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Aspects of eukaryotic-like signaling in Gram-positive cocci: a focus on virulence

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Abstract

Living organisms adapt to the dynamic external environment for their survival. Environmental adaptation in prokaryotes is thought to be primarily accomplished by signaling events mediated by two-component systems, consisting of histidine kinases and response regulators. However, eukaryotic-like serine/threonine kinases (STKs) have recently been described to regulate growth, antibiotic resistance and virulence of pathogenic bacteria. This article summarizes the role of STKs and their cognate phosphatases (STPs) in Gram-positive cocci that cause invasive infections in humans. Given that a large number of inhibitors to eukaryotic STKs are approved for use in humans, understanding how serine/threonine phosphorylation regulates virulence and antibiotic resistance will be beneficial for the development of novel therapeutic strategies against bacterial infections.

Keywords

antibiotic resistance; *Enterococcus faecalis*; serine/threonine kinase; *Staphylococcus aureus*; *Streptococcus agalactiae*; *Streptococcus mutans*; *Streptococcus pneumoniae*; *Streptococcus pyogenes*; virulence

In order to survive, all living organisms must respond to changes in their environment. Signal-transducing systems are essential for organisms to respond, survive and adapt to dynamic environmental changes. Protein phosphorylation is central to signal transduction, and is the most important of the 200 different post-translational modifications that have been described [1,2]. In eukaryotes, reversible protein phosphorylation mediates gene expression and protein function to regulate cellular processes, such as differentiation, cell cycle events, cell-type determination and hormone action [3,4]. Similarly, prokaryotes also utilize reversible protein phosphorylation as the primary mode of signal transduction to mediate gene expression and protein function for cellular processes, which include cell growth and division, metabolism, differentiation and adaptation to changes in environmental conditions [5–7].

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Despite the conservation of reversible protein phosphorylation in both prokaryotes and eukaryotes, the signal-transducing systems that mediate these events are distinct. Bacterial signal transduction is mainly accomplished through the action of two-component systems (TCSs) [5–7]. A typical TCS consists of a membrane-associated sensor histidine kinase and a cognate response regulator [5–7]. Environmental signals are detected by the sensor histidine kinase, which then phosphorylates its cognate response regulator at a conserved aspartate residue [5–7]. Aspartate phosphorylation often alters the activity of the response regulator, which binds to DNA and facilitates changes in gene expression or protein function for environmental adaptation [5–7]. By contrast, signaling in eukaryotes is primarily accomplished by a network of protein phosphorylation cascades that require the coordinate action of a number of serine/threonine and tyrosine kinases and their cognate phosphatases. Consequently, it is assumed that signaling in prokaryotic and eukaryotic organisms is mediated by distinct signaling mechanisms.

However, recent advances in genetic strategies and genome sequencing have revealed the presence of ‘eukaryotic-like’ serine/threonine kinases (STKs) and cognate phosphatases in a number of prokaryotes, including *Myxococcus xanthus* [8–10], *Streptomyces* spp. [11,12], *Anabaena* [13,14], *Cyanobacteria* [15], *Bacillus* spp. [16,17], *Streptococcus* spp. [18–21], *Mycobacteria* [22–24], *Yersinia* spp. [25,26], *Listeria monocytogenes* [27,28], *Pseudomonas aeruginosa* [29], *Enterococcus faecalis* [30] and *Staphylococcus aureus* [31–33]. The discovery of eukaryotic-like signaling systems, such as STKs and their cognate phosphatases (serine/threonine phosphatases [STPs]) in bacterial pathogens has sparked an interest in understanding their function. This is partly due to the fact that eukaryotic protein kinases are currently the largest group of drug targets, second only to G-protein-coupled receptors [34,35]. A large number of STK inhibitors are US FDA approved for use in humans [35]; approximately, another 150 kinase inhibitors are also in Phase I or higher level clinical trials [34–37]. In addition, STPs are also being pursued as targets in therapeutic strategies [38–40]. Therefore, studies on the importance of prokaryotic STK and STP in human pathogens have gained interest owing to the prospect that these signaling components may be useful in future anti-infective therapies. However, a complete understanding of their role is a prerequisite for future evaluation of these enzymes as antimicrobial targets.

The prokaryotic homologues of STKs and STPs resemble their eukaryotic counterparts by conserved amino acids (~34.6% amino acid identity to eukaryotic kinase) and, thus, are often referred to as eukaryotic-like enzymes. Typically, prokaryotes contain a single copy of STK and STP, and the genes encoding these enzymes are located within an operon and are cotranscribed. Most prokaryotic STKs are membrane-associated proteins, whereas the cognate phosphatase enzymes are cytoplasmic proteins. The catalytic region of the prokaryotic STKs is located in the N-terminal region and is predicted to be intracellular (within the bacterial cell). This catalytic domain contains 11 conserved subdomains (I–XI, also known as Hanks domains [3]), which form a conserved catalytic core structure (Figure 1) [4]. By contrast, the extracellular C-terminal region of the kinase exhibits a high degree of amino acid sequence diversity among Gram-positive bacteria (~24.5% identity between prokaryotic homologs [41]). Despite the diversity, a characteristic feature of the C-terminal region of the kinase is the presence of one–five repeated structural domains that are known as penicillin-binding protein and STK-associated (PASTA) domains (Figure 1). PASTA domains have been suggested to comprise the sensory component of the kinase [41,42], and are also found in penicillin-binding proteins. While the signals sensed by the PASTA domains of STK are not known, a recent study indicated that peptidoglycan is the ligand for the PASTA domains of the *Bacillus subtilis* STK and mediates exit from dormancy [41,43]. However, the sequence diversity of the C-terminal sensory domain of these kinases and the

disparate lifecycle of the pathogens/prokaryotes that encode these signaling enzymes suggest that the kinases may respond to different ligands for species specific signaling.

Prokaryotic STPs belong to the Mg^{2+} - or Mn^{2+} -dependent protein phosphatase family (PPM) based on structure, metal ion requirement and sensitivity to inhibitors [37,44,45]. These protein phosphatases are further classified as PP2C, based on the conservation of 11 subdomains in the active site of the protein [37,44,45]. Prokaryotic STPs share a nearly identical structure to the eukaryotic PP2C-type phosphatases except that they require a third metal ion and contain a loop above the active site, which may facilitate substrate binding and catalysis [45,46]. In eukaryotes, PP2C-type phosphatases are thought to regulate important biological processes, including stress signaling, cell differentiation, growth, survival, apoptosis and metabolism [45].

The goal of this article is to provide an overview of our current understanding of the role of STKs and their cognate phosphatases in Gram-positive cocci, such as *Streptococcus*, *Enterococcus* and *Staphylococcus* spp. A complete understanding of the substrates and the extracellular ligands of these novel signaling systems will provide greater insight into how they promote environmental adaptation. Conservation of eukaryotic-like signaling in pathogens that need to adapt to harsh environments and diverse host niches during their lifecycle highlights their contribution to the versatile nature of these bacteria and provides the foundation for their evaluation in antimicrobial strategies.

Nomenclature

To date, a number of STKs and phosphatases have been identified in prokaryotes and these are named with reference to the species in which they were identified. Consequently, STKs in prokaryotes have vastly differing names, that include Stk1, SP-STK, StkP, PknB, PrkC, BA-Stk1 and PrkA (discussed later). Similarly, prokaryotic STPs have also acquired various names that include Stp1, PppL, PstP, PrpC, Stp, BA-Stp1 and SP-STP (discussed later). These vastly differing names have caused confusion to researchers particularly to those who are not closely linked to the field. It misleads researchers and leads them to conclude that perhaps similarly named enzymes are homologous to each other in contrast to those with completely different names (e.g., Stks vs Prks). It would greatly benefit the field to have a consistent naming strategy for these conserved enzymes and will also promote their recognition in other prokaryotic organisms.

Streptococcus agalactiae.

Streptococcus agalactiae or group B *Streptococcus* (GBS) is commonly found as a commensal in the lower genital tract of approximately 30% of healthy women [47]. However, GBS is the most common cause of bacterial infections in human newborns, leading to diseases such as pneumonia, sepsis and meningitis [48–50]. GBS is also an emerging pathogen of adult humans; individuals at risk include the elderly, immunocompromised and those with diabetes and malignancies [51–54]. Given that GBS can efficiently transition from commensal environments to invasive niches, it is of interest to understand how the pathogen adapts to diverse environments during its disease cycle. Our studies have established that the STK Stk1 regulates growth and virulence factor expression, and may facilitate the transition of GBS from a commensal organism to an invasive, meningial pathogen [18,55–58].

The open reading frame (ORF) encoding the GBS STK *stk1* and STP *stp1* was identified in a screen for secreted or membrane-associated proteins of GBS that may regulate virulence [18]. Briefly, secreted or membrane-associated proteins that enabled secretion of enterococcal alkaline phosphatase that lacked its endogenous signal sequence were selected

[18]. Further characterization indicated that Stk1 is a membrane-associated, magnesium-dependent kinase, and Stp1 is a cytoplasmic, manganese-dependent phosphatase [18]. GBS deficient for Stk1 or both Stk1 and Stp1 (double mutant) exhibited a 25–100-fold decrease in virulence in the neonatal rat sepsis model of infection, and also showed an increase in lag-phase growth [18]. *In vitro* phosphorylation assays indicated that a manganese-dependent inorganic pyrophosphatase (PpaC) predicted to influence general metabolism is a Stk1 target [18]. The inability of the Stk1-mutant GBS strains to sustain *de novo* purine biosynthesis was later linked to altered function of PurA encoding adenylosuccinate synthetase [55].

Studies on the role of Stp1 and Stk1 in GBS resistance to host defenses revealed that Stk1 positively regulates the transcription of an important GBS toxin, known as β -hemolysin/cytolysin (β -H/C) [56]. Extensive studies have established the importance of β -H/C to GBS host cell invasion, induction of host inflammatory responses and virulence [59–68]. Transcriptional regulation of β -H/C by Stk1 was linked to threonine phosphorylation of the DNA binding response regulator CovR that alleviated CovR repression of β -H/C [56,69–71]. Threonine phosphorylation of CovR at a position 65 (T65) decreased CovR phosphorylation at the conserved aspartate residue in position 53 (D53) and, thus, prevented promoter binding and transcriptional repression of β -H/C [57]. These observations indicate how regulation of an important TCS (CovR/CovS) of GBS is achieved by the coordinate action of two distinct sensor kinases (i.e., CovS and Stk1). This novel mode of regulation enables GBS to fine-tune virulence factor expression that may be beneficial during its transition from commensal to invasive niches [57,58]. Crosstalk between STKs and TCS was also reported in other pathogens, such as *Streptococcus pneumoniae* (see section on *S. pneumoniae*) [72] and *Mycobacterium tuberculosis* [73].

In vivo phosphopeptide enrichment and mass spectrometric methods revealed that Stk1 also phosphorylates GBS proteins involved in cell division and segregation [74]. These observations are consistent with the extensive chain formation observed in the Stk1 mutants [18]. The role of Stk1 targets involved in cell division, namely DivIVA, DivIVA domain and FtsZ [74], in growth and virulence of GBS is currently under investigation. Although previous attempts to construct a GBS strain deficient only in *Stp1* expression were unsuccessful [18], advanced genetic strategies have recently enabled us to derive GBS mutants deficient only in *Stp1* [Rajagopal L *et al.*, Unpublished Data]. Characterization of this mutant's growth and virulence are in progress and will provide a comprehensive picture of eukaryotic-like signaling in environmental adaptation of this versatile pathogen.

Streptococcus pyogenes.

Streptococcus pyogenes, also known as group A *Streptococcus* (GAS), is an important human pathogen that causes a multitude of devastating diseases in humans, including toxic shock-like syndrome, rheumatic fever and necrotizing fasciitis [75–78]. Notably, GAS encodes eukaryotic-like signaling enzymes that are homologous to Stp1 and Stk1 of GBS. These are denoted as SP-STK for *S. pyogenes* STK and SP-STP for *S. pyogenes* STP [21]. Studies by Jin *et al.* have shown that SP-STK and SP-STP are functional, manganese-dependent kinase and phosphatase enzymes, respectively [21]. While the role of SP-STP in GAS is, as yet, unknown, SP-STK mutants exhibited altered cell morphology as manifested by an increase in colony size, distorted cell shape, incomplete septation, loosely associated electron-dense layer and a tendency to settle during growth in laboratory media [21]. The mutants also exhibit increased doubling time and a 50% increase in hemolysin activity [21].

Appropriate activity and localization of SP-STK are important for regulation of GAS virulence functions. GAS mutants lacking either the kinase or extracellular domain or the entire SP-STK gene exhibited decreased adherence to human pharyngeal cells (four- to 20-

fold) and decreased resistance to phagocytic killing (four- to 170-fold [21]). Consistent with these observations, SP-STK mutants had lower levels of the anti-phagocytic M1 protein, which also contributes to formation of the electron-dense layer [79]. These results indicated that SP-STK positively regulates expression of proteins important for GAS virulence [21]. Recently, Pancholi *et al.* suggested that the altered cell division observed in the GAS SP-STK mutants is due to a fourfold decrease in expression of a novel virulence-associated protein, CdhA, which facilitates early events in cell division and cell wall synthesis [80]. A possible mechanism for SP-STK-mediated regulation of virulence factors such as M1 protein, hemolysin and CdhA may be linked to its ability to phosphorylate a 10-kDa histone-like protein (SP-HLP) [21]. In bacteria, HLPs have been described to regulate DNA transcription and replication [81–85]. Taken together, these studies suggest that SP-STK is able to modulate transcription and expression of GAS virulence factors via post-translational regulation of a DNA-binding HLP [21,80]. This mode of transcriptional regulation appears to be a conserved function of STKs in certain Gram-positive pathogens. Support for this is provided by our recent findings in *S. aureus* where mutants deficient for Stk1 expression exhibit increased transcription of a hemolysin, known as α -toxin; this change in hemolysin expression is likely due to its ability to phosphorylate the DNA-binding HLP (HU; for details, see section on *S. aureus* and [86]).

Streptococcus pneumoniae.

Streptococcus pneumoniae is a versatile human pathogen that is the causal agent of pneumonia, meningitis, septicemia and otitis media [87]. Eukaryotic-like signaling in *S. pneumoniae* has been studied extensively and found to regulate pleiotropic functions that include virulence, competence, antibiotic resistance, growth and stress response of the pathogen [20,88–94]. The kinase StkP and the cognate STP PppL are manganese-dependent enzymes [88]. Interestingly, StkP has been described to promote persistence of *S. pneumoniae* *in vivo*. StkP-deficient *S. pneumoniae* showed decreased survival in pneumonia and sepsis models of infection (~ten- and 100-fold, respectively [20]). Furthermore, increased expression of StkP was observed in blood (5.3-fold), brain (3.9-fold) and lungs (5.9-fold) of infected mice compared with expression during *S. pneumoniae* growth in laboratory media [90]. Notably, antibodies to either the PASTA domain or the entire C-terminal region of StkP conferred significant protection from lethal sepsis (30 and 60% protection, respectively [91,92]). Thus, StkP is a vaccine candidate due to its pivotal roles in *S. pneumoniae* virulence and in immunity to infections.

StkP also regulates competence of *S. pneumoniae*. Competence was first discovered in *S. pneumoniae* and is important for pathogenesis as it enables the bacteria to acquire exogenous DNA containing either virulence or antibiotic resistance determinants [95]. Mutants of StkP have been described to exhibit decreased competence [20,89,94]. Despite the decrease in competence, Saskova *et al.* observed a ten- to 30-fold increase in expression of the central competence regulon *comCDE* in an StkP mutant [89]. However, as addition of the competence-stimulating peptide (CSP) did not rescue the decreased transformation of the StkP mutant, the authors predicted that expression of the competence regulon was not at sufficient levels to permit activation of the full competence cascade [89]. By contrast, Osaki *et al.* reported that CSP was indeed able to rescue the decreased transformability of the StkP mutant [94]. They further noted that the loss of StkP function led to a fivefold increase in mutation rate leading to the suggestion that the hypermutability of this strain may in part account for the discrepancy between the two studies [94]. In addition to regulating the competence regulon, StkP was also described to regulate the transcription of approximately 4% of the *S. pneumoniae* genome that includes genes involved in cell wall biosynthesis, oxidative stress, DNA repair, iron uptake and metabolism of glycerol, purines and

pyrimidines [89]. Consistent with these observations, StkP mutants showed increased sensitivity to acid pH, temperature, oxidative and osmotic stress [89].

In order to gain insight into the role of StkP in *S. pneumoniae*, multiple approaches were used to identify target proteins of StkP. Using phage display, the DNA-binding protein RitR was identified as a binding partner for PhpP (PppL) and StkP [72]. StkP and PhpP were then shown to reversibly phosphorylate RitR *in vitro* [72]. RitR is a repressor of the *S. pneumoniae* iron-transport system and is important for virulence of the pathogen [96,97]. Interestingly, RitR shares 45% similarity with CovR of GBS, which is regulated by the GBS Stk1 enzyme [56,57,72]. How serine/threonine phosphorylation affects RitR function is not known.

Using radio-labeled phosphoproteins, 2D gel electrophoresis and mass spectroscopy, phosphoglucosamine mutase (GlmM) and the α -subunit of RNA polymerase (RpoA) were also identified as substrates of StkP [88]. In order to function properly, bacterial transcriptional activators sometimes depend on their interaction with the C-terminal domain of RpoA [88,98]. Thus, StkP may regulate gene expression by controlling the interaction between RpoA and RitR in *S. pneumoniae*. As GlmM catalyzes the first step in cell wall biosynthesis [99,100], phosphorylation of GlmM suggests a role for StkP in *S. pneumoniae* cell wall biosynthesis. Support for the role of StkP in cell wall metabolism is also provided by observations that StkP mutants showed increased sensitivity to antibiotics, such as penicillin, vancomycin and ceftazidime [92,93]. Whether the changes in antibiotic resistance of StkP mutants are linked to altered GlmM function is not known. Likewise, although StkP can phosphorylate the prokaryotic tubulin homologue FtsZ *in vitro*, the *in vivo* relevance is not clear as there was no difference in phosphorylated FtsZ between *S. pneumoniae* strains proficient and deficient for StkP [91]. Collectively, the aforementioned studies provide evidence that StkP mediates virulence, competence, antibiotic resistance, cell division, gene expression and stress responses of *S. pneumoniae*. Since *S. pneumoniae* strains deficient only in expression of the phosphatase were not derived, it is thought to be an essential gene [94].

Streptococcus mutans.

Streptococcus mutans is the principle causative agent of dental caries [101,102]. Virulence of the bacteria has been attributed to its versatile nature as the pathogen can efficiently adapt to harsh environmental conditions and thrive in a mixed-species biofilm on tooth surfaces [103–105]. The *S. mutans* STK PknB and phosphatase PppL have been shown to contribute to virulence and competence functions, as observed in *S. pneumoniae* [19,20,89,94,106,107].

S. mutans deficient in PknB expression exhibit changes in traits that are important for virulence. These include weaker biofilm formation, increased sensitivity to oxidative and osmotic stress, decreased growth rate at acid pH (5.0) and reduced transformation efficiency [19,106,107]. Studies by Banu *et al.* also showed that kinase mutants exhibit reduced cariogenicity in a rat model of dental caries [106]. Significantly less initial dentinal fissures (score of 10.9 vs 12) and advanced dentinal fissures (score of 9.5 vs 11.2) were observed with PknB-deficient strains of *S. mutans* compared with the wild-type [106]. However, these mutants did not exhibit defects in colonization *in vivo* [106] despite a defect in biofilm formation *in vitro* [19,106,107]. By contrast, the phosphatase PppL mutant showed a 20% decrease in colonization in the rat caries model [106]. Together, these results suggest that PknB and PppL positively regulate growth and virulence functions of *S. mutans in vitro* and *in vivo*.

Microarray analysis revealed that PknB regulates the transcription of 67 genes involved in growth and virulence [106]. These include genes involved in bacteriocin production, competence regulation, osmotic stress and cell wall remodeling [106]. Decreased expression of bacteriocin genes was confirmed in competition assays where the *S. mutans* PknB mutant showed less inhibition of *E. faecalis* compared with wild type [106]. Decreased survival of the *S. mutans* PknB mutant was also observed in interspecies biofilms with *Streptococcus sanguis* (~55%) [107]. These results suggest that PknB-mediated regulation of virulence genes is important for survival *in vivo*.

The mechanisms of PknB regulation of *S. mutans* competence are not entirely clear. Earlier studies by Hussain *et al.* indicated that addition of the CSP failed to rescue the transformation deficiency of the PknB mutant [19,106]. Subsequent studies from the same group indicated that addition of CSP indeed did rescue the reduced transformability of the same PknB mutant [106]. Banu *et al.* suggested that the disparate results may be due to the hypermutability of the *S. mutans* PknB mutant as was observed with StkP-deficient strains of *S. pneumoniae* [94,106]. They also hypothesized that secondary mutations led to a loss of responsiveness to CSP; however, no experiments were performed to specifically address this hypothesis [106]. How StkP mediates hypermutability in *S. mutans* or *S. pneumoniae* is not known, and requires further investigation. Collectively, the studies describe that eukaryotic-like signaling enzymes regulate important virulence properties of *S. mutans* that include growth in an acidic environment, ability to form biofilms, response to stress and competence functions. Defining the molecular mechanisms of PknB-mediated regulation of gene expression and virulence is necessary to better understand the role of eukaryotic-like signaling in *S. mutans*.

***Enterococcus faecalis*.**

Enterococcus faecalis is commonly found as a commensal organism in the GI tract. However, *E. faecalis* is also a human pathogen that can cause urinary tract infections, surgical wound infections, bacteremia, sepsis and infective endocarditis [108]. Similar to *S. mutans*, *E. faecalis* can survive harsh environmental stresses, including antibiotics [109–112]. *E. faecalis* is intrinsically resistant to numerous cell-wall-acting antibiotics making treatment of infections problematic. Kristich *et al.* examined the role of the eukaryotic-like STK (PrkC) in intestinal persistence and antibiotic resistance of *E. faecalis* [30]. They observed that PrkC-deficient *E. faecalis* exhibited increased sensitivity to antibiotics, such as cefotaxime and broad-spectrum cephalosporins (two- to 768-fold) [30]. Increased sensitivity of the PrkC mutant was specific for the cell wall-acting antibiotics, as there was no difference in susceptibility of PrkC mutants to antibiotics that inhibit ribosomal function [30]. PrkC was also described to be important in preserving cell-wall integrity as *E. faecalis* deficient for PrkC displayed morphological defects, including ‘ghost cells’ that were devoid of genetic material and had cell wall lesions or collapsed cells [30]. Thus, PrkC may enable *E. faecalis* to mediate cell wall homeostasis and intrinsic resistance to antibiotics [30].

PrkC is also thought to promote bacterial persistence during infections. In orally inoculated mice, PrkC mutants showed increased sensitivity to bile and decreased intestinal persistence [30]. Thus, the STK PrkC of *E. faecalis* is thought to promote early intestinal colonization, mediate antibiotic resistance and facilitate persistence of the organism during infections [30]. Despite the crucial role of serine/threonine phosphorylation in antibiotic resistance and *in vivo* persistence of *E. faecalis*, a detailed understanding of the molecular mechanisms underlying these functions is still lacking. In addition, the role of a putative eukaryotic-like serine/threonine PP2C-type phosphatase (PrpC) in *E. faecalis* is not known.

Staphylococcus aureus.

Staphylococcus aureus is a commensal organism that asymptotically colonizes the skin, upper respiratory and GI tracts. *S. aureus* is also a major human pathogen that is currently the leading cause of invasive infections in community and healthcare settings [113–115]. Similar to *Enterococcus* spp., infections due to antibiotic-resistant *S. aureus* strains, such as methicillin-resistant *S. aureus* (MRSA), are increasing and are of major concern [113–120]. A number of studies have examined the role of the eukaryotic-like signaling enzymes in *S. aureus* and serine/threonine phosphorylation in *S. aureus* has been reviewed recently [31,86,121–125]. The *S. aureus* STK (known as Stk, Stk1 or PknB) and phosphatase (known as Stp or Stp1) are manganese-dependent enzymes. Growth of *S. aureus* in radiolabeled orthophosphate revealed that 11 phosphoproteins with predicted functions in metabolism are phosphorylated at serine/threonine residues [125]. However, the role of phosphorylation of these targets and the link to Stk1 has not been established *in vivo*. For the purposes of this article, we will compare the role of Stk1 and Stp1 in virulence and antibiotic resistance of both methicillin-resistant and -sensitive *S. aureus*.

Role in antibiotic resistance

Recent studies have revealed the role of Stk1 in antibiotic resistance of MRSA. Interestingly, MRSA strains N315, COL, USA300 and USA400 deficient in Stk1 (PknB) expression showed increased susceptibility to cell-wall-acting β -lactam antibiotics, including (but not limited to) nafcillin, cefazolin, cefotaxime, ceftriaxone and ertapenem [31,33,122,124] [Burnside K & Rajagopal L, Unpublished Data]. Transcription of the genes *mecA* or *pbp2a*, which is responsible for increased β -lactam or methicillin resistance in MRSA, was similar to the wild-type in the Stk1 mutants of USA300 [124]. These observations suggest that Stk1-mediated regulation of antibiotic susceptibility is independent of Pbp2a. Stk1 also regulates antibiotic susceptibility in certain methicillin-sensitive *S. aureus* (MSSA) strains, such as 8325, where mutants showed a modest twofold increase in susceptibility to methicillin and tunicamycin [122]. However, in the MSSA strain Newman, no significant differences in susceptibility to cell-wall-acting antibiotics were observed [86]. How Stk1 mediates resistance of MRSA to β -lactam antibiotics (similar to its role in *E. faecalis*, see previous section) is not known and is of interest. In addition, Stk1 has been described to positively regulate resistance to hydrophilic quinolone antibiotics in MSSA 8325-4 through phosphorylation of MgrA, which, in turn, upregulates expression of the NorA efflux pump [32]. Thus, Stk1 regulates multiple antibiotic resistance mechanisms in *S. aureus*.

Role in virulence

Stk1 and Stp1 also regulate virulence factor expression in MSSA and MRSA. A critical virulence factor of *S. aureus* is the hemolysin known as α -toxin. Extensive studies have described the role of α -toxin in virulence of *S. aureus* and also in induction of protective immunity [126–134]. Studies in MSSA Newman [86] and others on MRSA USA300 [124] indicated that hemolysin expression is increased in the Stk1 mutants. The increase in hemolysin expression correlated with an increase in transcription of the gene *hla*, encoding α -toxin [86,122,124]. Furthermore, Stp1 mutants of MSSA Newman had decreased α -toxin transcription and expression [86]. Therefore, Stk1 and Stp1 oppositely regulate the transcription of an important *S. aureus* virulence factor. Consistent with these observations, Stk1 and Stp1 were found to contribute to virulence of *S. aureus* [86]. Increased abscesses and cytokine IL-6 expression were observed in the kidneys of mice infected with Stk1 mutants [86]. By contrast, Stp1 mutants of *S. aureus* Newman showed severe virulence attenuation and decreased IL-6 expression in the kidneys of infected mice (for details, see [86]). Similar results on regulation of α -toxin expression and virulence were observed with

MRSA USA300 and USA400 [Burnside K & Rajagopal L, Unpublished Observations]. In addition, Tamber *et al.* recently described that Stk1 mutants of USA300 showed increased lesion size and severity in a murine skin abscess model of infection [124]. Taken together, these studies suggest that the absence of Stk1 increases the severity of *S. aureus* infections.

In order to understand Stk1 regulation of *S. aureus* toxin expression and virulence, phosphopeptide enrichment and mass spectroscopy were utilized to identify its targets [86]. These methods are superior as proteins/peptides that are phosphorylated *in vivo* (in the bacterium) are identified. A comparison of serine/threonine phosphorylated proteins in MSSA Newman to those identified in the kinase-deficient mutant indicated that threonine phosphopeptides corresponding to DNA-binding HLP HU, the serine/aspartate-rich bone sialoprotein binding protein, SdrE, and a hypothetical protein were unique to the Stk1-expressing strain (wild-type) [86]. As mentioned previously, histone-like DNA-binding proteins regulate prokaryotic DNA replication and transcription, similar to the role of histones in eukaryotes [81–85]. The phosphothreonine residue identified for HU is localized to the N-terminus of the protein, which is consistent with phosphorylation at a critical N-terminal serine residue in eukaryotic histones [86,135,136]. Given that SP-STK of *S. pyogenes* was also described to phosphorylate a HLP at an N-terminal threonine residue [21], it is likely that transcriptional regulation through reversible phosphorylation of DNA-binding HLPs is a conserved function of STKs in certain Gram-positive pathogens.

Contrary to observations that the presence of Stk1 dampens *S. aureus* virulence, Debarbouille *et al.* reported that Stk1 is important for virulence of *S. aureus* 8325-4. In a murine pyelonephritis model, a ten- to 100-fold decrease in bacteremia was observed during infection with the Stk1- or Stp1-deficient MSSA 8325-4, respectively [123]. As 8325-4 harbors a small deletion in a positive regulator of the σ B stress response *rsbU*, Debarbouille *et al.* also analyzed virulence potential of Stk1 mutants in *S. aureus* that was restored for σ B regulation (MSSA SH1000, 8325-4 *rsbU*⁺ derivative) [123]. Milder inflammatory lesions and decreased bacterial load were observed in the kidneys of mice infected with Stk1-deficient SH1000, suggesting that Stk1 positively regulates *S. aureus* virulence, independent of σ B [123]. Despite the observation of virulence attenuation in 8325-4, it is noteworthy that microarray analysis indicated that significantly upregulated genes in the Stk1 mutant included *hla* encoding α -toxin, as well as genes encoding β and γ hemolysins [122]. These results indicate that despite the increase in expression of certain toxins, Stk1 mutants of 8325-4 and SH1000 are attenuated for virulence.

Interestingly, Stk1 mutants of *S. aureus* 8325-4 and SH1000 show deficiencies in purine biosynthesis. Microarray analysis indicated that genes that were significantly downregulated in Stk1-deficient 8325 included those involved in nucleotide biosynthesis [122]. Consistent with these findings, the decreased growth of the 8325-4 Stk1 mutant in defined media (RPMI 1640) was complemented by the addition of exogenous purines [123]. Donat *et al.* further described that Stk1 phosphorylates the purine biosynthesis regulator PurA and phosphorylation inhibits PurA activity [122], similar to observations in GBS [55]. Although Debarbouille *et al.* suggest that the decrease in virulence is independent of purine auxotrophic requirements due to similar bacterial load in the bloodstream of infected mice during the course of infection [123], further studies are essential to understand the contribution of Stk1 to growth and virulence of *S. aureus* 8325-4 and SH1000. It is also noteworthy that Stk1- and Stp1-deficient mutants of MSSA Newman did not exhibit any requirement for purines or pyrimidines during growth in chemically defined medium [86]. Furthermore, no significant difference in expression of purine or pyrimidine biosynthetic genes was observed in Stk1-deficient *S. aureus* Newman [86]. Collectively, these observations suggest that signaling mediated by this novel class of signaling enzymes is multifaceted and can sometimes have strain-specific manifestation within a given bacterial

species. This feature is also being increasingly encountered with the role of TCSs in bacterial gene regulation and virulence [56,58,69,70,73,137–139].

Recent studies by Miller *et al.* provide an additional role for Stk1 in *S. aureus* pathogenesis. The authors indicate that PknB (Stk1) is secreted by *S. aureus* and thus has the potential to phosphorylate host cell proteins during infections [140]. Using *in vitro* phosphorylation of peptide microarrays, 68 potential human targets of PknB were identified and included proteins involved in apoptosis, immune response, transport and metabolism [140]. Based on the presence of a conserved proline residue in the identified phosphorylation targets of PknB, the authors suggest that PknB is a proline-directed 'MAPK-like' enzyme [140]. In addition, PknB was observed to directly phosphorylate the activating transcription factor-2 (ATF-2), which may permit *S. aureus* to evade intracellular killing [140]. Thus, PknB is potentially the first prokaryotic representative of the proline-directed kinases [140]. However, given that these studies were performed *in vitro*, identification of PknB specific host targets during *S. aureus* infection is necessary to determine how PknB-mediated phosphorylation of host proteins during infection benefits *S. aureus* pathogenesis. As changes in α -toxin expression were observed in Stk1 mutants of both MSSA and MRSA even in the absence of host cells [86,124], these results indicate that Stk1 regulates bacterial functions independent of the host. Furthermore, the increased virulence and abscess formation observed with the Stk1 mutants [86,124] suggests that the absence of the kinase promotes virulence in the animal model. Whether secretion of the kinase decreases the host immune response to *S. aureus* infection is not known. Further analysis will provide more insight into the role of these enzymes in virulence of a pathogen that is currently the leading cause of invasive infections.

Advances with other organisms

The purpose of this article is to highlight the role of STKs and phosphatases in pathogenic, Gram-positive cocci. However, it is important to emphasize that these signaling enzymes have been studied extensively in other pathogenic and nonpathogenic bacteria. These include *B. subtilis* [16,43,141–143], *B. anthracis* [17], *M. tuberculosis* [22,144–151] and *L. monocytogenes* [27,28]. A brief summary of the role of eukaryotic-like signaling enzymes in these organisms is provided (also see [150]). In the Gram-positive, spore-forming rod-shaped soil bacterium *B. subtilis*, the STK (PrkC) and phosphatase (PrpC) regulate biofilm formation, germination, growth and exit from dormancy, which, in part, is linked to reversible phosphorylation of elongation factor-G [16,43,141–143]. *B. anthracis* is a Gram-positive spore-forming rod that is the causal agent of anthrax. The STK, BA-Stk1 and phosphatase BA-Stp1 have been shown to regulate virulence of *B. anthracis* [17] suggesting that virulence regulation by STKs is a conserved function in Gram-positive pathogens. *M. tuberculosis*, the causative agent of tuberculosis, encodes eleven STKs; however, only PknB possess the extracellular PASTA domains commonly found in the homologous STKs reviewed here [22,144,150,151]. Unlike the STKs reviewed in this article, PknB is essential in *M. tuberculosis* [145–147], and is the first prokaryotic STK whose crystal structure has been resolved [148,149]. PknB has been described to mediate cell shape [146] and phosphorylated targets include proteins involved in cell wall synthesis and the stress response [150]. *L. monocytogenes* is a Gram-positive rod-shaped bacterium that is the causative agent of listeriosis. Recently, the STK PrkA has been shown to be functional and interacts with 62 bacterial proteins including those involved in cell wall synthesis [28]. While the role of PrkA in virulence of *L. monocytogenes* is not known, the STP, Stp, is necessary for growth in a murine model of infection [27] and elongation factor-Tu is a substrate for Stp in *L. monocytogenes* [27]. Collectively, these observations emphasize the importance of this novel class of signaling enzymes in prokaryotic organisms.

Conclusion & future perspective

The persistence of bacterial infections in humans and the emergence of antibiotic-resistant strains emphasize the need for novel therapeutic measures. Recent strategies have focused on the development of effective vaccines for prevention of bacterial infections. One disadvantage is that vaccines are not suitable in the treatment of infections. In order to sustain treatment of bacterial infections in humans, identification of alternate drug targets is pivotal. A greater understanding of molecular mechanisms underlying bacterial disease pathogenesis will be beneficial in the identification of novel drug targets and for further development of vaccines. Advances in genome sequencing have increased our understanding of virulence factors and their regulation in bacterial pathogens. Pathogenic bacteria commonly utilize signaling systems to regulate the expression of virulence factors during disease progression and for environmental adaptation. Previously, this was thought to be controlled mainly by TCSs [5–7]. The availability of whole genome sequencing has also revealed the presence of eukaryotic-like STK and phosphatase signaling enzymes in many bacterial pathogens. Signaling by STKs and STPs have been shown to mediate virulence of pathogens, including, but not limited, to the *Streptococcus*, *Enterococcus* and *Staphylococcus* spp., which are reviewed in this article (for a recent review on STK and STP in bacteria, see [150]). Regulation of important characteristics and their increasingly ubiquitous occurrence emphasizes the significance of these eukaryotic-like signaling systems in prokaryotes.

Although STKs and phosphatases regulate important functions in bacterial pathogens, our understanding of the signal transduction mechanism is still in its infancy. Despite the pleiotropic nature of mutants, we can conclude that serine/threonine phosphorylation regulates virulence, antibiotic resistance, nutrient biosynthesis and cell morphology of bacterial pathogens (Table 1). The contribution of these signaling enzymes to bacterial growth and pathogenesis is multifaceted as can be expected for any signaling system. The mechanism for how these signaling enzymes mediate diverse functions in a coordinated fashion remains to be completely understood. In *S. agalactiae* (GBS), *S. pyogenes* (GAS), *S. pneumoniae* and *S. aureus*, STK has been shown to regulate gene expression [20,56,80,86,89,122,124]. This can be attributed to post-translational regulation of DNA-binding proteins, such as two-component response regulators (CovR or RitR [56,72]) or HLPs (SP-HLP or HU [21,86]). Identification of extracellular signals that are sensed by these signaling systems for regulation of gene expression or protein function/s is necessary for a comprehensive view of serine/threonine phosphorylation in prokaryotic signal transduction.

A conserved function of STKs in the Gram-positive pathogens reviewed here is regulation of cell-wall biosynthesis and susceptibility to cell-wall-acting antibiotics. In *S. pneumoniae*, *E. faecalis* and *S. aureus*, STK mutants showed increased susceptibility to certain cell-wall-acting antibiotics [30,31,33,92,93,122,124]. An indirect association of GAS SP-STK with antibiotic resistance stems from observations that mutants of the putative SP-STK target CdhA showed increased susceptibility to cell wall-acting antibiotics [80]. As these pathogens are leading causes of invasive infections in humans, it is imperative to define how STKs regulate antibiotic resistance. Whether the changes in antibiotic resistance are linked to serine/threonine phosphorylation of proteins involved in cell division/morphology and/or regulation of PASTA domains function is yet to be understood. Elucidation of serine/threonine phosphorylation in antibiotic resistance of bacterial pathogens will be beneficial for evaluation of these signaling components in antimicrobial strategies.

Understanding the consequences of serine/threonine phosphorylation in Gram-positive pathogens is also critical for their evaluation as potential vaccines. Immunization with the

STK (StkP) from *S. pneumoniae* conferred protection against lethal challenge in numerous mouse models (sepsis and pneumonia) [91,92]. Furthermore, StkP-mediated protection of lethal pneumonia was highly significant and comparable to the currently available conjugated Prevnar vaccine for *S. pneumoniae* [91,92]. In addition, unlike Prevnar, StkP was immunogenic in all populations that are at risk [91,92]. Currently there are no approved vaccines available for GBS, GAS, *S. mutans*, *E. faecalis* or *S. aureus*. Thus, it would be beneficial to determine whether STKs from these Gram-positive pathogens elicit a protective immune response for prevention of severe disease. Given the pleiotropic nature of bacterial mutants deficient in eukaryotic-like signaling, a greater understanding of the signal transduction pathway will be beneficial to sustain our ability to combat bacterial infections.

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- ▪ of considerable interest

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Executive summary

Streptococcus agalactiae.

- Stk1 contributes to growth and virulence of group B *Streptococcus*.
- Proteins phosphorylated by Stk1 include the two-component regulator, CovR, purine biosynthesis enzymes, PurA, inorganic pyrophosphatase, PpaC and cell division proteins (DivIVA and FtsZ).
- Stk1 phosphorylation alleviates CovR repression of group B *Streptococcus* toxins, such as β -hemolysin/cytolysin.
- The extracellular signals for Stk1 signaling are not known.
- The role of Stp1 is not well understood.

Streptococcus pyogenes.

- *Streptococcus pyogenes* serine/threonine kinase (STK) regulates growth and virulence of group A *Streptococcus*.
- Regulated virulence factors include hemolysin, M1 protein and CdhA.
- DNA-binding histone-like protein (SP-HLP) is phosphorylated by *S. pyogenes* STK.
- The role of phosphorylation on SP-HLP function is not known.
- Extracellular signals for *S. pyogenes* STK and the role of serine/threonine phosphatases are not known.

Streptococcus pneumoniae.

- StkP regulates growth, competence, virulence, *in vivo* persistence, stress response and antibiotic resistance of *S. pneumoniae*.
- Expression of genes involved in cell wall biosynthesis, oxidative stress, DNA repair, iron uptake and metabolism are mediated by StkP.
- Proteins phosphorylated by StkP include the DNA binding protein involved in iron transport (RitR), phosphoglucosamine mutase (GlmM), cell division protein (FtsZ) and the α -subunit of RNA polymerase (RpoA).
- How phosphorylation affects protein function is not understood.
- The role of the phosphatase is not known.

Streptococcus mutans.

- PknB and PppL regulate growth, competence and virulence properties of *S. mutans*.
- PknB regulates the transcription of genes involved in growth, virulence, cell wall metabolism, stress response and bacteriocin production.
- Competence regulation by PknB requires further investigation.
- The role of PknB and PppL in dental caries is not completely understood.

Enterococcus faecalis.

- PrkC regulates antibiotic resistance, growth and virulence of *E. faecalis*.
- PrkC contributes to colonization and bacterial persistence during infections.

- Proteins phosphorylated by PrkC are not known.
- Mechanisms of PrkC-mediated antibiotic resistance, growth and virulence have not been described.
- PrpC has not been evaluated.

Staphylococcus aureus.

- Stk1 regulates growth, gene expression, virulence and antibiotic resistance of methicillin-resistant *S. aureus* (MRSA).
- In MSSA, Stk1 targets include the purine biosynthetic enzyme PurA, virulence gene regulator MgrA, serine/aspartate-rich bone sialoprotein binding protein SdrE and the DNA-binding histone-like protein HU.
- Stk1 has been suggested to phosphorylate host proteins.
- How Stk1 regulates antibiotic resistance of MRSA is unknown.
- Stp1 affects virulence gene expression and *S. aureus* pathogenesis.

Figure 1. The serine/threonine kinase in Gram-positive cocci

STKs of *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus mutans*, *Enterococcus faecalis* and *Staphylococcus aureus* along with their respective gene numbers. The position of the 11 (I–XI) conserved N-terminal Hanks domains, the TM region and the penicillin-binding protein and serine/threonine kinase-associated (PASTA) domains are denoted.

aa: Amino acids; STK: Serine/threonine kinase; TM: Transmembrane.

Table 1

Signaling by a serine/threonine kinase has pleiotropic effects in Gram-positive bacterial pathogens.

Pathogen	Proteins phosphorylated by the kinase	Virulence	Growth	Cell division	Antibiotic resistance	Gene expression	Competence
<i>Streptococcus agalactiae</i> /group B <i>Streptococcus</i>	CovR, PpaC, PurA, FtsZ DivIVA, DivIVA domain	✓	✓	✓	×	✓	NA
<i>Streptococcus pyogenes</i> /group A <i>Streptococcus</i>	SP-HLP	✓	✓	✓	?	✓	?
<i>Streptococcus pneumoniae</i>	RtrR, GlmM, RpoA, FtsZ	✓	✓	?	✓	✓	✓
<i>Streptococcus mutans</i>	Unknown	✓	✓	✓	?	✓	✓
<i>Enterococcus faecalis</i>	Unknown	✓	✓	?	✓	?	NA
Staphylococcus aureus							
Methicillin-sensitive <i>S. aureus</i>	PurA, HU, SdrE, MgrA, hypothetical	✓	SS✓	SS✓	SS✓	✓	NA
Methicillin-resistant <i>S. aureus</i>	Unknown	✓	✓	SS✓	✓	✓	NA

Extracellular activation signals are not known.

NA: Not applicable; SS✓: Strain-specific role; ✓: Stk1-mediated regulation; ×: Lack of serine/threonine kinase regulation.