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Do Shoot the Messenger: PASTA Kinases as Virulence Determinants and Antibiotic Targets

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Abstract

All domains of life utilize protein phosphorylation as a mechanism of signal transduction. In bacteria, protein phosphorylation was classically thought to be mediated exclusively by histidine kinases as part of two-component signaling systems. However, it is now well appreciated that eukaryotic-like serine/threonine kinases (eSTKs) control essential processes in bacteria. A subset of eSTKs are single-pass transmembrane proteins that have extracellular penicillin-binding-protein and serine/threonine kinase-associated (PASTA) domains which bind muropeptides. In a variety of important pathogens, PASTA kinases have been implicated in regulating biofilms, antibiotic resistance and ultimately virulence. Although there are limited examples of direct regulation of virulence factors, PASTA kinases are critical for virulence due to their roles in regulating bacterial physiology in the context of stress. This review focuses on the role of PASTA kinases in virulence for a variety of important Gram-positive pathogens and concludes with a discussion of current efforts to develop kinase inhibitors as novel antimicrobials.

Keywords

Bacterial protein kinase; bacterial pathogenesis; β -lactam resistance; kinase inhibitors; Firmicutes; Actinobacteria

Phosphorylation in Bacterial Pathogens

Protein modification by phosphorylation is a ubiquitous method of cellular regulation in all domains of life. Early studies of protein phosphorylation in Eubacteria focused on histidine

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kinases which transfer phosphoryl groups from themselves to a cognate response regulator [1]. Subsequent sequencing efforts have uncovered eukaryotic-like ser/thr kinases (eSTKs) which phosphorylate multiple protein substrates and affect many areas of the cell [2]. A subset of eSTKs, kinases with penicillin-binding-protein and serine/threonine kinase-associated (PASTA) domains (the structures of which were recently reviewed [3]) are highly conserved in sequenced Firmicutes and Actinobacteria in single copy. A phosphatase is also highly conserved in these phyla, often in the same operon. The cognate phosphatase may help regulate kinase activation [4,5] and has generally been found to remove phosphoryl groups from PASTA kinase substrates [6–8]. This reciprocal nature is evident in cases like the streptococci where the PASTA kinase and phosphatase are the only genomically-encoded eSTK and Ser/Thr phosphatase [7,9–11]. Importantly, cotranscribed-phosphatases can also function on non- PASTA kinase substrates in organisms such as *Mycobacterium tuberculosis* where multiple eSTKs, but only a single phosphatase are present [12].

For many pathogens, PASTA kinases are essential tools to sense and respond to the host environment and antibiotic stress. To cause disease, pathogens must survive and replicate within a host where they encounter stressful conditions that include nutrient limitation, immune-induced physiological stress, and sometimes antibiotic stress [13]. It is hypothesized that PASTA kinases regulate responses to these stresses via their ability to monitor cell wall homeostasis through recognition of muropeptides [14]. PASTA kinases contribute to virulence through regulation of basic physiological processes [3] and less frequently direct regulation of virulence factors [15]. In most cases, PASTA kinases are only required in the face of these environmental- or infection-associated stresses. An exception to this is *M. tuberculosis*, where the PASTA kinase PknB which is essential [16]. The mechanisms underlying selective essentiality and most other phenotypes of PASTA kinase mutants are still largely unknown. Although a significant source of our understanding of PASTA kinase biology comes from the study of the non-pathogen *Bacillus subtilis* [3], in this review we will focus on the roles of the PASTA kinases in the context of virulence and antibiotic resistance.

PASTA kinases are important determinants of virulence and biofilm formation

Likely owing to their central role in regulating metabolism and cell wall homeostasis during stress, PASTA kinases have been implicated in the virulence of a variety of pathogens (summarized in Table 1). The *Staphylococcus aureus* PASTA kinase, Stk1, is one of the most well-studied kinases with respect to its role in virulence and its direct impact on expression of validated virulence factors. Despite this, strain differences as well as multiple infection models have led to conflicting data such that the role of Stk1 in *S. aureus* virulence is unclear. Deletion of *stk1* in the SH1000 strain led to significant attenuation in a murine model of pyelonephritis, with the *stk1* mutant demonstrating ~100-fold lower bacterial burdens in the kidney [17]. In contrast, deletion of *stk1* in the methicillin-resistant *S. aureus* (MRSA) USA300 or methicillin-sensitive (MSSA) Newman strains resulted in no loss of virulence in skin and soft tissue infection (SSTI) and bacteremia models, respectively, and in fact may have resulted in increased virulence in the SSTI model [15,18]. Expression of

virulence factors in *S. aureus* are controlled through activity of the accessory gene regulator (*agr*) system [19,20]. The *stk1* mutant in the USA300 MRSA strain demonstrated enhanced *agr* activity, evident by elevated levels of several downstream virulence determinants including the virulence transcriptional regulator SarA [15]. Additionally, Stk1 was found to positively regulate activity of the alternate sigma factor SigB [15] which itself is inversely correlated with levels of SarA [21]. SarA itself may be further regulated directly by Stk1 *via* phosphorylation on threonine [22] and/or cysteine [23] residues. Threonine phosphorylation results in a reduced affinity of phosphorylated SarA for various gene promoters [22]. Failure to phosphorylate the conserved redox-sensing cysteine residues was found to increase α -hemolysin expression and ultimately virulence in the MSSA strain Newman [23]. Taken together, these studies highlight a role for Stk1 in negatively regulating virulence factor expression in multiple strains of *S. aureus*. It remains to be determined if the contradictory virulence phenotypes of *stk1* mutants in different studies is due to genuine differences in Stk1 function in different strains, or instead is due to differences in the infection model used in each study.

In addition to *S. aureus*, PASTA kinases and/or their cognate phosphatases have been implicated in the virulence of a variety of other pathogens. In *Listeria monocytogenes*, the PASTA kinase PrkA is required to promote survival in the host cytosol, and *prkA* mutants are ~5 logs attenuated *in vivo* [24]. Similarly, Shakir *et al.* analyzed the effect of a dual PASTA kinase/phosphatase knockout in *Bacillus anthracis* and found a minor survival defect in spore-infected macrophages [25]. It is not clear whether this defect is due to impaired spore germination as might be inferred from known germination defects in *B. subtilis* [14,26] or whether this defect is due to impaired survival in the macrophage environment similar to *L. monocytogenes*. Regardless, in an *in vivo* model of pulmonary infection the dual PASTA kinase/phosphatase mutant has a 10-fold higher LD₅₀ [25]. The PASTA kinases in a variety of *Streptococci* have been implicated in virulence. PASTA kinase (*stkP*) mutants in two different strains of *Streptococcus pneumoniae* exhibit reduced CFU burdens in the lungs of intranasally-infected mice, whereas only one of these strains also exhibited reduced survival in the blood after IV injection [27]. Similarly, *stkP* mutants in *Streptococcus pyogenes* exhibit reduced growth in whole blood [10] and reduced virulence *in vivo*, such that 100% of mice succumbed to intramuscular wild type infection whereas all mice survived infection with *stkP* mutants [28]. In *Streptococcus mutans* PASTA kinase mutants had no colonization defect in a rat model of dental caries but had significantly reduced caries and lesions [29]. *Streptococcus agalactiae* StkP is important for controlling toxin expression where cross-talk between StkP and the CovR two-component system regulates both β -haemolysin and CAMP factor expression [11]. Consistent with this, *S. agalactiae* *stkP* mutants exhibit 100-fold higher LD₅₀s than wild-type in a neonatal rat sepsis model [5]. In *Enterococcus faecalis*, the PASTA kinase IreK is necessary for optimal persistence in the gut, potentially due to the resulting sensitization to bile acids in its absence [30]. Finally, not surprisingly given its essentiality for viability *in vitro*, *M. tuberculosis* PknB is also essential for virulence [31]. Although these studies reliably demonstrate roles for PASTA kinases in virulence, in most cases the mechanism by which the kinase contributes to virulence is unknown and with rare exceptions, direct regulation of virulence factors has not been described.

In addition to direct roles in virulence, PASTA kinases also contribute to biofilm formation, which in many cases can either cause infection, contribute to disease progression, or lead to treatment failures [32,33]. Biofilm formation in *S. mutans*, which is an essential part of its pathogenesis, is impaired in the absence of either StkP or the cognate phosphatase StpP [29,9]. In *B. anthracis*, phosphorylation of the chaperone protein GroEL by PrkC positively regulates biofilm formation through an unknown mechanism [34]. Similarly, *Staphylococcus epidermidis* Stk1 is crucial for initial surface adhesion and the production of polysaccharide intercellular adhesin (PIA), the predominant constituent biomolecule in the *S. epidermidis* biofilm matrix [35]. Conversely, in *S. aureus* that make PIA-dependent biofilms, Stk1 negatively regulates PIA biofilm production through phosphorylation of residues in the DNA binding domain of the global transcription regulator carbon catabolite control protein A (CcpA) [36]. The underlying mechanism of this regulation is complex as both phosphomimetic and phosphoablative mutations to these residues were detrimental to biofilm formation. Whether this effect is specific to PIA biofilms is unknown as *S. aureus* biofilm composition is strain-specific [37], and the effect of Stk1 activity on strains which primarily produce DNA and/or protein matrices hasn't yet been investigated. Similarly, the role of PASTA kinases in biofilm formation by other pathogens has not been reported. In summary, PASTA kinases are important determinants of virulence or biofilm formation in at least 11 different species, however in most cases the specific substrates regulated by phosphorylation and how they contribute to these phenotypes is still unclear.

PASTA kinases regulate central metabolism

Metabolism involves the balance of nutrient acquisition and energy expenditure and is likely a major mechanism by which PASTA kinases contribute to virulence and antibiotic resistance. Carbon acquisition, utilization, and production of essential secondary metabolites are crucial for pathogenesis [38], while metabolic regulation is similarly critical for the bactericidal activity of β -lactam antibiotics [39,40]. PASTA kinases have been implicated as major regulators of all of these processes, although specific regulatory steps may be species-specific (Table 1).

Sugar uptake in bacteria is frequently performed by phosphotransfer systems (PTS) that coordinate uptake with many other cellular processes [41]. The essential PTS component HPr interacts with the *L. monocytogenes* PASTA kinase PrkA and is phosphorylated on threonines in addition to the canonical serine phosphorylated directly by HPr kinase, making it likely that, as in *B. subtilis*, HPr is a PASTA kinase substrate [42–44]. Importantly, PTS systems are shut down during intracellular growth and as such phosphorylation of HPr may be important for controlling PTS function to promote virulence [45]. In contrast, HPr regulates enzymes involved in glycerol utilization, a process that both activates the virulence master-regulator PrfA and provides an important carbon source for intracellular *L. monocytogenes* [41,46]. Phosphorylated HPr also contributes to metabolic regulation through the global metabolic transcriptional regulator CcpA [47] and in *S. aureus*, Stk1 directly phosphorylates CcpA to regulate gene expression [36], although this has not been assessed in *L. monocytogenes*. Finally, the metabolic transcriptional regulator Rex [48] both interacts with PrkA [42] and is serine phosphorylated in *L. monocytogenes* [43], further

demonstrating that PASTA kinases can regulate metabolism both at the level of carbon acquisition and through metabolic transcriptional regulators (Fig. 1) [49].

In addition to uptake systems and transcriptional regulators, metabolic enzymes are also directly regulated by PASTA kinases. The glycolytic enzymes pyruvate kinase PykA, pyruvate dehydrogenase PdhB, glyceraldehyde-3-phosphosate dehydrogenase Gap, and fructose-bisphosphate aldolase FbaA are Ser/Thr phosphoproteins in *B. subtilis* whose only eSTK is the PASTA kinase PrkC [43,50]. PykA and FbaA are also phosphorylated by Stk1 in *S. aureus* [51,52], and a *stp* phosphatase mutant in *S. aureus* exhibits higher FbaA activity than wild-type [53]. Control over the activity of these enzymes is crucial for adapting to particular growth conditions and avoiding futile cycling between glycolysis and gluconeogenesis [54]. Indeed, in *L. monocytogenes*, *prkA* mutants are able to grow on direct PTS-independent glycolysis substrates but have a significant growth defect on glycerol that requires gluconeogenesis [24]. Taken together, the PASTA kinases emerge as master regulators of cellular metabolism that coordinate metabolic strategies at levels ranging from regulation of carbon transport, to modification of metabolic enzymes to transcriptional regulation (Fig. 1).

Nucleotide metabolism is critical for virulence due to their critical role in energy balance and cell wall homeostasis [55–57], and metabolism of both purine and to a lesser extent pyrimidine nucleotides is affected by PASTA kinase activity. In *S. aureus*, Stk1 regulates transcription of both purine and pyrimidine biosynthetic pathways with the AMP synthetic enzyme PurA also being a phosphorylation target [49]. In agreement with these results, purine levels are drastically altered in *stk1* or *stp* mutants though changes in pyrimidine levels are only observed in a *stp* mutant at late growth stages [53]. Despite these findings, there is conflicting data whether or not *stk1* mutants are purine auxotrophs and the discrepancy could lie in strain differences or media formulations [17,18]. Regardless, how this phenotype would relate to virulence *in vivo* or in different models of infection is unclear. Similarly, *S. agalactiae* *stk1* mutants are purine auxotrophs and StkP phosphorylates both PurA and the IMP dehydrogenase GuaB to regulate flux of IMP precursor into either AMP or GMP synthesis [58]. These phosphorylation events are associated with drastically reduced levels of guanine nucleotides and somewhat elevated levels of adenine nucleotides (Fig. 1) [58].

In summary, PASTA kinases regulate metabolism at a variety of different levels ranging from regulation of carbon metabolism and uptake to nucleotide metabolism. Some of the mechanisms of metabolic regulation are highly conserved amongst multiple PASTA kinase-containing organisms, while others demonstrate species- and potentially even strain-specific differences. Importantly, despite our growing understanding of the mechanisms by which PASTA kinases regulate metabolism, little has been done to connect these phenotypes with virulence phenotypes in any of the pathogens discussed herein.

Roles of the PASTA Kinase in Cell Division and Cell Wall Homeostasis

Regulation of cell division and cell wall homeostasis are crucial for both virulence and β -lactam resistance and are among the most well-studied functions of PASTA kinases

(reviewed by Manuse *et al.* [3]). PASTA kinases physically interact with a variety of cell division proteins in a complex network to coordinate septal synthesis and elongation and the nature of the interactions is largely species-specific (Table 1). The cell division protein DivIVA (called Wag31 in *M. tuberculosis*) is a conserved phosphorylation target of PknB and other eSTKs in *M. tuberculosis* [59] and *Streptococcus pneumoniae* (Fig 2) such that phosphorylation coordinates septal vs elongation peptidoglycan synthesis [60,61]. A DivIVA-related protein, GpsB, is not a phosphorylation target in *S. pneumoniae* but controls localization of StkP. StkP also affects the stability of FtsZ structures through the phosphorylation of MapZ [62]. FtsZ, the bacterial tubulin homologue which controls bacterial septation, is itself a Stk1 substrate in *S. aureus* [63]. Another conserved substrate of various PASTA kinases is the protein of unknown function YvcK (CuvA in *M. tuberculosis*) [24,59,64]. Although the specific sites of phosphorylation are not conserved and the function of this protein is unknown, YvcK mutants in multiple species have defects in utilization of various gluconeogenic nutrients, cell wall homeostasis and resistance to cell wall stress [24,65,66]. Additionally, although not validated in any pathogens, a *B. subtilis* mutant lacking MreB, the bacterial actin homologue that coordinates peptidoglycan synthesis and cell morphology [67] can be functionally complemented by overexpression of YvcK in a phosphorylation-dependent manner [64]. Importantly, YvcK null mutants are highly attenuated in both *L. monocytogenes* [24] and *M. tuberculosis* [66], potentially connecting the regulation of YvcK functions to the role of PASTA kinases in virulence in these organisms.

Production of cell wall precursors is important for dealing with cell wall stress encountered during infection [68] and as a result of β -lactam antibiotic activity [69,70]. Uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), an essential peptidoglycan and wall teichoic acid (WTA) precursor, is produced by the GlmSMU pathway and in *M. tuberculosis*, phosphorylation of GlmU by PknB reduces its acetyl transferase activity (Fig. 2) [71]. Similarly, StkP from *S. pneumoniae* phosphorylates GlmM although the consequence of phosphorylation is unknown [72]. If regulation of this pathway is conserved in *S. epidermidis* and *S. aureus*, it could provide another explanation for the PIA biofilm defect as UDP-GlcNAc is the primary component of PIA biofilms. Downstream of the GlmSMU pathway, several enzymes in the Mur pathway are eSTK substrates in *L. monocytogenes*, although again this is not validated to be mediated by the PASTA kinase PrkA [43]. In *M. tuberculosis*, PknB-dependent phosphorylation of CwlM contributes to its regulation of MurA activity, and thus peptidoglycan precursor synthesis (Fig. 2), through unknown changes in protein-protein interactions [73]. Similarly, in *S. aureus*, the levels of several metabolic intermediates in the Mur pathway are significantly altered in PASTA kinase or phosphatase mutants, strongly suggesting altered levels of MurB, MurC, and MurF activity [53]. In *M. tuberculosis*, phosphorylation of Wag31 by PknB indirectly alters the activity of the MurG and MraY enzymes at the end of this pathway (Fig. 2) [74]. The final steps of flipping the lipid II precursor from the cytosol to the extracellular space are also regulated at several steps by PknB. MviN, a membrane protein essential for flipase activity, is phosphorylated by PknB on its pseudokinase domain to recruit another PknB substrate, the cell wall regulatory protein FhaA [75], which aids in localization of flipase activity [76]. In addition to direct regulation of biosynthetic enzymes in mucopeptide synthesis, PASTA

kinases likely regulate cell wall homeostasis at the transcriptional level. In *S. aureus*, Stk1 directly phosphorylates the essential cell wall-related two-component response regulator WalR [63], whereas in *S. pneumoniae* the PASTA kinase regulates its cognate histidine kinase WalK through protein-protein interactions to alter transcription of cell wall synthesis genes [77]. Another example of crosstalk between PASTA kinases and two-component systems has been reported in *S. aureus* where Stk1 phosphorylates GraR to increase its DNA binding activity, likely leading to differential expression of the *dlt* operon [78]. The *dlt* operon modifies lipoteichoic acid (LTA) with D-Ala-D-Ala thus altering the overall charge of the cell wall [79,80] and contributing to antimicrobial peptide resistance during infection [81]. Comprehensive examination of changes in levels of peptidoglycan and WTA precursors led Liebeke *et al.* to propose that Stk1 and the phosphatase Stp play alternating roles in peptidoglycan and WTA synthesis with Stk1 activity promoting greater peptidoglycan synthesis [53]. As maintenance of cell wall homeostasis and appropriate cell wall stress responses are critical for resistance to lysozyme and localization of key virulence factors in a variety of pathogens, determining the relative impact of PASTA kinase-driven cell wall regulation on virulence will be key to our understanding of the role of these kinases during infection.

PASTA kinases as regulators of β -lactam resistance

With such a deep involvement in cell division and cell wall homeostasis, it is little surprise that the PASTA kinases are crucial for resistance to a variety of cell envelope stresses including, but not limited to, β -lactams [17,24,49,82–84]. Specifically, the PASTA kinases are important for β -lactam resistance in *S. aureus* [15,49,82], *L. monocytogenes* [24,85], the streptococci [28,86], and the enterococci [30,87–89]. However, the mechanism(s) by which the PASTA kinases regulate β -lactam resistance are unknown, and the extent and patterns of β -lactam resistance vary between species and amongst strains. This is particularly apparent in *S. aureus*, where *stk1* mutants have been studied in 4 different strains. Deletion of Stk1 in the MRSA strain N315 has minor effects on select cephalosporin and carbapenem MICs [82]. On the other hand, both hospital-acquired MRSA strain COL and community-acquired strain LAC *stk1* mutants are significantly sensitized to nafcillin and cloxacillin [15]. Finally, deletion of Stk1 in the MSSA strain NCTC 8325 has minimal effects on β -lactam resistance, resulting in a modest 2-fold sensitization to methicillin and no effect on the MICs of the cephalosporins ceftriaxone and cefepime [49]. What mediates differences in β -lactam sensitivity between strains or to specific β -lactams is currently unknown.

Contrary to results in *S. aureus*, PASTA kinase mutants in *L. monocytogenes* and *E. faecalis* display robust sensitization to cephalosporins, reaching MICs 10–100-fold lower than their wild type parental strains [24,85,88]. Importantly, both *L. monocytogenes* and the enterococci are inherently resistant to cephalosporins [90,91] suggesting that the PASTA kinases may specifically regulate penicillin-binding proteins (PBPs) that confer resistance to cephalosporins. Intrinsic cephalosporin resistance in *L. monocytogenes* was originally attributed to Pbp3 (*Imo1438*), which has low affinity for the cephalosporins [92]. However, recent studies demonstrating that overexpression or deletion of Pbp3 does not affect the MICs of cephalosporins have challenged this model [93,94]. In *E. faecalis*, IreK and its cognate phosphatase IreP act in a reciprocal manner to regulate IreB, a protein of unknown

function, which itself represses cephalosporin resistance in a phosphorylation-dependent manner [87].

While it is well established that the PASTA kinases regulate antibiotic resistance, efforts to identify the underlying signaling circuits are currently in their infancy. One hypothesis is that the extracellular PASTA domains may directly sense antibiotic insult. The PASTA domains of both PBPs and PASTA kinases have been shown to bind certain β -lactam antibiotics in addition to peptidoglycan [95,96]. Furthermore, overexpression of the transmembrane and PASTA domains of PknB in *M. smegmatis* leads to increased sensitivity to β -lactams [97]. However, studies in the MRSA strains COL (whose truncated *stk1* gene lacks the PASTA domains) and LAC by Tamber *et al.* found no PASTA domain-dependent effects on β -lactam sensitivity [15]. In addition, work by Dias *et al.* shows that antibiotic resistance status does not appear to be a selective pressure for the evolution of the PASTA domains in *S. pneumoniae* strains [86]. This suggests that even if β -lactams do bind to PASTA domains, they do not impede PASTA kinase function. Alternatively, Stk1 was found to phosphorylate the β -lactamase regulator protein BlaR1 in the MRSA strain NRS70 which resulted in increased β -lactamase activity [98]. However, various MRSA strains (such as COL) [15] and other bacterial species (such as *L. monocytogenes*) [24] that lack a β -lactamase are considerably sensitized to β -lactams when their respective PASTA kinases are compromised, suggesting that cell wall synthesis and metabolic regulation may be at work. Future studies defining how PASTA kinases regulate β -lactam resistance will be critical for targeting PASTA kinases as a component of combination therapies as discussed below.

PASTA kinases as pharmacologic targets

Initial efforts to pharmacologically target the PASTA kinases were focused on targeting PknB in *M. tuberculosis* due to its essentiality. Inhibitors identified in these early efforts included the indolocarbazoles (i.e. staurosporine, K252a, and K252b) [16,99,100] and the type II topoisomerase inhibitor mitoxantrone (Table 1) (Fig. 3) [101]. Although these early hits had biochemical activity against PknB and revealed valuable insights into PASTA kinase biochemistry, they had little activity against *M. tuberculosis* with the most promising MIC being 20 μ M. Later efforts incorporated comprehensive medicinal chemistry campaigns to identify effective PknB inhibitors with improved microbiologic activity [102,103]. After screening ~54,000 compounds, Loughheed *et al.* identified 12 lead compounds with incremental increases in antibacterial activity with the lowest MICs being 16 μ M and 5 μ M in broth and an *ex vivo* macrophage model of infection, respectively [102]. Chapman *et al.* pushed these limits even further with the identification of an aminopyrimidine pharmacophore, yielding a compound with a biochemical IC₅₀ of 0.176 μ M and an MIC of 8 μ M (Fig. 3) [103].

A common hurdle in the development of PknB-targeting compounds continues to be a lack of correlation between biochemical and microbiologic activity [102]. The issue may stem, in part, from the unique structural features of the mycobacterial cell wall that can deny access to the target [104]. Strategies to breach the cell envelope such as weakening the cell wall with sublethal antibiotics [105–107] or targeting efflux pump systems [108–111] have been met with mixed results for various drug families. Loughheed *et al.* determined that these

strategies were capable of improving the activity of some of their lead inhibitors, though their most potent compounds remained unaffected [102]. Alternatively, differences in biochemical and microbiologic activity could be due to differences in kinase activation in the *in vitro* versus *in vivo* environment. To activate, PASTA kinases are predicted to form both back-to-back homodimers to stabilize the active conformation [112] as well as front-to-front homodimers [113] resulting in phosphorylation of the activation loop and juxtamembrane region [113,114,4]. The effect of phosphorylation on the juxtamembrane region has not been thoroughly investigated, but it is known to affect the interaction of PknB with the cell wall regulatory protein FhaA [75], and phosphorylation of residues in the activation loop is required for kinase activity [114,4]. *In vitro* kinase reactions including only the kinase cytoplasmic domain may not fully account for the strength of these interactions in the context of PASTA domain function and association to the membrane. Additionally, other molecular interactions can alter kinase activity [115]. These works highlight need for a better understanding of drug delivery to bacterial cytoplasmic targets and *in vivo* activation mechanisms that could explain the discrepancies between biochemical efficacy and antitubercular activity.

Despite their lack of essentiality, the critical role of the non-essential PASTA kinases in β -lactam resistance and virulence has led to interest in identification of PASTA kinase inhibitors as either combination therapies to augment β -lactam activity or as antivirulence therapies. The highly non-selective indolocarbazole staurosporine inhibits PASTA kinases from *L. monocytogenes*, *B. subtilis*, *S. epidermidis* and *E. faecalis* [14,35,85,87] while activity against *S. aureus* Stk1 is controversial [85,116]. In some of these cases staurosporine has been demonstrated to potentiate β -lactam activity as has been observed with PASTA kinase mutant bacteria. Based on these preliminary studies, combination activity with other structurally distinct scaffolds has been achieved in various MRSA strains and in *L. monocytogenes* (Fig. 3) [85,98,116]. The structural diversity of effective PASTA kinase inhibitors improves the odds of identifying effective inhibitors that also possess favorable pharmacologic properties while avoiding toxicity associated with inhibition of host kinases. Current efforts focused on identifying additional novel scaffolds as well as targeted medicinal chemistry on existing scaffolds hold great promise for the development of potent and specific inhibitors of PASTA kinases that could function either in isolated or combination therapies.

An alternative approach to inhibiting PASTA kinase activity is to target the extracellular PASTA domains rather than the intracellular kinase domain. Benefits to this approach include the ease of targeting extracellular domains and the divergent nature of the PASTA domains [117] which may enhance specific targeting of pathogen kinases over commensal or host kinases. Drawbacks to this approach include instances such as the *S. aureus* MRSA strain COL which doesn't require PASTA domains for activity [15], and a general lack of understanding of how PASTA domains function. Structures for *S. aureus* Stk1 and *M. tuberculosis* PknB PASTA domains have been solved [100,118–121] and structural features of these domains have been reviewed by Manuse *et al.* [3]. Likely functions for the PASTA domains include activation of the intracellular kinase domain, potentially through initiation of dimerization [122,120], and localization of the PASTA kinase to sites of peptidoglycan synthesis for proper control of peptidoglycan synthesis activity [63,123]. Each of the 4

PASTA domains in PknB are essential and three residues in a putative ligand binding pocket in PASTA domain 4 are required for PknB function [119]. Self muropeptide fragments have been considered to be likely ligands for PASTA domains as the *B. subtilis* PASTA kinase PrkC induces spore germination in response to binding its own muropeptide fragments but not muropeptide fragments with alternate peptide structures [14]. Similar muropeptide binding constraints have been observed with StkP in *S. pneumoniae* [96] and in *M. tuberculosis* the MurNAc moiety, peptide stem composition and length all contribute to PASTA domain binding [123]. These results suggest that binding ligands are species-specific and that PASTA kinases may function as a quasi-quorum sensing system. In contrast, it was recently demonstrated that the ultimate peptidoglycan precursor Lipid II is the activating ligand for Stk1 in *S. aureus*, and that pure muropeptides are unable to compete away bound Lipid II [63] suggesting Stk1 primarily functions to monitor and regulate the division of an individual cell. To date, no successful targeting of PASTA kinase activity through the PASTA domains has been reported, but β -lactams themselves can bind PASTA domains [96] and we anticipate that improved understanding of PASTA domain structure and function will aid the design and/or identification of small molecule inhibitors in the future.

Concluding Remarks

PASTA kinases play essential roles in virulence and β -lactam resistance in a variety of important pathogens through their regulation of metabolism, cell division, and cell wall homeostasis and as such have been identified as high value antibiotic targets. Physiological differences between species make it likely that although some PASTA kinase and cognate phosphatase functions are conserved, others are likely to be species-specific. Key to the development of PASTA kinases as antibiotic targets is understanding the activating signals and downstream signaling cascades by which they regulate physiology and stress responses to influence virulence and antibiotic resistance (see Outstanding Questions). Finally, despite extensive attempts to identify and develop novel kinase inhibitors as new antibiotics, to date, no studies have demonstrated PASTA kinase inhibitor efficacy *in vivo*. Therefore, to successfully make it to the clinic, discovery- and mechanism-driven studies need to be coupled to robust medicinal chemistry programs (such as the study published by Chapman *et al.*) [103] and *in vivo* studies whose focus is to optimize these molecular scaffolds for better pharmacokinetic and pharmacodynamic properties. Once an *in vivo* proof of concept is successfully established, we will be one step closer to turning these “promising targets” into a clinical reality.

Outstanding Questions

- What proteins are phosphorylated by PASTA kinases in the context of infection and how does phosphorylation of these proteins mediate virulence?
- How is expression of the PASTA kinase and its cognate phosphatase regulated? Additionally, how is activity of the PASTA kinase regulated through ligand binding, post-translational modifications, or other structural changes?

- What PASTA kinase substrates regulate antibiotic resistance, and how do they differentially mediate resistance to specific β -lactams in unique species or strains?
- How can small molecule PASTA kinase inhibitors be optimized not only for biochemical activity, but also to promote entry into, and retention within the bacteria?
- What structural characteristics of PASTA kinases could facilitate the development of species-specific kinase or PASTA domain inhibitors to minimize off-target effects on both the host and the microbiome?

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Trends

- In addition to traditional two-component systems, phosphorylation by eukaryotic-like ser/thr kinases (eSTKs) has emerged as a key signal transduction system in bacteria.
- PASTA kinases have emerged as a highly conserved subset of eSTKs that sense muropeptides in Firmicutes and Actinobacteria.
- Recent studies demonstrate that both broadly conserved and species-specific functions of PASTA kinases connect metabolism and cell wall homeostasis to virulence and antibiotic resistance.
- Advances in proteomics have led to the identification of many phosphosubstrates, however the functional consequences of phosphorylation in many cases remain unknown.
- Due to their conserved role in virulence and antibiotic resistance PASTA kinases have emerged as high value targets for antibiotic development.

Fig. 1. Overview of PASTA Kinases in central metabolism

Phosphorylation events are shown by colored bold lines where solid lines are confirmed phosphorylation events and hashed lines are suspected interactions. Red lines represent inhibition by phosphorylation, green lines are activation, and yellow lines are undetermined. Small blue lines indicate metabolic interconversions while black arrows indicate other interactions.

Fig. 2. PASTA Kinases in cell division and synthesis of cell wall precursors

Phosphorylation events are shown by colored bold lines where solid lines are confirmed phosphorylation events and hashed lines are suspected interactions. Red lines represent inhibition by phosphorylation, green lines are activation, and yellow lines are undetermined. Small blue lines indicate metabolic interconversions while black arrows indicate other interactions.

Staurosporine
 Targets: *PknB*, *PrkA*, *IreK*, *Stk1*(?)
 IC_{50} : 0.6 μM
 MIC: 25 μM < MIC < 50 μM
 Family: Indolocarbazole

K252a
 Targets: *PknB*
 IC_{50} : 0.096 μM
 MIC: 20 μM
 Family: Indolocarbazole

K252b
 Targets: *PknB*
 IC_{50} : 0.106 μM
 MIC: > 40 μM
 Family: Indolocarbazole

Mitoxantrone
 Targets: *PknB*
 IC_{50} : 0.8 μM
 MIC: 400 μM
 Family: anthracenedione

Aminopyrimidine 16c
 Targets: *PknB*
 IC_{50} : 0.176 μM
 MIC: 8 μM
 Family: Aminopyrimidine

ST085384
 Targets: *Stk1*
 Family: Sulfonamide

Triarylimidazole 12
 Targets: *Stk1*
 Family: Triarylimidazole

AZD5438
 Targets: *PrkA*
 Family: Imidazole sulfone

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Fig. 3. Drugs with anti-PASTA kinase activity

Structures are shown for compounds with published efficacy against bacterial PASTA kinases *in vitro* and against live bacteria. For antitubercular compounds, MICs and IC_{50} s are displayed where available.

Table 1

PASTA Kinase Substrates and Associated Phenotypes

Species	PASTA Kinase /Phosphatase	Kinase Essentiality	Virulence Phenotypes	Biofilm Phenotypes	Central Metabolism Substrates	Cell Wall Metabolism Substrates	Antimicrobial Sensitivity Phenotypes	Identified Inhibitors
<i>Mycobacterium tuberculosis</i>	PknB/PtsP	Yes ¹⁶	PknB mutant attenuated ³¹	Unknown	Unknown	Yes (CwIM ⁷³ , MviN ⁷⁶ , FhaA ⁷⁵ , GlmU ⁷⁷ , DivIVA ⁵⁹)	Unknown	Indolocarbazoles ^{16,98,99} , mitoxantrone ¹⁰⁰ , aminopyrimidine ^{101,102}
<i>Staphylococcus aureus</i>	Stk1/Stp	No ⁴⁹	Strain and model specific Stk1 mutant attenuated in pyelonephritis ¹⁷ Stk1 mutants hypervirulent in SST model ^{15,18} (SarZ ^{22,23} SigB ¹⁵).	Stk1 inhibits biofilm formation (CcpA) ³⁶	Yes (CcpA ³⁶ , FhaA ^{50,51} , PykA ⁵² , PurA ⁴⁹)	Yes (WalR ⁶³ , FtsZ ⁶³ , GraRA ⁷⁸ , BlaR1 ⁹⁸)	Stk1 mutant sensitive to β -lactams ^{15,49} , tunicamycin ⁴⁹ and more resistant to fosfomycin ¹⁷ . Stp mutant sensitive to vancomycin in VISA strains ⁸³	Staurosporine (inconclusive) ^{85,111} Sulfonamides ¹¹¹
<i>Staphylococcus epidermidis</i>	Stk1/Stp	No ³⁵	Unknown	Stk1 mutant is attenuated for biofilm formation ³⁵	Unknown	Unknown	Unknown	Staurosporine ³⁵
<i>Listeria monocytogenes</i>	PrkA/Stp	Inconclusive ⁸⁷	PrkA mutant attenuated ²⁴	Unknown	Unknown	Unknown	PrkA mutant sensitive to β -lactams ^{24,85} , tunicamycin, bacitracin, lysozyme, LL-37 ²⁴	Staurosporine ⁸⁵ , AZD5438 ⁸⁵
<i>Streptococcus mutans</i>	StkP/PhpP	No ⁹	StkP mutant attenuated ²⁹	StkP mutant is attenuated for biofilm formation ^{9,29}	Unknown	unknown	Unknown	Unknown
<i>Streptococcus pneumoniae</i>	StkP/PhpP	No ⁷²	StkP mutant attenuated in pulmonary model strain specific phenotypes in bacteraemia model ²⁷	Unknown	Unknown	Yes (DivIVA ^{60,61} , MapZ ⁶² , GlmM ⁷²)	StkP mutant sensitive to β -lactams ⁸⁶	Unknown
<i>Streptococcus pyogenes</i>	StkP/PhpP	No ¹⁰	StkP mutant attenuated ²⁸	Unknown	Unknown	Unknown	StkP mutant sensitive to β -lactams ²⁸	Unknown
<i>Streptococcus agalactiae</i>	StkP/PhpP	No ⁵	StkP mutant attenuated ⁵	Unknown	Yes(PurA, GuaA ⁵⁸)	Unknown	unknown	Unknown
<i>Enterococcus faecalis</i>	IreK/IreP	No ⁸⁹	IreK mutant attenuated for GI colonization ³⁰	Unknown	Unknown	Unknown	IreK mutant sensitive to β -lactams ^{30,87,88}	Staurosporine ⁸⁷
<i>Bacillus anthracis</i>	PrkC/PrpC	No ²⁵	PrkC mutant attenuated ²⁵	PrkC promotes biofilm formation (GroEL) ³⁴	Unknown	Unknown	Unknown	Unknown