INTRODUCTION

A considerable number of proteins are involved in building and splitting the bacterial cell wall during cell growth and division (Table 1). The peptidoglycan part of the wall consists of a continuous network of circumferentially oriented glycan chains cross-linked by short peptide bridges. It is assembled from lipid-linked disaccharide-peptide subunits (Lipid II), which are flipped across the cytoplasmic membrane by MurJ (Meeske et al., 2015; Ruiz, 2008; Sham et al., 2014). Peptidoglycan glycosyltransferases polymerize the disaccharide units into glycan strands, while peptidoglycan transpeptidases cross-link and process the peptide side chains to produce a mesh-like structure (Lovering et al., 2012; Vollmer, Blanot, et al., 2008). The disaccharide units consist of β-1,4-linked N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). All bacterial peptidoglycan contains the same carbohydrate backbone of alternating MurNAc and GlcNAc residues, but the length of the glycan strands varies between species (Vollmer & Seligman, 2010). In addition, the glycan strands can be further modified by addition or removal of acetyl residues (Crisóstomo et al., 2006; Moynihan & Clarke, 2010; Vollmer, 2008; Vollmer & Tomasz, 2000). The pentapeptide linked to MurNAc is not universally conserved among bacteria. Its amino acid composition varies, especially at the second and third positions (Vollmer, Blanot, et al., 2008). In *Streptococcus pneumoniae* and many other Gram-positive bacteria, the pentapeptide chain has the following sequence: L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala. Instead of D-iso-Gln and L-Lys, *Escherichia coli*, for instance, has D-iso-Glu and meso-diaminopimelic acid (meso-DAP) in the corresponding positions (Morlot et al., 2018; Vollmer, Blanot, et al., 2008).
| Proteins | Function of proteins |
|----------|---------------------|
| **PBP1a** | Most important class A PBP(s) in the species listed. They are bifunctional and have transpeptidase as well as transglycosylase activity |
| **PBP2a** | |
| **PBP1b** | Class A PBPs that can be inactivated without appreciable effect on growth and morphology |
| **PBP2b** | Class B PBPs that work together with RodA in the elongosome to synthesize peptidoglycan along the lateral cell body. Class B PBPs are monofunctional and have only transpeptidase activity |
| **PBP2x** | Class B PBPs that work together with FtsW in the divisome to synthesize the septal cross-wall |
| **1PBP3** | Nonessential class B PBP of unknown function. Becomes essential when PBP2b of B. subtilis is catalytically inactive |
| **FtsW** | SEDS family peptidoglycan polymerase (GTase). Part of the divisome where it works in conjunction with its accompanying class B PBP |
| **RodA** | SEDS family peptidoglycan polymerase (GTase). Part of the elongosome where it works in conjunction with its accompanying class B PBP |
| **FtsB** | FtsB, FtsL, and FtsQ form a subcomplex that constitutes the core of the bacterial divisome. The FtsBLQ subcomplex interacts with several divisomal proteins and has a regulatory role in the initiation of septal peptidoglycan synthesis. It inhibits the FtsW/bPBP machinery until the time is right to divide |
| **FtsQ** | FtsN relieves FtsBLQ-mediated inhibition of the FtsW/bPBP machinery |
| **FtsL** | Transporter that flips lipid II across the cytoplasmic membrane |
| **MurJ** | Broadly distributed polytopic membrane protein that together with PBP1a and MreCD coordinates cell elongation in S. pneumoniae. In S. aureus, it has been reported to control cell division |
| **CozE** | Homolog of CozE that contributes to cell size homeostasis in S. pneumoniae |
| **CozEb** | In S. pneumoniae and B. subtilis, GpsB acts as an adaptor that coordinates peptidoglycan synthesis with other processes in a cell cycle-dependent manner. In S. aureus, GpsB stabilizes the Z-ring at the onset of cell division and stimulates cytokinesis through direct interaction with FtsZ |
### Proteins

|          | S. p. | S. a. | B. s. | E. c. | Function of proteins |
|----------|-------|-------|-------|-------|----------------------|
| MreB     | -     | -     | MreB  | MreB  | The actin homolog MreB directs lateral cell wall synthesis in rod-shaped bacteria |
| MreC     | MreC  | MreC  | MreC  | MreC  | Associated with the elongasome. May act as a scaffold for other components of the elongasome machinery |
| MreD     | MreD  | MreD  | MreD  | Polytopic membrane protein that is part of the elongasome. Regulatory and/or scaffolding function |
| RodZ     | RodZ  | RodZ  | RodZ  | Required for cell-elongation in rod-shaped and ovoid bacteria. Forms a supramolecular complex with elongasome proteins such as MreB (if present), MreC, MreD, and peptidoglycan synthases. The role of RodZ in S. aureus has not been determined |
| MacP     | -     | -     | -     | Membrane-anchored substrate of StkP, and an activator of PBP2a |
| PcsB     | -     | CwlO  | AmiA  | Peptidoglycan hydrolases that split the septal cross-wall during cell division and are regulated by FtsEX (PcsB) or FtsEX/EnvC (AmiA and AmiB). CwlO is regulated by FtsEX and are involved in regulating growth and cell elongation |
| FtsE     | 4FtsE | FtsE  | FtsE  | ATPase required for the transmission of a conformational signal from the cytosol through the membrane via FtsX. In S. pneumoniae and E. coli, FtsEX regulates the activity of cell wall hydrolases that cleave the septum to release daughter cells after cell division. Instead of controlling cell division, B. subtilis FtsEX controls the peptidoglycan hydrolase CwlO, which plays a central role in cell wall elongation during growth. Little research has been conducted to investigate the presence and potential role of FtsEX-like proteins in S. aureus. It is therefore uncertain whether S. aureus contains a FtsEX-system corresponding to those regulating cell division in S. pneumoniae and E. coli and cell wall elongation in B. subtilis |
| FtsX     | 4FtsX | FtsX  | FtsX  | The transmembrane protein FtsX mechanically transduces a conformational signal from the cytoplasmic FtsE that provokes the activation of peptidoglycan hydrolases |
| -        | -     | -     | MepS(Spr) | Endopeptidase that cleaves peptidoglycan during cell wall expansion to allow insertion of new glycan strands |
| MltG     | -     | MltG  | MltG  | Lytic transglycosylase proposed to be responsible for glycan strand termination during peptidoglycan synthesis |
| CbpD     | -     | -     | -     | Competence induced peptidoglycan hydrolase. |
| StkP     | PknB  | PrkC  | -     | Eukaryotic-like serine/threonine kinase that has been reported to sense lipid II and peptidoglycan fragments. StkP and PknB regulate cell division and peptidoglycan synthesis in S. pneumoniae and S. aureus, respectively. In B. subtilis, PrkC does not affect cell division, morphology, or cell growth, but alters stationary phase physiology and induces spore germination. No ortholog with a PASTA domain is present in E. coli |
| -        | -     | -     | LpoA  | Outer-membrane-anchored lipoprotein that regulates the function of PBP1a |
| -        | -     | -     | LpoB  | Outer-membrane-anchored lipoprotein that regulates the function of PBP1b |
| FtsZ     | FtsZ  | FtsZ  | FtsZ  | Structural homolog of tubulin that forms a cytokinetic ring at midcell and recruits the division machinery to orchestrate cell division |

Note: Only proteins that are mentioned in this review have been included.

*a* *Streptococcus pneumoniae*.

*b* *Staphylococcus aureus*.

*c* *Bacillus subtilis*.

*d* *Escherichia coli*.
In most bacterial species, pentapeptides from different glycan strands are cross-linked by transpeptidation between the fourth D-alanine residue of the donor chain and the third residue (e.g., Lys or meso-DAP) of the acceptor chain. Alternatively, in some species the stem peptides can be linked together by means of interpeptide bridges, which in the case of *S. pneumoniae* consists of L-Ser-L-Ala or L-Ala-L-Ala (Vollmer et al., 2019). The enzymes catalyzing the transpeptidation reaction are called penicillin-binding proteins (PBPs). PBPs come in three different classes: A, B, and C (Table 1) (Sauvage et al., 2008). aPBPs are bifunctional enzymes that catalyze transglycosylation as well as transpeptidation, while bPBPs are monofunctional and possess only transpeptidase activity. Class C PBPs (cPBPs) are peptidoglycan hydrodases with D,D-carboxypeptidase or endopeptidase activity. It strongly suggests that aPBPs are not the central players in the biosynthesis of bacterial peptidoglycan, FtsW and RodA work in conjunction with monofunctional bPBPs (Emami et al., 2017; Meeske et al., 2016; Sjödt et al., 2019). These proteins were originally reported to be lipid II flippases (Mohammadi et al., 2011), a function later assigned to MurJ (Sham et al., 2014). However, it remains a possibility that FtsW and RodA have a double role, that is, flippase as well as glycosyltransferase activity (Egan et al., 2020). To synthesize peptidoglycan, FtsW and RodA work in conjunction with monofunctional bPBPs (Emami et al., 2017; Meeske et al., 2016; Sjödt et al., 2020; Taguchi et al., 2019). It is now firmly established that FtsW and RodA together with their cognate transpeptidase partners form the core peptidoglycan-synthesizing machineries of the divisome and elongosome, respectively. The recent shift in our understanding of peptidoglycan synthesis has important implications. It strongly suggests that aPBPs are not the central players in septal cross-wall synthesis and cell elongation. What, then, could be the role of aPBPs in the construction of the bacterial cell wall?

Over the past decades, a vast amount of information on aPBPs from various bacterial species has been published. For the sake of simplicity and clarity, this short review will mainly focus on the aPBPs of *S. pneumoniae* and *E. coli* as representatives of Gram-positive and Gram-negative bacteria, respectively.

### 2 | THE VARIABLE ESSENTIALITY OF aPBPs

aPBPs seem to be present in most peptidoglycan-producing bacteria. Hence, they must perform an important function. However, their numbers vary between bacterial species (Table 1). *B. subtilis* produces four different aPBPs, *S. pneumoniae* and *E. coli* have three, while *S. aureus* survives and thrives with only one (Sauvage et al., 2008). Interestingly, bacteria producing several different aPBPs, often need only one of them for survival. In *S. pneumoniae*, single knockouts of *pbp1a*, *pbp2a*, and *pbp1b* can be obtained, but it is not possible to construct a *pbp1a/pbp2a* double-knockout strain (Paik et al., 1999). The pneumococcus must, therefore, produce either PBP1a or PBP2a to be viable. PBP1b, on the contrary, cannot substitute for the other two and is not essential. Similarly, in *E. coli*, PBP1c is dispensable while the bacterium must produce either PBP1a or PBP1b to survive. Studies on the properties of aPBPs from model bacteria such as *S. pneumoniae*, *B. subtilis*, and *E. coli* have revealed that many bacterial aPBPs are nonessential, while others have partly overlapping functions. Notably, it has been reported that *B. subtilis* as well as *Enterococcus faecalis* are viable even when all their aPBPs have been deleted (Arbeloa et al., 2004; McPherson & Popham, 2003). Deletion of the bifunctional PBPs in these species led to a significant increase in generation time, and to a modest decrease in cross-linking of their peptidoglycan. In the case of *B. subtilis*, the quadruple class A-less mutant was obtained at a near normal transformation frequency, indicating that the survival of this mutant does not depend on the acquisition of additional suppressor mutations (McPherson & Popham, 2003). Another interesting case is *Chlamydia trachomatis*, which produces peptidoglycan but not aPBPs. It does, however, harbor genes encoding bPBPs and SEDS proteins (Cox et al., 2020; Meeske et al., 2016). The same holds true for members of *Francisella*, *Wolbachia*, and some other genera, demonstrating that bPBPs and SEDS proteins are more widely conserved than aPBPs (Meeske et al., 2016). Thus, although aPBPs play an important and usually indispensable role in the biosynthesis of bacterial peptidoglycan, SEDS proteins together with bPBPs seem to constitute the basic cell wall-building machinery.

### 3 | PROPERTIES OF PNEUMOCOCCAL aPBPs

In addition to the three aPBPs (PBP1a, PBP1b, and PBP2a), *S. pneumoniae* produces two bPBPs (PBP2x and PBP2b), and a single cPBP (PBP3) (Sauvage et al., 2008). PBP2x and PBP2b are essential transpeptidases (Kell et al., 1993) that work together in pairs with FtsW and RodA in the divisome and elongosome, respectively (Perez et al., 2019; Sjödt et al., 2020; Taguchi et al., 2019). PBP3 (DacA) functions as a D,D-carboxypeptidase that converts pentapeptide into tetrapeptide moieties by cleaving the terminal D-alanyl-D-alanine bond (Hakenbeck & Kohiyama, 1982; Severin et al., 1992). Presumably, PBP3 regulates the extent of cross-linking between glycan strands by limiting the amount of D-alanyl-D-alanine donor groups required for transpeptidation (Barendt et al., 2011; Morlot et al., 2004). The enzyme is distributed across the entire cell surface but is absent from the future division site (Morlot et al., 2004).
Deletion of PBP3 gives rise to severe morphological defects including misplaced division septa (Barendt et al., 2011; Schuster et al., 1990).

PBP1a primarily localizes to the midcell of S. pneumoniae (Land et al., 2013). The divisome as well as the elongosome operate in this region. Several lines of evidence connect PBP1a with proteins involved in lateral cell wall elongation. The essentiality of MrcE, MreD, RodZ, and MltG is suppressed in a S. pneumoniae D39 strain lacking PBP1a (Fenton et al., 2016; Land & Winkler, 2011; Tsui et al., 2016). MrcE, MreD, RodZ, and MltG are all associated with the pneumococcal elongosome. MltG is a lytic transglycosylase, while MrcE, MreD, and RodZ are structural elements required to assemble a functional elongosome in rod-shaped and ovoid bacteria (Stamsås et al., 2017; Tsui et al., 2016; Winther et al., 2021). Similarly, a polytopic membrane protein named CoxE (for coordinator of zonal elongation) can be deleted in a Δpbp1a background, but not in a Δpbp2a background (Fenton et al., 2016). PBP1a has been shown to directly interact with MrcE, MreD, CoxE, and its paralog CoxEb, all of which appear to be involved in controlling the activity of PBP1a (Fenton et al., 2016; Land & Winkler, 2011; Stamsås et al., 2020). As experimental data suggest that PBP1a forms a complex with the four abovementioned proteins, PBP1a is assumed to be part of the pneumococcal elongosome. This is in accordance with the observation that PBP1a, PBP2b, and MrcE colocalize throughout the division cycle. In contrast, PBP2x colocalizes with PBP1a during the early stages of pneumococcal cell division, but at later division stages these PBPs occupy different positions in constricting division septa (Land et al., 2013; Tsui et al., 2014).

By screening for mutants synthetically lethal with a pbp1a deletion, Fenton and coworkers (2018) identified a protein termed MacP for membrane-anchored cofactor of PBP2a. MacP was shown to form a complex with PBP2a, and to be required for its in vivo function. Furthermore, MacP is phosphorylated by StkP, a eukaryotic-type serine-threonine kinase which is a regulator of cell division and morphogenesis. Pneumococcal StkP possesses a cytoplasmic catalytic domain and an extracellular PASTA domain consisting of four repeats. The PASTA domain is required for midcell localization of StkP and is involved in cell division as well as ligand sensing. It has been reported to sense muropeptides, lipid II levels, and to control septal cell wall thickness (Beilharz et al., 2012; Fenton et al., 2018; Fleurie et al., 2012; Hardt et al., 2017; Maestro et al., 2011; Sun & Garner, 2020; Zucchini et al., 2018). MacP apparently constitutes a link between signals sensed by StkP and peptidoglycan synthase activity. Similar to deletion of the macP gene, deletion of gpsB is synthetically lethal with a Δpbp1a mutation, but not with Δpbp2a and Δpbp1b mutations. GpsB interacts with PBP2a, MrcE, and other cell wall and cell cycle proteins. It is an adaptor protein that regulates septal and peripheral peptidoglycan synthesis in S. pneumoniae and other low-GC Gram-positive bacteria (Cleverley et al., 2019; Fleurie et al., 2014; Rued et al., 2017). It is of interest to note that deletion of pbp1a reduce cell size in the D39 strain, while cell dimensions are not significantly changed in cells lacking pbp2a (Land & Winkler, 2011). This demonstrates that the two PBPs affect the morphology of the pneumococcal cell differently. Furthermore, considering that PBP1a and PBP2a are regulated by and interact with different proteins, the synthetic lethality of the pbp1a/pbp2a double mutation cannot be explained by simple functional redundancy. In sum, experimental data strongly indicate that PBP1a and PBP2a carry out overlapping functions as well as functions specific to each PBP.

aPBPs were recently shown to function autonomously in vivo in S. pneumoniae (Straume et al., 2020). It was discovered that bifunctional PBPs are still active in cells lacking a functional divisome or elongosome, demonstrating that they can operate independently of the two multiprotein complexes. This insight derives from experiments performed with a peptidoglycan hydrolase CbpD, which splits pneumococcal cells at the septum and in a poorly understood way is able to differentiate between peptidoglycan synthesized by the divisome (PBP2x/FtsW) and aPBPs. It was demonstrated that the activity of pneumococcal PBP1a or PBP2a is required to establish resistance against the lytic activity of CbpD. The finding that PBP2x/FtsW-synthesized peptidoglycan is sensitive to CbpD while class A-synthesized peptidoglycan is resistant, shows that the two types of peptidoglycan must differ in composition and/or architecture. Furthermore, it was demonstrated that peptidoglycan synthesis by aPBPs lags a few minutes behind the synthesis carried out by the PBP2x/FtsW machinery (Straume et al., 2020). Together, these facts show that mature pneumococcal peptidoglycan is synthesized by three independent entities: the divisome, the elongosome, and the bifunctional aPBPs. Lateral cell wall expansion and synthesis of the septum are carried out by the elongosome and divisome, respectively, while the exact function of aPBPs is still a matter of debate.

4 | PROPERTIES OF E. COLI aPBPs

The peptidoglycan wall of a Gram-negative bacterium, such as E. coli, differs fundamentally from the peptidoglycan wall of Gram-positive bacteria (Egan et al., 2020). It is only one or a couple of layers thick and is surrounded by an outer membrane composed of phospholipids and lipopolysaccharide (Sperandeo et al., 2017; Turner et al., 2013). The genome of E. coli encodes 12 PBPs: three class A (PBP1a, PBP1b, and PBP1c), two class B (PBP2 and PBP3), and seven class C (PBP4, PBP4b, PBP5, PBP6, PBP6b, PBP7, and AmpH) PBPs (Table 1) (Sauvage et al., 2008). The essential bPBPs, PBP2, and PBP3, are closely associated with the SEDS family peptidoglycan polymerases of E. coli. The FtsW/PBP3 pair makes up the core peptidoglycan-synthesizing machinery of the divisome, while RodA/PBP2 have a corresponding function in the elongosome (Cho et al., 2016; Leclercq et al., 2017). Six of the cPBPs (PBP4, PBP4b, PBP5, PBP6, PBP6b, and AmpH) have D,D-carboxypeptidase activity, while three (PBP4, PBP7, and AmpH) have D-endopeptidases activity (Denome et al., 1999; Ghosh et al., 2008; Pazos & Peters, 2019; Typas et al., 2012). It is not clear whether PBP4 and AmpH, which have dual activities, carry out both activities in vivo, but enzymological data support that PBP4 primarily functions as a D,D-endopeptidases (Gonzáles-Leiza et al., 2011; Korat et al., 1991; Meberg et al., 2004).
D,D-endopeptidases cleave the bond between D-Ala and meso-DAP in cross-linked stem peptides, whereas D,D-carboxypeptidases remove the terminal residues from stem peptides. In *E. coli*, none of the class C PBPs are essential for viability. Bacteria lacking all seven of them grow nearly as well as the parental strain and display only modest morphological defects (Denome et al., 1999).

Among the three different aPBPs produced by *E. coli*, the best-studied and most important are PBP1a and PBP1b. Despite having the same name as two of the pneumococcal class A PBPs, they belong to different subclasses and are not functionally equivalent or closely related to their pneumococcal counterparts. *E. coli* cells must produce either PBP1a or PBP1b to be viable (Yousif et al., 1985). Like PBP1a and PBP2a from *S. pneumoniae* they are semi-redundant enzymes, that is, they can substitute for each other with regard to viability under standard laboratory growth conditions but in addition have specific nonoverlapping functions. An interesting observation regarding the properties of PBP1a and PBP1b is that their enzymatic activity is influenced by pH. Hence, maximal fitness across a wide pH range (pH 4.8–8.2) seems to require the function of both PBPs (Mueller et al., 2019). PBP1c, the third aPBP encoded in the genome of *E. coli*, cannot substitute for the double loss of PBP1a and PBP1b. Moreover, a PBP1c deletion mutant is viable, and does not show any obvious phenotype (Schiffer & Höltje, 1999). The transpeptidase and transglycosylase activities of PBP1a and PBP1b are regulated by their cognate outer-membrane lipoproteins LpoA and LpoB (Egan et al., 2014; Jean et al., 2014; Paradis-Bleau et al., 2010; Typas et al., 2010). PBP1a, which localizes to foci in the lateral cell wall, has been reported to interact with the elongation-specific class B transpeptidase PBP2 (Banzhaf et al., 2012). Hence, this aPBP appears to have an important role in cell elongation. Despite that it is found throughout the cell envelope, PBP1b is considered to be specialized for cell division. It is reported to interact with several divisome proteins, namely PBP3, FtsW, FtsN, FtsQ, FtsL, and FstB (Bertsche et al., 2006; Boes et al., 2019; Leclercq et al., 2017; Müller et al., 2007). Thus, all in all, there is considerable evidence that PBP1b is an intrinsic component of the divisome, while PBP1a is an intrinsic component of the elongasome in *E. coli*.

Curiously, despite the low amino acid sequence identity of PBP1a and PBP1b (27%), and all the specific interactions reported for these aPBPs with partners in the divisome and elongasome, PBP1a and PBP1b are largely interchangeable in their capacities to support growth. Presumably the change of partners involved would require that both PBPs are able to interface productively with members of both complexes. In other words, both must be able to function as integrated cogwheels in the elongasome as well as divisome machinery. It takes a stretch of the imagination to envisage how this could be possible. In a single-molecule study, Cho et al. (2016) present results that conflict with the model predicting that aPBPs are intrinsic components of the elongasome. Under the experimental conditions used, they found that the RodA/PBP2 complex, but not aPBPs, displays MreB-guided circumferential motion in *E. coli*, demonstrating that the elongasome and aPBPs are spatially distinct. Hence, class A PBPs can be envisioned as dynamic and relatively autonomous entities that are not directly involved in synthesizing the cell wall, but rather have auxiliary functions such as repair and/or fortification of the peptidoglycan layer. A similar view has been put forward by Vigouroux and colleagues (2020). In a recent article, they reported that aPBPs have no role in maintaining the cell shape but were crucial for mechanical cell wall integrity (Vigouroux et al., 2020). Hence, aPBPs evidently function to strengthen the cell wall of *E. coli*. The authors found evidence that this strengthening is due to an adaptive class A-mediated repair mechanism that senses and repairs cell wall defects. In support of this, Lai et al. (2017) found that PBP1b-mediated peptidoglycan synthesis increases following overexpression of the space-maker endopeptidase MepS (Spr), an enzyme that makes room for localized insertion of new material during peptidoglycan matrix expansion (Singh et al., 2012). Their findings strongly indicate that aPBPs and their auxiliary proteins detect and fill gaps in the peptidoglycan network. Taken together, available data suggest that aPBPs serve dual roles. They operate in conjunction with the divisome and elongasome during synthesis of the primary cell wall, but in addition function as autonomous entities that maintain and repair the peptidoglycan sacculus.

## 5 | THE SYNTHETIC LETHALITY PARADOX

As discussed above, aPBPs often form synthetic lethal pairs such as PBP1a/PBP2a in *S. pneumoniae* and PBP1a/PBP1b in *E. coli*. Each member of these pairs can be individually deleted, but the bacterium must produce one of them to be viable. Moreover, members of each pair have overlapping as well as nonoverlapping functions, demonstrating that they are semi-redundant. It follows from this that it must be their overlapping function that is essential for cell survival. What could this essential function be? The many studies reporting close interactions between aPBPs and proteins associated with the divisome or elongasome, suggest that aPBPs are intrinsic components of these multiprotein complexes (Bertsche et al., 2006; Boes et al., 2019; Claessen et al., 2008; Fenton et al., 2016; Leclercq et al., 2017; Scheffers & Errington, 2004; Steele et al., 2011). On the contrary, strong recent experimental evidence shows that aPBPs are able to function autonomously (Lai et al., 2017; Straume et al., 2020; Vigouroux et al., 2020). How can this apparent paradox be explained? Perhaps aPBPs can operate both autonomously and in a context where their actions are coordinated with the activities of the elongasome and divisome. When they detect and repair damage to the peptidoglycan sacculus localized outside areas of active peptidoglycan synthesis, aPBPs probably function as autonomous entities, possibly together with accessory proteins that help regulate and direct their activity. However, in areas where synthesis of new cell wall peptidoglycan takes place, aPBPs may operate in conjunction with the divisome and elongasome to repair defects made during primary peptidoglycan synthesis. Members of synthetic lethal pairs of aPBPs have low amino acid identity. Hence, it is unlikely that they can substitute for each other in cases where their function depends on close interactions with several divisome or elongasome proteins.
However, it is conceivable that they can perform overlapping repair functions when operating as autonomous entities. We therefore propose that the essential function of class A PBPs is to detect and repair gaps and imperfections in the cell wall peptidoglycan localized outside areas of active peptidoglycan synthesis.

A TWO-LAYERED CELL WALL IN GRAM-POSITIVE BACTERIA?

In a newly published study by Pasquina-Lemonche and coworkers (2020), atomic force microscopy was used to study the cell wall architecture of the Gram-positives *B. subtilis* and *S. aureus*. Interestingly, the paper reports evidence that nascent septa in both species consist of two different peptidoglycan layers with distinct architectures that indicate two synthesis regimes (Figure 1). The inner layer, in the following referred to as the core or primary cell wall, has a highly ordered structure composed of concentric rings. The core is sandwiched between the outer layers, which constitute the bulk of the septum thickness. In contrast to the core, the outer layers (the secondary cell walls) are made up of randomly oriented material (Figure 1). As the core peptidoglycan in nascent septa acts as a scaffold for the outer layers, the core material must be synthesized first. Hence, the authors suggest that the core peptidoglycan is formed at the leading edge of the growing septum, while the outer layers are subsequently added on top of the core. During cell wall growth and maturation, the internal cytoplasm-facing surface of *S. aureus* cells remains a disordered mesh with relatively small pores. In contrast, the post-divisional outer surface, which originally had an ordered ring-like architecture, gradually matures into a porous mesh due to the action of various peptidoglycan hydrolases. Similarly, the outer surface of *B. subtilis* cells reorganizes into a randomly oriented porous network, while the poles retain some of the ring structure originating from the inner core of the split septum. The inner surface of the *B. subtilis* cell wall cylinder, on the contrary, consists of material that has been deposited in a circumferential orientation (Figure 1). This is due to the circumferential movement of MreB filaments which guide the movement of the elongosome (Dion et al., 2019; Dominguez-Escobar et al., 2011; Garner et al., 2011; Pasquina-Lemonche et al., 2020). Very recently, a similarly structured two-layered cell wall has also been reported for *Staphylococcus warneri* (Su et al., 2020).

How are the two different layers of nascent septa synthesized? A likely solution to this question involves the function of aPBPs. In the paper by Straume et al. (2020) several possible functions for aPBPs were proposed. One of these was that aPBPs make the primary peptidoglycan produced by the PBP2x/FtsW machinery stronger and denser by adding peptidoglycan that is more heavily cross-linked. This hypothesis agrees well with the data presented in the paper by Pasquina-Lemonche et al. (2020). Hence, we postulate that the PBP2x/FtsW machinery synthesizes the ordered rings deposited at the leading edge of the growing septum, while class A PBPs subsequently adds the randomly oriented peptidoglycan deposited on top of the core material (Figure 2). The finding that the activity of class A PBPs occurs subsequent to and separate in time from divisome-mediated peptidoglycan synthesis fits nicely with this model (Straume et al. 2020).

A two-layered septal cross-wall has not been demonstrated for the pneumococcus, yet, but as *B. subtilis*, *S. aureus*, *S. warneri*, and *S. pneumoniae* all belong to class *Bacilli* it is reasonable to assume...
that their cross-walls are built in a similar manner and have the same architecture (Figure 1). As stated in Table 1, PBP2 is the only aPBP in *S. aureus*. It is active at the septum as well as in peripheral regions and is thought to cooperate with the PBP1/FtsW machinery to synthesize the septal cross-wall. Thus, from what is currently known, it cannot be excluded that PBP2 is responsible for synthesizing the secondary layers sandwiching the core layer of the *S. aureus* septal disk (Figure 1). On the contrary, as PBP2 is the only peptidoglycan-synthesizing entity in *S. aureus* apart from the PBP1/FtsW and PBP3/RodA complexes, and only PBP2 and PBP1/FtsW are essential, it is difficult to envision a two-layer-generating synthesis mechanism that does not involve PBP2. Alternatively, the divisome, using different modus operandi, might synthesize two architecturally different layers of peptidoglycan. However, since septum synthesis in *S. aureus* does not take places exclusively at the leading edge but occurs across the whole septal surface (Lund et al., 2018), the latter mechanism seems to be the less likely of the two alternatives.

Presumably, the question could be settled by examining the *B. subtilis* class A-less mutant by atomic force microscopy along the lines described by Pasquina-Lemonche and coworkers (2020). The fact that *B. subtilis* is able to grow and multiply without aPBP5 shows that the FtsW/PBP2b and RodA/PBP2a/PBPH machineries (Table 1) are capable of synthesizing a functional cell wall on their own. SEDS- and aPBP-type glycosyltransferases must have evolved independently at different times during evolution. Thus, early prokaryotes possessing an outer peptidoglycan layer may well have used only SEDS-type glycosyltransferases to build their cell wall. It is conceivable that PBPs with glycosyltransferase domains evolved later in evolution to work in conjunction with the SEDS-type peptidoglycan polymerases as auxiliary proteins. Different theories have been put forward to explain the transition between monoderm (Gram-positive) and diderm (Gram-negative) bacteria. Although this question has not been settled, the latest phylogenetic analyses support a scenario where diderms represent the ancestral type. The monoderm phenotype appears to have arisen independently multiple times due to the loss of key genes involved in the synthesis of the outer membrane (Megrian et al., 2020). Thus, it is likely that the thick peptidoglycan layer surrounding many Gram-positive bacteria has evolved to compensate for the loss of the outer membrane. As outlined above, recent studies on the function of aPBPs in *E. coli* favor a model in which their primary role is to repair and maintain the integrity of the peptidoglycan sacculus. In the Gram-positives, class A PBPs may have acquired a new or additional function to compensate for the stress generated by the loss of their outer membranes.
namely to build a thicker cell wall by synthesizing a secondary peptidoglycan layer on top of the primary cell wall made by the FtsW glycosyltransferase and its cognate bPBPs.

The recently discovered two-layered architecture of *B. subtilis* and *S. aureus* cell walls raises a pertinent question: how is cell elongation conducted in bacteria with a heterogeneous double-layered cell wall? Most likely, elongation of the primary and secondary cell walls would be carried out by separate protein complexes. Different peptidoglycan-cleaving enzymes (space-maker enzymes) are probably required to make room for insertion of new material in each of the two different peptidoglycan layers. Based on the hypothesis that aPBPs synthesize the cytoplasm-facing secondary layer of the septal cross-wall, it is conceivable that bifunctional PBPs fill in the gaps made by space-maker enzymes in this part of the cell wall while the RodA complex inserts new material in the primary cell wall. If so, the aPBPs and the RodA complex most likely coordinate their activities to synchronize the elongation process between the two layers.

Bacteria produce a large number of peptidoglycan hydrolases which are essential for maintaining the architecture and function of the bacterial cell envelope. They are involved in processes such as cell separation, cell enlargement, recycling of peptidoglycan, and assembly of trans-envelope structures too large to pass through the natural pores of the peptidoglycan sacculus (Scheurwater & Burrows, 2011; Typas et al., 2012; Vollmer, Bernard, et al., 2008). It is not clear how bacteria control the potential suicidal activity of these enzymes, but several different mechanisms are undoubtedly applied. One advantage of evolving a two-layered architecture may be that it enables the cell to better control the activity of peptidoglycan-degrading enzymes in order to avoid autolysis and cell death. Pasquina-Lemonche et al. (2020) reports that in *S. aureus*, the external side of the wall has significantly larger pores than the internal side, and that the large external pores taper off as they traverse the wall (Figure 1). Perhaps the activity of the enzymes creating these pores is controlled, at least in part, by the architecture of the double-layered cell wall. The same may be the case for potential suicide enzymes involved in cell separation. PcsB, for example, is the peptidoglycan hydrolase that splits the septal cross-wall during pneumococcal daughter cell separation (Bartual et al. 2014). Although PcsB is strictly regulated by the membrane associated FtsEX complex (Alcorlo et al., 2020; Sham et al., 2011; Sham et al., 2013; Rued et al., 2019), its activity may also be controlled by a structurally heterogeneous cell wall. Hence, it is conceivable that PcsB specifically cleaves peptide bridges connecting glycan strands in the circumferentially oriented core layer, while being unalowed to attack the disordered flanking layers (Figure 2).

7 | CONCLUSION

The traditional paradigm of peptidoglycan biogenesis states that aPBPs are intrinsic key components of the divisome and elongasome. However, after it was discovered that the SEDS proteins FtsW and RodA have glycosyltransferase activity and work in conjunction with bPBPs, aPBPs were in principle no longer critical components of these multiprotein complexes. Moreover, aPBPs are not essential for survival in some Gram-positive bacteria, strongly indicating that at least in monoderms, bifunctional PBPs are not directly involved in synthesizing the primary cell wall. It is therefore time to rethink and revise the role of aPBPs. Several recent studies in *E. coli* present evidence that aPBPs are important for repair and maintenance of the peptidoglycan matrix. Perhaps *E. coli* produce three different bifunctional PBPs in order to detect and repair different types of damages to the peptidoglycan meshwork. It is likely that aPBPs play a similar repair and maintenance role in Gram-positive bacteria (Figure 2). In addition, we postulate that aPBPs are responsible for synthesizing the cytoplasm-facing section of the two-layered cell wall described for *B. subtilis*, *S. aureus*, and *S. warneri* (see Figure 2) (Pasquina-Lemonche et al., 2020; Su et al., 2020). So far, a two-layered cell wall has only been demonstrated for these species, but there is no reason why it should not be widespread among Gram-positive bacteria.

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