Fig. 16), cell wall and membrane regions of N315ΔSTK and N315ΔSTP/STK cells revealed characteristically weak, electron dense, wavy and often interrupted membranes with a fragile appearance, as opposed to the thickly stained, distinctly visible and uninterrupted cell walls seen in N315 and N315ΔSTP cells. Additionally, the cell wall thickness of N315ΔSTP mutants was nearly double that of the other three strains, as seen in a representative cell in Fig. 16. Notably, in all images, deletion of both stk and stp resulted in a more conspicuous phenotype than deletion of stk or stp alone, suggesting a coordinated role for STK and STP in determination of cell wall structure.

**Discussion**

To propagate an infection, bacteria must be able to grow and replicate effectively. *S. aureus* divides by forming septa and dividing sequentially along three perpendicular planes, resulting in a cluster formation [146]. This process occurs approximately once per hour *in vivo* and nearly three times as quickly under ordinary laboratory conditions, under which cells reach stationary phase in 4-5 hours after 1:100 dilutions of overnight (18 hour) cultures (Fig. 12). Upon deletion of stp and stk in *S. aureus*, septa formed along parallel planes (Fig. 15, arrows) and multiple septa were seen forming simultaneously in a single cell (Figs. 14-17). These findings suggest a coordinated role for STK and STP in *S. aureus* cell division.
In multiple organisms, ESTKs have been implicated in cell division processes. This has been substantiated primarily through over-expression and gene knockout studies, with extensive work being done on the 11 ESTKs of mycobacteria. Over-expression of two essential ESTKs (PknA and PknB) in *M. smegmatis* and *M. bovis* BCG results in significantly slowed growth and reduced viability [99]. These genetic manipulations also cause incomplete septation, bulging, and even branching within cells [99], suggesting a clear role for these two mycobacterial ESTKs in septation. When mycobacterial PknA is expressed in *E. coli*, cells similarly exhibit incomplete septum formation, and are as a result elongated to more than 60μm [110], again providing evidence for a link between ESTK signaling and cell division. It is believed that this elongation is due to PknA-mediated phosphorylation of *M. tuberculosis* FtsZ, which inhibits its GTPase activity [110]. FtsZ is a homolog of eukaryotic tubulin which comprises the foundation of the contractile ring in most bacteria, including *S. aureus*, and is responsible for initiating the septation process [147]. When PknA and PknB are over-expressed in *C. glutamicum*, bacteria shift from a rod to coccoid shape, whereas deletion of the ESTKs PknG or PknL in the same organism does not have any obvious effect on cellular morphology [98]. Incomplete septation and bulging has been shown in *S. pyogenes* strains lacking ESTK expression [73], although the mechanism behind this phenomenon is not clear. These bacteria appear to divide with little regard for division planes, resulting in a loss of chain formation that can only be restored upon supplementation with the extracellular PASTA-containing portion of the bacteria’s sole ESTK, SP-STK [73]. The extracellular PASTA domain is thus likely critical for proper
regulation of cell division and may explain why similar disregard for division planes was seen in N315ΔSTP/STK cells. Similarly, *S. agalactiae* cells that lack *stp1, stk1*, or both are unable to segregate, forming abnormally long chains up to 100 cells in length [71], demonstrating a role for ESTPs in these processes as well.

Interestingly, deletion of *stp* or *stk* alone in *S. aureus* did not cause significant differences in cell division morphologies as compared to wild type cells. However, deletion of *stp* did cause an obvious increase in cell wall thickness (Fig. 16). This thickened layer appears on the outermost portion of N315ΔSTP cells, thus is likely composed of peptidoglycan, although this cannot be confirmed by microscopy alone. This effect was not seen in bacteria lacking *stk* cells. Thus, it is possible that STP is responsible for mediating crosslinking and turnover of peptidoglycan in *S. aureus*, whereas STK provides an extracellular sensor (by way of PASTA repeats) to initiate these processes. In the absence of *stk* there is no outside-in signal to mediate changes in peptidoglycan crosslinking and formation, thus no obvious phenotype is seen. However upon deletion of *stp*, this signal is functioning properly but peptidoglycan crosslinking and accumulation is unregulated.

Affecting the expression levels of ESTKs in various organisms has been shown to contribute to an overall growth defect. In *S. aureus*, growth was not significantly affected upon deletion of *stk* or *stp* alone, but N315ΔSTP/STK growth was slightly delayed during exponential phase (Fig. 12A) as has been seen in *S. agalactiae* [71]. Notably, bulging of N315ΔSTP/STK cells may have contributed to the observed optical densities, resulting in a more subtle difference between wild type and N315ΔSTP/STK curves than viable cell.
counting. Regardless, the delay of entry of these cells into exponential phase is consistent with growth patterns of other ESTK mutants. Down-regulation of PknA and PknB in *C. glutamicum*, and deletion of ESTK genes in *S. agalactiae* [71] and *S. pneumoniae* [97] causes delayed entrance into exponential phase during *in vitro* growth, similar to what was observed in N315ΔSTP/STK. Additionally, entrance into death phase occurs earlier in *B. subtilis* bacteria lacking the ESTK PrkC [100]. However, deletion of the gene encoding the ESTP PrpC in the same organism delays bacterial entry into death phase, suggesting opposing mechanisms for ESTKs and ESTPs in *B. subtilis* [100]. Notably, not all ESTKs are implicated in bacterial growth and division.

Taken together, these findings suggest that ESTK- and ESTP-mediated signaling is critical for proper cell division in *S. aureus*, just as in many other Gram-positive organisms. Cell division is closely linked to peptidoglycan formation. Thus we next sought to identify any differences in peptidoglycan structures in each mutant strain as compared to wild type, which could account for the observed morphological defects.
CHAPTER 4

TOWARDS A ROLE FOR STP AND STK IN S. AUREUS PEPTIDOGLYCAN STRUCTURE DETERMINATION

As seen in the previous chapters, STK- and STP-mediated reversible phosphorylation is critical for maintenance of proper cell wall morphology in S. aureus. Since the cell wall is comprised primarily of peptidoglycan, we investigated the influences of STK and STP on peptidoglycan structure. The basic structure of S. aureus peptidoglycan consists of rigid glycan chains linked together by short peptides [22]. We hypothesized that the aberrations in cell wall morphology seen under microscopy upon deletion of stp and stk may be the result of alterations in this structure.

Lysostaphin is a highly charged, two (catalytic and peptidoglycan-binding) domain peptidase produced by Staphylococcus simulans [148] which specifically targets peptidoglycan, lysing the target bacterium. Specifically, it functions as a glycylglycine endopeptidase, cleaving the pentaglycine crossbridges unique to staphylococcal peptidoglycan, but it has been shown to exhibit endo-β-N-acetyl glucosamidase and N-acetyl-muramyl-L-alanine amidase activities [149] on species other than S. aureus [150]. It has been periodically considered to be a viable therapeutic for staphylococcal
infections, although is not presently used in common clinical practice [149]. By measuring the susceptibility of each mutant to lysostaphin, we were able to indirectly analyze substrate (pentaglycine crosslink) availability.

We purified peptidoglycan from the wild type and isogenic mutant *S. aureus* strains and subjected each sample to LC/MS/MS to directly identify core structure(s). Since its initial development by Bernd Glauner in 1988 [151], this method has been well established by many groups as a reliable approach to high-resolution peptidoglycan analysis [152-155]. For this, we first fragmented purified peptidoglycan using the recombinant muralytic enzyme mutanolysin. Released muropeptides were then separated by liquid chromatography (LC), and each separated peak was subjected to MS/MS analysis in tandem. Using this method, we were able to specifically identify peptide sequences and structures in peptidoglycan from each mutant, and compare these to established structures found in wild type *S. aureus*.

**Materials and Methods**

**Lysostaphin Susceptibility Assays.** Lysostaphin susceptibility assays were performed similarly to those described previously [133, 148]. Overnight cultures of *S. aureus* N315 (control), N315ΔSTP, N315ΔSTK and N315ΔSTP/STK and their corresponding complemented strains were pelleted, washed once in lysostaphin buffer (20mM Tris/HCl pH 7, 150mM NaCl, 1mM EDTA) and resuspended in fresh buffer to the original culture volume (OD$_{600nm}$≈1.0). Lysostaphin (Sigma) was added to each culture at a final concentration of 0.5μg/mL. OD$_{620nm}$ of each culture was read (BMG POLARstar Galaxy)
at the indicated time points and plotted as percent of the initial reading, which was set at 100% and 0 minutes. As a negative control and to measure autolysis, lysostaphin buffer alone (without lysostaphin) was used in identical assays. Indicated $p$ values were determined using the student’s unpaired $t$-test and significance determined as $p<0.05$.

**Peptidoglycan preparation**

Peptidoglycan from N315, N315ΔSTP, N315ΔSTK and N315ΔSTP/STK was isolated similarly to published methods [152]. Each strain was grown to late stationary phase (20 hours) in 600mL TSB, chilled, and pelleted at 4°C. 50mL boiling 4% SDS was added to each cell pellet and the samples boiled for an additional 30 minutes. Cell lysates were then spun down at 3500rpm for 25 minutes at 4°C, and washed three times in sterile water under the same centrifuge conditions. Final pellets were resuspended in 1mL water and sonicated on ice for 7 minutes (10s on/10s off pulses, 75% amplitude) with a Vibra-Cell VCX 130 and 2mm microtip (Sonics). Samples were then again pelleted at max speed, 4°C, and stored at -20°C. The next day, pellets were resuspended up to 10mL in 100mM TrisHCl (pH 7.5) containing 100μg/mL α-amylase (Sigma) and incubated with shaking at 37°C. After 2 hours, 50μg/mL RNase H, 10μg/mL DNase, and 20mM MgSO$_4$ were added and samples were incubated for another 2 hours at 37°C with shaking. Samples were further incubated for 8 hours at 37°C with 10mM CaCl$_2$ and 100μg/mL trypsin (Sigma) to remove surface protein contamination. To account for enzyme degradation, fresh trypsin (final concentration 100μg/mL) was then added and the incubation proceeded for an additional 8 hours. Next, 375μL 20% SDS was added to each sample (final concentration ~1%) and the samples were boiled for 15 minutes. Samples were...
spun at max speed, 4°C, for 30 minutes to collect wall fragments and washed sequentially in water (2x), 8M LiCl, 100mM EDTA, water (2x), and acetone. Pellets were then spun in an Eppendorf Vacufuge™ until dry. A small portion (15-70mg) of peptidoglycan was used for further manipulations and remaining pellets stored at -80°C. Samples were weighed and 48% hydrofluoric acid added at a ratio of 2mL/5mg peptidoglycan followed by gentle rocking at 4°C for 48 hours to remove teichoic acid. Samples were then pelleted at max speed, 4°C, washed twice in water and once in 100mM Tris/HCl (pH 7.5) before being incubated in 100mM (NH₄)₂CO₃ containing 0.5U/μL alkaline phosphatase (Sigma) for 16 hours at 37°C with shaking. Finally, samples were boiled for 5 minutes to inactivate any remaining enzymes and washed twice in water. Peptidoglycan samples were stored in 0.05% sodium azide at 4°C. For digestion, 1mg peptidoglycan was incubated overnight with 120U mutanolysin (Sigma) and 1mM phenylmethylsulphonyl fluoride (PMSF) in 1mL mutanolysin buffer (50mM 2-(N-morpholino)ethanesulfonic acid (MES, Sigma), 1mM MgCl₂, pH 6.0) at 37°C with shaking.

**LC/MS/MS analysis**

This assay was performed by Liwen Zhang at the Mass Spectrometry and Proteomics Facility at the Ohio State University. Samples were first concentrated by spin-vac centrifugation until final volume was ~30μL. Sample were separated on an UltiMate™ 3000 HPLC system (LC-Packings). Each sample (5μL) was first injected into the trapping column (LC-Packings), and washed with 50mM acetic acid. The injector port was switched to inject, and the peptides were eluted off the trap onto a capillary column (0.2x150mm Magic C18AQ 3μ 200Å, Michrom Bioresources Inc.). Mobile phases used
were A: 0.1% formic acid in water, and B: 0.1% formic acid in acetonitrile. Flow rate was set at 2µL/min. Different gradient settings were tested for the best separation and the final gradient was set as below: mobile phase B was set at 2% at 0 minutes and kept at 2% for 10 minutes, mobile phase B was continually increased to 35% from 10-40 minutes and then increased to 90% from 40-45 minutes. Mobile phase B was then kept at 90% for 1 minute before being brought back quickly to 2% in 1 minute. The column was then equilibrated at 2% of mobile phase B (or 98% A) for 20 minutes before the next sample injection. MS data was acquired on an Orbitrap LTQ mass spectrometer (Thermo Scientific). The scan sequence of the mass spectrometer was programmed for a full scan (resolving power=30000) and MS/MS scans of the 10 most abundant peaks in the spectrum. Dynamic exclusion was used to exclude multiple MS/MS of the same peptide after detecting it three times. Accurate molecular weights were calculated manually.

Results

N315ΔSTP mutants display alterations in lysostaphin susceptibility. Based on the results obtained by TEM, we suspected that deletion of stp and/or stk may directly affect cell wall structure. We therefore determined the susceptibility of each mutant and its corresponding complemented strain to the peptidoglycan-targeting cell lysis agent lysostaphin by measuring a decline in cell density as described in Materials and Methods. Upon addition of lysostaphin, wild type (N315), double mutant (N315ΔSTP/STK), and all complemented strains (triangles) reached 50% lysis by approximately 20 minutes (Fig. 18), while N315ΔSTP took a significantly longer incubation time (35min, p<0.002 as
**Fig. 18: Lyostaphin sensitivity assay.** Results indicate susceptibility of (A) N315, (B) N315ΔSTP, (C) N315ΔSTK, (D) N315ΔSTP/STK and their appropriate complemented strains (triangles) to lysostaphin. Autolysis (no lysostaphin) controls are shown in closed squares. Data shown are the averages of three independent experiments. Error bars indicate standard deviations. Data are presented as a percentage of the original culture turbidity. *p*<0.05, **p*<0.002 by student’s unpaired *t* test.

compared to wild type, Fig. 18B). All N315ΔSTP (+ lysostaphin) data points between 10 and 40 minutes were very significant (*p*<0.002) as compared to wild type (Fig. 18B).

Notably, N315ΔSTK reached 50% lysis by approximately 25 minutes (Fig. 18C), lysing significantly more slowly than N315 (*p*<0.05 at 20 and 25 minutes as compared to wild type), but faster than N315ΔSTP. We further calculated the slope of the N315,
N315ΔSTP, N315ΔSTK, and N315ΔSTP/STK (+ lysostaphin) curves using a linear regression analysis of the data points between 5 and 25 minutes (the time period in which most lysis occurred). The slopes and their standard deviations were -2.354 ± 0.105, -1.481 ± 0.0313, -1.903 ± 0.0599, and -2.171 ± 0.151, respectively. R² values were all >0.94. No significant differences were observed between strains in negative control reactions which did not incorporate lysostaphin (Fig. 18, dark squares), indicating that the aforementioned differences in lysis kinetics were not due to changes in autolysis. Based on these analyses, we concluded that N315ΔSTP, and to a lesser degree N315ΔSTK, bacteria are more resistant to the effects of lysostaphin as compared to the parent strain N315 as a direct result of the deletion of stp or stk.

**LC/MS/MS**

We next determined if the altered susceptibilities of N315ΔSTP, and to a lesser extent N315ΔSTK, bacteria to lysostaphin were due to alterations in the lysostaphin substrate, the glycylglycine crosslink. To do this, we analyzed peptidoglycan digested with mutanolysin from N315, N315ΔSTP, N315ΔSTK, and N315ΔSTP/STK bacteria using LC/MS/MS as described in Materials and Methods. The basic peptidoglycan structure is shown in Fig. 19, where \( n \) indicates a repeating unit, and the pentaglycine crosslink joins the third amino acid in the stem peptide (Lys) to the fourth amino acid in a neighboring peptide (Ala). The predicted M+H score for this structure (where \( n=1 \) is 2413.0853 (Table 6).
Fig. 19: Basic peptidoglycan structure in *S. aureus*. Primary monomer in *S. aureus* peptidoglycan. Sites of action for lysostaphin and mutanolysin are indicated. G: N-acetylglucosamine, M: N-acetylmuramic acid, *n* indicates repeating unit. Figure adopted from Liwen Zhang.

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Table 6: Theoretical masses and m/z scores of peptidoglycan multimers.
Theoretical M+H and m/z scores were calculated for multimeric peptidoglycan structures from \( n=1 \) to \( n=8 \) (Table 6). Lysostaphin-digested peptidoglycan samples from each strain were eluted separately from 0-55 minutes, although the majority of relevant peptides eluted between 21-29 minutes (see full representative LC pattern, Fig. 20A).

Using the predicted M+H and m/z values and read values, we were able to match spectra to structures, as seen in Fig. 20B, where \( n \) values are indicated above the appropriate peak (corresponding to multimers of the structure shown in Fig. 19). Resolution decreased with higher multimers, as indicated by increasing mass errors (Table 7), thus multiple structures were assigned to common peaks in some instances. Fig. 21 shows the alignment of LC patterns from each strain. All structures \( (n=1-8) \) could be identified in each strain’s peptidoglycan with the exception of \( n=8 \) in the wild type N315 (Table 7). We attribute this to difficulty in higher order multimer resolution rather than an absence of this structure in this sample. Additionally, there were not reproducible differences in fragment masses between the strains, as seen by an overlay of all spectra during the period of relevant elution (Fig. 21) and the m/z values collected (Table 7). Taken together, these data suggest that mutant and wild type peptidoglycan contain similar stem peptide sequences.

Discussion

The structure of \( S. \text{ aureus} \) peptidoglycan has been established by many groups, and is seen in Fig. 19. The rigid portion of the peptidoglycan is comprised of alternating N-acetylglucosamine and N-acetylmuramic acid sugars, linked by \( \beta-(1,4) \) glycosidic
**Fig. 20: Matching spectra to structure.** (A) A representative full elution profile (0-55min) for lysostaphin-digested *S. aureus* peptidoglycan. Circle indicates area shown in (B). (B) Limited elution profile (21-29min) for lysostaphin-digested *S. aureus* peptidoglycan. *n* values and elution times (in minutes) are indicated above the corresponding peaks. Figure adopted from Liwen Zhang.
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Table 7: Observed m/z scores of peptidoglycan monomers.
Fig. 21: Alignment of spectra from mutant strains. Representative LC patterns from each indicated strain. Overlay of all spectra is shown at the bottom. Figure adopted from Liwen Zhang.
bonds. Chains of these sugars can reach nanometers in length, varying most notably by the *Staphylococcus* species being examined [156, 157]. Attached to the muramic acid groups are short, flexible peptides of the sequence D-alanine, D-isoglutamine, L-lysine, D-alanine, and D-alanine [22]. These amino acids are added to the growing peptidoglycan by Mur ligases [158]. The terminal alanine residue is cleaved prior to pentapeptide crosslinking, during which five glycine moieties are attached sequentially by the Fem (factors essential for methicillin resistance) family of proteins [158] at the L-lysine residue, terminating at the fourth residue of a neighboring peptide chain, or D-alanine. The frequency of this crosslinking is extremely high in *S. aureus*. It is estimated that 75-95% of its peptidoglycan is linked by pentaglycine chains, which is quite significant when compared to 25% in a Gram-negative bacterium, such as *E. coli* [23, 148]. This tight meshwork imparts stability and strength to the *Staphylococcus*.

In the current study, N315ΔSTP bacteria were significantly more resistant to the effects of lysostaphin than the parent strain, N315 (Fig. 18A and B). Taken together with the thickened cell wall observed in this mutant strain (Fig. 16), it is reasonable to consider this effect is not due to an alteration in the enzymatic substrate, but rather simply an overabundance of it. An overabundance of crosslinks implies a more oligomeric structure for N315ΔSTP peptidoglycan as compared to that of wild type, suggesting a role for STK in crosslink formation and STP in crosslink regulation. However, a moderate increase in resistance to lysostaphin as observed in N315ΔSTK is not so easily explained. This mutant does not display noticeably thickened cell walls as compared to wild type (Fig. 16). Thus, this resistant phenotype is more likely attributable to changes
in crosslinking frequency and/or substrate. Despite the increased lysostaphin resistance observed upon deletion of \( stk \) or \( stp \), deletion of both enzymes did not have an additive effect in the lysostaphin susceptibility assays. We attribute this primarily to the presence of an additional ESTK and multiple ESTPs in the genome that may become activated upon deletion of the primary ESTK and ESTP. Since the only other ESTK in the genome (SA0077) does not contain a PASTA domain and the other ESTPs contain different catalytic domains than STP (Fig. 5), these enzymes likely cannot fully compensate for a loss of \( stp \) and \( stk \), as was observed by considerable division defects in N315ΔSTP/STK cells (Figs. 13-19). However, they may be able to achieve partial compensation, resulting in an N315ΔSTP/STK phenotype consistent with the wild type in regards to lysostaphin susceptibility. An additional factor which may inhibit lysostaphin activity is capsule production. If capsule production differs upon deletion of \( stk \) and/or \( stp \), this may affect substrate availability in the lysostaphin susceptibility assays by masking the peptidoglycan [159].

We employed an LC/MS/MS technique to characterize peptidoglycan isolated from \( S. aureus \) N315 bacteria and its cognate mutants lacking \( stp \) and/or \( stk \). Upon digestion with mutanolysin (N-acetylmuramidase), peptidoglycan fragments in \( S. aureus \) are predicted to consist primarily of monomers \( (n=1, \text{ see Fig. 19}) \) and oligomers of this basic structure. This prediction is consistent with the results, whereby the most abundant peaks in each strain’s elution spectra correspond to the monomeric form (Figs. 20B and 21). Oligomers, which elute later due to their size, were least abundant in all samples and much more difficult to resolve. The elution profiles of all samples were seemingly
identical (Fig. 21), as were the corresponding m/z scores for each major peak (Table 7). As LC/MS/MS is an extremely sensitive application, identical masses obtained for each peak across strains (Table 7) indicates identical structures with no amino acid substitutions or alterations in crossbridge composition. Thus, the alterations in lysostaphin susceptibility seen in N315ΔSTP and N315ΔSTK are not due to changes in substrate (pentaglycine bridge) biochemistry, but more likely are due to crosslinking frequency or abundance, which cannot readily be measured by LC/MS/MS.

The basic core structure of a bacterium’s peptidoglycan normally remains unaltered across a given species. Rather, glycan chain length, frequency and location of crosslinking, and composition of the interpeptide bridge (although the latter was not seen in the current study) may change in response to environmental conditions. In *E. coli*, transition from logarithmic to stationary phase signals a halving of sugar chains and notable increases in crosslinking frequency (from ~28.5% to ~39%) [22]. Across bacterial species, this crosslinking varies from 1-7 amino acids, with smaller interpeptide bridges normally associated with Gram-negative bacteria, and pentaglycine crosslinks associated with staphylococcal species [22, 152]. Notably, inclusion of amino acids (particularly D-amino acids) in the growth media or genetic manipulations of *fem* or *mur* genes can result in alterations in amino acid sequences within the peptidoglycan, and significant reductions in crosslinking in *S. aureus* specifically [22, 157]. Selective pressure from antibiotics can also affect peptidoglycan structure. Glycopeptides, such as vancomycin, utilize the terminal Ala-Ala residues of the peptidoglycan peptide chain as a substrate, thus vancomycin-resistant strains of *S. aureus* have been isolated with novel
muropeptide species, presumably evolved to evade such antibiotic treatment. Almost invariably, these isolates utilize tetrapeptide, rather than pentapeptide, crosslinks [157], however alterations in crosslink composition were not observed in any of the mutant strains in the current study. Changes in peptidoglycan core structure also been seen in vancomycin-resistant *E. faecalis*. Upon expression, the Van gene cluster reconfigures peptidoglycan assembly to include D-Lactate at the terminal position of the peptide chain, rather than D-Alanine, eliminating the substrate for vancomycin [160].

Thus, N315, N315ΔSTP, N315ΔSTK, and N315ΔSTP/STK all have identical peptidoglycan core structures, with alternating N-acetylglucosamine and N-acetylmuramic acid sugars linked to short peptides of the sequence D-alanine, D-isoglutamine, L-lysine, D-alanine, and D-alanine which are crosslinked by 5 glycine residues. However, subtle differences in N315ΔSTP and N315ΔSTK peptidoglycan crosslinking beyond structure (e.g. frequency, abundance) likely result in the differences in lysostaphin susceptibilities observed in these strains as compared to wild type. The major class of enzymes responsible for assembling peptidoglycan includes PBPs, each of which has precise transpeptidase and/or transglycosylase activities. PBPs and their substrates are often the target of antibiotics as well. Thus, we next questioned the effect of *stp* and/or *stk* deletions on PBP expression and antibiotic susceptibility.
CHAPTER 5

EFFECTS OF STK AND STP ON ANTIBIOTIC RESISTANCE AND PENICILLIN-BINDING PROTEIN EXPRESSION

After considering the unusual cellular morphology observed in *S. aureus* N315 cells lacking *stp* and *stk* and the common core peptidoglycan structure seen in all strains, we predicted that STP and/or STK may play a role in modulating peptidoglycan assembly, rather than its composition.

Cell wall synthesis is a common pathway targeted by antibiotics, which interfere by mimicking various cell wall components [6]. These (β-lactam) antibiotics bind to penicillin-binding proteins (PBPs) present on the cell surface which normally facilitate the assembly, or “stitching,” of the cell wall [161-164]. Thus, changes in peptidoglycan assembly by PBPs would manifest in alterations in antibiotic susceptibility. To see if this was the case in the current study, we measured the minimum inhibitory concentrations (MICs) of various antibiotics when exposed to *S. aureus* N315, N315ΔSTP, N315ΔSTK, or N315ΔSTP/STK bacteria. By determining susceptibility profiles for mutants and complemented strains against a variety of antibiotic classes we were able to identify
specific antibiotic substrates whose availabilities were affected by the absence of STP and/or STK.

Using quantitative real-time PCR, we investigated \( pbp \) transcript abundance in each strain as well. Each PBP in \( S. aureus \) has a specific role and substrate, based on their catalytic domains. These enzymes are responsible for transglycosylation of the rigid sugar backbones of the peptidoglycan, cleavage of the terminal alanine on the flexible peptide chains, as well as attachment of the pentaglycine crosslink to the newly exposed residue [157, 162, 165]. Alterations in \( pbp \) expression levels can have severe repercussions as a result of peptidoglycan crosslinking imbalance, including septum disruption [166] and heightened antimicrobial sensitivity [165]. Therefore, by performing both antibiotic sensitivity assays and measuring \( pbp \) transcript abundance, we were able to better understand the source of morphological defects observed in strains lacking STP and/or STK.

**Materials and Methods**

**Antibiotic Susceptibility Assays:** The MICs of various antimicrobial agents for each \( S. aureus \) isolate were initially determined using the MicroScan WalkAway system (Siemen’s Healthcare Diagnostics). MicroScan is an automated, commercially available system for rapid identification and susceptibility testing of Gram-negative and -positive bacteria. The species identification was performed by using the Gram-Positive ID/AST Combo PC29 panel. The MIC determinations and quality control protocols were followed in accordance with standards established by the Clinical and Laboratory Standards.
Institute (formerly National Committee for Clinical Laboratory Standards) [167]. Microscan was performed at the Clinical Microbiology Facility at the Ohio State University Medical Center. MICs of the wild type, mutant, and corresponding complemented mutant strains were determined using Etest® strips (AB Biodisk) on triplicate samples as per the manufacturer’s instructions where indicated. Briefly, the appropriate fresh cultures were normalized to an OD\(_{600nm}\)=1.0 by dilution with fresh TSB and a sterile cotton swab (dipped in and removed of excess culture) used to spread the cultures on TSA containing 5% sheep’s blood (Hemostat Laboratories). Etest® strips were placed in the center of the plates, and plates were incubated for 18 hours at 37ºC before reading. Description of this assay has also been described previously [133].

**Real-time PCR** RNA was isolated from N315, N315ΔSTP, N315ΔSTK and N315ΔSTP/STK cells and cDNA synthesized as described (see Chapter 1, Cotranscription assay). The cDNA was diluted at least 1:100 in water and 1μL used as template in 25μL real-time PCR reactions containing 100mM each of two primers (Table 5), 12.5μL iQ™ SYBR® Green Supermix (Biorad), and 9.5μL sterile, RNase-free water. Primers used were specific to each gene as follows: #105/106: staphylococcal 16s rRNA (endogenous control, [168]), #97/98: \textit{SA1024} (\textit{pbpA}), #99/100: \textit{SA0038} (\textit{pbp2x}), #101/102: \textit{SA1283} (\textit{pbp2}), #103/104: \textit{SA1381} (\textit{pbp3}), and #95/96: \textit{SA0598} (\textit{pbp4}). Real-time PCR was performed using an iQ5 cycler (Biorad) and the protocol as follows: 95ºC (9 minutes) followed by 40 cycles of amplification (95ºC 15 seconds, 58ºC 30 seconds, 72ºC 30 seconds). Real-time PCRs were terminated with a melt curve (81 30-second cycles of 55-95ºC, increasing set point temperature every cycle after the first by 0.5ºC) to
confirm the absence of primer self-annealing. Standard curves were created for each primer set in each run using 2-fold dilutions of control (N315) cDNA as template. These curves were used to calculate reaction efficiencies. Control reactions with water in place of cDNA template were also included in each run to ensure no exogenous nucleic acid contamination. PBP copy numbers in mutant strains were expressed as a ratio to those of the wild type by the Pfaffl method [169], and converted to percentages and relative fold changes for ease of interpretation. Relative expression ratios were determined by:

\[
\text{Ratio} = \frac{(E_{\text{pbp}} \Delta C_{\text{pbp}})}{(E_{16S} \Delta C_{16S})}
\]

where \( C \) indicates copy number, \( \Delta C \) indicates deviation in copy number (\( C_{\text{N315}} - C_{\text{mutant}} \)), and \( E \) indicates efficiency. Statistical significance (\( p<0.05 \)) of mutant expression levels as compared to wild type were determined by two-tailed, unpaired student’s \( t \) tests.

**Results**

**Role of STP/STK in modulation of antibiotic susceptibility in *S. aureus*** Changes in cell division and wall structures (Fig. 14-17) and altered lysostaphin susceptibilities (Fig. 18) in STP and/or STK-specific mutants suggest that *S. aureus* N315 STP and STK may be involved in cell division and/or peptidoglycan structure. Changes in the cell wall of *S. aureus* have been shown to affect staphylococcal susceptibility to certain antimicrobials, particularly those which target cell wall structure or synthesis [170]. Based on this, we determined the susceptibility of N315 and its isogenic mutant strains to 22 antibiotics with various known targets. N315ΔSTP did not show any change in susceptibility as
compared to wild type (Table 8). However, N315ΔSTK, and to a greater degree N315ΔSTP/STK, mutant strains displayed increased susceptibility to multiple antibiotics tested (Table 8, bold). Most appreciably, N315ΔSTP/STK bacteria displayed susceptibility increases ranging from 2-fold in the case of cefazolin to more than 50-fold in the case of ertapenem, as compared to N315. The affected antibiotics in both N315ΔSTK and N315ΔSTP/STK included cephalosporins (cefazolin, ceftriaxone, cefotaxime) and carbapenems (ertapenem, imipenem), all of which act by interfering with bacterial cell wall synthesis. To verify these effects, complementation studies were performed using Etest® strips containing gradients of representative antibiotics from each of these groups, specifically those in which the MIC values changed in both N315ΔSTK and N315ΔSTP/STK (cefotaxime, ceftriaxone, and ertapenem). For these studies, N315ΔSTKΩSTK and N315ΔSTP/STKΩSTK were used in place of N315ΔSTK and N315ΔSTP/STK respectively, and MIC values returned to those of wild type (Table 8). As a control, MICs of N315ΔSTK and N315ΔSTP/STK were also determined using Etest® strips, and values were equivalent to those obtained by MicroScan. All experiments involving E-test® strips were performed in triplicate. Representative plates are shown in Fig. 22. Increased selective susceptibility of N315ΔSTK and N315ΔSTP/STK to cell wall-acting antibiotics indicates an important (direct or indirect) role of STK in staphylococcal cell wall biosynthesis and structure. Results and interpretation of these data are adopted from a previous publication [133].

**STP/STK mutants display altered levels of PBP transcripts.** Many of the antibiotics whose susceptibilities were affected in the mutant strains target PBPs. Therefore, we
<table>
<thead>
<tr>
<th>Antibiotics most affected</th>
<th>N315</th>
<th>N315ΔSTP</th>
<th>N315ΔSTK</th>
<th>N315ΔSTK/STK</th>
<th>N315 ΔSTP/STK</th>
<th>N315 ΔSTP/STK</th>
<th>N315 ΔSTP/STK</th>
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</thead>
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<tr>
<td>Cefazolin</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>≥8</td>
<td>&gt;256*</td>
<td>12</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>43</td>
<td>≥8</td>
<td>&gt;32</td>
<td>≥8</td>
<td>&gt;32</td>
</tr>
<tr>
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<td>32</td>
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<td>&gt;32</td>
<td>≥8</td>
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<td>≥8</td>
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<tr>
<td>Ertapenem</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>18</td>
<td>&gt;32</td>
<td>≥8</td>
<td>0.6</td>
<td>&gt;32</td>
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<td>&gt;32</td>
<td>≥4</td>
<td>&gt;32</td>
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<tr>
<td>Ampicillin/Sublactam</td>
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<tr>
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<tr>
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<td>&gt;8</td>
<td>&gt;8</td>
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<td>≤1</td>
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<td>≤1</td>
<td>≤1</td>
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<tr>
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<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

Continued on next page
### Table 8: Minimum inhibitory concentrations (µg/mL).

*Complementation MIC values shown are the average of three independent experiments using Etest® strips. Antibiotic gradients on Etest® strips were 0.016-256µg/ml (cefotaxime and ceftriaxone) and 0.002-32µg/ml (ertapenem). Clav = clavulanic acid.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>≤4</th>
<th>≤4</th>
<th>≤4</th>
<th>≤4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethoprim/Sulfa</td>
<td>≤0.5/9.5</td>
<td>≤0.5/9.5</td>
<td>≤0.5/9.5</td>
<td>≤0.5/9.5</td>
</tr>
</tbody>
</table>

**Fig. 22: E-test® strip assay.** An example of cefotaxime susceptibility testing using N315, N315ΔSTP/STK and the corresponding complemented strain, N315ΔSTP/STKΩSTPSTK. Note the area of inhibition in the mutant strain.
hypothesized that the altered antibiotic susceptibilities observed in N315ΔSTK and N315ΔSTP/STK bacteria may be a direct result of \(pbp\) expression changes. Using cDNA created from total RNA preps from exponential phase N315, N315ΔSTP, N315ΔSTK, and N315ΔSTP/STK bacteria, we measured the transcript levels of each of the 5 staphylococcal \(pbps\) (\(pbps\) A, 2, 2x, 3, and 4) using quantitative real-time PCR. Transcripts of all \(pbps\) appeared to be up-regulated in N315ΔSTP bacteria as compared to wild type, with significant up-regulation of \(pbps\) A and 4 in the absence of \(stp\) (Fig. 23). Bacteria lacking \(stk\) (N315ΔSTK or N315ΔSTP/STK) displayed decreased levels of \(pbpA\) and \(pbp2\) transcripts, and increased levels of all other \(pbp\) transcripts (\(pbp2x\), \(pbp3\), and \(pbp4\)) (Fig. 23). The largest effects were seen in \(pbp4\), where all mutant strains showed increased transcript levels (up to 4-fold, in the case of N315ΔSTP).

**Discussion**

The assembly of *S. aureus* peptidoglycan (shown in Fig. 24) is a multi-step process. The first step is the transglycosylation of N-acetylmuramic acid and N-acetylglucosamine into chains. This is performed by PBP2, and provides the substrate (N-acetylmuramic acid) for cooperative attachment of short (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) peptide chains (assembled by Mur ligases) by PBPs A, 2, 2x, and/or 3. The final step utilizes the carboxypeptidase activity of PBP4 to cleave the terminal alanine residue in these peptides and expose the fourth alanine residue for pentaglycine crosslinking to a neighboring peptide chain at lysine [162]. Many antibiotics act by interfering with this cell wall synthesis process. This is the classic mechanism of penicillins (\(\beta\)-lactams) a
Fig. 23: Penicillin binding protein (PBP) expression. Transcript abundance from the same experiments are expressed as a (top panel) percent of wild type (set at 100%) and (bottom panel) relative fold change from wild type (set at 0). Data shown are averages of results obtained using three independent RNA preparations. Error bars indicate standard deviations. *p<0.05 as determined by student’s t test.
**Fig. 24: S. aureus peptidoglycan structure.** PBP enzymatic activities and sites of action are indicated. PBP2 transglycosylase activity assembles the rigid glycan chains to which 5 amino acids are added by Mur ligases (not shown). The terminal D-Ala moiety is cleaved by PBP4 to enable cross-linking by one or more of PBPs A, 2, 2x, and 3. MurNAc, N-acetylmuramic acid. GlcNAc, N-acetylglucosamine.
large family of antibiotics which encompasses cephalosporins, carbapenems and monobactams. These are the antibiotics whose MICs were most affected upon deletion of stk or stp and stk. Members of this family interfere with cell wall synthesis by mimicking peptidoglycan fragments and binding with varying affinities to PBPs, which are exposed on the surface of Gram-positive bacteria due to a lack of an outer membrane [6]. This binding involves an acylation step, which inhibits PBP function [162]. Glycopeptides, such as vancomycin, also inhibit cell wall synthesis, although in a different manner. These antibiotics bind directly to D-Ala-Ala components of the short peptide chains within bacterial peptidoglycan, inhibiting their reticulation. These mechanisms are distinct from those with intracellular targets, such as tetracyclines, macrolides, aminoglycosides, and fluoroquinolones, which act by directly inhibiting nucleic acid or protein synthesis, and whose MICs were not affected by stp and/or stk deletion (Table 8).

Resistance to cell wall-acting antibiotics is normally conferred by enzymatic cleavage of the antibiotic (e.g. β-lactamase production), over-expression of the antibiotic substrate, or low-affinity binding between the antibiotic and its substrate [6]. The S. aureus N315 genome contains 11 genes annotated as β-lactamases (www.tigr.org), enabling it to digest penicillin quite easily before it has a chance to act on the PBPs. During activation of these enzymes, the β-lactam antibiotic binds to a transmembrane sensor protein BlaR1, which in turn undergoes autocleavage and triggers hydrolysis of the β-lactamase transcriptional repressor protein BlaI, initiating enzyme production [171]. Interestingly, BlaI can also repress mecA expression [171], one of the exogenously
acquired genes responsible for methicillin (a penicillin derivative resistant to the actions of β-lactamases) resistance. The mec operon encodes PBP2x, a PBP with decreased affinity for β-lactams [9]. PBP2x can assume the transpeptidase responsibilities of PBPs A, 2, and 3 when these enzymes are bound by penicillin derivatives, although PBP2x is less efficient at crosslinking [157].

Increased sensitization to cell wall-acting antibiotics has been observed in E. faecalis strains lacking the ESTK PrkC [74]. We observed similar results in the current study, as MICs to antibiotics that specifically target cell wall synthesis were affected upon deletion of stk or stk and stp. N315ΔSTP bacteria remained resistant to all antibiotics tested. Importantly, levels of resistance cannot be measured by the methods we employed. Thus, an increase in resistance in this strain as compared to wild type should not be discounted as a possibility, particularly in light of the thickened cell wall observed under microscopy and increased levels of all pbp transcripts in N315ΔSTP (Fig. 23).

While all antibiotics with affected MICs were β-lactams, cefazolin, cefotaxime, and ceftriaxone are cephalosporins, while ertapenem and imipenem fall under the category of carbapenems. Both classes of antibiotics were developed to evade the effects of bacterial β-lactamases. Cephalosporins are not normally effective against MRSA, consistent with the resistant phenotypes observed for such antibiotics in the wild type strain (Table 8). These antibiotics contain a 6-member dihydrothiazine ring in lieu of the 5-member thiazoladine ring found in penicillins [172]. Carbapenems substitute a carbon for sulfur group in their β-lactam ring, also rendering them resistant to the effects of β-
lactamase [173]. *S. aureus* species have varying susceptibilities to carbapenems [173], although strain N315 was found to be resistant in our testing. Meropenems and cephalosporins both are known to directly bind to PBPs, albeit with varying affinities [163, 174-176].

Increased sensitivities to these antibiotics upon deletion of *stk* (and more so upon deletion of *stk* and *stp*), suggest that STK/STP-mediated signaling plays a role in modulation of antibiotic substrate (PBP) availability. Quantitative real-time PCR confirmed this, as transcript levels of multiple *pbps* were significantly (*p*<0.05) altered upon deletion of *stp* and/or *stk* (Fig. 23). This includes *php2x* transcripts, which were up-regulated in all mutant strains, explaining the consistent oxacillin resistance observed (Table 8). In *S. aureus*, ceftriaxone binds to PBPA [163], while cefotaxime binds preferentially to PBPA and PBP2 [175, 176]. Transcripts of both of these *pbps* were down-regulated in N315ΔSTK and N315ΔSTP/STK bacteria as compared to wild type (Fig. 23), which may partially account for the increased sensitivities to ceftriaxone and cefotaxime in these strains as less antibiotic is required when the substrate concentration has decreased. Transcripts of all *pbps* were up-regulated in bacteria lacking *stp*, consistent with its antibiotic-resistant phenotype. Effects of *stk* or *stp* and *stk* deletions on *php3* and *php4* transcript levels were non-significant, although these manipulations appear to result in up-regulation, with the exception of the *stk* deletion and *php3* (Fig. 23).

Together, these data show that STP and STK modulate expression levels of *pbps* in *S. aureus*, leading to alterations in antibiotic susceptibilities in their absence. The
mechanism behind this modulation is unknown. For this reason, we next sought to identify a phosphorylation target for STK and STP to begin to understand the signaling cascades responsible for the observed cell wall defects in the mutant strains.
CHAPTER 6

REVERSIBLE PHOSPHORYLATION OF AN ESSENTIAL CELL DIVISION PROTEIN BY STK AND STP

Real-time PCR analysis revealed that \textit{pbpA} is significantly down-regulated in the absence of STK. This gene is encoded on a highly conserved \textit{cell wall/division} gene cluster (termed the \textit{dcw} gene cluster) in \textit{S. aureus} [177]. We hypothesized that \textit{pbpA} down-regulation is due to changes in the functional status of transcription factors affected by the absence of STP and/or STK.

In \textit{B. subtilis}, several genes in the \textit{dcw} gene cluster are regulated by the TCRS YycF/G [178], and all of the genes in this conserved cluster are predicted to have similar regulatory mechanisms [177]. YycF and YycG serve as a response regulator and histidine kinase, respectively, with orthologs found in various other Gram-positive bacteria. Interestingly, the orthologous response regulator and histidine kinase in \textit{S. aureus} (WalR/K) are essential, but only the response regulator is required for \textit{S. pneumoniae} viability [179]. Similarly, in \textit{S. pyogenes} VicR (YycF ortholog) function is not affected by the absence of WalK (YycG ortholog) [180]. These findings indicate the presence of
alternative phosphoryl group sources (free acyl phosphate, ESTKs, etc.) capable of phosphorylating WalR [179].

Phosphorylation of response regulators by ESTKs affects their ability to bind target DNA [107, 122, Jin, et al. unpublished data]. In fact, in *S. pyogenes*, a tighter association between the response regulator CovR and its target DNA is seen after threonine phosphorylation by an ESTK than is seen after aspartic acid phosphorylation by its cognate histidine kinase, resulting in increased target gene expression (Jin, et al. unpublished data).

We therefore hypothesized that in *S. aureus*, the phosphorylation of the transcriptional activator WalR by STK may have a function supportive to that of the cognate histidine kinase (WalK). In the absence of STK, this supportive function may not be executed, resulting in less/inefficient binding of WalR to its specific promoter and reduced transcription of *pbpA*. As the first step to address this hypothesis, we questioned whether *S. aureus* STK can utilize WalR as a phosphorylation substrate, and whether this phosphotransfer can be reversed by STP. Similarly to Chapter 2, we performed *in vitro* kinase assays using radiolabelled $[^{32}\text{P}]ATP$ and investigated this hypothesis by autoradiography as outlined in the following section.

**Materials and Methods**

**Production of recombinant STP/STK.** Recombinant STP was produced exactly as described in Chapter 2. For recombinant STK, the catalytic domain (STKcat, base pairs 1-807) alone was amplified using primers STK-F and #125 and cloned into pET14B.
before subsequent transfer into DH5α and BL-21(DE3) pLysS. This protein was then soluble upon induction and could be purified under native conditions and dialyzed as described in Chapter 2. Membranes exposed for autoradiography were stained with 0.1% methylene blue in 0.01% methanol.

Production of recombinant WalR. Recombinant WalR was produced similarly to recombinant (full length) STK as described in Chapter 2, with the exception of FPLC which was not required. Primers WalR-F and WalR-R were used to amplify walR (SA0017) from the N315 genome before insertion into pET14B and subsequent transfer into E. coli XL-1-Blue and BL-21(DE3) pLysS. Upon over-expression, WalR was insoluble. Therefore it was purified under denaturing conditions and dialyzed as described in Chapter 2. Recombinant WalR production was performed by Vijaya Srinavasan.

In vitro kinase assays. These assays were performed exactly as described in Chapter 2, using 3µg of each recombinant protein per reaction.

Results

WalR is an in vitro substrate of STK. To determine if STK can phosphorylate the response regulator WalR, we performed in vitro kinase assays. As shown in Fig. 25A, the recombinant catalytic domain of STK (rSTKcat, 31.5kDa) is capable of phosphotransfer to rWalR (27.1kDa). We tested this reaction using two different cation cofactors (magnesium or manganese) in the kinase buffer. Although autophosphorylation of rSTKcat appears to favor the use of manganese (Fig. 25A) as was seen in full length
Fig. 25: Reversible phosphorylation of rWalR by rSTKcat. (A) The catalytic domain of STK (rSTKcat) and rWalR were incubated separately and in the indicated combinations in *in vitro* kinase assays using [γ-32P]ATP. (B) Reversibility of rSTKcat-mediated phosphorylation of WalR was measured by the addition of rSTP. Shown are representative autoradiographs.
rSTK (Fig. 8B), bands corresponding to phosphorylated rWalR appeared identical regardless of manganese or magnesium inclusion (Fig. 25A). A faint band was observed in kinase reactions containing rWalR alone and manganese, which we attribute to free acetylphosphate present upon partial \( [\gamma-^{32}\text{P}]\text{ATP} \) degradation.

**Phosphorylation of WalR is reversible.** Since **stk** and **stp** are encoded together on a common RNA transcript (Fig. 9) and able to reversibly phosphorylate the non-specific substrate MBP (Fig. 10A), we predicted that they may partner to phosphorylate and dephosphorylate other substrates. To explore this, we incubated rSTKcat and rWalR in the presence and absence of rSTP. As seen in Fig. 25B, two bands corresponding to rSTKcat and WalR were seen in the autoradiogram, with rSTKcat appearing more intense than the substrate rWalR, as was seen in Fig. 25A. Upon addition of rSTP to the reaction mixture, both of these bands were abolished, demonstrating that rSTP is capable of reversing rSTKcat-mediated phosphorylation of rWalR.

**Discussion**

YycG/F is the most highly conserved TCRS in low-G+C Gram-positive bacteria [181]. It is named differently depending on the organism involved, and is termed WalK/R in the case of *S. aureus* (Fig. 26). The protein pair is essential in *S. aureus* and *B. subtilis* [178, 182]. In functional assays, the response regulator WalR has been shown to activate transcription of cell wall hydrolases, resulting in increased levels of peptidoglycan crosslinking and glycan chain length when it and its cognate histidine kinase are depleted [183]. A mutation in *walR* causes increased susceptibility to macrolide antibiotics,
purportedly due to defects in the permeability of the cell wall [182]. A nucleotide recognition motif has been proposed for WalR. Interestingly, it is found in the promoter regions of not only genes involved in cell wall synthesis, but also virulence genes such as antigenic proteins (SsaA and IsaA) and transport systems [181], suggesting that this protein may have widespread regulatory effects. This may explain in part why VicR and VicK mutants in *S. pyogenes* are significantly less virulent than the wild type in mouse models [180].

The response regulator YycF and WalR from *B. subtilis* and *S. aureus* respectively are 74% identical in their amino acid sequences and are predicted to bind the same DNA sequences [181]. Many of the genes controlled by this system in *B. subtilis*

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**Fig. 26: Nomenclature of YycF/G orthologs.** The TCRS YycF/G is encoded by overlapping genes corresponding to the response regulator (RR) and histidine kinase (HK). Although highly conserved, each gene has been given a different name depending on the organism involved. A relevant selection of these is shown here.
are implicated in cell wall metabolism [184]. These include \textit{ftsA} and \textit{ftsZ}, encoded by the \textit{ftsAZ} operon. YycF can directly bind to the \textit{ftsAZ} P1 promoter, resulting in transcriptional activation as shown by a \(\beta\)-galactosidase reporter assay [178]. In Gram-positive bacteria, the \textit{ftsAZ} operon is consistently found at the 3’ end of a highly conserved collection of genes known as the cell wall-cell division (\textit{dcw}) gene cluster [177]. \textit{Dcw} clusters also contain genes involved in peptidoglycan biosynthesis, particularly Mur ligases and PBPs [177].

Regulation of \textit{dcw} gene clusters is not well understood, as they vary in size and content [177]. However, commonalities between them (such as the presence of \textit{ftsAZ}) imply that they share regulatory mechanisms. In \textit{E. coli}, there is an abundance of promoters within the \textit{dcw} cluster, but just a single predicted terminator, suggesting that many of these genes are transcribed into a single long polycistronic transcript [185]. Indeed, transcription of \textit{ftsAZ} is controlled by factors both within and outside the \textit{dcw} cluster, with “significant contributions” originating in the upper half of the \textit{dcw} cluster [186]. Thus it is reasonable to predict that regulators of \textit{ftsAZ} may also affect transcription of other genes within these clusters.

The \textit{S. aureus} genome contains two \textit{dcw} clusters, the most highly conserved of which encodes \textit{pbpA} at its 5’ end [177], a gene that was down-regulated in the absence of \textit{stk} or \textit{stp} and \textit{stk} (Fig. 23). We hypothesized that this may be due to modulation of the \textit{dcw} cluster-acting transcriptional activator WalR. Interestingly, orthologous VicRs from \textit{S. pneumoniae} and \textit{S. pyogenes} are essential for growth, but their cognate activating histidine kinases (VicKs) are not [187, 188, Jin, \textit{et al.} unpublished data]. However,
phosphorylation of VicR is absolutely essential for it to elicit its regulatory effects [187]. In the absence of VicK, the source of VicR phosphorylation is not known but could originate from free acetyl phosphate [189] or another kinase.

ESTKs are known to phosphorylate transcription factors in prokaryotes, impacting their ability to bind the target DNA [107, 122, Jin, et al. unpublished data]. Threonine phosphorylation of the response regulator CovR by the ESTK SP-STK in S. pyogenes results in stronger binding to target DNA than aspartic acid phosphorylation (Jin, et al. unpublished data). Mycobacterial ESTK PknL is not only capable of phosphorylating the transcriptional regulator Rv2175c, but they are both encoded on the dcw gene cluster, suggesting a role for ESTKs in the regulation of such cell division genes [121]. In the current study, rSTK and rSTP were capable of reversibly phosphorylating rWalR in vitro (Fig. 25). Based on this, we propose a model in which STK-mediated phosphorylation of WalR results in activation of dcw cluster gene transcription (Fig. 27). In this model, the intracellular catalytic domain of STK autophosphorylates and catalyzes phosphotransfer to the transcription factor WalR, allowing it to activate transcription of dcw cluster genes. In the absence of stk (N315ΔSTK, Fig. 27), WalR is not properly phosphorylated, thus transcription of dcw genes, such as pbpA, is decreased as was observed in this strain (Fig. 23). Similarly, in the absence of stp, this system is unregulated and WalR is constitutively phosphorylated, resulting in up-regulation of these genes, including pbpA (Fig. 23).
Fig. 27: Proposed model of dcw gene cluster regulation in *S. aureus*. STK autophosphorylates and catalyzes phosphotransfer to WalR, enabling it to bind tightly to promoters within the dcw gene cluster (P1, P2, P3) and activate transcription.
CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

Summary

In the previous six chapters, we have investigated the role of the one-component regulatory protein STK and its cognate phosphatase (STP) in staphylococcal biology, specifically cell division and peptidoglycan synthesis. While many previous studies have reported ESTK- and ESTP-specific mutants in Gram-positive pathogens, ours is the first to characterize both single (N315ΔSTP and N315ΔSTK) and double (N315ΔSTP/STK) mutants in the multidrug-resistant pathogen *S. aureus*. Among published reports on Gram-positive ESTK and ESTPs, estp-specific mutants were either made but not characterized, found to be non-viable, or never attempted.

Driving the study is the initial finding of abnormal cell division in the absence of stp and stk as well as altered cell wall morphologies in the absence of either gene on its own, as observed by transmission electron microscopy. As N315 is a multidrug resistant *S. aureus* strain, these results led us to investigate the antibiotic susceptibility profiles of each mutant. Upon doing so, it became clear that STK plays a major role in modulation antibiotic resistance in *S. aureus*, as N315ΔSTK and N315ΔSTP/STK strains were
progressively more sensitive than N315 to cell wall-acting antibiotics. PBPs, enzymes responsible for peptidoglycan assembly, are major targets of such antibiotics. We predicted that the regulation of PBPs (either transcriptionally or post-translationally) by STK and/or STP may be contributing to the observed alterations in cell wall-acting antibiotic susceptibilities. Using real-time PCR, we quantified the transcript levels of \( pbps \) in each mutant, which correlated well with the antibiotic susceptibility patterns. We observed increased expression of all \( pbps \) in the antibiotic resistant strain N315ΔSTP, and in accordance with increased antibiotic susceptibilities, we observed a significant decrease in \( pbpA \) transcript levels in the absence of \( stk \) (Fig. 23). These results suggest that STK positively regulates expression of PBPA. Based on these findings, we investigated the possibility of a novel regulatory pathway linking ESTK to \( pbpA \) transcription.

In \textit{S. aureus}, \( pbpA \) is encoded by the cell wall/cell division (\textit{dcw}) gene cluster, which has been shown to be regulated by the TCRS WalR/K. We therefore propose a mechanism whereby direct serine and/or threonine phosphorylation of the response regulator and transcriptional activator WalR by STK facilitates transcription of \( pbpA \) (Fig. 27). This is in contrast to the existing paradigm, which dictates the activation of TCRS response regulators occurs exclusively via phosphorylation of aspartic acid residues by histidine kinases. Cumulatively, our study has provided several novel findings and a better understanding of the critical nature of ESTK-mediated signaling in \textit{S. aureus} cell biology and drug resistance. The conclusion and proposed mechanism open
several new research areas, which, in addition to the implications of this work in the context of public health, certainly merit discussion.

**Future Directions**

The proposed model suggests a novel mechanism of STK-mediated drug resistance in *S. aureus*. Further investigation on the role of STK-phosphorylated WalR in transcriptional regulation of *dcw* genes will certainly help define this regulatory mechanism. As outlined in Chapter 6, we propose that STK-phosphorylated WalR serves as a transcriptional activator of *pbpA* (and other *dcw* genes) by binding to specific promoter regions. The corresponding increase in translation products is predicted to then facilitate cell wall peptidoglycan synthesis and cell division. The ability of *S. aureus* STK to directly phosphorylate WalR (Fig. 25) is the first step towards confirmation of this model. Future directions may include investigations relating the effect of this phosphorylation to WalR function, specifically its ability to bind DNA. Additionally, development of *in vitro* transcription assays would be essential to test the hypothesis that WalR drives *pbpA* transcription. Finally, particularly to apply the findings of this study translationally, future experiments should determine if STK indeed phosphorylates and functionally regulates WalR *in vivo*.

Although the effects of histidine kinase (WalK)-mediated phosphorylation of WalR are known, ours is the first study to investigate the effects of serine/threonine phosphorylation on this enzyme. The presence of both histidine kinases and ESTKs in the *S. aureus* genome suggests that phosphorylation by each can occur in tandem and/or
cooperatively. Aspartic acid phosphorylation is relatively unstable as compared to serine/threonine phosphorylation. In light of this, we propose that, at least in the case of WalR, STK phosphorylation may play a role in stabilizing or enhancing WalK-mediated phosphorylation. Thus, on a broader scale, the implication of both histidine kinase and ESTK phosphorylation on common substrates warrants further investigation.

Despite the fact that PBPs play an essential role in staphylococcal cell wall synthesis, their regulation is not clearly understood. In fact, even the mechanisms of action of PBPs 3 and 4 in peptidoglycan synthesis remain relatively enigmatic. In this regard, understanding the regulation of pbps other than pbpA as described above is another important research avenue to consider, and the current study has provided a starting point for this.

**STK as a Therapeutic Target**

Ultimately, the goal of this work is to identify and characterize novel therapeutic targets (i.e. STP and STK) to help prevent *S. aureus*-mediated disease. The resurgence of MRSA in the community and failure of all available broad spectrum and “front-line” antibiotics in many cases has created a daunting task for researchers and clinicians alike. Additionally, antibiotic development is not high on the priority list for pharmaceutical companies. Since 2000, two thirds of the top fifteen pharmaceutical companies have either cut back or completely eliminated research in this area [190]. This may be partly attributable to the lack of return on investment due to the acute nature of antibacterial therapy, which is not likely to change. The estimated cost of bringing a drug from
development and through testing vacillates between 71 and 900 million U.S. dollars, depending on the study [190]. Obviously new strategies are desperately needed to fight infections caused by \textit{S. aureus} and other pathogenic organisms.

Despite the availability of several antibiotic classes, dozens of formerly potent antistaphylococcals are now considered ineffective [191]. If therapies could be developed to sensitize resistant \textit{S. aureus} isolates to already well-established drugs, the economic benefits would be substantial. Towards this aim, perhaps the most important finding of our study is the observation of increased susceptibility of \textit{S. aureus} to cell wall-acting \(\beta\)-lactam antibiotics in the absence of STK. Using a combinatorial chemical biology approach, it is possible to create specific inhibitors against \textit{S. aureus} STK to block its catalytic activity or ability to sense external stimuli via its PASTA domains. If such inhibitors are developed, based on the current study, we believe STK inhibition may result in dysregulation of PBPs, reviving the effects of formerly obsolete antibiotics. Additionally, as ESTKs have minimal sequence homology to their eukaryotic counterparts [192], STK- and other ESTK-specific inhibitors may even serve as broad-spectrum therapeutics in treating other Gram-positive infections. This would be a unique approach, as current antibiotics primarily target cell wall, DNA, or protein synthesis. As \textit{S. aureus} are quick to alter their cell wall structure to evade the effects of even third generation \(\beta\)-lactam antibiotics, targeting a potentially central enzyme such as STK may prove to be a more long-term option for antibacterial therapy. Our study characterizing \textit{stp} and/or \textit{stk} deletions in the clinical \textit{S. aureus} isolate N315 has already carved out the foundation for the development of such inhibitors.
Final Thoughts

It has only been in recent decades that we have begun to understand the mechanisms behind antimicrobial resistance and the behaviors that facilitate its emergence. In particular, hospital-associated bacteria such as *S. aureus* are developing resistance at an alarming rate. Experts agree that while we cannot stop bacterial evolution, we should not allow it to dampen our efforts to develop new broad-spectrum antimicrobials to treat infected individuals. Examples of broad-spectrum antibiotic targets are investigated in the current study. An equally important approach includes monitoring the transmission of pathogenic organisms. In vulnerable settings such as hospitals, clinics, and other densely-populated areas (detention facilities, nursing homes, etc.), bacterial infections are rampant, and not surprisingly, so is antimicrobial resistance. These are areas where the first resistant staphylococcal strains were identified, as well as many of the most recent. Bacteriologists have identified the *mecA* operon and meticulously outlined the mechanisms of *S. aureus* resistance, but research has only begun to examine the contributing social behaviors in at-risk environments. By understanding and addressing these, we have the potential to drastically reduce the spread of *S. aureus* within and between high-risk populations. Furthermore, just as antimicrobial resistance in HA-MRSA led to its emergence as CA-MRSA, addressing the transmission and accelerated evolution of bacteria in healthcare settings has the potential to retard the dissemination of resistant pathogens into the general public.
The data included in this document provide a foundation for understanding a novel signaling cascade in *S. aureus* which, due to the highly conserved nature of the enzymes involved, can potentially be applied to other organisms, specifically low G-C content Gram-positive bacteria. Basic science studies such as this continue, and are critical to identification of new therapeutic targets. Additionally, to address the ongoing problem of *S. aureus* resistance, the administration of antimicrobials and all interactions with MRSA, and particularly VRSA, should be buttressed with solid policy and thoughtful oversight. Together, this will provide a holistic approach to our continued battle against drug resistant microorganisms, including *S. aureus*. 
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