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Evolution of Transmembrane Protein Kinases Implicated in Coordinating Remodeling of Gram-Positive Peptidoglycan: Inside versus Outside[▽]

Greg Jones and Paul Dyson*

School of Medicine, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, United Kingdom

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Members of a family of serine/threonine protein kinases (STPKs), unique to gram-positive bacteria, comprise an intracellular kinase domain and reiterated extracellular PASTA (for "penicillin-binding protein and serine/threonine kinase associated") domains. PASTA domains exhibit low affinity for β-lactam antibiotics that are structurally similar to their likely normal ligands: stem peptides of unlinked peptidoglycan. The PASTA-domain STPKs are found in the actinobacteria and firmicutes and, as exemplified by PknB of *Mycobacterium tuberculosis*, they are functionally implicated in aspects of growth, cell division, and development. Whereas the kinase domains are well conserved, there is a wide divergence in the sequences of the multiple PASTA domains. Closer inspection reveals position-dependent evolution of individual PASTA domains: a domain at one position within a gene has a close phylogenetic relationship with a domain at a similar position in an orthologous gene, whereas neighboring domains have clearly diverged one from one another. A similar position-dependent relationship is demonstrated in the second family of proteins with multiple PASTA domains: the high-molecular-weight type II penicillin-binding protein (PBP2x) family. These transpeptidases are recruited to the division site by a localized pool of unlinked peptidoglycan. We infer that protein localization is guided by low-affinity interactions between structurally different unlinked peptidoglycan stem peptides and individual PASTA domains. The STPKs possess a greater multiplicity and diversity of PASTA domains, allowing interactions with a wider range of stem-peptide ligands. These interactions are believed to activate the intracellular kinase domain, allowing an STPK to coordinate peptidoglycan remodeling and reproduction of a complex cell wall structure.

Of all infectious diseases, tuberculosis is the biggest killer worldwide. The causative agent, the gram-positive bacterium *Mycobacterium tuberculosis*, can lie quiescent in unidentified sites in the human host for years without producing overt disease and then revive to cause lesions and, in many cases, progressive tuberculosis. To improve the outlook for combating this pathogen, it is particularly important to understand the genes that govern the cell cycle and latency state of infection. Serine/threonine protein kinases (STPKs) play an important part in bacterial signaling pathways, particularly in more complex prokaryotes such as *Mycobacterium* and *Streptomyces*, which possess multiple STPK genes (reviewed in reference 1). One family of these STPKs, unique to gram-positive bacteria, consists of transmembrane proteins, with the N-terminal kinase domain inside the cell and a C-terminal sensory component outside the cell (Fig. 1). The extracellular component is made up of three or four reiterated PASTA (for "penicillin-binding protein and serine/threonine kinase associated") domains (30). These domains were first identified associated with a high-molecular-weight type II penicillin-binding protein, PBP2x, in another medically important gram-positive bacterium *Streptococcus pneumoniae*. Indeed, PBP2x, which has two C-terminal PASTA domains, is the only protein for which the PASTA domain structure has been determined by X-ray crystallography (25). Each domain consists of an alpha helix and three beta strands, with a loop region of variable length between the first and second strands. A further crystal structure was determined in the presence of cefuroxime, a β-lactam antibiotic (13). Surprisingly, two molecules of the antibiotic were bound: one as a covalent complex with the active-site serine residue, the second associated by Van de Waal's interactions between the β-lactam ring and the first PASTA domain. This part of the antibiotic structurally resembles an unlinked peptidogylcan stem peptide, the likely normal ligand of a PASTA domain.

The multiple PASTA domain STPKs are found in the firmicutes and actinobacteria. PknB of *M. tuberculosis* is representative of one of the two different PASTA domain STPKs commonly found in the latter and has been the most extensively studied. A *pknB* (Rv0014c) ortholog is found in all sequenced actinomycete genomes in a highly conserved gene context and location, close to the chromosomal origin of replication, *oriC*. The gene is linked with others involved in signaling: its cognate phosphatase gene and two genes coding for fork-head-associated (FHA) proteins, mediators of serine and threonine phosphorylation (9, 24). In addition, the conserved gene cluster contains critical morphogenetic genes: *pbpA*, encoding a penicillin-binding protein important for growth and cell division (5); a *rodA* gene; and an ortholog of *crgA*, a gene implicated in the control of cell division in *Streptomyces* (6, 7). PknB undergoes autophosphorylation and is dephosphorylated by its cognate phosphatase in vitro (3). It can in turn phos-

* Corresponding author. Mailing address: School of Medicine, University of Wales Swansea, Singleton Park, Swansea SA2 8PP, United Kingdom. Phone: 44 1792 295667. Fax: 44 1792 295447. E-mail: p.j.dyson@swansea.ac.uk.

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TABLE 1. Organisms and genes discussed in this study[a]

Gene type and organism	STPK gene	Ortholog(s)	
		pknB	*pbp2x*
Actinomycetes genes		Rv0014c	
Mycobacterium tuberculosis H37Rv		ML0016	
Mycobacterium leprae TN		MAP0016c	
Mycobacterium avium subsp. *paratuberculosis*		Mb0016c	
Mycobacterium bovis AF2122/97		MSMEGPKNB	
Mycobacterium smegmatis MC2 155		SAV4338	
Streptomyces avermitilis MA-4680		SCO3848	
Streptomyces coelicolor A3(2)		CE0033	
Corynebacterium efficiens YS-314		DIP0053	
Corynebacterium diphtheriae NCTC 13129		CG0057	
Corynebacterium glutamicum ATCC 13032		BL0589	
Bifidobacterium longum NCC2705		NFA800	
Nocardia farcinica IFM 10152		LXX00210	
Leifsonia xyli subsp. *xyli*		PPA0184	
Propionibacterium acnes KPA171202			
Bacillus genes			
Bacillus clausii KSM-K16	ABC2315		ABC2362
Bacillus halodurans C-125	BH2504		BH2573
Bacillus licheniformis ATCC 14580	BL02302		−
Bacillus anthracis strain Ames	BA4000		BA4055
Bacillus cereus ATCC 14579	BC3860		BC2662, BC3916
Bacillus thuringiensis serovar Konkukian strain 97-27	BT9727_3603		BT9727_2431, BT9727_3568
Bacillus subtilis subsp. *subtilis* strain 168	BSU15770		BSU15160
Other firmicute *pbp2x* orthologs			
Clostridium acetobutylicum ATCC 824			CAC2130
Clostridium perfringens strain 13			CPE0564, CPE1863, CPE1880
Clostridium tetani E88			CTC01633
Enterococcus faecalis V583			EF0991
Lactobacillus johnsonii NCC 533			LJ0969
Lactobacillus plantarum WCFS1			LP_2200
Lactococcus lactis subsp. *lactis* Il1403			L89079
Listeria innocua Clip11262			LIN2145
Listeria monocytogenes EGD-e			LMO2039
Oceanobacillus iheyensis HTE831			OB1464
Staphylococcus aureus subsp. *aureus* MRSA252			SAR1157
Streptococcus agalactiae 2603V/R			SAG0287
Streptococcus mutans UA159			SMU.455
Streptococcus pneumoniae R6			SPR0304
Streptococcus pyogenes SSI-1			SPS0461
Symbiobacterium thermophilum			STH1205, STH2392
Thermoanaerobacter tengcongensis MB4			TTE1651

[a] Actinobacterial STPK genes are known either by the identifiers shown or as "*pknB*". The STPK gene of bacilli was originally characterized in *B. subtilis* as "*prkC*" (formerly "*yloP*") and the *pbp2X* ortholog was characterised as "*ftsI*". Other firmicute *pbp2X* ortholog gene names include *pbpA* (SAR1157), *pbpB* (LIN2145), *pbpC* (EF0991), *pbp2B* (LJ0969 and some bacilli), *spoVD* (all clostridia), and *pbp2B2* (LP_2200). −, Not present.

phorylate FHA domains, including that of the neighboring gene Rv0020c in vitro (14), and also two threonine residues of PbpA (5). Overexpressing PknB in mycobacterial cells alters cell division and cell wall synthesis to produce swollen cells with incomplete division septa that also show occasional branching, whereas partial depletion results in long, thin cells (16). *pknB* and the neighboring *rodA* are both essential genes in *M. tuberculosis* (27).

The firmicutes generally contain a single multiple-PASTA-domain STPK. A null mutation of the *Bacillus subtilis* kinase gene *prkC* results in a decrease in stationary-phase cell density and of loss of efficiency in the formation of spores and biofilms (11, 19). In *Streptococcus pneumoniae*, the orthologous kinase (StkP) is implicated in virulence (10). A likely target for phosphorylation by StkP is phosphoglucosamine mutase, GlmM, involved in cell wall synthesis (23).

We have undertaken the first study of how these STPKs have evolved. Our analysis reveals an unexpected diversity among the PASTA domains and allows us to predict how they may function.

MATERIALS AND METHODS

Sequence selection. Bacterial species containing multiple PASTA domain proteins were identified by using the Conserved Domain Architecture Retrieval Tool (CDART [http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd = rps]). The protein sequence of the PASTA domains from *S. coelicolor* was used as a query sequence. The same sequence was used to perform BLAST searches against the individual genomes. A representative sample of genomes was selected by using the genomes list at the GOLD database (http:

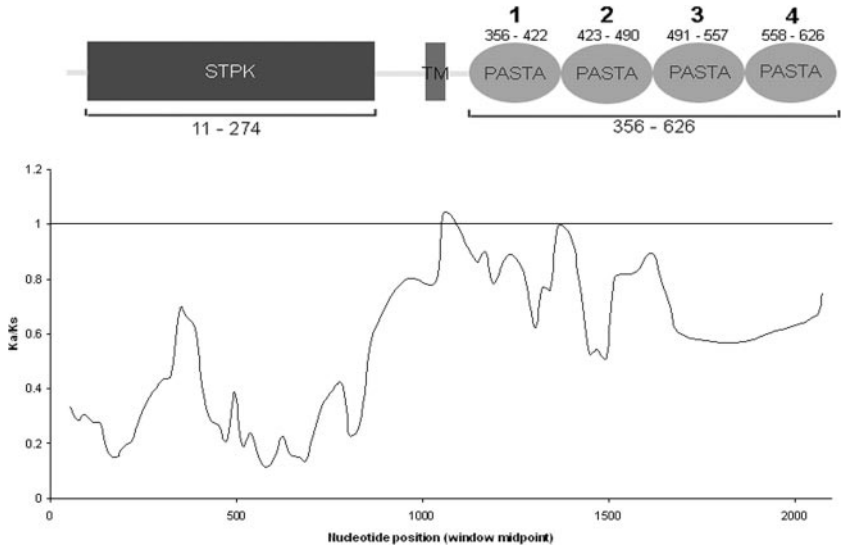

FIG. 1. Sliding-window analysis of 14 actinobacterial *pknB* orthologs, using K_a/K_s as a measure of selective pressure (window = 72, step = 21 nucleotides). Values are averages of pairwise comparisons between all individual sequences in the data set. Shown above the graph is the domain structure for PknB of *M. tuberculosis* aligned to the same scale as the *x* axis. Small numbers refer to amino acids constituting each domain. The numbering system for the four individual PASTA domains is also indicated. TM, transmembrane region.

//www.genomesonline.org/) (2). *Mycobacterium smegmatis* sequences were obtained by using BLAST searches at TIGR (http://www.tigr.org). Sequences giving an e-value of ≤0.0001 were selected, and the domain architecture was checked by using the SMART tool (18). Only proteins showing N-terminal kinase, transmembrane and C-terminal PASTA domains were selected, as well as PBP2x orthologs. Representative samples of firmicute and actinomycete multiple PASTA domain proteins were selected for further detailed analysis (Table 1). Transmembrane domains were confirmed by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Sequences of individual domains were retrieved from the SMART alignment. The DNA sequences encoding these domains were obtained by aligning the reverse-translated protein sequences with the actual gene sequence.

Data analysis. Alignments were obtained by using MEGA3 software (17) for analysis in DNASP (26); these alignments were checked with the version of CLUSTALX current at the time. Maximum-likelihood trees were obtained by using PHYML (15) and visualized by using MEGA3. Sliding window analysis was achieved by using DNASP, comparing each sequence against all others in the data set in a pairwise manner. K_a/K_s values were calculated by using the Nei-Gojobori method (22), using the Jukes-Cantor correction for substitutions at multiple sites. Average K_a/K_s values were calculated from individual values of all pairwise comparisons between all members of each data set. Some comparisons were made impossible due to a high (>75%) proportion of synonymous substitutions, thus preventing the use of the Jukes-Cantor correction. These comparisons were excluded from the analysis. For *pknB* orthologs, the differences between K_a/K_s values for kinase and extracellular domains were tested for significance by using a paired *t* test, while the K_a/K_s values for the PASTA domains of *pbp2x* orthologs and *pknB* orthologs were compared by using a Mann-Whitney U test. These statistical analyses were done by using SPSS.

Structural analysis. Structures of PASTA domains were determined by using the NPS server (4), using the MLRC, DSC, and PHD programs to give a consensus secondary structure prediction.

RESULTS AND DISCUSSION

Evolutionary comparison of the intracellular and extracellular components of actinomycete *pknB* orthologs. A comparison of the amino acid sequences of the cytoplasmic kinase domains of four actinomycete (*S. coelicolor*, *S. avermitilis*, *M. tuberculosis*, and *Corynebacterium diphtheriae*) PknB orthologs revealed 55% conservation of identical residues and 11.8% conservation of similar residues, with an overall similarity of 66.8%. In contrast, the predicted extracellular portion of these proteins, consisting of four reiterated PASTA domains, has only 24.5% overall similarity, with 10.7% conserved residues and 13.8% similar residues. This suggests that different selection pressures are acting on either side of the plasma membrane. To quantify these differences, we examined the ratio of nonsynonymous (K_a) and synonymous (K_s) nucleotide changes in 14 orthologs of *pknB* (Table 1). Figure 1 illustrates the average values for K_a/K_s ratios using a window size of 72 and a step size of 21 nucleotides. When the sliding-window K_a/K_s ratios are mapped against the domain structure of the protein, clear differences in the values either side of the membrane are evident. Overall, the intracellular STPK domain is under much tighter selective constraint than the juxtamembrane, transmembrane, or extracellular (PASTA) domains. Indeed, the average K_a/K_s value for *pknB* kinase domains is 0.266, whereas for the extracellular reiterated PASTA domains the value is 0.787. When the two sets of pairwise comparisons for PASTA and kinase domains were examined, the values were found to be significantly different at the 95% confidence level using the paired *t* test (df = 90, $t = -16.5$).

Position-dependent evolution of individual PASTA domains. Phylogenetic comparison of the individual PASTA domains from actinobacterial STPKs reveals that they cluster according to their position in orthologous genes, rather than with neighboring domains in the same gene. This is clearly shown in the maximum-likelihood tree drawn for five mycobacterial *pknB* orthologs (Fig. 2), and trees drawn for other genera give a similar outcome. A large subset of the actinobacteria contains a second transmembrane kinase gene that lies within 20 genes of the division cell wall gene cluster. In these organisms each PASTA domain is more related to its orthologous domain than its homologous domain (results not shown). The positional clustering of PASTA domains is also true for *pbp2x* orthologs. Comparison of the nucleotide sequences of PBP2X- and

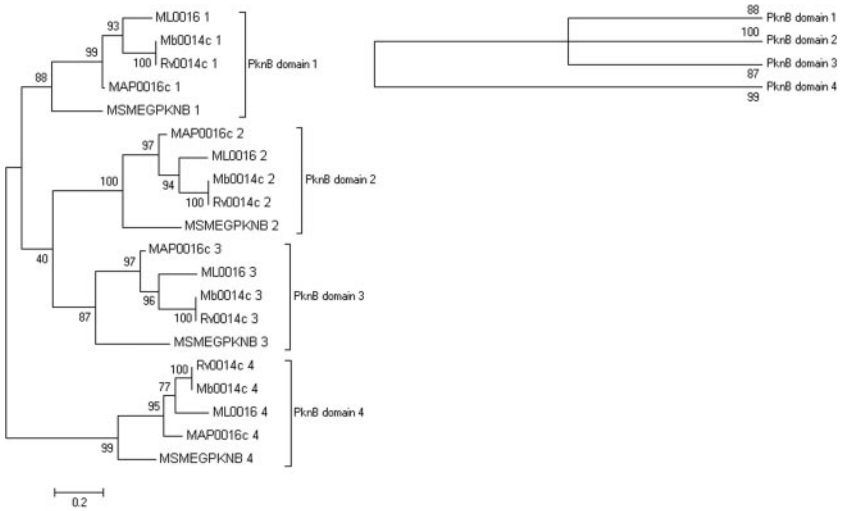

FIG. 2. Phylogenetic tree drawn for mycobacterial PASTA domains associated with *pknB* orthologs drawn using maximum likelihood. PASTA domains are numbered according to the scheme shown in Fig. 1. Percent bootstrap values from 500 replicates are shown on branches from the main nodes. Also shown (top right) is the topology tree obtained when bootstrap values of <70% are excluded.

STPK-associated PASTA domains of seven bacilli species (Table 1) reveals orthologous clustering of PASTA domains (Fig. 3), with PBP2X-associated domains forming their own unique clusters. However, the nucleotide diversity for the STPK-associated PASTA domain 2 is too great to allow grouping together with any degree of confidence. The nucleotide diversity is also too great to allow comparisons between PASTA domains from the same protein family from different genera.

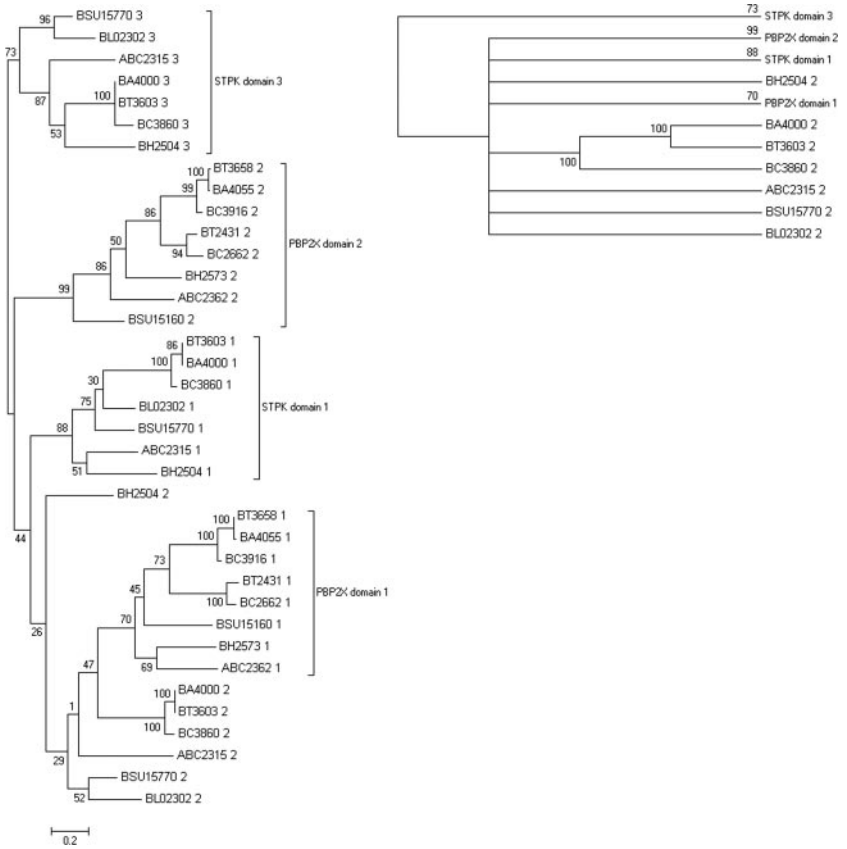

FIG. 3. Phylogenetic tree for PASTA domains associated with *Bacilli* STPKs and *pbp2X* orthologs. The scheme for numbering individual PASTA domains is sequential, starting with the most N terminal, similar to the scheme used for PknB in Fig. 1. The percent bootstrap values from 500 replicates are shown on branches from the main nodes. Also shown (top right) is the topology tree obtained when bootstrap values of <70% are excluded.

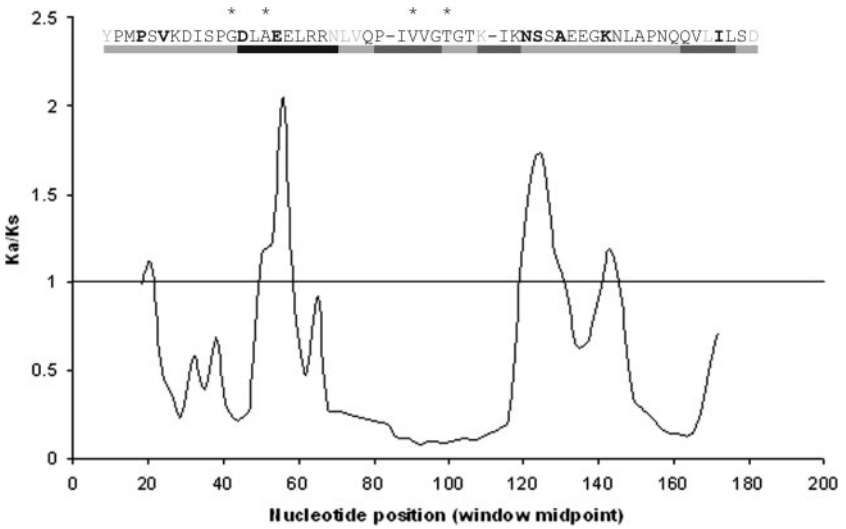

FIG. 4. Sliding-window analysis of *S. pneumoniae pbp2x* PASTA domain 1 compared to the same domain of 27 other firmicute *pbp2x* orthologs. The corresponding protein structure (black bars, α-helix; gray bars, β-sheet) drawn to scale is shown above the graph, together with the *S. pneumoniae* PBP2x amino acid sequence alignment. Amino acids residues implicated in interactions with cefuroxime are denoted by asterisks, and residues shown in boldface are mutated in penicillin-resistant isolates. Amino acids shown in gray are not included in the sliding window analysis since they correspond to gaps in the alignment. The sliding-window analysis starts at position 18 due to "overhanging" amino acids from other sequences in the alignment.

The significantly higher average K_a/K_s value for multiple STPK-associated PASTA domains compared to that for the kinase domains could be explained by either positive selection or relaxation of purifying selection. However, the phylogenetic analysis indicating position-dependent evolution of individual PASTA domains could only be consistent with positive selection favoring PASTA domain diversity. We hypothesize that each domain has evolved a binding affinity toward a specific stem peptide ligand. The complement of PASTA domains associated with a particular protein is specific to each genus, and this is likely to reflect differences in peptidoglycan composition existing between families of bacteria (see below). Moreover, the complement of PASTA domains is specific to each family of gene orthologs within a genus. For example, the actinomycete *pknB* orthologs have a distinct complement of PASTA domains compared to the division cell wall-linked STPKs from the same bacteria. This is consistent with a particular complement of PASTA domains recognizing a specific set of the stem peptide ligands. In this way, the diversity of gram-positive peptidoglycan composition may influence the function of the various PASTA domain proteins in different ways.

Identification of variable regions within PASTA domains. The variability of peptidoglycan structure in gram-positive bacteria is likely to provide a selection pressure for the rapid evolution of PASTA domains. Indeed, the *pbp2x*-associated PASTA domains of penicillin-resistant *S. pneumoniae* isolates are mutational hotspots (8, 30). More than 70% of the stem peptides in these isolates can consist of abnormal branched structures (12), and PASTA domain mutations presumably allow these structures to be recognized. Uniquely for the first PASTA domain of this *pbp2x*, there is experimental data on amino acid residues that contact the β-lactam cefuroxime (13, 30) and also the positions of amino acid substitutions present in variants that recognize abnormal stem peptides in penicillin-resistant strains (8). To identify regions of variability in this domain that could be evolving to recognize different ligands, a sliding-window analysis of K_a/K_s ratios was performed for the first PASTA domain sequence of *S. pneumoniae pbp2x* compared to the same domain of 27 other firmicute *pbp2x* orthologs (Table 1). Using a window size of 12 and a step size of three nucleotides, codon-by-codon analysis of variability could be mapped against the corresponding domain structure, with attention to amino acid residues implicated in Van der Waal's interactions with cefuroxime and the positions of amino acid substitutions present in *pbp2x* genes from penicillin-resistant isolates (Fig. 4). Of particular note is a correlation between the highly variable regions of this domain ($K_a/K_s > 1$) with the locations of amino acid substitutions. Moreover, the most divergent sequence aligns to the first half of the α-helix containing two amino acids involved in interactions with the β-lactam antibiotic. The analysis is consistent with the evolution of PASTA domain diversity to recognize different peptidoglycan stem peptides.

The overall average K_a/K_s values for the first and second PASTA domains of 28 firmicute *pbp2x* orthologs belonging to 11 genera are 0.659 and 0.524, respectively. In contrast, the overall average K_a/K_s values for the 14 actinomycete *pknB*-associated PASTA domains from 7 genera are 0.895 (domain 1), 0.722 (domain 2), 0.779 (domain 3), and 0.859 (domain 4). With the exception of the comparison between the values for *pknB*-associated PASTA domain 2 and *pbp2x*-associated PASTA domain 1, in all other comparisons the diversity of the *pknB*-associated PASTA domains is significantly greater than that of the *pbp2x*-associated PASTA domains (at the 95% confidence level using a Mann-Whitney U test). A sliding-window codon-by-codon analysis of each *pknB*-associated domain revealed many more regions within each domain with K_a/K_s values greater than 1 than were present in either *pbp2x*-associated PASTA domain (results not shown). When mapped against domain structure, no consistent patterns of variability

could be identified, although in general regions with K_a/K_s values of greater than 1 mapped to linker regions. Hence, regions of high variability appear to be largely constrained by protein tertiary structure.

The complexity of peptidoglycan in gram-positive bacteria: a clue to PASTA domain multiplicity, diversity, and function. Peptidoglycan is a highly complex and essential macromolecule of bacterial cell walls (except mycoplasma, which coincidentally lack PASTA domain proteins) that contributes to cell shape and confers rigidity and resistance to osmotic pressure. It is constantly remodeled to allow cell growth and division. Whereas gram-negative bacteria have a single or few peptidoglycan layers composed of a uniform primary structure, gram-positive bacteria have up to 40 layers that can exhibit a great variation in composition and structural arrangement (28). Species-to-species differences are attributed to variation in the composition of the stem peptide linked to the glycan backbone. For example, the diamino acid component at position 3 of the peptide that is critical for cross-linking peptidoglycan chains can be any one of six different amino acids, or it can be unsubstituted, requiring that cross-linking is via a trifunctional amino acid at position 2. Moreover, a further increase in structural complexity is provided by frequent cross-bridges that link two chemically distinct stem-peptides. A corollary to this is that, in contrast to gram-negative peptidoglycan, the composition of murein in a gram-positive species can be nonuniform. In addition, the composition can be modified in response to environmental pressures. For example, up to 22 novel stem peptides are observed after the induction of vancomycin resistance in *S. aureus* (29), and heritable changes to peptidoglycan structure are a basis for resistance to β-lactam antibiotics (8).

Given the chemical diversity of gram-positive peptidoglycan, how is the cross-linked polymer assembled reproducibly? Mechanisms are required during cell growth and division to ensure that peptidoglycan is remodeled in a manner to reproduce a specific template. De novo peptidoglycan synthesis is preceded by localized murein hydrolysis, involving the breakage of existing cross-bridges, to allow the insertion of newly synthesized polymers that can then be cross-linked with "old" strands. For growth (elongation), the extent of incorporation of newly synthesized peptidoglycan is limited, but occurs at many sites, whereas multiple new strands are inserted at the two poles of a cell during septation. Genetic and cytological evidence suggests that these two processes are mediated by separate enzyme machinery. It is vital that appropriate peptidoglycan-synthesizing machinery is produced at the requisite time in the cell cycle and correctly positioned to allow for either growth or division.

The PASTA domains of PBP2x are likely to have a critical role in the positioning of this FtsI ortholog at the division site in *S. pneumoniae*. In this organism, deletion of the small carboxypeptidase, PBP3, thought to be responsible for generating a tripeptide stem transpeptidation substrate of PBP2x, leads to frequent incorrect positioning of the transpeptidase (20). Normally, PBP2x colocalizes with FtsZ and FtsW, and this positioning is due to recruitment by the localized pool of substrate stem peptides. Low-affinity binding to the substrate by a PASTA domain can thereby guide the localization of PBP2x to the division site. A second PASTA domain, recognizing an alternative stem peptide ligand, can provide greater specificity for PBP2x guidance, contributing to the reproduction of a nonuniform peptidoglycan template.

In comparison to the PBP2x orthologs, the STPKs possess a greater number of PASTA domains, and these exhibit even greater diversity (higher overall K_a/K_s ratios), suggesting evolution to recognize a wider spectrum of ligands. In actinobacteria, PknB is likely to function by sensing unlinked peptidoglycan in order to direct synthesis and/or localization of the machinery required for cell wall modification to permit growth. Likely ligands for the four PASTA domains of the STPK are unlinked stem peptides of different composition resulting either from the action of modifying endopeptidases on cross-linked peptidoglycan or from the synthesis of new peptidoglycan. Another potential source of ligands is the release of muropeptides. Recent research on resuscitation-promoting factors of actinobacteria has revealed that their biological activity in stimulating the revival of quiescent bacteria correlates with their murolytic activity (21). Extremely low concentrations of these cytokines are required for their biological activity, indicating that a limited release of muropeptides is likely to trigger a signaling cascade that amplifies the response. PknB is a good candidate as a kinase that can trigger such a phosphorelay and thus may be crucial in signaling the switch between latency and the onset of progressive tuberculosis.

In addition to growth and cell division, various developmental processes in prokaryotes also demand peptidoglycan remodeling. The PASTA domain STPKs of firmicutes appear to be dedicated to these changes, with evidence for a coordinating role in developing competence, biofilm formation, and sporulation. We postulate that different unlinked stem peptides may participate in the activation of these STPKs, leading to remodeling of the peptidoglycan appropriate to a specific developmental pathway.

Concluding remarks. Even for the uniform and relatively simple cell wall structure of *E. coli*, our understanding of peptidoglycan remodeling associated with growth and division is limited. The increased chemical complexity and variety of the gram-positive cell wall would appear to demand a higher-order coordination of peptidoglycan remodeling. The evolution of proteins with multiple diverse PASTA domains that can recognize different unlinked stem peptides is likely to have been driven by the complexity and variety of gram-positive murein. The multiple PASTA domains of the STPKs can contribute to the reproduction of complex peptidoglycan by interaction with diverse stem-peptide ligands. As a consequence, signaling pathways can be triggered that coordinate the remodeling machinery. Since these interactions are important in pathogenic organisms such as *M. tuberculosis*, this underlines the importance of investigating the nature of the ligands that individual PASTA domains recognize.

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