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PEPTIDOGLYCAN POLYMERASE FUNCTION AND REGULATION

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ABSTRACT

Most bacterial species possess two distinct types of glycosyltransferases (GTases or GTs), each with unique structural folds, which catalyze the addition of lipid II monomers to the anomeric reducing end of a growing glycan chain, ultimately forming β−1,4 glycosidic bonds. These bonds link the GlcNAc-MurNAc-peptide disaccharide subunits of the peptidoglycan (PG) polymer. The first type belongs to the carbohydrate-active enzyme (CAZy) GT51 family, which includes a lysozyme-like domain typically associated with a transpeptidase domain in bifunctional class A penicillin-binding proteins (aPBPs) and is occasionally found as a monofunctional GTase in certain bacteria. The second type, a C1-type GTase from the CAZy GT119 family, has a distinctly different structural fold and is composed of polytopic membrane proteins. These proteins also belong to the SEDS (shape, elongation, division, and sporulation) family and are characterized by 10 transmembrane segments and a large extracellular loop. In a single bacterial cell, multiple representatives of each family (aPBPs and SEDS) are typically present, often performing semi-redundant or distinct physiological functions. This review focuses on the structure-activity relationship of these two crucial PG GTases, the coordination between their GTase and the transpeptidase activities, and the regulatory mechanisms controlling these enzymes during cell growth and division within the elongasome and divisome complexes.

INTRODUCTION

Bacterial cell wall peptidoglycan (PG) is a large mesh-like macromolecule that surrounds the cytoplasmic membrane of most bacterial species, allowing them to withstand the high osmotic pressure of the cytoplasm and to maintain their characteristic cell shape. Gram-positive bacteria have a thick, multilayered cell wall, while gram-negative bacteria contain a much thinner PG composed of 1–3 layers, which are encompassed between the inner and the outer membranes in the periplasmic space. The PG structure consists of glycan strands composed of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) cross-linked by short peptides (1). The final stages of PG polymerization require the combined action of the glycosyltransferase (GTase) and the DD-transpeptidase (DD-TPase; also known as penicillin-binding protein or PBP) enzymes, which catalyze the glycan chain elongation from the lipid-linked precursor, lipid II {undecaprenolpyrophosphoryl-Mur*N*Ac(L-Ala^{1}-γ-D-Glu^{2}-[*m*-Dap/L-Lys]^{3}-D-Ala^{4}-D-Ala^{5})-Glc*N*Ac} – *m*Dap is *meso* diaminopimelic acid in *Escherichia coli* and other gram-negative bacteria, and L-Lys is

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found in many gram-positive bacteria and their cross-linking via the stem peptides, respectively (1). The 3→4 type cross-links between the D-Ala[4] and a diamino acid[3] represent the most frequent linkage. However, minor 3→3 type cross-links, catalyzed by LD-TPases, have been detected in *E. coli* and other species as a result of the cross-linking between two diamino acids at the third position. The 3→3 cross-links represent the major linkage in some bacteria, such as *Mycobacteria* and *Clostridioides difficile*, and play an important role in PG synthesis and antibiotic resistance (2–4).

Two biosynthetic polymerase systems are involved in PG assembly, the bifunctional class A penicillin-binding proteins (aPBPs), which catalyze both GTase and TPase reactions (5, 6), and the pair formed by a SEDS (shape, elongation, division, and sporulation) GTase and a monofunctional TPase PBP of class B (bPBP), which together form a bifunctional heterodimeric enzyme complex (7, 8) (Fig. 1). The GTase domain of class A PBPs belongs to the GT51 family, and the SEDS is a member of the GT119 family (a member of the C-type superfamily, group C_B-GT) according to the carbohydrate-active enzymes (CAZy, https://www.cazy.org/) classification database (9, 10), and both use the lipid II as a substrate.

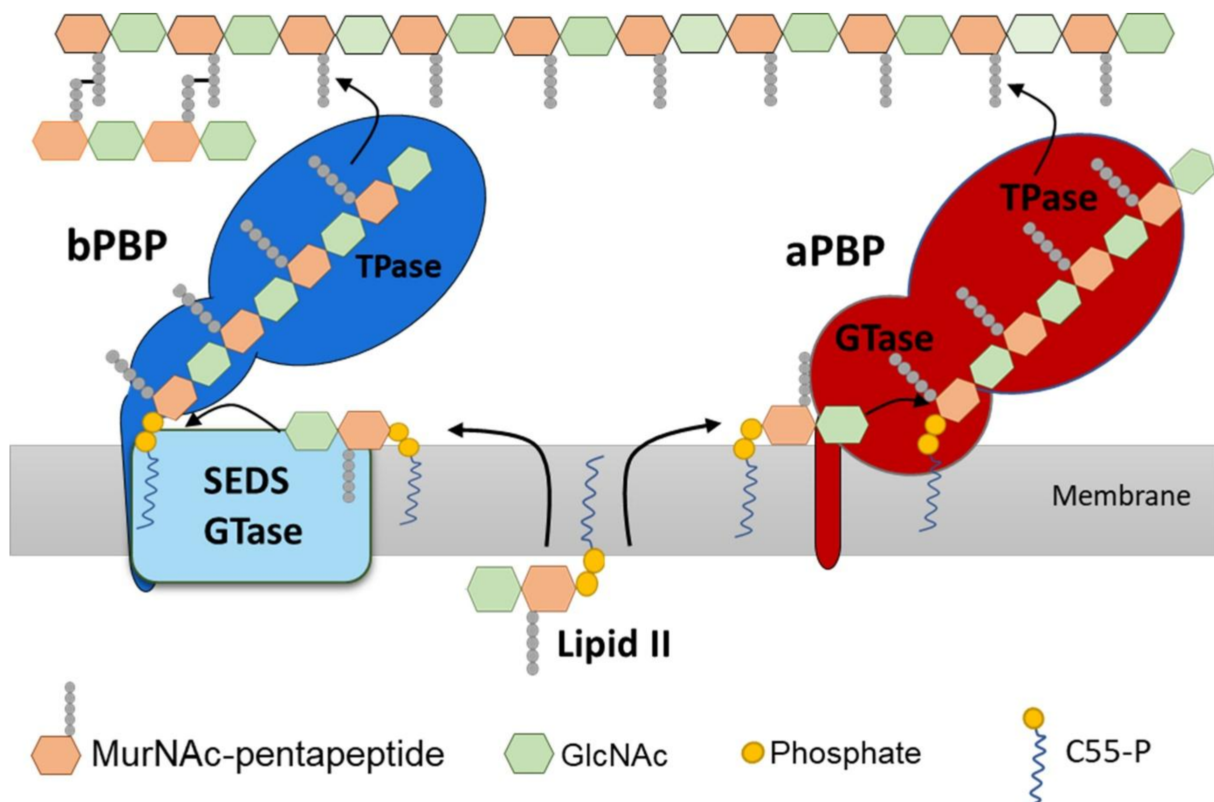

Fig 1. Overview of the final stages of PG polymerization. The PG precursor lipid II is assembled in the inner face of the cytoplasmic membrane and translocated to the outer face of the membrane by a flippase. Lipid II is then polymerized by the glycosyltransferase activities of the class A PBPs and SEDS and cross-linked to the preexisting PG sacculus by the TPase activities of aPBP and bPBPs.

In addition to PG synthases, PG sacculus enlargement, remodeling, and recycling during cell growth and division also require the contribution of PG hydrolases. Their role is to provide space for the insertion of new material for cell wall expansion and cell separation during cell division (11, 12). PG hydrolases include the DD-carboxypeptidase and DD-endopeptidase of the class C PBPs with various

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specificities (5, 11, 13), which are involved in the cleavage of the terminal D-Ala of the pentapeptide and the hydrolysis of cross-links between two peptides, respectively. Amidases, which remove the peptide stems from the glycan chains, also play an important role in PG biogenesis and remodeling. Finally, PG glycosidases, which cleave within the glycan strands, include the glucosaminidases, which cleave between GlcNAc and MurNAc, the muramidases and lytic TGases, which cleave between MurNAc and GlcNAc. While the muramidases are hydrolytic enzymes, lytic TGases catalyze non-hydrolytic cleavage of the PG leading to a 1,6-anhydroMurNAc product (11, 14, 15). Lytic TGases, particularly, have been involved in the release of newly synthesized PG strands (see below) and PG turnover during growth and cell separation (15, 16).

The proteins involved in PG metabolism are organized and coordinated within multiprotein assemblies called the elongasome and divisome (Fig. 2). In *E. coli*, the elongasome organizes PG synthesis during the elongation phase and contains core proteins such as MreB, RodZ, MreD, MreC, and RodA-PBP2 (17). On the other hand, the divisome governs PG insertion at mid-cell for septum formation by mobilizing a core group of a dozen proteins, including FtsZ, FtsA, ZipA, FtsE-X, FtsK, FtsB-L-Q, FtsW-FtsI (FtsI is also called PBP3, the specific TPase of the divisome), and FtsN (18).

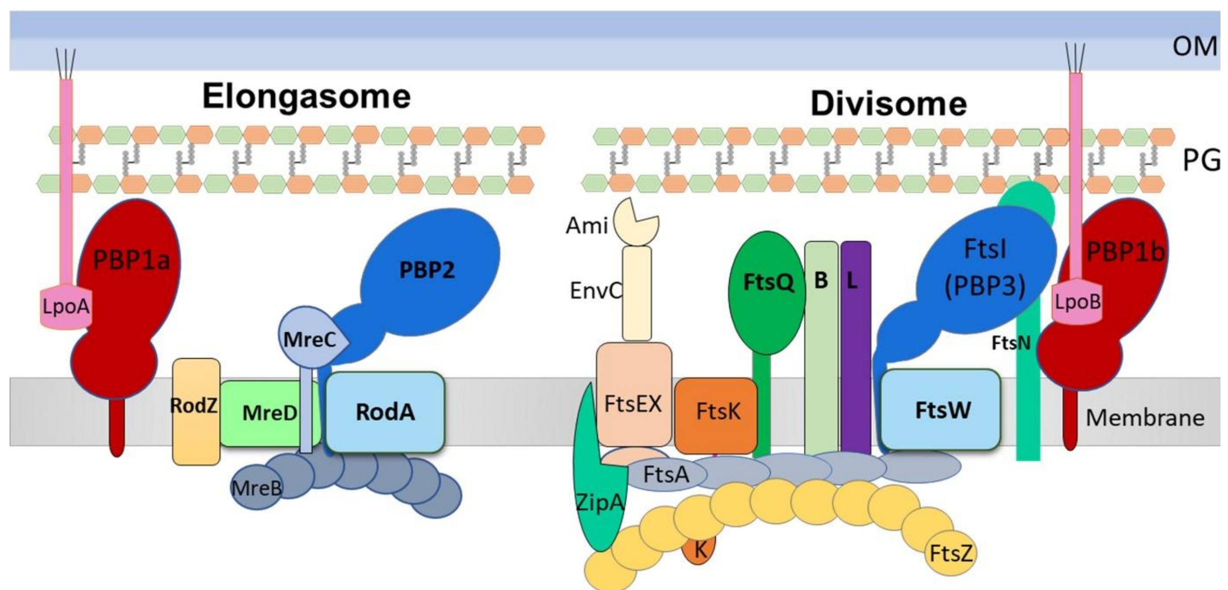

Fig 2. Schematic representation of the elongasome and the divisome complexes. The core proteins of each complex are shown in addition to the aPBPs synthases and their lipoprotein activators PBP1a/LpoA and PBP1b/LpoB.

LIPID II SUBSTRATE SYNTHESIS AND POLYPRENYL-PHOSPHATE CYCLE

Bacterial cell surfaces contain a variety of polysaccharide polymers, such as peptidoglycan (PG), lipopolysaccharide (LPS) O-antigen, wall teichoic acids (WTAs), capsular polysaccharides (CP), and

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exopolysaccharides. The late stages of bacterial cell wall PG polymerization, like those of other polymers, occur at the cell surface and are separated from the synthesis of the building blocks, which takes place in the cytoplasm, by the cytoplasmic membrane (Fig. 3). Therefore, the precursors assembled in the inner leaflet of the membrane must be transported across the membrane. The carrier responsible for this transport is the undecaprenyl-phosphate (Up-P, C_{55}-P, or bactoprenyl-P) lipid carrier. The precursor undecaprenyl-pyrophosphate (Up-PP) is synthesized by the Up-P synthase (UppS) from eight C5- isopentenyl pyrophosphate and a C15 farnesyl pyrophosphate (19). Up-PP, originating from *de novo* synthesis or released after glycan chain elongation, must be dephosphorylated to the monophosphate form before it can be used for sugar transport. Two families of Up-PP phosphatases are known to catalyze this reaction, BacA and type 2 phosphatidic acid phosphatases (PAP2), including PgpB, LpxT, and YbjG in *E. coli* (20, 21). Notably, the active site of these phosphatases is located on the external face of the cytoplasmic membrane. Therefore, how Up-PP and Up-P cross the membrane remains an open question. Recently, members of two protein families, DedA and DUF368-containing membrane proteins, have been shown to be involved in polyprenyl-phosphate transport (22, 23).

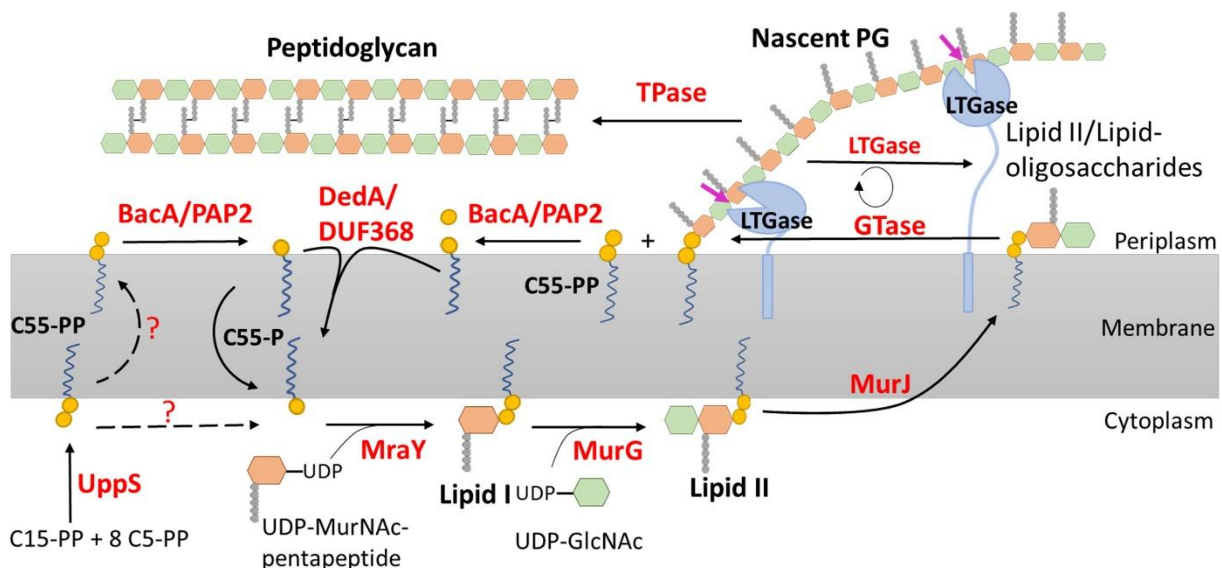

Fig 3. Overview of the undecaprenyl-phosphate lipid carrier (Upp-P or C55-P) cycle in the PG metabolism pathway. C55-PP is formed *de novo* by UppS and gets dephosphorylated by an unknown mechanism. The Upp-P is also recycled by the Up-PP phosphatases BacA and PAP2 and translocated to the cytoplasmic side of the membrane by DedA/DUF368 after Up-PP release during PG polymerization. Upp-P is used as a carrier of P-MurNAc-(pentapeptide)-GlcNAc added by MraY and MurG to form lipid II. The latter is translocated by the flippase MurJ to be polymerized by the GTases. The nascent glycan chain elongation is cleaved by specific PG hydrolases (MltG) that release a lipid-free glycan chain that is cross-linked to the existing PG sacculus by the TPases, whereas the lipid-linked oligosaccharide can be elongated again by the GTase.

The PG building block phospho-Mur*N*Ac(pentapeptide)-Glc*N*Ac is assembled on the Up-P at the inner face of the cytoplasmic membrane by MraY and MurG transferases, which add P-Mur*N*Ac(pentapeptide) and Glc*N*Ac, respectively, yielding lipid II. Lipid II is then transported by a flippase (MurJ) (24) to the extracellular surface of the membrane where PG polymerization occurs.

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PG SYNTHASES AND PG ARCHITECTURE IN DIFFERENT BACTERIA

A large, unbiased analysis of approximately 5,000 genomes showed that the majority of bacteria (80%) possess genes coding for aPBPs, bPBPs, and SEDS. However, a small fraction (20%) had lost aPBPs, and less than 1% had either lost all aPBPs, bPBPs, and SEDS or had lost bPBPs and/or SEDS but retained aPBPs (25).

The correlation between the repertoire of PG polymerases and the content and the stiffness of the PG sacculus in various bacteria reveals that species lacking aPBPs but possessing almost all the genes required for the PG synthesis pathway (lipid II synthesis, at least one SEDS and one bPBP; e.g., *Rickettsiales*, *Planctomycetes*, *Verrucomicrobia*, and *Chlamydiae*), although many were found to be not essential in planctomycetes (26), have a low amount of PG-like cell wall (also designated as intermediate PG or PGi). This structure confers less rigidity and provides less osmotic protection to the cells compared to the authentic PG sacculus of bacteria containing both aPBPs and SEDS/bPBPs (Fig. 4) (25). Additionally, these bacteria often lack a uniform or regular shape, further supporting the dominant role of PG in bacterial cell shape determination and stability (27). Notably, bacteria with PGi are often host-associated species with an obligate intracellular (or endosymbiotic) lifestyle. It has been suggested that they have lost the bulk of their structural PG to evade PG-recognizing proteins (Nod1/Nod2, PGRP) of the host-immune system (25). However, free-living bacteria such as *Planctomycetes*, which most likely contain PGi in their cell wall (as indicated by the presence of SEDS and bPBP proteins), may additionally rely on Cys-rich protein structures for their cell wall stability (28). These bacteria use a budding mechanism for multiplication, which may be dependent on PGi (29).

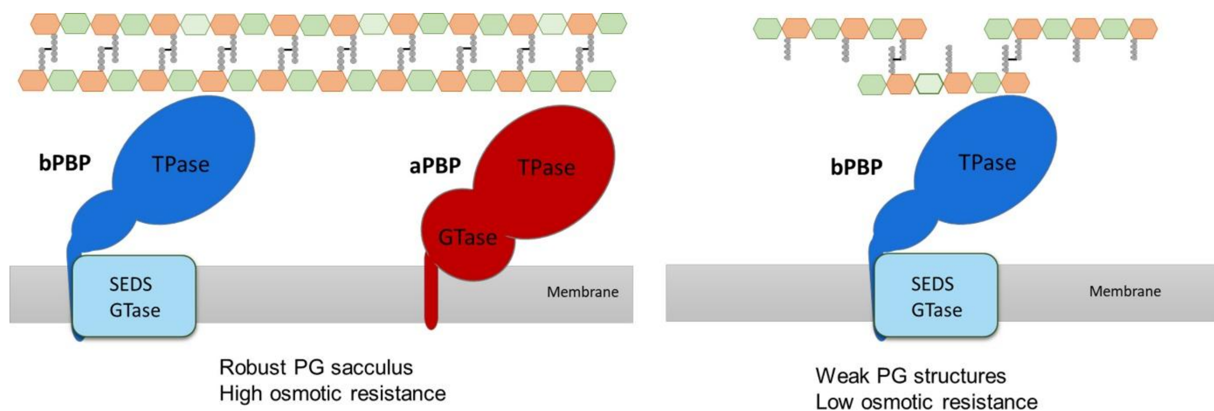

Fig 4. Correlation between PG structure and the PG synthases content. The presence of both SEDS-bPBP and aPBP provides the cell with a robust PG sacculus that can withstand high osmotic pressure. In the absence of aPBP, the PG structure is weaker, and the cells are unable to cope with high osmotic pressure, unless they are protected in an osmo-protective environment.

Chlamydia trachomatis is the most studied bacterial species with PGi. For a long time, it was considered a PG-less bacterium because of the absence of a detectable PG polymer and the lack of aPBPs, despite the presence of PG synthesis machinery, including one bPBP, one SEDS protein

(whose function was unknown at the time), and its sensitivity to penicillin (30, 31). This apparent paradox was known as the *Chlamydia* anomaly (32, 33). Thanks to modern techniques of PG labeling and super-resolution microscopy, it has been shown that *Chlamydia* indeed lacks the classical PG sacculus that surrounds most bacteria, but it synthesizes a narrow PG-like ring structure (PGi) at the division site during the replicative intracellular phase of its cell cycle (34). On the other hand, the non-replicative infectious form does not contain detectable PG. This agrees with the observation that replicative intracellular cells (PGi species) have a much higher level of PG than the infectious host-associated extracellular form (25). Interestingly, the minimal PGi was found to be required mainly for cell division and not for general osmotic stability due to their lifestyle (34). The septal PGi (sPGi) ring thus facilitates cell constriction, as in most bacteria. The synthesis of the sPGi ring, which occurs in the presence of β-lactam antibiotics that inhibit the TPase and CPase, was incomplete and susceptible to lysozyme, indicating that it was most likely composed of uncross-linked PG. This led to cell enlargement but failed to initiate cell division or maintain cell size, showing that the coordination between GTase and TPase is essential for mature, cross-linked sPGi synthesis and for cell division (34). PBP3 was localized to the septum of *C. trachomatis* (35), suggesting that its potential partner, FtsW, may also localize to the septum and that the two proteins are responsible for the synthesis of the sPGi ring. A similar observation was made in the unstable *E. coli* L-form, where minimal PG synthesis was required for cell division when the cells were protected from lysis using osmoprotective medium (36). Indeed, the specific inhibition of the class A PBPs 1a and 1b using cefsulodin β-lactam converted *E. coli* into L-form-like (spherical and osmo-sensitive) cells that could grow in rich hypertonic medium. Interestingly, these cells required a minimal cell wall synthesis (~7% of the normal amount) for growth, most likely to achieve cell division. However, further work showed that stable *E. coli* L-forms generated by the inhibition of cell wall precursor synthesis and lacking FtsZ (and therefore unable to assemble the division machinery) are viable in osmoprotective medium, indicating that these cells are completely independent of PG for growth (37). PGi identified in the environmental endosymbiotic *Protochlamydia amoebophila* was not limited to the septum but forms a PG-like sacculus surrounding the entire cell (38).

STRUCTURE AND PROPERTIES OF THE GLYCOSYLTRANSFERASES OF FAMILY 51

The glycosyltransferases of family 51 (GT51) exist in two forms: as an N-terminal domain of bifunctional aPBPs and as an independent monofunctional GTase (MGT) domain found in some bacteria, such as *E. coli* and *Staphylococcus aureus* (5, 39, 40). Both forms contain an N-terminal transmembrane (TM) segment, and in the case of aPBPs, the N-terminal GTase domain is followed by a TPase domain at the C-terminus, which binds penicillin (Fig. 5; Fig. S1). Certain sub-groups of aPBPs also possess additional domains that interact with regulatory proteins (see below) (6). MGTs are not essential, and their exact role is not fully understood. However, aPBPs are essential in most bacteria with a classical PG sacculus, with two known exceptions: the deletion of all four aPBP genes in *Bacillus subtilis* and all three aPBP genes in Enterococci, *Enterococcus faecalis*, and *Enterococcus*

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faecium did not result in lethality under laboratory conditions (41–43). In these mutants, PG synthesis is likely supported by a SEDS protein. Interestingly, overexpression of RodA rescued defects of *B. subtilis* quadruple mutant, and RodA from both *B. subtilis* and *E. faecalis* was active in the absence of cognate bPBPs, unlike other SEDS proteins, suggesting that these proteins fulfill this role (6, 7, 44, 45).

The number of aPBPs orthologs varies between species, from one (e.g., *S. aureus* and *Neisseria gonorrhoeae*) to several genes (e.g., four in *B. subtilis*) encoding aPBPs (5). These proteins are named based on their molecular weight and the SDS-PAGE profile of labeled PBPs with radioactive or fluorescent penicillin. Although aPBPs seem to play redundant or semi-redundant roles (as some aPBPs are interchangeable with minimal or no effect on cell growth and morphology, e.g., *E. coli* PBP1a and PBP1b), careful analysis of mutants under more stringent conditions revealed that they play specific roles at different stages of the cell cycle and are required for optimal fitness in changing environments. In *E. coli*, PBP1a and PBP1b have been linked to the elongation and division phases, respectively (46–48). They have also been shown to exhibit optimal activity at distinct pH levels (defects in PBP1a or PBP1b affect the growth under alkaline or acidic conditions, respectively), enabling the cells to adapt to fluctuating pH conditions (49). Similarly, the pathogenic *Salmonella enterica* has an additional PBP3 (bPBP), called PBP3sal, which is only active at acidic pH to promote cell division within host cells, where the regular PBP3 is inactive (50).

All GT51 enzymes share five conserved motifs in their amino acid sequence, including two invariable glutamate residues in motifs 1 and 3 (51, 52). The 3D structures of all solved GT51 enzymes are very similar and primarily consist of α-helices, folding into a large and a small lobe, and they share structural similarities with the phage λ-lysozyme (Fig. 5C; Fig. S1) (53, 54). The catalytic cleft lies between the two lobes and can be divided into two sub-sites: a donor site that accommodates the growing glycan chain (this site also binds the moenomycin antibiotic) and an acceptor site for the feeding of the enzymatic reaction with lipid II substrate (Fig. 5C; Fig. S1). This reaction is processive (55) and can be described in two phases: an initial and a slow phase, where both the donor and acceptor sites are occupied by lipid II molecules (Fig. 5D; Fig. S1). The deprotonation of the 4-OH group of the GlcNAc of the acceptor substrate by the glutamate (general base) in motif 1 is followed by a nucleophilic attack at the reducing end (anomeric carbon C1) of the donor substrate, leading to the formation of a β−1-4 linkage with inversion of the configuration through a simple S_N2 displacement reaction (51, 56). Successive additions of disaccharide-peptide units elongate the donor substrate, which likely accelerates the reaction rate through a cooperative mechanism (57). The glycan chain length is an intrinsic property of each GTase enzyme (58), probably related to the enzyme's affinity for the donor substrate and the reaction's processivity. A comparison of the aPBPs in *E. coli* revealed that while PBP1b requires lipid IV {Up-PP-(Mur*N*Ac[pentapeptide]-Glc*N*Ac)$_2$} to be present alongside lipid II, PBP1a can couple two lipid IV molecules even in the absence of lipid II (59, 60). Furthermore, lipid IV and longer substrates in the donor site were shown to initiate polymerization at a significantly faster rate than lipid II (61).

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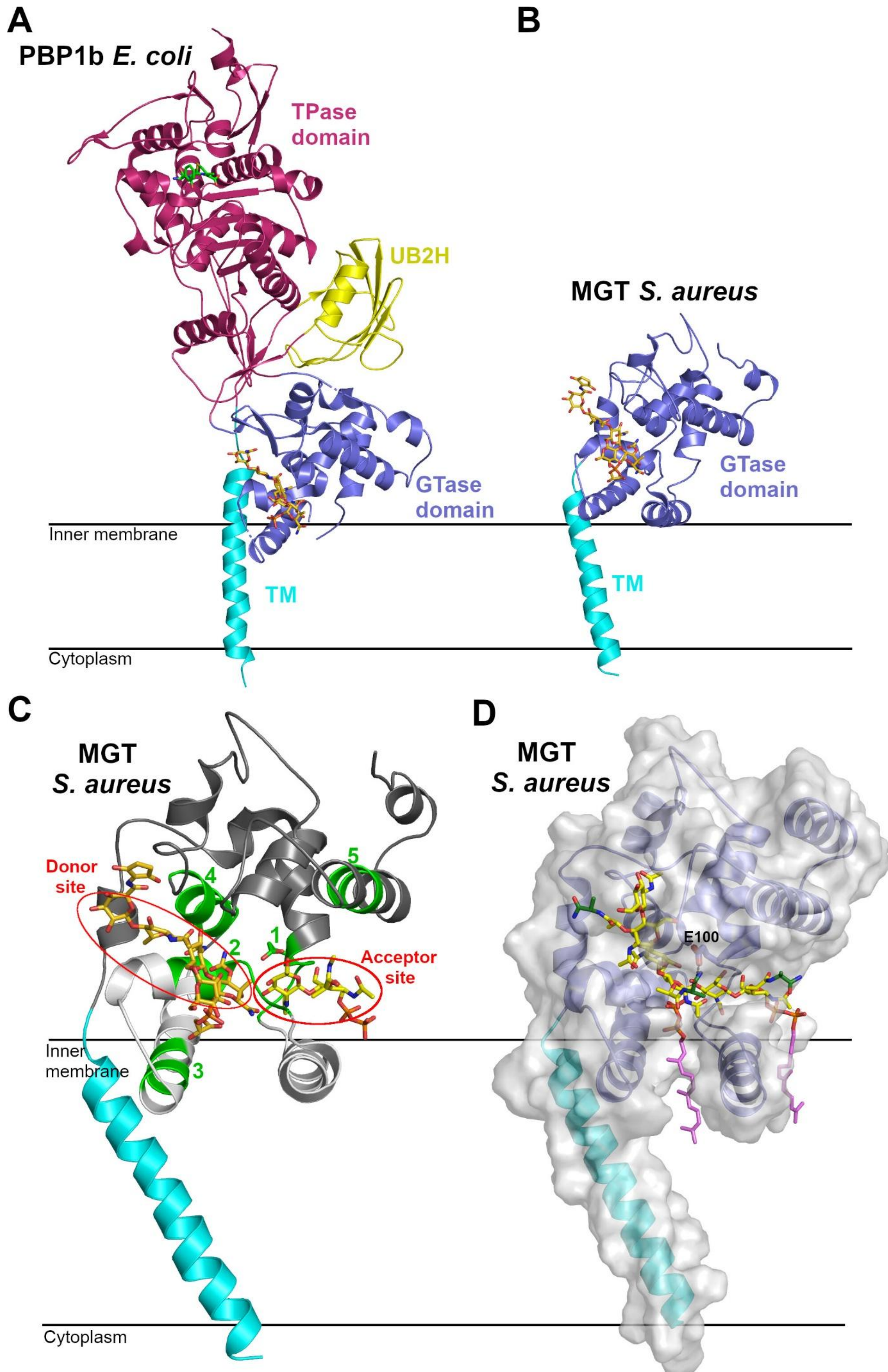

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Fig 5. 3D structures of PG polymerases from the GT51 family. (A) Cartoon representation of the Class A PBP1b from *E. coli* (PDB code 5HLA). (B) MGT from *S. aureus* (PDB code 3VMR). The GTase domain of both enzymes binds moenomycin (gold sticks), while the TPase domain of PBP1b is acylated by the cephalexin antibiotic (green sticks). (C) Same structure of MGT showing the five conserved motifs in green. The donor site binds moenomycin, and the acceptor site contains a lipid II analog from the superposed 3VMT structure. (D) AlphaFold3 model of MGT (gray surface and blue cartoon representations) in complex with a transition state analog made of a lipid II and a lipid IV molecule with 15 carbon long acyl chains (pink sticks) and the peptide moieties constituted of a single Ala (green sticks). The catalytic Glu100 is shown as blue sticks. The quality of the model can be seen in the version of the figure (Fig. S1A) colored according to the predicted local distance difference test. The details of the interactions between MGT and moenomycin, lipid II analog, and the transition state analog can be seen in Fig. S2A through C.

The donor and acceptor sites of the GTase have distinct lipid length requirements: the donor site requires a minimum of 20 carbon atoms, while the acceptor site can accommodate shorter lipids. Notably, a lipid length exceeding 20 carbon atoms is crucial for maintaining the processivity of the GTase (55). A minimal peptide moiety, consisting of D-lactate and L-Ala, also appears to be essential for lipid II substrate binding and its processing by the GTase (62). While the GTase activity can occur when the TPase is inhibited, the TPase reaction requires an ongoing GTase reaction in the same enzyme molecule (51, 63).

REGULATION OF BIFUNCTIONAL CLASS A PBPS

The aPBPs interact with various essential components of the divisome and the elongasome complexes, hydrolases, and non-essential proteins. Below we will discuss some of these interactions, particularly those that have a potential or proven modulation effect on the enzymatic activities.

E. coli PBP1a and PBP1b each contain small domains, termed ODD and UB2H, respectively, which have been shown to bind the outer membrane-bound lipoproteins LpoA and LpoB, essential for their activation. LpoA stimulates the TPase activity of PBP1a, while LpoB stimulates both the GTase and TPase activities of PBP1b (6,64) (Fig. 6B; Fig. S1). The effect of LpoB on the TPase activity of PBP1b is modulated by CpoB, which binds between the TPase and UB2H domains, while TolA further enhances the GTase activity (65). In *Pseudomonas aeruginosa*, PBP1b regulation involves an UB2H-like domain and LpoP, a protein structurally related to CpoB, which uses a similar binding mechanism to LpoB to stimulate both the GTase and TPase activities (66, 67).

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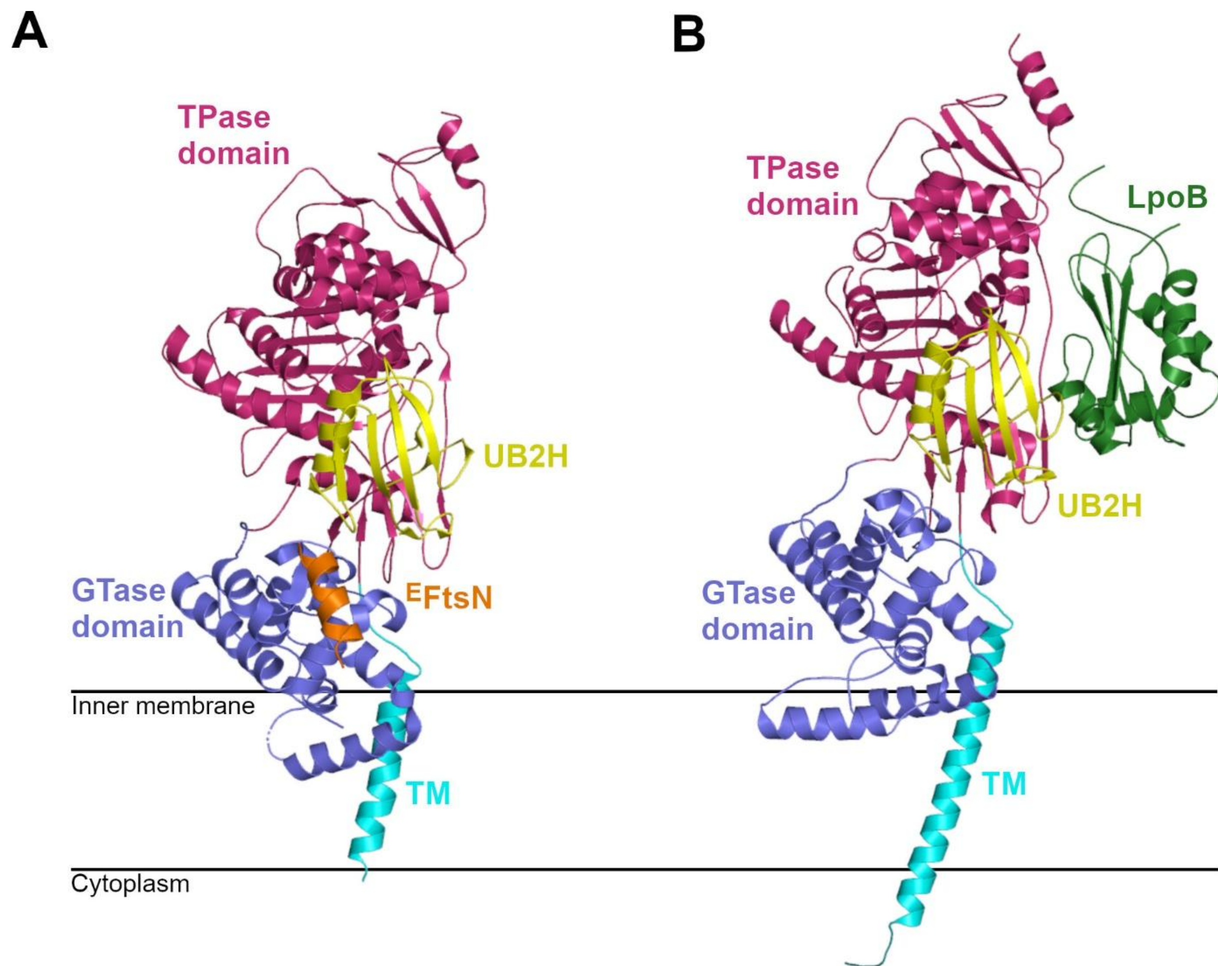

Fig 6. (A) Cartoon representation of the *E. coli* PBP1b structure in complex with the essential fragment of FtsN, ^{E}FtsN (PDB code 6YN0). (B) AlphaFold3 model of *E. coli* PBP1b in complex with LpoB (green cartoon). The quality of the model can be seen in the version of the figure colored according to the predicted local distance difference test (Fig. S1B).

The GTase activity of PBP1a is stimulated by PBP2 (bPBP) *in vitro*, and both collaborate in PG synthesis and its attachment to a purified sacculus (68). PBP1b forms a ternary complex *in vitro* with the cell division proteins FtsW and FtsI; PBP1b is active in the presence of FtsWI, but FtsW alone has an inhibitory effect (69). The GTase activity of *E. coli* PBP1b has been shown to be repressed by the divisome protein FtsBLQ (70, 71), and the presence of FtsN (or LpoB) counterbalances the inhibitory effect of FtsBLQ and restores PBP1b activity (72). The stimulation of PBP1b activity by FtsN involves the essential α-helix fragment of FtsN (^{E}FtsN), which is sufficient to bind to and activate the GTase of PBP1b (72). The binding site was shown using X-ray crystallography to occur between the GTase and the UB2H domains, distinct from the LpoB binding site (Fig. 6A; Fig. S1).

PBP1b also interacts with the Up-PP phosphatases PgpB and BacA, and both proteins stimulate its glycosyltransferase activity. These findings suggest that during PG synthesis, the coupling of PG polymerization with Up-P recycling prevents the inhibition of the synthase by the reaction product Up-PP (73).

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STRUCTURE AND PROPERTIES OF THE SEDS GTASE 119/bPBP COMPLEX

SEDS proteins are polytopic integral membrane proteins of the GT119 family (10), characterized by 10 multi-pass TM helices, cytoplasmic N- and C-termini, and a large extra-cytoplasmic loop. This topology (though the number of TM helices may vary) is shared with other integral membrane proteins of the C-type superfamily GTases with C_{55}-PP-sugar processing activity, including O-antigen ligase (WaaL), the O-antigen polymerase (Wzy), and PglB oligosaccharide transferase (7, 74). Two closely related SEDS proteins in terms of amino acid sequence and structure, RodA and FtsW, have been characterized and shown to specialize in cell elongation and cell division, respectively (7, 75). In addition, sporulating bacteria such as *B. subtilis* contain an additional SEDS member (SpoVE) involved in sporulation (76). The number of SEDS proteins varies among bacterial species, from one or two (RodA and FtsW in *E. coli*) to several genes present (three in Enterococci and four in *Listeria monocytogenes*) (77).

The overall structure of SEDS proteins is well conserved. RodA (from *Thermus thermophilus*) shows 10 transmembrane helices (TM1–TM10). The extracellular loops (ECLs 2, 4, and 5) display several conserved and essential residues, with ECL2 forming a conserved hairpin and ECL5 being buried within the core of the protein. ECL4 is the largest loop (~80 amino acids); it was not well resolved in the structure of RodA alone (7, 78) (Fig. 7; Fig. S1). A central cavity between TM2 and TM4 is surrounded by highly conserved residues and forms the putative binding site for the lipid moiety of the substrate. Adjacent to it, there is a hydrophilic groove open to the extracellular surface of the protein and capped by a highly conserved salt bridge.

A study of *E. coli* FtsW identified a series of dominant-negative mutants, including the putative catalytic residue D297, that blocked septal PG synthesis but did not affect FtsW localization to the division site or its interaction with its partners or with its substrate lipid II. These mutations reside in extracellular loops that are highly conserved in the SEDS family and involve residues that are scattered around a central cavity in the FtsW model structure. Taken together, these results suggest that the residues corresponding to the dominant-negative mutations likely constitute the putative active site of FtsW (79) (Fig. 7; Fig. S1).

The active sites of most GTase and glycosidase enzymes contain a conserved acidic amino acid (Glu or Asp) that acts as a catalytic general base. Sequence alignments and mutagenesis studies of SEDS pointed out a highly conserved aspartate residue in ECL4 (D297 in *E. coli* FtsW and its equivalents in other SEDS proteins) as the potential catalytic residue (7, 8, 80). Like GT51, SEDS enzymes add disaccharide-peptide units to the reducing end of the growing glycan strand (81). However, kinetic parameters for the reactions catalyzed by SEDS proteins, for comparison with GT51, are not yet available. The donor and acceptor substrates seem to have different lipid moiety requirements, similar to GT51 (81). However, in the absence of enzyme-lipid II substrate complex structures, the positions of the donor and the acceptor sites are not well defined. Nonetheless, careful inspection of SEDS structures, combined with molecular dynamics simulations in the presence of lipid II

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substrates and mutagenesis studies, pointed to two potential substrate-binding cavities, containing conserved positively charged residues suggested to coordinate the pyrophosphate moiety, which were proposed as the donor and acceptor sites (82) (Fig. 7; Fig. S1).

The reported activities of SEDS proteins were measured in the presence of divalent cations (7, 8). Analysis of the activity of *Streptococcus thermophilus* FtsW-PBP2x in the presence and absence of Ca^{2+}, Mg^{2+}, Mn^{2+}, or EDTA showed that divalent cations (except for Zn^{2+}) are required for activity (7, 8). However, it was reported that *E. coli* RodA-PBP2 activity was not perturbed in the presence of EDTA, and the authors concluded that the GTase activity of RodA was metal independent (82). Similarly, the activity of most GT51 enzymes is stimulated by divalent cations (39, 56, 83), but SgtB of *S. aureus* and *Streptococcus pneumoniae* PBP1a was active in the presence of EDTA (8). These discrepancies are likely due to different metal-binding affinities in the presence of substrates, which prevent their complete chelation, suggesting that metal ions are required for the optimal activity of both GTase classes. Metal ions were not observed in the crystal structures of GT51 or SEDS, indicating that their binding is mediated by the pyrophosphate of the substrates, where they play a role in catalysis or in stabilizing the released pyrophosphate-lipid moiety.

SEDS GTases coordinate their glycan chain polymerase activity with a cognate bPBPs TPases, which catalyze the cross-linking of nascent PG to preexisting sacculi (8, 84). The structure of RodA-PBP2 from *Thermus thermophilus*, along with other structures, revealed a 1:1 stoichiometry complex with a compact conformation (85). The main interactions between the two proteins occur in the TM domains, between the TM segment of PBP2 and TM8 and TM9 of RodA via hydrophobic interactions. The second and major interaction occurs between the N-terminal domain (pedestal domain), through the anchor subdomain of PBP2, and the large ECL4 (connects TM7 and TM8) of RodA, which contains essential functional residues. ECL4, which was disordered in RodA alone, becomes ordered upon binding to the pedestal domain of PBP2, which functions as an allosteric stimulator of RodA GTase activity (85). The interaction of PBP2 with RodA induces an outward displacement of TM7 by approximately 10 Å, exposing a large cavity within the membrane, which may serve as the entry or exit site for the lipid II substrate. This brings the TPase domain almost parallel to the membrane surface, with the active serine at approximately 80 Å away from RodA's potential catalytic site. This distance is similar to that between the GTase (GT51) and TPase catalytic residues in bifunctional PBPs, which is around 70 Å (53). However, negative stain electron microscopy showed that the *Tt*RodA-PBP2 complex adopts a variety of different conformations, indicating its dynamic nature. Similarly, comparisons of different structures of *S. aureus* PBP2 (class A) revealed variations in the orientation of the TPase domain relative to the GTase domain (86). In the *Tt*RodA-PBP2 structure, the TP domain oscillates between a closed state (40–50 Å above the membrane) and an open state (70–80 Å above the membrane), which is within reach of the PG layer to perform crosslinking. The pedestal domain of PBP2 plays a major role in this dynamic, which likely reflects different states adopted by the polymerases during catalysis (87). Using engineered *E. coli* RodA-PBP2, the extended conformation exhibited higher GTase activity than the closed state, and this dynamic is essential for the function of the complex *in vivo* (87). Moreover, a study of *E. coli* RodA-PBP2 dynamics using molecular dynamics simulations and double electron-electron resonance experiments confirmed the dynamics and flexibility of PBP2 relative to RodA and the membrane surface (82). This may help

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guide the growing glycan chains toward the TPase active site and their attachment to the sacculus. Alternatively, glycan chain elongation may induce these conformational changes.

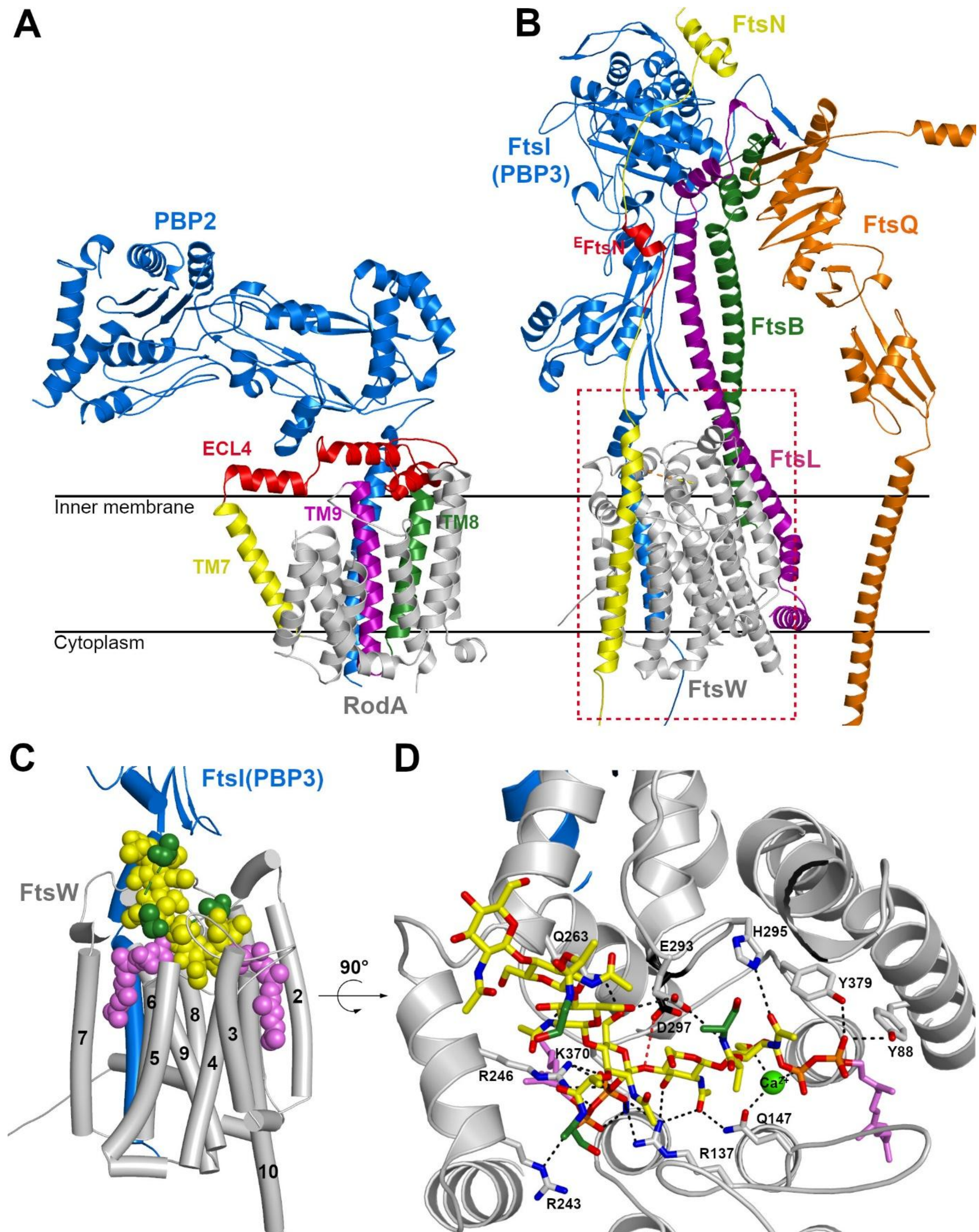

Fig 7. Structure and models of SEDS/PBPb complexes. (A) Structure of the *E. coli* RodA/PBP2 complex (PDB

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code 8TJ3). (B) Cartoon representation of the AlphaFold3 model of the *E. coli* FtsBLQWIN complex. (C) AlphaFold3 model of the *E. coli* FtsWI complex (approximately corresponding to the red dashed rectangle in B) in the presence of a transition state analog made of a lipid II and a lipid IV molecule (glycan as yellow spheres) with 15 carbon long acyl chains (pink spheres) and peptide moieties constituted of a single Ala (green spheres). (D) Similar to C with a 90° rotation, the transition state analog is shown as sticks. Residues of FtsW making putative polar contact (dashed lines) are displayed as gray sticks except for the catalytic D297 in black. The putative Ca2+ ion required for activity is shown as a green sphere. The quality of the models can be seen in the version of the figure colored according to the predicted local distance difference test (Fig. S1C and D).

Except for some RodA (*B. subtilis* and *E. faecalis*), which exhibit low GTase activity (7, 44), most tested SEDS, including all FtsWs, were only active in the presence of the cognate bPBPs (8, 44, 80, 88). Thus, the SEDS-bPBP reconstitutes a bifunctional enzyme, like the class A PBPs, regulated by allosteric interactions between the two subunits to ensure an optimal coordination between the GTase and the TPase activities. This regulatory mechanism could serve to avoid the release of glycan chains before their attachment to the sacculus or in the rational use of lipid II substrate (8).

Surface complementarity between interacting proteins is important for activation, as SaFtsW is only active with SaPBP1; StFtsW is also active with StPBP2x or with the close relative SpPBP2x but not with the distant SaPBP1. This demonstrates that a certain degree of sequence conservation is required to ensure functional complementarity (8). The interaction between the TM segment of bPBP and cognate SEDS plays an essential role *in vivo*, not only in GTase activation but also in the proper assembly and function of divisome and elongasome complexes. The swapping of TM segments between elongasome and divisome-specific bPBPs (in StPBP2b/2x and SpPBP2b/2x) shows that only bPBPs with a compatible TM of a divisome protein activate the corresponding FtsW (8). Mutations of the PBP2 TM-binding site in RodA cause a dominant negative effect in *E. coli*, but this does not abolish the *in vitro* GTase activity of RodA (8, 78). On the other hand, *in vitro*, both StPBP2x and the ΔTM variant were able to cross-link glycan chains formed by SaMGT or the aPBP SpPBP1a* GTase (TPase inactive) in addition to StFtsW. Thus, the TPase activity of StPBP2x does not depend on its specific FtsW partner (8).

REGULATION OF SEDS-bPBP

In addition to the activation of the GTase activity of SEDS by a cognate bPBP, it has been shown using purified proteins from *P. aeruginosa* that FtsBLQ interacts with FtsWI and further enhances the GTase activity of FtsW (80, 88). However, FtsN, which is proposed as the ultimate activator of PG synthesis and cell constriction, had no additional effect on the complex activity (88, 89). Notably, FtsN homologs are not found in gram-positive species.

The cryo-electron microscopy (EM) structure and AlphaFold2 modeling of the FtsWIBLQ complex showed that, in addition to the interactions of transmembrane segments of FtsLB with those of FtsWI (FtsQ TM does not interact with any protein of the complex), an extensive network of interactions occurs between the C-terminal domains of FtsI, FtsB, FtsL, and FtsQ (80). The binding of FtsBLQ to FtsWI induces conformational changes that may be responsible for the observed stimulation of the

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GTase activity of FtsW and probably also of FtsI, but the overall cryo-EM structure is relatively closed (~54 Å above the membrane surface) and out of reach of the PG layer (at ~80 Å) to achieve TPase activity by FtsI (80, 90). This suggests that *in vivo*, additional factors are probably required for full activation of PG synthesis and cross-linking. Interestingly, the predicted models of FtsWIBLQ using AlphaFold2 showed an extended conformation (at ~80 Å), which may reflect the native active state of the complex (80).

FtsN is also known to interact and regulate the activity of FtsWI. AlphaFold2 modeling, molecular dynamics simulations of the FtsWIBLQ complex with and without the addition of an N-terminal segment of FtsN containing the essential domain ^{E}FtsN, and mutagenesis studies of FtsWIBLQ reveal that ^{E}FtsN interacts with FtsI and FtsL (at a so-called AWI [Activation of FtsWI] region), but only minor global conformational changes of FtsWIBLQN were observed compared to the complex without FtsN (Fig. 7; Fig. S1). However, the presence of ^{E}FtsN triggered extensive local conformational changes in the interface regions (LBI^{E}N) that propagate to affect the interactions between the anchor domain of FtsI and ECL4 of FtsW and the active site. Based on mutant analysis, the binding of ^{E}FtsN seems to reduce inhibitory interactions and strengthen activating interactions in the FtsWIBLQ complex (90), which probably further stimulates the polymerase activities of the FtsWI complexed with FtsBLQ (90–92). Mutagenesis studies also suggested that ^{AWI}FtsL-FtsI interaction is not required for maintaining FtsI in the open state but is only required for activation, suggesting that the open state is maintained by multiple interactions between these proteins and that glycan chain elongation also plays a role (91). In addition, the sporulation-related repeat (SPOR) domain of FtsN, which binds the PG, although not essential, may contribute to the extended conformation, competent for optimal PG polymerization and cross-linking (71). However, the absence of FtsN in Gram-positive bacteria suggests that an alternative model is also possible.

The core synthase complex of the elongasome RodA-PBP2 is regulated through protein-protein interaction with MreBCD (Fig. 2). The crystal structures of the soluble PBP2:MreC complex from *Helicobacter pylori* show MreC interacting via its C-terminal β-domain with the N-terminal domain of PBP2 (93). This interaction induces conformational changes of PBP2, which affect the interaction between PBP2 and RodA and activate PG synthesis (84, 94). MreD also interacts directly with MreC, and the balance between MreC and MreD is suggested to determine the activity of RodA-PBP2 (94).

ORGANIZATION OF PG SYNTHESIS BY CYTOSKELETAL PROTEINS DURING GROWTH AND DIVISION

PG synthases are components of large multiprotein complexes, the divisome and the elongasome. The cellular dynamics of SEDS-bPBPs and class A PBPs during PG synthesis have been studied using single-molecule tracking in different bacterial models (95–99). All these studies point to a cytoskeletal (MreB or FtsZ)-guided PG synthesis, either directly or indirectly, by SEDS-bPBP, with RodA-PBP2 following MreB tracks during elongation and FtsWI moving with FtsZ during cell division (100). However, PBP2 and MreB were found to only partially overlap (101), and active PG synthesis

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influences MreB polymer formation (84). Similarly, slow-moving active synthase populations at the divisome were independent of FtsZ treadmilling in *E. coli* but depended on PG precursor pools and the GTase-TPase reactions of the septal machinery. This suggests that other factors, such as associated proteins, the availability and localization of lipid II substrate (102–104), which is shared by both GTase classes, and specific cell wall structures (105) (e.g., open breaches by hydrolases), also exert some constraints on the localization, dynamics, and the activity of the PG synthases.

PG synthesis mediated by aPBPs, in contrast, has been shown to operate semi-autonomously of the cytoskeletal complexes (99)—single-molecule tracking of *E. coli* PBP1a or 1b suggested that possible associations with the rod complex were short-lived—and is thought to contribute to the repair or fortification of the cell wall (106). It should be noted that the term "PG repair" had been used differently in the literature: as a system needed for survival under cell envelope stress, such as in response to defect in outer membrane assembly, where *E. coli* used the LD-TPase LdtD, PBP6a, and the PBP1b to straighten its PG (107), and in other occasions as occurring during normal growth (106), and were both shown to involve aPBPs.

The mechanisms of cell division are relatively well conserved. However, the mechanisms of cell elongation are diverse and include lateral, unipolar, bipolar, and medial growth, reflecting diverse modes of PG synthesis during elongation (108). Polar-growing bacteria include actinomycetes (Mycobacterium, Corynebacterium, and Streptomyces) that grow by bi- or multi-polar PG synthesis and rhizobia that elongate by unipolar PG synthesis. The rhizobial *Agrobacterium tumefaciens* lacks rod complex proteins (PBP2/RodA and MreBCD) and contains three FtsZ homologs, only one of which is essential and plays a role in the regulation of both polar growth and cell division (109). PG synthesis that drives cell elongation at the pole mainly relies on the class A PBP1a polymerase, while FtsW-PBP3 is involved in cell division (109–111). Interestingly, the polar-growing bacteria encode a large number of LD-TPases, and their PG exhibits an unusually high percentage of 3–3 cross-linking (30%), indicating that aPBP and/or a SEDS protein cooperate with LD-TPases to build a significant amount of the sacculus in these bacteria (110, 111).

The sporulating *C. difficile* undergoes two types of cell division: an asymmetric polar division during sporulation and a vegetative division at mid-cell, both requiring septal PG synthesis. The repertoire of PG synthases of *C. difficile* is composed of one aPBP (PBP1), two SEDS proteins (RodA and SpoVE), and three bPBPs (PBP2, PBP3, and SpoVD) in addition to one MGT. The analysis of the function of these proteins revealed that SpoVD-SpoVE and PBP3 are involved in asymmetric cell division during polar endospore formation, RodA-PBP2 constitutes the core elongasome synthase, and PBP1 is the major septal PG synthase during vegetative mid-cell division (112). Of note, the SpoVD-SpoVE encoded by the *dcw* cluster—which constitutes the core divisome proteins in most bacteria—is, in this case, specialized in sporulation, whereas the core divisome PG synthase during vegetative cell division is the aPBP, PBP1. Furthermore, FtsBLQ was essential for sporulation (asymmetric division), but deletion mutants had no growth or morphological defects in vegetative cells, indicating that these proteins have a dedicated role in sporulation in *C. difficile*. Therefore, as shown here from the findings in *A. tumefaciens* and *C. difficile*, it becomes evident that the mode of PG synthesis during growth and division, inferred from the most studied model species *E. coli*, *B. subtilis*, and others, is

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not universal as previously thought, and substantial variations exist in the bacterial world, revealing the necessity of studying the mechanisms of PG synthesis in different model organisms to understand the existing alternative models used by bacteria and their extent. In fact, a recent study pointed out the huge gap that exists between known and studied bacteria. Half of all the articles in the PubMed repository mention only 10 species, with *E. coli* at the top of the list; 90% of the papers deal with barely 1% of species, and 74% of all known species were not mentioned in any research paper at all (113).

COORDINATION BETWEEN SEDS-bPBPS AND aPBPS

Both aPBPs and SEDS-bPBP bi-functional enzymes are, as mentioned above, clearly required for the assembly of a robust functional peptidoglycan wall in most bacterial species, as the inhibition of either synthase system drastically reduces PG synthesis by about 70% (8, 99, 100, 106). A study using high-resolution imaging, cryogenic electron tomography, and selected mutants (114, 115) to visualize the architecture of the septum of dividing *E. coli* cells demonstrated that substantial sPG material (so-called wedge-like sPG structure adjacent to the ingrowing septum) is not made by FtsWI, suggesting that it is probably made by the aPBP, PBP1b. This is consistent with previous findings that cells lacking PBP1b have 50% reduced fitness and show increased sensitivity to osmotic stress at the stationary phase (106, 116, 117). Thus, the emerging picture points to a model where aPBP and FtsWI coordinate their activities to safely build the septum, with FtsWI making the inward leading edge (structural element) and PBP1b synthesizing a triangular wedge (PG bridge) structure at the outer edge of the septum, which constitutes a transient fortifying layer that protects the septum at the site of active hydrolysis and prevents cell lysis during cell division (Fig. 8). This localized protective layer is particularly important to prevent cell lysis due to hydrolase activities that promote cell constriction concomitant with septum progression and cell separation. Thus, the function of aPBP, which may also apply during elongation (e.g., synthesis of a PG layer at sites opened by hydrolases at the lateral PG wall to allow the rod complex to insert new material), is fundamental for homeostatic sacculus growth and division in unstressed cells and is not limited to a repair function that can occur under stress conditions such as in the presence of antibiotics (6). The essentiality of aPBPs in certain species like *E. coli* and not in others (*B. subtilis* and *Enterococci*) is probably related to PG thickness, to the shape of septation, with or without constriction (V-shape), and the timing of hydrolases intervention relative to septum progression and their relative activity (114).

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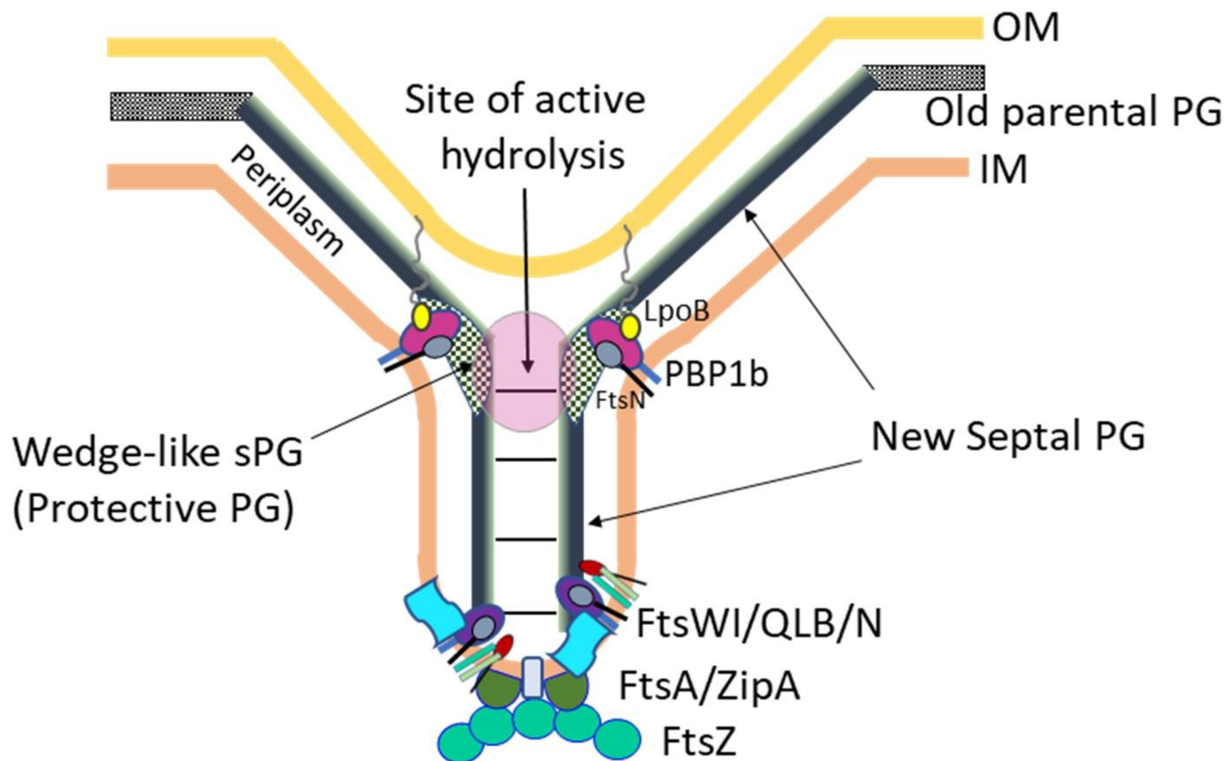

Fig 8. Model of *E. coli* cell division by septation-constriction. Model of the progression of septal PG synthesis and cell constriction depicting the respective role of PG synthase FtsW-FtsI and the class A PBP1b and their regulatory proteins. In this model, FtsW-FtsI drives septum synthesis inward at the leading edge, and PBP1b synthesizes a protective PG structure at the outer edge of the septum, where active hydrolysis and cell constriction take place that prevents cell lysis.

GLYCAN CHAIN RELEASE

One important question is how the elongating glycan chains are released from the C55-PP-lipid anchor in the GTase active site after polymerization. Cytoplasmic membrane-anchored glycosidases have been suggested to specifically terminate glycan elongation in different bacteria by cleaving the nascent growing chain and promoting their release from both classes of PG synthases (118, 119). The cleavage site within nascent PG relative to the lipid anchor is determined by the distance between the active site of the hydrolase and the membrane surface (Fig. 3) (120). In *E. coli*, MltG is a lytic transglycosylase (LTG, releases *anh*Mur*N*Ac products), which, unlike the seven other LTGs, is able to suppress lethal deficits in either aPBP or SEDS/bPBP PG synthase activities (118, 119). MltG interacts with PBP1b, and the ortholog from *B. subtilis* also interacts with the class A PBP1 and the class B PBP2b (121). Like *Ec*MltG, *Bs*MltG is also an LTG. Their activities were higher during ongoing glycan chain polymerization and produced glycan strands of seven disaccharide units, ~70 Å in length, which is about the same distance between the GTase and the TPase active sites of aPBPs and SEDS-bPBPs (121). MltG competes with the TPase activity of class A PBPs but has no effect on their GTase activity. In *Streptococcus pneumoniae*, MpgA (homolog of *E. coli* MltG) and MpgB, a PG muramidase that generates reduced ends unlike MltG, cleave nascent PG at different sites from the lipid anchor

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(120, 122). MpgA cuts nascent PG and releases it from a seven-disaccharide-lipid-linked product. MpgB cleaves at the closest site relative to the lipid moiety, generating lipid II and free glycan chains. MpgA includes a LysM domain that determines the cleavage site and thus the length of the released products. Point mutations in the catalytic domain of MpgA suppress lethality due to deletion of PBP2b, an essential bPBP of the elongasome (123). In *S. aureus*, SagB is a membrane-bound glucosaminidase that cleaves PG polymers from their lipid anchor and was proposed as a PG terminase in this organism (124). Mutation of *mltG* in *N. gonorrhoeae* resulted in increased PG turnover, releasing increased amounts of muropeptides and larger oligomers. In contrast, the mutation of either *ltgA* or *ltgD*, encoding lytic transglycosylases, resulted in wild-type levels of PG monomer release. MltG interacts with PBP1 and PBP2 and the endopeptidase PBP4 (PbpG). These data indicate that MltG acts as a glycan chain terminase in *N. gonorrhoeae* (125).

The observation that lipid IV and longer substrates in the donor site initiate PG polymerization by a class A PBP much faster than lipid II (61) suggests that the cleavage that leaves a compatible lipid-linked oligosaccharide unit bound at the donor site generates an enzyme complex ready to start a new polymerase cycle at a high rate. Therefore, the cleavage and release of glycan chains of nascent PG can be considered an integral part of the global PG polymerization process through the coordination between the synthases and hydrolases (Fig. 3). In addition, the TPase-mediated attachment of the glycan chain to the existing sacculus will promote its release from the GTase after cleavage.

CONCLUDING REMARKS

Activation or inhibition of PG synthesis results in the perturbation of cell cycle synchronization and leads to cellular defects and eventually to cell death. Therefore, any compound that interferes with substrate binding or protein-protein interactions, disturbs the spatial and/or temporal coordination, or stimulates or inhibits the enzymatic activity is a potential antibacterial agent.

Moenomycin A is a potent antibiotic against most gram-positive bacteria but is not exploited clinically because of poor pharmacokinetic properties (126). It is the only natural compound known to inhibit the GTase activity of family 51. It acts as a substrate analog inhibitor and binds to the donor site with high affinity in the nanomolar range. Despite the fact that moenomycin mimics the elongating glycan chain (donor substrate), this inhibitor is not active on SEDS proteins (FtsW and RodA), suggesting that the structure of the binding site, its access, and/or mechanism are different. The identification of GTase activity of SEDS and the development of assays will certainly stimulate the search for inhibitors of these essential enzymes (127, 128). The current accumulated knowledge of the function of the divisome and elongasome, along with the development of tools such as *in situ* monitoring of PG synthesis using fluorescent D-amino acids (129) and fluorescence resonance energy transfer (FRET)-based assays both *in vitro* and *in vivo* for monitoring protein-protein interactions (130) and the GTase activity using membrane-reconstituted enzymes (131), could help identify new classes of antibacterials targeting the substrate, the polymerase activity, or the protein-protein interaction networks of these supramolecular assemblies. These developments, combined

with the explosion of information technology tools such as Alphafold 3 and artificial intelligence, could be a game-changer in the discovery of next-generation antibiotics targeting bacterial cell wall biogenesis.

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AUTHOR CONTRIBUTIONS

Mohammed Terrak, Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review and editing | Frédéric Kerff, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

SUPPLEMENTAL MATERIAL

Figures S1 and S2 (MMBR00070-25-s0001.pdf). Structural models and detailed description.

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