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## Critical Review

# $\beta$ -Lactam Resistance: The Role of Low Molecular Weight Penicillin Binding Proteins, $\beta$ -Lactamases and LD-Transpeptidases in Bacteria Associated with Respiratory Tract Infections

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## Abstract

Disruption of peptidoglycan (PG) biosynthesis in the bacterial cell wall by  $\beta$ -lactam antibiotics has transformed therapeutic options for bacterial infections. These antibiotics target the transpeptidase domains in penicillin binding proteins (PBPs), which can be classified into high and low molecular weight (LMW) counterparts. While the essentiality of the former has been extensively demonstrated, the physiological roles of LMW PBPs remain poorly understood. Herein, we review the function of LMW PBPs,  $\beta$ -lactamases and LD-transpeptidases (Ldts) in pathogens associated with respiratory tract infections. More specifically, we explore their roles in mediating  $\beta$ -lactam resistance. Using a comparative genomics approach, we identified a high degree of genetic redundancy for LMW PBPs which retain the motifs, SxxN, SxN and KTG required for catalytic activity. Differences in domain architecture suggest

distinct physiological roles, possibly related to bacterial cell cycle and/or adaptation to various environmental conditions. Many of the LMW PBPs play an important role in  $\beta$ -lactam resistance either through mutation or variation in abundance. In all of the bacterial genomes assessed, at least one  $\beta$ -lactamase homologue is present, suggesting that enzymatic degradation of  $\beta$ -lactams is a highly conserved resistance mechanism. Furthermore, the presence of Ldt homologues in the majority of species surveyed suggests that alternative PG crosslinking may further mediate  $\beta$ -lactam drug resistance. A deeper understanding of the interplay between these different mechanisms of  $\beta$ -lactam resistance will provide a framework for new therapeutics, which are urgently required given the rapid emergence of antimicrobial resistance. © 2018 IUBMB Life, 00(0):000–000, 2018

**Keywords:** peptidoglycan; penicillin; low molecular weight penicillin binding protein; DD-carboxypeptidase;  $\beta$ -lactamase; LD-transpeptidase

**Abbreviations:** BLNAR,  $\beta$ -lactamase-nonproducing ampicillin resistance; BLPACR,  $\beta$ -lactamase-producing amoxicillin-clavulanic acid-resistant; DD-CPase, DD-carboxypeptidase; HMW, high molecular weight; Ldts, LD-transpeptidases; LMW, low molecular weight; PBPs, penicillin binding proteins; PG, peptidoglycan; TG, transglycosylase

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## INTRODUCTION

The respiratory tract is constantly exposed to air containing potential infectious agents and as a result is equipped with various innate defense mechanisms (1). These include mechanisms such as activation of autophagy pathways and pro-inflammatory cytokine regulation. To bypass these, pathogens may mask detection from immune cells and/or avoid lysosomal enzymes (2–4). Despite the availability of antibiotics, respiratory tract infections remain a major cause of morbidity and mortality on a global scale (5). Common to all bacterial respiratory infections (*e.g.*, epiglottitis, laryngotracheitis or pharyngitis in the upper tract and bronchitis, bronchiolitis or pneumonia in the lower) is the requirement for bacterial colonization to different compartments of the lung and nasopharynx. This may occur either via attachment to the epithelial lining and utilization of nutrients in the mucosa or, as is the case with tuberculosis, uptake and proliferation in resident alveolar macrophages which facilitate transport to deeper lung tissue (5,6).

Antibiotics have revolutionized the practice of medicine, providing a multitude of therapeutic modalities that accelerate clearance of invading bacteria and the best example of this is the widespread use of penicillin and other  $\beta$ -lactam-based antibiotics. However, the rise of antimicrobial resistance now threatens to undermine these hard-won gains and a mechanistic understanding of how  $\beta$ -lactam resistance emerges in bacteria is paramount to combat the increasing threat of drug resistant bacterial infections. Penicillin binding proteins (PBPs) play a central role in  $\beta$ -lactam resistance. Comprehensive reviews regarding general PBP molecular structure, role in peptidoglycan (PG) biosynthesis, hydrolysis and bacterial shape have previously been published (7–9). Herein we review the function of low molecular weight (LMW) PBPs and  $\beta$ -lactamases in modulating  $\beta$ -lactam resistance in respiratory tract infections. In addition, our analysis includes the function of LD-transpeptidases (Ldts) as certain LMW PBPs remodel PG stem peptides to allow for 3→3 crosslink formation by these enzymes, which are also susceptible to new derivatives of  $\beta$ -lactams. The most well-known etiological agents of respiratory infections typically include *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*. All of these adopt a somewhat similar modality of colonization involving intracellular growth in macrophages and stimulation of adaptive immunity.

Among the variety of bacterial products that are recognized by the host immune system, cell wall derivatives such as PG play an important role in modulating the immune response. Bacteria possess cell walls which prevent cell lysis due to internal turgor pressure and act as an intrinsic barrier against environmental stresses (10–13). One important difference between Gram-positive and -negative bacteria is the amount of PG surrounding the cytoplasmic membrane (7,14). In the case of mycobacteria, the cell wall is more complex and comprises PG linked to arabinogalactan and mycolic acid. These components are surrounded by a glycan-rich capsule which is required to

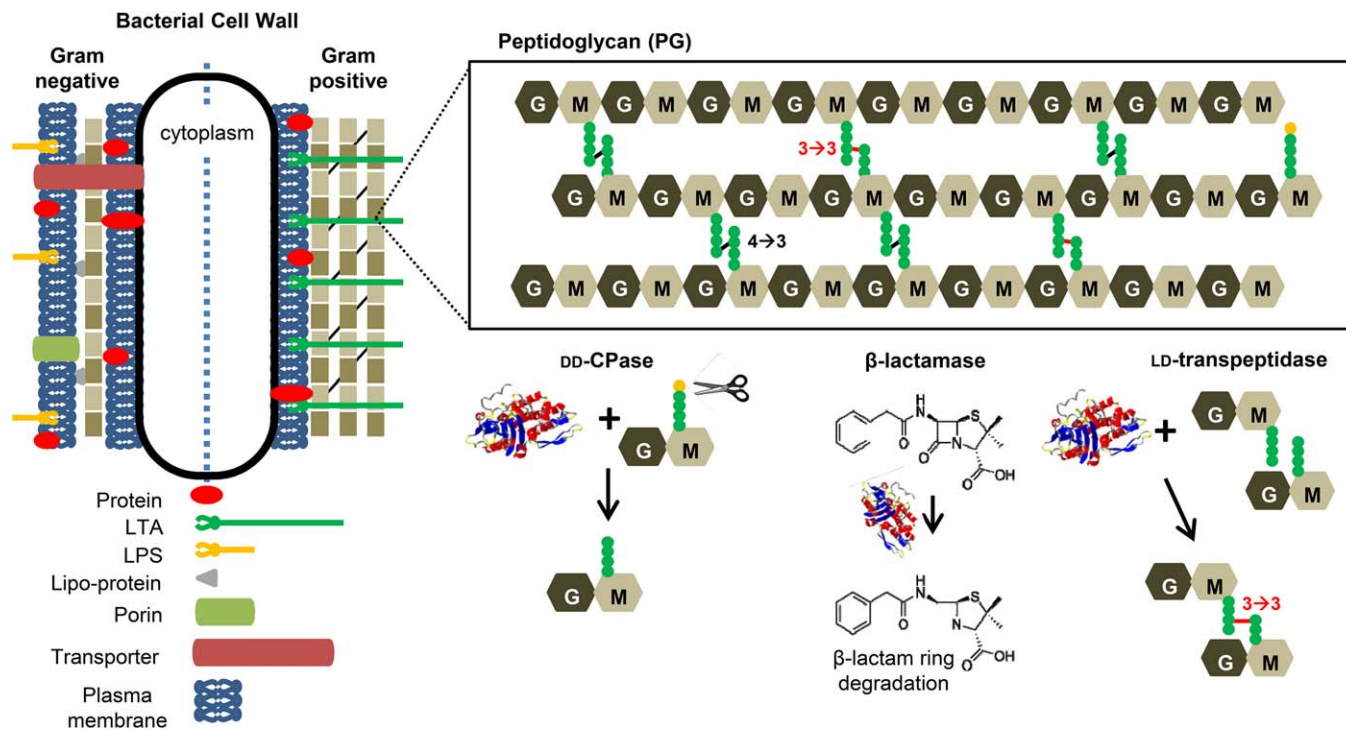
mediate pathogenicity (15–17). In all bacteria, PG is constantly remodeled during growth by PBPs and other enzymes for the insertion and incorporation of new material at defined cellular locations. These remodeling processes are targeted by  $\beta$ -lactam antibiotics as they represent a central vulnerability in the bacterial cell cycle. Before remodeling in the periplasm, PG biosynthesis occurs in the cytoplasm to yield lipid II, a PG subunit comprised of an undecaprenyl-linked disaccharide-pentapeptide containing  $\beta$ -linked *N*-acetylglucosamine and *N*-acetylmuramic acid. Incorporation is facilitated by the PBPs, thus termed for their affinity and binding of penicillin and other  $\beta$ -lactams (18). High molecular weight (HMW) PBPs, which are essential for growth, link the saccharide component via a transglycosylase (TG) domain and form crosslinks between neighboring stem peptides via a transpeptidase domain (19). In *Escherichia coli*, crosslinking of L-Ala<sup>1</sup>-D-Glu<sup>2</sup>-mesoDAP<sup>3</sup>-D-Ala<sup>4</sup>-D-Ala<sup>5</sup> stem peptides mainly occur between the  $\alpha$ -carboxyl of D-Ala<sup>4</sup> of one subunit and the  $\epsilon$ -amine of mesoDAP<sup>3</sup> of another subunit to form 4→3 crosslinks (20). In contrast, LMW PBPs are dispensable but regulate PG crosslinking (21,22). As PBPs are vital for bacterial survival, the discovery of penicillin in the early 1940s proved to be a ground-breaking tool in combating bacterial infections (23). The primary target of  $\beta$ -lactam antibiotics is the transpeptidase domain of HMW PBPs. Due to the structural resemblance to the terminal D-Ala<sup>4</sup>-D-Ala<sup>5</sup> motif present in PG stem peptides, these antibiotics effectively act as potent inhibitors by irreversibly modifying the transpeptidase domain (18). Structurally, this class of antibiotics is defined by the presence of a  $\beta$ -lactam ring which is directly targeted for hydrolysis (24–26).  $\beta$ -Lactamase enzymes are functionally diverse but structural evidence suggest that these enzymes are evolutionarily related to PBPs (25). Four molecular classes (A–D) exist, with class A, B and C containing active-site serine enzymes and class D, zinc-dependent or “EDTA-inhibited” enzymes. As PBPs contain a serine amino acid in the active site, any subsequent comparisons herein will exclude class D  $\beta$ -lactamases.

In addition to the multiplicity of LMW PBPs and  $\beta$ -lactamases (22), bacteria can also modulate  $\beta$ -lactam resistance via Ldts, which catalyze the formation of alternative PG 3→3 crosslinks between two mesoDAP<sup>3</sup> units (20,27). Ldts use cysteine in their active-sites and are structurally unrelated to DD-transpeptidases, which use a serine residue (28). Carbapenem derivatives of  $\beta$ -lactams are active against Ldts, which makes their application particularly exciting with the emergence of extensively and multi-drug-resistant bacterial strains (29).

## MOLECULAR FEATURES AND DOMAIN ARCHITECTURE OF LMW PBPS (DD-CPASES AND $\beta$ -LACTAMASES) AND LDTS

### Low Molecular Weight Penicillin Binding Proteins

A variety of PG remodeling enzymes exist but homologues vary in activity and according to species (30). An important



**FIG 1**

General structure of Gram-negative and Gram-positive cell walls. Complexity of each cell wall varies but the fundamental difference is the level of PG present. PG is comprised of alternating  $\beta$ -linked N-acetylglucosamine (G) and N-acetylmuramic acid (M) (hexagons in shades of brown). In *E. coli*, stem-peptides (green dots), comprised of L-Ala<sup>1</sup>-D-Glu<sup>2</sup>-mesoDAP<sup>3</sup>-D-Ala<sup>4</sup>-D-Ala<sup>5</sup> are attached to (M). Composition of the stem peptide varies according to bacterial species but in general, they are crosslinked by HMW PBPs in a 4-3 configuration (black bars between stem peptides linking D-Ala<sup>4</sup> and mesoDAP<sup>3</sup> of adjacent stem peptides). DD-CPases regulate the level of crosslinking by cleaving the terminal D-Ala (orange dot) to limit substrate for HMW PBPs.  $\beta$ -Lactamases degrade the cyclic amide bond ( $\beta$ -lactam ring) rendering the antibiotic ineffective. An alternative crosslink can be formed by Ldts to yield a 3-3 configuration (red bars between stem peptides linking two mesoDAP residues) using tetra-stem peptides a substrate.

biological role attributed to these enzymes is the modulation of cell shape and underlying PG structure to mediate growth (cell extension and division), pathogenesis, survival/transmission in adverse environmental conditions and host-pathogen interactions. Readers are referred to a comprehensive review summarizing the aspects (7). There are two main classes of PG hydrolases: (i) glycosidases, which cleave the glycan backbone and (ii) amidases, which cleave the stem peptide (30). Peptidases are either endopeptidase, which cleave the amide bond between two amino acids within or between stem peptides, or carboxypeptidase, which cleave the C-terminal amino acid of the stem peptide (Fig. 1) (31). Cleavage between two D-amino acids or between the D- and L-amino acid is mediated by DD-peptidases and LD-peptidases, respectively (7). *E. coli* encodes a multiplicity of class C LMW PBPs and several studies have attempted to describe the physiological roles of these enzymes (8,9,11). In this case, DD-carboxypeptidases (DD-CPases) and endopeptidases are encoded by *pbp5/pbp6/pbp6B/dacD* and *pbp4/pbp7*, respectively. A model elucidating the carboxypeptidase reaction, that is, cleavage of the C-terminal amino acid residue from the stem peptide, and the amino acid residues required was determined using site-directed mutagenesis (31-34). Essentially, D-alanine residues on the penta-peptide

sidechain interact with Ser<sub>44</sub> and Lys<sub>213</sub> of the Ser-Xaa-Xaa-Lys (SxxK) and Lys-Thr-Gly (KTG) motifs of PBPs (Supporting Information Fig. S1). A mutation in either of these residues eliminates DD-CPase activity (33,34). During acylation, Lys<sub>47</sub> of the SxxK tetrad acts as a proton acceptor for the nucleophilic attack by Ser<sub>44</sub>, which aids in the formation of an acyl-enzyme intermediate (35-38). During deacylation of this intermediate, Ser<sub>110</sub> of the Ser-Xaa-Asn (SxN) and Lys<sub>213</sub> of the KTG motif form a hydrogen bridge with a water molecule. This assists to properly orientate the hydrolytic water molecule toward the carbonyl carbon of the acyl-enzyme complex to dissociate the complex during DD-carboxypeptidation (37). Moreover, the Lys<sub>213</sub> of KTG motif acts as a general base in polarizing Ser<sub>110</sub> during deacylation of the acyl-enzyme intermediate and acts as an electrostatic anchor for substrate binding (36,37). Additional residues such as Asp<sub>175</sub>, His<sub>216</sub> and Thr<sub>217</sub> are all in close proximity to the KTG motif and assist in regulating enzyme activity (31,33). These residues are required for activity and appear to be grouped around a central peptide-binding pocket in almost all homologues assessed (Supporting Information Fig. S1), suggesting that this region is crucial in mediating enzyme kinetics and substrate specificity (31).

### LD-Transpeptidases

Ldts have been associated with  $\beta$ -lactam resistance in *Enterococcus faecium* (39,40). These enzymes catalyze the formation of 3 $\rightarrow$ 3 crosslinks to replace the 4 $\rightarrow$ 3 crosslinks, which are formed by HMW PBPs (41). The mechanism was not linked to any variation in Ldt activity but rather with the production of a DD-CPase that generates the tetrapeptide donor stems (27,40). Therefore, it was proposed that the physiological role of Ldts in  $\beta$ -lactam-susceptible strains was their involvement in PG maturation (27).

The crystal structures of two related Ldts, Ldt<sub>fm</sub> from *E. faecium* (42) and Ldt<sub>Bs</sub> from *B. subtilis* (43) failed to elucidate the mechanism of inactivation by carbapenems. However, the Ldt<sub>fm</sub> contained a mixed  $\alpha$ - $\beta$ -fold and a structural domain associated with *E. coli* Ldts, that is, ErfK\_YbiS\_YhnG (44). A single cysteine residue (Cys<sub>442</sub>), in a Cys-His-Gly catalytic triad, serves as the catalytic residue as a Cys<sub>442</sub>Ala substitution abrogated function (42). Metallic ions were detected in the active sites of the two crystallized Ldts, that is, Cd<sup>2+</sup> in Ldt<sub>Bs</sub> and Zn<sup>2+</sup> in Ldt<sub>fm</sub>. Lecoq et al. subsequently showed that the Ldt<sub>Bs</sub> is comprised of two domains, a N-terminal LysM PG-binding domain (from residues 1 to 5) and a C-terminal catalytic domain from residues 55 to 169 (45). Moreover, it was demonstrated that the cysteine residue (Ldt<sub>Bs</sub>) remains protonated under neutral pH conditions and hydrogen bonds to His<sub>126</sub> (45). Gly<sub>127</sub>, the third residue in the Ldt<sub>Bs</sub> active site, serves as a hydrogen bond acceptor for the histidine through its backbone carbonyl. Therefore, in contrast to the canonical thiolate-imidazolium (S<sup>-</sup>/NH<sup>+</sup>) ion pair found in the active site of prototypical cysteine proteases, Ldt<sub>Bs</sub> uses a neutral, hydrogen bonded thiol-imidazole pair (SH/N) for catalysis (28).

To assess the conservation of the abovementioned catalytic residues/mechanisms, amino acid sequences of the *E. coli* LMW PBPs and Ldts were retrieved from genome databases (<http://genolist.pasteur.fr/or> [www.genome.jp](http://www.genome.jp)) and used for comparative genomics with common respiratory tract pathogens. Sequences alignments comparing LMW PBPs and  $\beta$ -lactamases identified motifs characteristic of PBPs, that is, SxxK, SxN and KTG (Supporting Information Fig. S1), with some exceptions (Table 1). Interrogation of the *E. coli* (K12 substr. MG1665) homologues detected all three motifs and the sequential order of the motifs is generally consistent. However, in *apmH*, the KTG motif precedes SxN. In *S. aureus* (subsp. aureus N315 MSRA/VSSA), the proteins annotated as  $\beta$ -lactamases or probable  $\beta$ -lactamases lacked one or two motifs entirely. Hence, the structural basis for  $\beta$ -lactamase function in these proteins remains unclear. Similarly, the sole  $\beta$ -lactamase of *S. pneumoniae* (TIGR virulent serotype 4), SP0010, lacked all three motifs. One of the three *H. influenzae* (10810 serotype B) LMW PBP homologues, HIB\_01590, lacked the SxN and KTG motifs, whereas its sole  $\beta$ -lactamase, HIB\_01180, lacked both SxxK and SxN motifs. PA4110, the only  $\beta$ -lactamase of *P. aeruginosa* (PA01), lacked SxN. The  $\beta$ -lactamase homologue of *M. tuberculosis* (H37Rv), Rv2068c (BlaC), only maintained the SxxK and KTG motifs but

upon further comparison with other SxN homologues, the asparagine of the SxN motif appeared to be replaced with glycine (Supporting Information Fig. S1).

Further analysis of domain architecture (using tools at <https://www.ebi.ac.uk/interpro/search/sequence-search>) suggested that the *E. coli* genes predominantly encoded LMW PBPs (PBP5, PBP6 and PBP6b) with S11\_PBP5C domains, (Table 2). PBPG maintained the S11 domain but lacked PBP5C, the physiological significance of which remains unknown. PBP4 was classified as a S13 family protein, while the two  $\beta$ -lactamases, AmpC and AmpH, contained  $\beta$ -lactamase-related domains. In *S. aureus*, the sole PBP (annotated as PBP4) contains an S11\_PBP4 domain and of the four potential  $\beta$ -lactamases identified, only two (BlaZ and SA2230) were previously annotated as proteins related to  $\beta$ -lactamase families or domains. From our analysis, the remaining two (SA1633 and SA1818) were classified as “probable”  $\beta$ -lactamases. However, of the three motifs necessary for activity, SA1633 contains only the SGN and KTG residues, whereas SA1818 contains none of these residues (Table 1). Furthermore, domain architectures for SA1633 and SA1818 matched to proteins of unknown function (DUF4888). Therefore, we propose that these may not necessarily function as  $\beta$ -lactamases that use serine in the active site.

The sole LMW PBP of *S. pneumoniae* and *H. influenzae* displayed significant homology to *E. coli* *dacA* and retained the S11\_PBP5C domain. Similarly, their  $\beta$ -lactamase counterparts aligned with  $\beta$ -lactamase/transpeptidase family proteins. The two additional LMW PBPs of *H. influenzae*, HIB\_01590 and HIB\_14940 (PBP4) encoded S11 and S13 domains, respectively, with the latter aligned with the PBP4 homologue of *E. coli*. The domain architectures of three *P. aeruginosa* LMW PBP and two  $\beta$ -lactamase homologues also aligned with *E. coli* counterparts, suggesting that they may perform similar physiological roles. Analyses of the *M. tuberculosis* homologues indicated that DacB1 and DacB2 are similar to each other and they both encode S11 domains. In contrast, Rv3627c encodes a S13 family protein which implies that it is the direct homologue of *E. coli* PBP4. Interestingly, the mycobacterial BlaC showed greater homology to a class A  $\beta$ -lactamase family protein unlike AmpC and AmpH of *E. coli*.

Using the *E. coli* Ldt homologues in BLAST analyses revealed the absence of these proteins in *S. aureus* and *S. pneumoniae* (Table 2). In contrast, *H. influenzae* and *P. aeruginosa* encoded one (HIB\_18450) and two homologues (PA2854 and PA3756), respectively. Our bioinformatics analysis for domain architecture revealed that, as for the *E. coli* homologues LdtC and LdtE, an additional LysM domain was present in the PA2854 homologue. The genome of *M. tuberculosis* encodes five distinct homologues but, surprisingly, all lacked LysM or PG-binding domains (41). Moreover, sequence alignments showed the very strict conservation of the proposed active-site cysteine residue in all of these homologues (Supporting Information Fig. S2). Given the variations in PBP complement, canonical  $\beta$ -lactamase motifs, and differential abundance of Ldt in the select

**TABLE 1****Presence of PBP signature domains/motifs in serine-type LMW PBP and  $\beta$ -lactamase homologues**

Strain and Locus name	SxxK motif	SxN motif	KTG motif
<i>E. coli</i> str. K-12 substr. MG1665			
<i>pbp4</i> ( <i>dacB</i> )	STQK	SDN	KTG
<i>pbp5</i> ( <i>dacA</i> )	SLTK	SGN	KTG
<i>pbp6</i> ( <i>dacC</i> )	SLTK	SGN	KTG
<i>pbp6B</i> ( <i>dacD</i> )	SLTK	SGN	KTG
<i>pbp7</i> ( <i>pbpG</i> )	SISK	SEN	KTG
EG10040 ( $\beta$ -lactamase– <i>ampC</i> )	SVSK	SDN	KTG
EG12867 ( $\beta$ -lactamase– <i>ampH</i> )	SLTK	SGN <sup>a</sup>	KTG
<i>S. aureus</i> subsp. aureus N315 (MSRA/VSSA)			
SA0598 ( <i>pbp4</i> )	SMTK	SKN	KTG
SAP010 ( $\beta$ -lactamase– <i>blaZ</i> )	STSK	SDN	–
SA1633 ( $\beta$ -lactamase)	–	SGN	KTG
SA1818 ( $\beta$ -lactamase)	–	–	–
SA2230 ( $\beta$ -lactamase)	SNTK	–	–
<i>S. pneumoniae</i> TIGR (virulent serotype 4)			
SP0872 ( <i>dacA</i> )	SITK	SAN	KTG
SP0010 ( $\beta$ -lactamase)	–	–	–
<i>H. influenzae</i> 10810 (serotype B)			
HIB_00290 ( <i>dacA</i> )	SLTK	SGN	KTG
HIB_01590	SKFK	–	–
HIB_14940 ( <i>dacB</i> )	STQK	SDN	KTG
HIB_01180 ( $\beta$ -lactamase)	–	SDN	–
<i>P. aeruginosa</i> PAO1			
PA3047 ( <i>pbp4</i> )	STMK	SNN	KTG
PA3999 ( <i>pbp5</i> )	SLTK	SGN	KTG
PA0869 ( <i>pbp7</i> )	SITK	SEN	KTG
PA4110 ( $\beta$ -lactamase– <i>ampC</i> )	SVSK	–	KTG
PA5514 ( $\beta$ -lactamase)	STYK	–	KTG
<i>M. tuberculosis</i> H37Rv			
Rv3330 ( <i>dacB1</i> )	SVIK	SGN	KTG

**TABLE 1** (Continued)

Strain and Locus name	SxxK motif	SxN motif	KTG motif
Rv2911 ( <i>dacB2</i> )	STIK	SGN	KTG
Rv3627c	STNK	SDN	KTG
Rv2068c ( $\beta$ -lactamase– <i>blaC</i> )	STFK	SDG <sup>b</sup>	KTG

Amino acid sequences were interrogated for the presence of each motif. The sequential order of detection in any given PBP sequence is usually SxxK, SxN and KTG.

<sup>a</sup>A KTG motif occurs upstream of SxN in the *AmpH* homologue of *E. coli*.

<sup>b</sup>An SDG sequence aligned with KTG of the  $\beta$ -lactamases in *HIB\_01180*, *Rv2068c* (*blaC*) and *SAP010* (*blaZ*). Despite the absence of the SxN motif, *Rv2068c* is a functional  $\beta$ -lactamase.

group of organisms assessed, we next reviewed the literature for the roles of these proteins in drug resistance.

## $\beta$ -LACTAM RESISTANCE IS MEDIATED IN PART BY LMW PBPS AND LDTS

The *E. coli* PBP5 homologue, *dacA* (Tables 1 and 2), has a demonstrated role in the maintenance of cell morphology (46–48). In addition, deletion of PBP5 resulted in a four- to eight-fold increase in susceptibility to all  $\beta$ -lactams tested (49). Complementation with PBP5 restored  $\beta$ -lactam sensitivity to wildtype levels suggesting that PBP5 plays a specific role in mediating  $\beta$ -lactam resistance. Interestingly, when mutant strains were heterologously complemented with *dacA* homologues from *Salmonella enterica*, *Vibrio cholerae* and *H. influenzae*, sensitivity to  $\beta$ -lactams decreased either completely or partially, implying a similar role for PBP5 in other bacterial species (49). Below, we review the literature to determine the contribution of PBP5 homologues, and other LMW PBPs, to  $\beta$ -lactam resistance. Possible mechanisms are summarized in Fig. 2.

### *Staphylococcus aureus*

Staphylococci possess two distinct mechanisms for  $\beta$ -lactam resistance, namely the expression of PBP2A which is insensitive to  $\beta$ -lactams and the production of  $\beta$ -lactamases (50). The contribution of LMW PBPs to resistance, if any, remains unclear. PBP4 has previously been shown to be non-essential for growth *in vitro* despite being associated with low levels of resistance to vancomycin and several  $\beta$ -lactam antibiotics (51). PBP4 plays a negligible role in high-level  $\beta$ -lactam resistance, particularly to drugs such as nafcillin, methicillin, cefoxitin and imipenem. Assessment of PBP profiles for four vancomycin intermediately susceptible clinical isolates showed PBP4 to be undetectable in three and significantly decreased in the fourth (52). However, the protein was easily detectable in vancomycin-sensitive strains suggesting that PBP4 activity directly influences vancomycin resistance in *S. aureus* (52).

More recent evidence has linked community-acquired methicillin resistance in *S. aureus* specifically to PBP4. Methicillin-resistant *S. aureus* (MSRA) strains may acquire, by horizontal gene transfer and integration of mobile genetic elements, an additional HMW PBP gene (*mecA*) encoding PBP2A, which is sufficient for high-level resistance to almost all  $\beta$ -lactams (53). Loss of PBP4 resulted in a 16-fold reduction in oxacillin and nafcillin resistance, demonstrating that the HMW PBP, PBP2A, is not the only determinant of methicillin resistance (54). Growth of MRSA at low pH (~5) completely suppressed drug resistance, suggesting that PBP2A-mediated resistance is dependent on environmental pH (55). Therefore, it is plausible that PBP4 is affected similarly. In a mutant derived from a susceptible clinical strain (SG511 Berlin), the only differences were a single nucleotide change and a 90-nucleotide deletion, comprised of a 14-nucleotide inverted repeat in the non-coding *pbp4* gene promoter proximal region (56). Similarly, *in vitro* acquired methicillin resistance was associated with increased production of PBP4 (57). The loss of PBP4 is also associated with adverse effects on transcription of PBP2 in cells after exposure to oxacillin leading to decreased PG crosslinking (54,58). PBP4 is adjacent to but divergently transcribed relative to the putative ABC-type transporter gene *abcA* (SA0599). Disruption of *abcA* resulted in elevated resistance to  $\beta$ -lactams, which was associated with increased PG crosslinking. Promoter fusion studies demonstrated that the *abcA* mutation led to dramatic increases in both the PBP4 and *abcA* promoter activities. These genes have long, untranslated leader sequences with an 80 bp transcriptional overlap. When a 26 bp region containing an inverted repeat sequence was deleted, expression in both promoters (*i.e.*, for *abcA* and PBP4) was abolished. Thus, both genes are under the control of a common regulatory mechanism that may use the transport function of the *abcA* gene product (59).

### *Streptococcus pneumoniae*

Streptococci include species that display variable resistance to  $\beta$ -lactams ranging from high-level resistance to susceptible, an effect that may be related to inherent properties of PBPs (60).

TABLE 2

Representative homologues of serine-type LMW PBPs,  $\beta$ -lactamases and cysteine-type Ldts<sup>a</sup> in major bacterial pathogens infecting the respiratory tract

Bacterial strain	Locus name	LMW (DD-CPase and/or DD-endopeptidase)	
		Serine-type peptidases; $\beta$ -lactamases and Cysteine-type Ldts (Domain and/or Family architecture)	
		Domain structure	
<i>Escherichia coli</i> str. K-12 substr. MG1665	EG10201 (PBP5, <i>dacA</i> )		(D) S11_PBP5C
	EG10203 (PBP6, <i>dacC</i> )		(D) S11_PBP5C
	EG11893 (PBP6b, <i>dacD</i> )		(D) S11_PBP5C
	EG12015 (PBPG, PBP7)		(D) S11
	EG10202 (PBP4, <i>dacB</i> )		(F) S13
	EG10040 ( <i>ampC</i> )		(D) $\beta$ -lactamase-related
	EG12867 ( <i>ampH</i> )		(D) $\beta$ -lactamase-related
	EG12682 ( <i>erfK/srfK/ltdA</i> )		(D) Ldt
	EG13324 ( <i>ybiS/ltdB</i> )		(D) Ldt
	EG13437 ( <i>ycfS/ltdC</i> )		(D) LysM; Ldt
	EG11253 ( <i>ycbB/ltdD</i> )		(D) PG binding, Ldt
	EG14015 ( <i>ynhG/ltdE</i> )		(D) LysM; Ldt
	EG13148 ( <i>yafK</i> )		(D) Ldt
<i>Staphylococcus aureus</i> subsp. aureus N315 (MSRA/VSSA)	SA0598 (PBP4)		(D) S11_PBP4
	SAP010 ( <i>blaZ</i> )		(F) $\beta$ -lactamase class A
	SA1633 ( $\beta$ -lactamase) <sup>b</sup>	-	(F) DUF4888
	SA1818 ( $\beta$ -lactamase) <sup>b</sup>	-	(F) DUF4888
	SA2230 ( $\beta$ -lactamase) <sup>b</sup>		(D) $\beta$ -lactamase-related
<i>Streptococcus pneumoniae</i> TIGR (virulent serotype 4)	SP_0872 (PBP5/6, <i>dacA</i> )		(D) S11_PBP5C
	SP_0010		(F) $\beta$ -lactamase/transpeptidase-like
<i>Haemophilus influenzae</i> 10810 (Serotype b)	HIB_00290 (PBP5/6, <i>dacA</i> )		(D) S11_PBP5C
	HIB_01590		(D) S11
	HIB_14940 (PBP4, <i>dacB</i> )		(D) S13
	HIB_01180 ( $\beta$ -lactamase)		(F) $\beta$ -lactamase; class A
	HIB_18450		(D) Ldt
<i>Pseudomonas aeruginosa</i> PAO1	PA3047 (PBP4, <i>dacB</i> )		(D) S13
	PA3999 (PBP5/6, <i>dacC</i> )		(D) S11_PBP5C
	PA0869 (PBP7, <i>pbpG</i> )		(D) S11
	PA4110 ( $\beta$ -lactamase- <i>ampC</i> )		(D) $\beta$ -lactamase-related; class C
	PA5514 ( $\beta$ -lactamase)		(D) PBP, Transpeptidase; class D
	PA2854 ( <i>erfK/srfK/ltdA</i> )		(D) LysM, Ldt
	PA3756		(D) Ldt
<i>Mycobacterium tuberculosis</i> H37Rv	Rv3330 ( <i>dacB1</i> )		(D) S11
	Rv2911 ( <i>dacB2</i> )		(D) S11
	Rv3627c		(F) S13
	Rv2068c ( $\beta$ -lactamase- <i>blaC</i> )		(F) $\beta$ -lactamase; class A
	Rv0116c ( <i>ldt<sub>Mt1</sub></i> )		(D) Ldt
	Rv2518c ( <i>ldt<sub>Mt2</sub></i> )		(D) Ldt
	Rv1433 ( <i>ldt<sub>Mt3</sub></i> )		(D) Ldt
	Rv0192 ( <i>ldt<sub>Mt4</sub></i> )		(D) Ldt
	Rv0483 ( <i>ldt<sub>Mt5</sub></i> ; <i>lprQ</i> )		(D) Ldt

Data were accessed in January 2018. Proteins were identified by BLASTp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and KEGG ([www.genome.jp](http://www.genome.jp)), using *E. coli* and *M. tuberculosis* homologues as query sequences. Gene names are represented as annotations used in KEGG for standardization. Domain annotation was obtained at InterPro (<https://www.ebi.ac.uk/interpro/>): Blue peptidase\_S11, PF00768; red PBP5\_C, PF07943; purple peptidase\_S13, PF02113; green  $\beta$ -lactamase/transpeptidase-like, PF0144; grey PBP4, IPR037091; orange  $\beta$ -lactamase class A, IPR000871; Olive green, L,D-transpeptidase catalytic domain IPR005490; black, LysM domain IPR018392; pink, PG binding-like IPR002477. Schematic diagrams are not drawn to scale.

<sup>a</sup>Ldts possess an active-site cysteine residue as opposed to serine but are integral in PG processing and  $\beta$ -lactam resistance.

<sup>b</sup>Annotated as a probable  $\beta$ -lactamase.



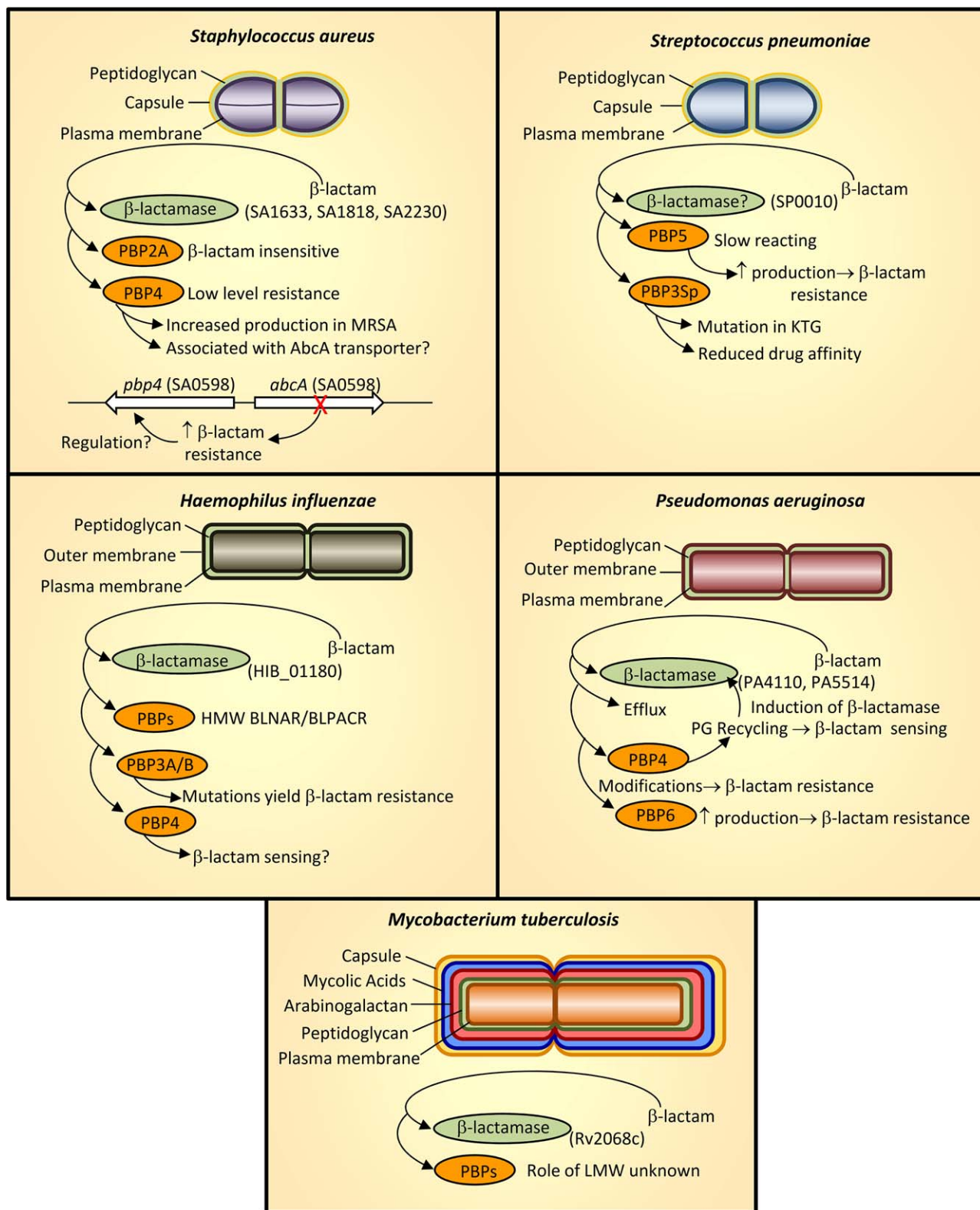


FIG 2

Mechanisms of  $\beta$ -lactam resistance in respiratory tract pathogens.  $\beta$ -Lactamases are shown in green and PBPs are displayed in orange. All pathogens encode at least one  $\beta$ -lactamase that is involved in resistance through different mechanisms. With the exception of PBP2A in *S. aureus* and the HMW PBPs in *H. influenzae*, all PBPs shown are LMW counterparts. Abbreviations: BLNAR,  $\beta$ -lactamase-nonproducing ampicillin resistance; BLPACR,  $\beta$ -lactamase-producing amoxicillin-clavulanic acid-resistant.

An early study addressed this possibility by assessing the affinities of PBPs from various streptococcal strains to penicillin and demonstrated that PBP5 isolated from *S. faecium*, reacted slowly (61). Overproduction of this slow-reacting PBP was associated with strains with natural insensitivity to penicillin and, moreover, the protein was always absent from penicillin sensitive strains. Interestingly, a PBP with similar enzyme kinetics was not detected in *S. pneumoniae*. A subsequent study assessed variation in PBP patterns of penicillin-resistant clinical isolates of pneumococci. In susceptible strains, PBP patterns did not vary whereas resistant strains were associated with greater, strain-specific variations (62). Several reports suggested that PBP3 of *S. pneumoniae* possesses DD-CPase activity, plays an important role in cell division and is homologous to the *E. coli* and *Bacillus subtilis* PBP5 and PBP6, confirming that it is a LMW PBP (63,64). To prevent confusion with other HMW PBPs, which carry a similar annotation, we refer to the LMW *S. pneumoniae* homologue as PBP3Sp. Selakovitch-Chenu et al. observed that decreased amounts of PBP3Sp confer sensitivity to high temperature, excess glycine and certain D-amino acids suggesting a possible role in membrane stability. Moreover, lower levels of PBP3Sp could be correlated to increased sensitivity to cefotaxime (65,66).

Cefotaxime resistance in a laboratory strain was shown to be independent of modifications in HMW PBPs. Instead, a lower affinity for penicillin and decreased susceptibility was associated with a point mutation in the DD-CPase PBP3Sp. This mutation, Thr-242 → Ile, was significant because it was located next to the KTG motif which is required for interaction with  $\beta$ -lactams. However, this mutation was absent in clinical isolates with high levels of penicillin resistance but there was evidence for a site-specific recombination event downstream of PBP3Sp, possibly mediating resistance (67). Resistance is thus thought to be a result of stepwise mutations or alterations in LMW PBP patterns that affect drug affinity and not necessarily, the production of  $\beta$ -lactamases (68–71). The level of resistance is determined by how many PBPs are modified and to what extent (72). In addition, other proteins, not related to PBPs, may also contribute (70).

### ***Haemophilus influenzae***

In 2007, Tristram et al. comprehensively reviewed antimicrobial resistance in *H. influenzae*, addressing among others factors, clinical significance and resistance mechanisms (73). Therefore, we only focus on the subsequent advancements in the understanding of  $\beta$ -lactam resistance. Two primary mechanisms are implicated, which are alteration in PBPs and enzymatic hydrolysis via  $\beta$ -lactamases. Hydrolysis is the most common mechanism, usually involving TEM-1 or ROB-1 type enzymes (74,75). Resistance as a result of amino acid substitutions in PBPs is referred to as the  $\beta$ -lactamase-nonproducing ampicillin resistance (BLNAR) mechanism and was reported in the literature as early as the 1980s (76–79). Substitutions in the HMW PBP3 (FtsI) homologue, which regulates septal PG biosynthesis, resulted in decreased  $\beta$ -lactam affinity (80).

These two phenomena have been reported simultaneously in clinical isolates where strains are referred to as  $\beta$ -lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) (81,82). Interrogation of the *H. influenzae* genome suggests that at least 8 PBPs are present, originally named PBP1 to PBP8 of decreasing molecular weight (83). These were subsequently renamed PBPs IA, IB, 2, 3A, 3B, 4, 5 and 6, respectively, based on comparison with the binding affinities of the *E. coli* homologues (84). Proteins referred to as PBP4 (*dacB*) and PBP5 are now known as PBP3A and 3B, respectively (73,80,85). Mutations in PBP3 were previously associated with resistance to aminopenicillins, amoxicillin/clavulanic acid and cephalosporins (85,86). Interestingly, an extensively drug-resistant strain of *H. parainfluenzae* was identified with mutations in PBP3, L4, GyrA and ParC and displayed resistance mechanisms for Mef(A), Tet(M) and CatS, which collectively confer resistance to a broad range of antibiotic classes including  $\beta$ -lactams (87). Crystal structures of PBP4 and PBP5 indicate that PBP4 is more easily inhibited by  $\beta$ -lactams than PBP5, suggesting that PBP4 is a prime candidate for  $\beta$ -lactam sensing (13). Similarly, in *P. aeruginosa*, defects in PBP4 (*dacB*) also led to an induction of  $\beta$ -lactamase expression corroborating the role of PBP4 as a sensor. Variability in PBPs was observed in both ampicillin-susceptible and resistant strains but decreased binding capacity was consistently seen in PBP5 of all the resistant isolates (88).

### ***Pseudomonas aeruginosa***

*P. aeruginosa* is a major opportunistic pathogen with an intrinsic resistance to several antibiotics, including  $\beta$ -lactams. It causes severe infections in a variety of clinical settings such as pneumonia, septicemia and chronic destructive lung infection in cystic fibrosis, with the ability to develop resistance during treatment (89). In addition to natural resistance in the form of an impermeable cell wall, *P. aeruginosa* also produces a  $\beta$ -lactamase, alters outer-membrane permeability, has active efflux pumps and can modify PBPs (90). The presence of an inducible  $\beta$ -lactamase has been reported as early as the 1970s and serves as the main mechanism of mediating  $\beta$ -lactam resistance (91,92). Moreover, AmpC itself can be mutated to adapt to efficacious  $\beta$ -lactams (93). We identified two chromosomally encoded  $\beta$ -lactamase homologues, that is, PA4110 (*ampC*), a class C enzyme linked to the regulatory repressor gene *ampR* (94) and PA5514, a class D enzyme (Table 1). The acquisition of secondary  $\beta$ -lactamases encoded by mobile genetic elements is thought to be bacterial specific (90). Modification of PBP targets, particularly LMW PBP members, has been reported following exposure to antibiotic treatment *in vivo* (95). After high piperacillin doses in a cystic fibrosis patient, decreased penicillin G affinity was correlated with increased  $\beta$ -lactam resistance and increased PBP6 expression (96). Following PBP4 inhibition by  $\beta$ -lactams,  $\beta$ -lactamase expression was activated through an unknown mechanism which the authors postulated to be via the detection of an altered pattern of recycled PG fragments (88). This resistance

mechanism was further elucidated by Zamorano et al. a year later (97). Under *in vitro* conditions, induction of the  $\beta$ -lactamase (*ampC*) expression following exposure to  $\beta$ -lactams is regulated by several genes linked to PG recycling including *ampG*, *nagZ*, *ampR* and *ampD* (97–99). Deletion of *nagZ* in the background of a *dacB* (PBP4) mutant restored  $\beta$ -lactam susceptibility, suggesting an interplay between PG recycling components and LMW PBPs to mediate drug tolerance (97). The mechanisms that underpin this have yet to be resolved. In clinical strains, antibiotic resistance has also been associated with LMW PBPs, where in the absence of PBP4, expression of *ampC* is induced (100–102). In addition, mutations in PBP4 were detected in a high proportion of AmpC-hyper-producing clinical isolates (88). Recent structural analysis of PBP4 in *H. influenzae* points to a potential role as a  $\beta$ -lactam sensor involved in regulating AmpC expression suggesting that the *P. aeruginosa* homologue may potentially have a similar role (13). In this regard, a structural analysis of the *P. aeruginosa* PBP5 homologue showed that in addition to DD-CPase activity, sufficient  $\beta$ -lactamase activity was also detected which is unique relative to its *E. coli* counterpart (103). Ropy et al. investigated the role of the LMW PBPs (4, 5 and 7) in PG composition,  $\beta$ -lactam resistance and *ampC* regulation (104). No significant morphological changes were observed in the respective deletion mutants relative to the wildtype strain. Of the single mutants, only a PBP5 mutation resulted in increased penta-peptide levels and PBP4 and PBP7 is able to compensate in its absence, suggesting that this is the major DD-CPase in *P. aeruginosa*. Consistent with the earlier literature, inactivation of PBP4 resulted in the induction of *ampC* expression which increased significantly following combinatorial LMW PBP inactivation. Moreover, PBP4 inactivation also resulted in a significant increase in the MICs for  $\beta$ -lactams. In contrast, inactivation of PBP5 and PBP7 failed to increase drug sensitivity. MICs for all  $\beta$ -lactams tested were lowest when all LMW PBPs were inactivated in combination with AmpC, suggesting that these enzymes, particularly PBP5, are intricately involved in mediating  $\beta$ -lactam resistance (104).

### *Mycobacterium tuberculosis*

Unlike the etiological agents of respiratory infections discussed above which are inherently sensitive to  $\beta$ -lactams, this class of antibiotics has not been used to treat tuberculosis or any other mycobacterial infections. Mycobacteria are inherently resistant to  $\beta$ -lactams, a finding reported in the late 1940s (105–108). This is due to three distinct mechanisms, acting singularly or in combination, which affect resistance outcomes: (i) the production of a  $\beta$ -lactamase, (ii) an impermeable, complex cell envelope (15,109) and (iii) the inherent resistance of target PBPs (110). Early studies attempted to establish whether clinically achievable drug concentrations could be used to inhibit PBPs in *M. tuberculosis* (109). Ampicillin, amoxicillin and imipenem were previously reported to possess activity *in vitro* against *M. tuberculosis* and are able to bind several PBPs. As tolerance to  $\beta$ -lactams could be reversed with a  $\beta$ -lactamase

inhibitors, the mycobacterial  $\beta$ -lactamase was thought to be the major determinant of resistance (109). Characterization of the  $\beta$ -lactamase of *M. tuberculosis* in clinical isolates and via recombinant expression identified one major homologue, *blaC*, as a class A  $\beta$ -lactamase. Another minor  $\beta$ -lactamase was also detected (111–113). However, our BLAST analysis only identified Rv2068c (*blaC*) (Table 1). Consistent with our analysis, a genetic analysis of the  $\beta$ -lactamases of *M. tuberculosis* also detected only one  $\beta$ -lactamase, which was identical to *blaA* of the avirulent *M. tuberculosis* strain, H37Ra (110,112,114). Characterization of mycobacterial strains lacking the major  $\beta$ -lactamase showed an increased susceptibility to most  $\beta$ -lactams, especially the penicillins. However, basal levels of resistance were observed in the mutants against certain penicillins and the susceptibilities to some cephalosporin-based  $\beta$ -lactams were comparable to wildtype (115). The authors further characterized mutants derived from the initial  $\beta$ -lactamase deletion mutants that were hypersusceptible to  $\beta$ -lactams and identified insertions in genes associated with PG biosynthesis (*ponA2*, *pbpX* and *dapB*). Interestingly, the first two are PBPs and the protein product of *dapB* is involved in the synthesis of DAP and L-lysine (116).  $\beta$ -lactamase inhibitor combinations, particularly clavulanate, have thus emerged as a potential option for the treatment of multi- and extensively drug-resistant strains (117–121). The mycobacterial HMW PBPs, PonA1 and PonA2 in *M. tuberculosis* mediate various responses to cell wall targeting antibiotics but the role of LMW PBPs remains unclear (122,123). Of the three distinct *M. tuberculosis* LMW PBP homologues, Rv3330 (*dacB1*) and Rv2911 (*dacB2*) are known to be dispensable, whereas Rv3627c is essential (124,125). Deletion and overexpression of DacB2 in *M. smegmatis* resulted in decreased growth rates and altered colony morphology, with defects in sliding motility and biofilm formation. Deletion in *M. tuberculosis* led to diminished growth under acidic and low oxygen conditions, but the effect on  $\beta$ -lactam resistance was not tested (126). PknI was then deleted in the background deletion of DacB2 and the mutant strain displayed similar colony defects (127). Moreover, the double deletion mutant strain  $\Delta dacB2 \Delta pknI$  was hypersensitive to several cell wall damaging agents, including lysozyme, malachite green, ethidium bromide and isoniazid (a first line anti-tuberculosis drug). Susceptibility to several  $\beta$ -lactams was not significantly different to the wildtype and single deletion mutant strain suggesting that DacB2 does not play a role in mediating  $\beta$ -lactam resistance. Meropenem treatment of *M. tuberculosis* results in dramatic lysis, together with an accumulation of unlinked stem penta-peptides and no change in tetra-peptide pools (128). The changes in the stem peptide profiles that occurred upon meropenem treatment, suggested that this antibiotic inhibits Ldts, which catalyze the formation of 3–3 crosslinks in PG.

### LD-Transpeptidases

Ldt homologues of *E. coli*, *B. subtilis* and *E. faecium* have previously been associated with maintenance of cell wall

integrity (29,44,45,129). The structure of *M. tuberculosis* PG is atypical due to a high abundance of crosslinks in the 3→3 configuration. However, the physiological significance of these crosslinks with respect to virulence and antibiotic resistance remain poorly understood. Recently, Ldts have been intensively studied in *M. tuberculosis* (128,130). Five distinct homologues have been identified and are annotated as Ldt<sub>Mt1</sub> – Ldt<sub>Mt5</sub> (Ldt *M. tuberculosis* 1–Ldt *M. tuberculosis* 5, Table 2). The Ldt<sub>Mt1</sub> of *M. tuberculosis* is sensitive to various carbapenems and cephalosporins (41). Similarly, deletion of one Ldt, *ldtC*, in *Mycobacterium smegmatis* results in increased susceptibility to imipenem. Moreover, a strain simultaneously lacking three Ldts had altered colony morphology and was associated with hyper-susceptibility to antibiotics and lysozyme (131). Similarly, loss of the predominant Ldt homologue, Rv2518c (*ldt<sub>Mt2</sub>*), was associated with aberrant colony morphology as well as susceptibility to amoxicillin-clavulanate (132). Despite the functional and structural similarity of Ldt<sub>Mt5</sub> to Ldt<sub>Mt2</sub>, which was consistent with our analyses (Table 2), mutant strains lacking Ldt<sub>Mt5</sub> displayed slower growth relative to the wildtype and increased susceptibility to crystal violet, osmotic shock and certain carbapenem antibiotics (133). Moreover, strains simultaneously lacking Ldt<sub>Mt1</sub> and Ldt<sub>Mt2</sub> displayed increased susceptibility to vancomycin (134). Due to their susceptibility to carbapenem derivatives, the role of Ldts in modulating  $\beta$ -lactam susceptibility remains an exciting area for future investigation, with the possibility of yielding new therapeutic options for tuberculosis.

## CONCLUSION AND FUTURE DIRECTION

PG is an integral component in all eubacterial cell walls. This is evidenced by the fact that perturbation of mechanisms governing its biosynthesis and remodeling have been associated with aberrant morphology as well as altered host-pathogen interactions, of which ultimately affect virulence. Serine-type PBPs are PG-associated enzymes required for biosynthesis and remodeling of the cell wall and are the targets of  $\beta$ -lactam antibiotics. However,  $\beta$ -lactam resistance is modulated by two main factors including enzymatic degradation via  $\beta$ -lactamases and modification of PBP targets either via mutations, altering drug affinity, or alteration in PBP profiles and/or abundance. Ldts provide an alternative mechanism of PG crosslinking (3→3 instead of 4→3) and due to their fundamental differences in domain architecture, can bypass susceptibility to  $\beta$ -lactam antibiotics. However, despite this dissimilarity, they are susceptible to a newer class of  $\beta$ -lactams. Therefore,  $\beta$ -lactam antibiotics, and carbapenems in particular, that target both PBPs and Ldts, may provide an attractive alternative to combat the emergence of drug resistance in major pathogens.

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