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Don't let sleeping dogmas lie: new views of peptidoglycan synthesis and its regulation

Heng Zhao,[1] Vaidehi Patel,[1] John D. Helmann[1] and Tobias Dörr[1,2]*

[1]*Department of Microbiology, Cornell University, Ithaca, NY 14853-8101, USA.*

[2]*Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY 14853, USA.*

Summary

Bacterial cell wall synthesis is the target for some of our most powerful antibiotics and has thus been the subject of intense research focus for more than 50 years. Surprisingly, we still lack a fundamental understanding of how bacteria build, maintain and expand their cell wall. Due to technical limitations, directly testing hypotheses about the coordination and biochemistry of cell wall synthesis enzymes or architecture has been challenging, and interpretation of data has therefore often relied on circumstantial evidence and implicit assumptions. A number of recent papers have exploited new technologies, like single molecule tracking and real-time, high resolution temporal mapping of cell wall synthesis processes, to address fundamental questions of bacterial cell wall biogenesis. The results have challenged established dogmas and it is therefore timely to integrate new data and old observations into a new model of cell wall biogenesis in rod-shaped bacteria.

Introduction

Most bacteria surround themselves with a cell wall, a complex biopolymer with a crucial role in maintaining cellular integrity and cell shape. Due to its essentiality for bacterial growth and survival, the bacterial cell wall constitutes an ideal target for antibiotics, and there has been a longstanding scientific interest in the mechanisms of its synthesis and turnover. Pioneering work beginning over 50 years ago established the general composition of the cell wall (or sacculus) as a single large molecule made primarily of peptidoglycan (PG). The Gram-positive cell wall also contains a large amount of teichoic acid, including wall teichoic acids covalently linked to PG (Reichmann and Grundling, 2011; Brown *et al.*, 2013). PG is a mesh-like macromolecule comprising roughly parallel glycan strands of polymerized disaccharide (*N*-acetylglucosamine-*N*-acetylmuramic acid; or NAG-NAM), which are intermittently crosslinked with neighboring strands by peptide bonds between short oligopeptides (pentapeptides) attached to the NAM residues (Strominger *et al.*, 1959, 1971; Anderson *et al.*, 1966, 1967).

This basic PG structure is conserved amongst essentially all Bacteria, although there are variations in the details. These include differences in glycan strand chain length and a diversity of peptide crosslinks. The peptide crosslinks vary in amino acid composition and modifications (e.g., amidation), the addition in some species of interstrand bridging peptides, the precise site of interstrand linkage and their overall density (Quintela *et al.*, 1995; Espaillat *et al.*, 2016). The PG sacculus can be further modified after synthesis by occasional additions to the glycan strands, for example of acetyl residues [O-acetylation, (Moynihan *et al.*, 2014)]. Despite these subtle variations, the overall PG structure is highly conserved when compared to other bacterial surface layers (including capsules, S-layers and enterobacterial O-antigen), and therefore PG and its derivatives serve as effective pathogen-associated molecular patterns (PAMPs) for host recognition of bacterial infection (Mogensen, 2009).

Despite being the focus of intense research, conspicuous gaps in our knowledge of PG biogenesis have persisted over decades, and some long-entrenched ideas have been found to be either incorrect or incomplete. Recent studies of the enzymology, genetics and cell biology of PG synthesis have challenged many long-standing assumptions. Here, we review recent insights

Accepted 27 September, 2017. *For correspondence. E-mail: tdoerr@cornell.edu; Tel. 607-255-1903; Fax 607-255-1903.

into PG synthesis, largely from studies of the rod-shaped model organisms *Escherichia coli* and *Bacillus subtilis*. The focus will be the enzymology and cell biology of proteins involved in cell elongation and division. We conclude by proposing a new model of cell wall biogenesis that incorporates these recent findings.

Enzymology of PG synthesis: variations, nuances and the key points of controversy

Assembly of the PG layer requires three major stages: precursor synthesis in the cytoplasm to generate the key intermediate lipid II, the lipid II cycle (translocation, transglycosylation and recycling of the carrier lipid) and glycan strand crosslinking and maturation. As would be expected for such a central process in bacterial cell biology, the key enzymes for PG synthesis are generally well-established. However, the overall process displays more plasticity than originally envisioned, some key enzymes have remained elusive or controversial and several puzzling genetic observations have only recently been resolved.

PG synthesis starts in the cytoplasm, where the precursor molecule UDP-NAM-pentapeptide is produced by enzymes encoded by the *mur* genes as well as the D-Ala-D-Ala ligase Ddl as the last soluble precursor (Lovering *et al.*, 2012). Ligation of this precursor to an undecaprenyl (C_{55}) carrier lipid by the membrane-associated enzyme MraY generates lipid I, the first membrane-associated intermediate. MurG ligates a NAG residue to lipid I to generate the final, lipidated disaccharide-pentapeptide precursor referred to as lipid II (Scheffers and Tol, 2015). Once synthesis is complete on the cytoplasmic face of the inner membrane, the lipid II precursor must be translocated (flipped) to the outer face of the membrane by a flippase, where the final steps of PG assembly occur. Following assembly, PG may be further modified and serves as a scaffold for the anchoring of wall teichoic acids (in Gram-positive bacteria), proteins and other surface structures and appendages (Brown *et al.*, 2013; Guest and Raivio, 2016; Siegel *et al.*, 2016).

Lipid II provides the subunits that are polymerized into glycan strands via a transglycosylation (TG) reaction. The lipid carrier is released as undecaprenyl-pyrophosphate and recycled into the cytoplasm by a putative, as yet unidentified C_{55}-pyrophosphate flippase, which may or may not be the same as the lipid II flippase. The TG reaction has historically been thought to be mediated solely by bifunctional (class A) penicillin binding proteins, here designated as aPBPs (Goffin and Ghuysen, 1998; Sauvage *et al.*, 2008). The polysaccharide strands resulting from the TG reaction are subsequently covalently linked via D, D transpeptidation (TP) reactions to form peptide bond crosslinks between the glycan strands (Sauvage *et al.*, 2008). The acceptor amino group derives from the side chain of the third amino acid (typically diaminopimelic acid, DAP_3 or lysine, Lys_3, depending on the species) with D-Ala_4 as the donor (generating a 4-3 crosslink); this results in the release of the terminal D-Ala_5 from the donor strand (McDonough *et al.*, 2002; Mainardi *et al.*, 2008). The TP reaction can be mediated by either aPBPs (which possess both TG and TP activity) or by monofunctional D, D-transpeptidases (class B PBPs, designated here as bPBPs).

This basic, textbook version of PG synthesis provides a framework for a more detailed consideration of how this process may differ between organisms or be modified in response to stress. Moreover, some of the central steps in PG synthesis have retained an aura of mystery, with a lack of consensus about the identity of key enzymes and some confounding genetic observations. Recent excitement centers on three major advances. First, the proteins that translocate the lipid II precursor from the cytosolic to the external face of the membrane are now becoming clear. Second, a long predicted but elusive PBP-independent TG activity has been defined. Third, variations in the nature of the PG intra-strand crosslinking reactions, and in particular the presence and impact of 3-3 in place of 4-3 crosslinks, is an emerging area of focus.

MurJ and functionally redundant lipid II flippases

After its generation in the cytoplasm, the PG precursor lipid II must be translocated (flipped) across the cytoplasmic membrane to provide the substrate for cell wall synthesis enzymes. The identity of the lipid II flippase(s) has been the subject of a longstanding controversy. Using a reductionist bioinformatics approach, Ruiz first proposed the membrane-anchored protein MurJ as the lipid II flippase in *E. coli* and supported this notion by demonstrating that MurJ is essential and required for PG synthesis (both of which would be expected of a flippase) (Ruiz, 2008). This was later challenged by Mohammadi *et al.*, who used an *in vitro* assay to demonstrate flippase activity of purified FtsW protein, and thus speculated that SEDS (shape, elongation, division and sporulation) family proteins (including RodA, FtsW and SpoVE in *B. subtilis*), rather than MurJ, were flippases (Mohammadi *et al.*, 2011). Another key point of their argument was that while a flippase is expected to be universally essential, all MurJ homologues could be deleted in *B. subtilis* (Fay and Dworkin, 2009).

Two recent studies have shed some more light on this controversy. Using an *in vivo* biochemical assay, Sham *et al.* demonstrated that MurJ does have lipid II flippase activity (Sham *et al.*, 2014). Importantly, in the same

study, depleting FtsW in a Δ*rodA* background [essentiality of *rodA* was suppressed by overexpression of the *ftsQAZ* operon (Kruse *et al*., 2005)] did not affect precursor translocation, suggesting that RodA and FtsW are entirely dispensable for this process. Another recent study addressed the important question of why MurJ proteins were (collectively) non-essential in *B. subtilis*. Using a synthetic lethal screen (via transposon insertion sequencing), Meeske *et al*. searched for genes that become essential in the absence of all MurJ homologs, arguing that an alternative flippase must exist and should be synthetic lethal with MurJ (Meeske *et al*., 2015). The screen was answered by a locus that was renamed *amj* (alternate to MurJ); intriguingly, the predicted Amj protein bears no sequence or structural homology to MurJ. Using the *in vivo* biochemical assay mentioned above (Sham *et al*., 2014), it was demonstrated that both MurJ and Amj can mediate lipid II translocation across the inner membrane; in addition, Amj could functionally replace MurJ in *E. coli*. Interestingly, *amj* is induced in the absence of MurJ, and its expression depends on the cell wall stress responsive alternative sigma factor SigM (Eiamphungporn and Helmann, 2008; Meeske *et al*., 2015; Helmann, 2016). Thus, *B. subtilis* can respond to inhibition of one of its flippases, perhaps by currently unknown antibiotics, with the expression of an alternative, structurally unrelated enzyme. In summary, there are now strong data supporting the role of MurJ and Amj as lipid II flippases. The role of FtsW remains controversial; however, recent revelations about the similar SEDS family protein RodA provide us with some room to speculate on FtsW function (see next section).

Important open questions remain concerning the reverse side of the flippase reaction; after transglycosylation, the undecaprenyl pyrophosphate (UPP) portion of lipid II remains on the outer leaflet of the cytoplasmic membrane. UPP molecules in the cell membrane are limited and UPP must therefore be efficiently recycled. This is accomplished by known, membrane-associated enzymes (UPP phosphatases) that convert UPP to undecaprenyl phosphate (UP), which can be reintroduced into the lipid II cycle (El Ghachi *et al*., 2005; Zhao *et al*., 2016). Due to the size and charge of the lipid carrier, it is generally expected to be translocated back into the cytoplasm by an enzyme facilitator rather than via spontaneous flipping, but the identity of this putative facilitator remains unknown.

The SEDS protein RodA has TG activity

The transglycosylation (TG) reaction is a crucial step in periplasmic cell wall assembly. Until recently, two classes of enzymes were known or predicted to perform the TG reaction: monofunctional transglycosylases (MTGs) and the TG domains of aPBPs. While MTGs have a demonstrated role in cell wall synthesis in some coccoid Gram-positive bacteria like *Staphylococcus aureus* (Reed *et al*., 2011), they are not widely conserved (absent for example in *B. subtilis*) and their physiological role in rod-shaped bacteria is unclear as there are no strong phenotypes associated with deletion or overexpression mutants (Di Berardino *et al*., 1996; Denome *et al*., 1999). Hence, the aPBPs were generally considered as the principal TGases during cell wall biosynthesis.

This dogma was challenged over a decade ago, when David Popham's group found that *B. subtilis* was able to grow (albeit poorly) in the absence of all aPBPs (McPherson and Popham, 2003). This striking finding strongly suggested that an unidentified TGase could compensate for the loss of aPBPs by collaborating with the TP function of a bPBP. Other groups have reported similar observations in *Enterococcus* spp. (Arbeloa *et al*., 2004; Rice *et al*., 2009). Intriguingly, a study from more than 30 years ago had already provided a candidate for Popham's 'missing' transglycosylase. Ishino *et al*., while conducting studies on PG synthesis processes mediated by the bPBP2 (for clarity, we will add the a/b class prefix to specific PBPs throughout the text), found that crude membrane extracts of *E. coli* produced cell wall material when they were isolated from a strain in which bPBP2 as well as RodA were overproduced (the aPBPs were at the same time inactivated using antibiotics) (Ishino *et al*., 1986). Using bPBP2-specific antibiotics and thermosensitive variants of both bPBP2 and RodA, these authors dissected the contribution of each protein to the PG synthesis process and found that while bPBP2 was, as expected, required for the crosslinking part of assembly, RodA was required for chain elongation. They then discussed the possibility that RodA itself possessed transglycosylase activity, but dismissed this as 'unlikely' and rather concluded (in light of what was known about PBPs in 1986) that bPBP2 itself had TG activity that was somehow stimulated by RodA. These observations were thus not integrated into later models of cell wall synthesis. Later, the idea that RodA possessed TG activity was further obscured by the proposal (as noted above) that another SEDS protein, FtsW, functioned as a lipid II flippase based on an *in vitro* biochemical assay (Mohammadi *et al*., 2011), fueling the assumption that this was true for RodA as well. FtsW and RodA were thus tentatively assigned as flippases, as noted above.

Several recent papers from the Bernhardt, Ruiz, Rudner and Errington labs have provided new insights into the roles of SEDS proteins. First, the identification of MurJ (and Amj in *B. subtilis*, see previous section) as a lipid II flippase (Meeske *et al*., 2015), re-established the possibility that RodA and FtsW have activities other than

(or in addition to) precursor translocation. Then, using independent approaches [homology search (Meeske *et al*., 2016) or candidate gene elimination (Emami *et al*., 2017)], it was discovered that RodA has TGase activity *in vitro* (Meeske *et al*., 2016), and that overexpression of RodA rescued the strong growth defect of the *B. subtilis* strain lacking all aPBPs (Meeske *et al*., 2016; Emami *et al*., 2017). Possible natural molecule inhibitors of RodA were also identified (Emami *et al*., 2017).

Interestingly, these data provided an explanation for another curious feature of *B. subtilis*: its resistance to moenomycin. Moenomycin is a potent aPBP transglycosylase inhibitor (Welzel, 2007; Gampe *et al*., 2013; Rebets *et al*., 2014) whereas RodA TG activity was found to be unaffected by moenomycin *in vitro* (McPherson and Popham, 2003; Meeske *et al*., 2016). In *B. subtilis*, resistance to moenomycin depends on the SigM dependent cell envelope damage response, and SigM induces expression of *rodA* (Mascher *et al*., 2007; Eiamphungporn and Helmann, 2008; Meeske *et al*., 2016). Thus, similar to Amj (see above), or PBP2a in Methicillin Resistant *Staphylococcus aureus* (Hao *et al*., 2012), *B. subtilis* enhances the expression of one cell wall synthesis enzyme (RodA) upon inhibition of another (aPBPs) (Meeske *et al*., 2015; Helmann, 2016).

RodA was also shown to contribute significant TG activity to cell wall synthesis mediated by the 'elongasome' in *E. coli* (Cho *et al*., 2016). However, unlike its Gram-positive counterpart, this activity does not suffice to sustain growth in the absence of aPBPs. This might be a common feature in Gram-negative bacteria, as in these organisms depletion or inhibition of aPBPs typically leads to cessation of growth and/or lysis and death (Yousif *et al*., 1985; Satta *et al*., 1995; Dorr *et al*., 2014).

Whether FtsW possesses TG activity has not been completely resolved. Recent biochemical evidence suggests that in *E. coli*, FtsW forms a complex with bPBP3 and aPBP1B at the division site (Leclercq *et al*., 2017). FtsW was also shown to bind lipid II and to negatively regulate aPBP1b activity using *in vitro* assays, and this inhibition was alleviated by the presence of bPBP3 (Leclercq *et al*., 2017). Importantly, FtsW did not exhibit TGase activity under these experimental conditions.

L,D-transpeptidases and diversification of PG architecture

D-Ala$_4$-D-DAP$_3$ or D-Ala$_4$-D-Lys$_3$ (D,D) crosslinks (generally referred to as 4,3 crosslinks), whose formation is mediated by D,D transpeptidases (the PBPs), have been established as the major type of PG crosslink. However, many bacteria also harbor L,D transpeptidases (LDT) (Mainardi *et al*., 2000; Magnet *et al*., 2007; Lavollay *et al*., 2008; Magnet *et al*., 2008; Lam *et al*., 2009; Bramkamp, 2010; Cava *et al*., 2011; Hernandez *et al*., 2015). These enzymes also catalyze TP reactions between two amino acids, for example between two DAP molecules in neighboring PG strands (using the energy stored in the DAP$_3$-D-Ala$_4$ bond), which at least in principle could lead to fully crosslinked PG. Intriguingly, even mutants deleted in multiple or all L,D transpeptidases exhibit only minor phenotypes (Sanders and Pavelka, 2013) and the types of crosslinks these enzymes create (DAP-DAP or 3,3 crosslinks) are typically too rare to provide full structural integrity (Glauner *et al*., 1988; Desmarais *et al*., 2013). A notable exception is *Agrobacterium tumefaciens*, whose PG naturally consists of ~45% L,D crosslinks (Quintela *et al*., 1995). An increase in 3,3 crosslinks has been observed in multiple other species when cells enter stationary phase [where in *Mycobacterium tuberculosis*, up to 80% of PG can be crosslinked via DAP-DAP (Lavollay *et al*., 2008)], and under envelope stress conditions: activation of the Cpx response in *E. coli* for example led to a ~1.5-fold increase in DAP-DAP crosslinks (Lavollay *et al*., 2008; Bernal-Cabas *et al*., 2015). It is therefore possible that L,D transpeptidation serves a supporting role for D,D crosslinks to further strengthen the PG meshwork under certain conditions.

Early evidence that L,D transpeptidases could assume a more fundamental role in cell wall biogenesis came from the work of the Gutmann and Arthur labs. In a series of papers (Mainardi *et al*., 2000, 2002, 2005), these authors described the selection for a β-lactam resistant mutant of *Enterococcus faecalis*, whose cell wall was found to be essentially devoid of the classical D,D crosslinks mediated by PBPs. This mutant could grow in the presence of β-lactam antibiotics by substituting the D,D crosslinks formed by the β-lactam sensitive PBPs with those formed by a β-lactam-insensitive L,D transpeptidase named Ldt$_{fm}$, which catalyzes transpeptidation between D-asparagine and L-lysine residues situated in neighboring PG strands, resulting in L,D (3,3) bonds (Mainardi *et al*., 2005). Interestingly, the activity or abundance of the Ldt$_{fm}$ or PBPs was unaltered in the resistant mutant. Instead, this strain showed an increase in the activity of a carboxypeptidase that removes the terminal D-Ala$_5$; the resulting tetrapeptide sidestem is recognized by L,D transpeptidases, but not PBPs as a substrate. Thus, *E. faecalis* provides intrinsic substrate cues to reprogram the activity of PG crosslinking enzymes and thus the nature of its PG crosslinks.

Another study recently reported that a similar mechanism of β-lactam resistance can evolve in *E. coli* (Hugonnet *et al*., 2016). After multistep selection on β-lactam antibiotics, a mutant emerged that had upregulated one of its L,D transpeptidases (YcbB) as well as the stringent response, a starvation response that leads to the

accumulation of the alarmone ppGpp and subsequent reprogramming of transcription. While the connection between the stringent response and L,D-TP activity is unclear, this strain utilizes the TG activity of the aPBP1B in conjunction with TP activity of YcbB for cell wall synthesis and crosslinking. Like in *E. faecium*, the ability to form L,D crosslinks depended on the presence of a carboxypeptidase, in this case PBP5. Although these experiments involved mutants that were generated under severe and artificial selection conditions, these results clearly demonstrate that L,D-TPase activity can, at least in principle, contribute significantly to the structural integrity of the cell wall. It remains to be seen whether the primary reliance on L,D transpeptidation for bacterial growth is an oddity resulting from stringent conditions of mutant selection or can be an adaptive response (for example as a stress response mechanism in the presence of β-lactam antibiotics) in nature as well.

Cell biology of PG synthesis: new insights into the roles of the cytoskeletal proteins MreB and FtsZ

The basic enzymology of PG synthesis was established in early studies following conventional approaches that integrated *in vitro* enzyme assays with chemical and structural characterization of reaction mechanisms and products. However, efforts to decipher the larger scale coordination of PG synthesis with cell growth and division did not make great strides until the advent of bacterial cell biology. The introduction of fluorescently labeled proteins, high resolution light microscopy methods and, more recently, single-molecule tracking approaches has invigorated the field and enabled the development of new models of PG synthesis and its coordination. It is not enough to just be able to stitch together new PG; the newly synthesized glycan strands must be integrated into the existing sacculus in a manner that does not compromise the overall integrity and load-bearing properties of the wall, and old wall material must be simultaneously shed and recycled. How new areas of synthesis are defined in a manner appropriate for the maintenance of cell shape, as seen for example in rods, cocci and helically shaped bacteria, has been a challenging problem. Here, we focus on the emerging view of the two primary biosynthetic, macromolecular complexes involved in synthesis of rod-shaped bacteria: the 'elongasome' and the 'divisome'.

MreB and the 'elongasome'

The transmembrane and periplasmic proteins associated with cell wall synthesis processes have been at least implicitly assumed to be part of a single multiprotein complex called the 'elongasome' (Errington, 2015; Laddomada *et al*., 2016; Egan *et al*., 2017) that contains structural components, as well as aPBPs, bPBPs, cell wall lytic enzymes (autolysins) and presumably a flippase. The 'elongasome' was assumed to be spatiotemporally directed by the cytoskeletal protein MreB, a homologue of eukaryotic actin (van den Ent *et al*., 2001) that localizes to the lateral wall of the bacterial cell (Jones *et al*., 2001). One model suggests that MreB mediates the formation of regions with increased fluidity (RIFs), which affect distribution and diffusion of membrane proteins and may contribute to the organization of the 'elongasome' (Strahl *et al*., 2014). Until recently, a generally accepted model of cell wall synthesis proposed that MreB serves to guide the aPBPs, which in turn produce peptidoglycan strands via their TG domains while the aPBPs and the bPBPs crosslink these strands into a tight PG mesh, fitting new material into cell wall gaps provided by the cleavage activity of autolysins (Fig. 1A). However, the existence of an 'elongasome' protein complex could never be demonstrated *in vivo* and recent single molecule tracking experiments revealed that MreB and aPBPs operate in distinct complexes (Cho *et al*., 2016). This, in addition to the recent revelation that RodA itself possesses TG activity, calls for a re-evaluation of MreB's contribution to cell wall synthesis (Meeske *et al*., 2016; Emami *et al*., 2017).

MreB is found in most rod-shaped bacteria and loss of MreB generally leads to the cessation of lateral cell wall synthesis and concomitant loss of rod-shape, establishing the cytoskeleton's crucial role in directional PG insertion during cell elongation. MreB strongly interacts with RodA and RodZ in *E. coli* (Morgenstein *et al*., 2015) and the latter mediates the indirect interaction between MreB and a bPBP. This established complex (MreB-RodAZ-bPBP) will be referred to hereafter as the 'Rod complex'.

Early localization studies using immunofluorescence and epifluorescence microscopy to visualize fluorescently tagged proteins in *B. subtilis, E. coli* and *Caulobacter crescentus* suggested that MreB localizes in helical filaments along the inner face of the cytoplasmic membrane spanning the lateral cell (Jones *et al*., 2001; Shih *et al*., 2003; Figge *et al*., 2004). However, using microscopy techniques that allowed for higher spatiotemporal resolution, it was later shown that instead of forming continuous filaments, MreB rotates around the cell in patches (arcs) whose motion depends on bPBP transpeptidation activity and the presence of RodA, but, at least in *E. coli*, not on the activity of aPBPs (Dominguez-Escobar *et al*., 2011; Garner *et al*., 2011; van Teeffelen *et al*., 2011). More recent data have

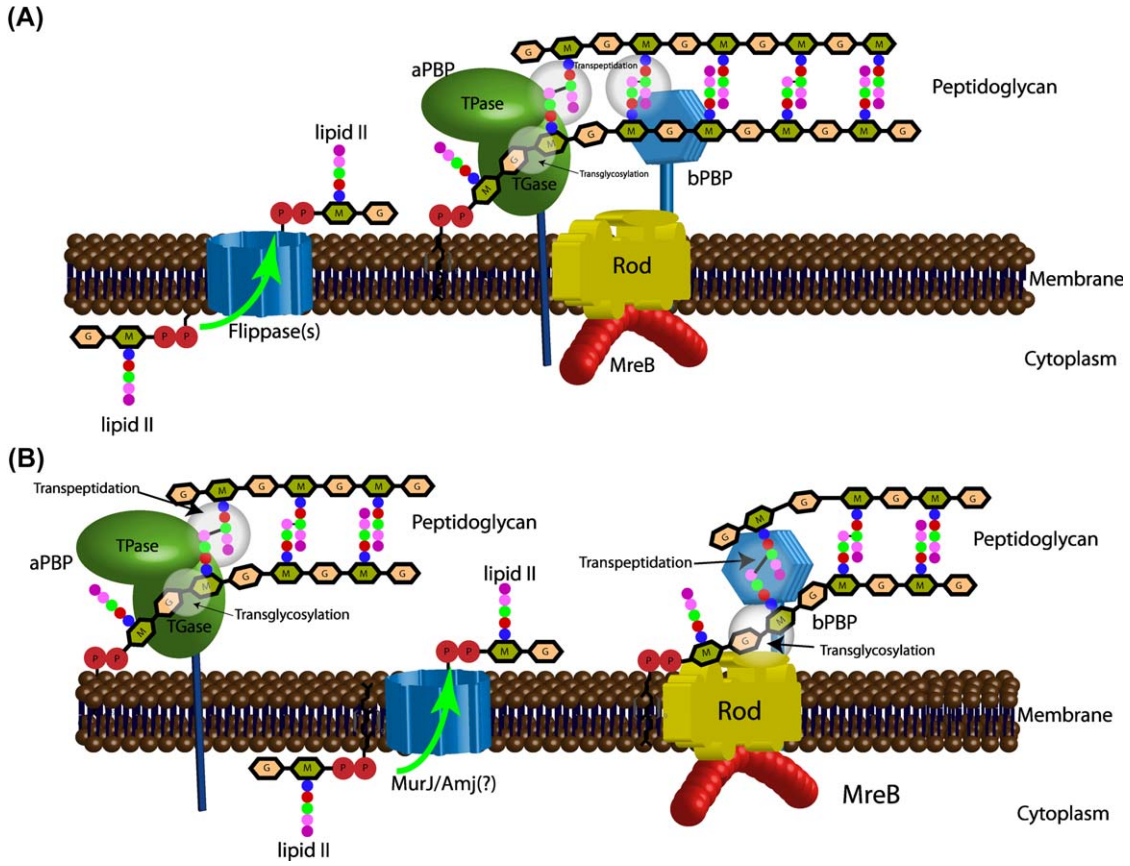

Fig. 1. Unified (A) and Interdependent (B) models of peptidoglycan (PG) synthesis complexes.
A. In the unified model, RodAZ, MreB, aPBPs and bPBPs form one protein complex: guided by MreB, the aPBPs produce peptidoglycan strands via their TG domains while both aPBPs and bPBPs crosslink these strands into a tight PG mesh.
B. In the interdependent model, RodAZ, bPBP and MreB form one complex, while aPBPs work in a different spatial and temporal frame. Glycan strands are produced by the transglycosylase RodA and are crosslinked by bPBP to existing PG. PG synthesis provides the force for pushing circumferential MreB movement. aPBPs exhibit a different movement pattern distinct from MreB, including two modes of movement: fast diffusion and slow movement (pause). These two systems are spatially distinct, but functionally interdependent for PG synthesis.

shown that MreB locates to regions of negative curvature, and 'corrects' this negative curvature by filling this region with newly synthesized PG, suggesting a self-correcting feedback mechanism for cells to maintain rod shape (Ursell *et al.*, 2014). Overall, these data strongly support a model in which cell wall synthesis during cell elongation is mediated primarily by the Rod complex.

Importantly, these single molecule studies have provided evidence for a spatial independence of the Rod complex and aPBPs (Fig. 1B). The Rod complex and bPBPs appear to move along the same trajectories, suggesting that they may be coupled (Cho *et al.*, 2016) (although it has to be noted that another study had previously found that MreB and bPBP2 move at different velocities (Lee *et al.*, 2014); this can be attributed to differences in imaging parameters and/or intrinsic differences between different fusion constructs, and may suggest that bPBP2's circumferential motion is not essential for its function). In contrast, aPBPs showed a bimodal pattern of movement, with two distinct subpopulations: one exhibiting fast, diffusive motion and another moving at a speed an order of magnitude slower (Cho *et al.*, 2016; Lee *et al.*, 2016). When considering the behavior of a single PBP molecule, these data can be interpreted as short periods of fast diffusion interspersed with temporary pauses. Although spatially independent, the partially redundant TG activities of the Rod complex and aPBPs are functionally coupled, as inactivation of one or the other leads to the same dramatic (~80%) decrease in incorporation of new cell wall material (Cho *et al.*, 2016). This, together with the observation that in *E. coli* a bPBP interacts directly with aPBP1A (Banzhaf *et al.*, 2012), suggests that the two seemingly independent activities of aPBPs and the Rod complex are somehow synergistic, and that they may at least transiently interface. A recent paper has exposed an additional layer of complexity about the relationship between the Rod complex and aPBPs. Using TIRF, Billaudeau *et al.*

showed that in *B. subtilis*, MreB not only shows rotational movement but has also subpopulations that, similar to aPBPs, diffuse slowly or stop altogether (Billaudeau *et al*., 2017). This, coupled with indirect evidence of an interaction between MreB and aPBPs (Kawai *et al*., 2009) opens up the possibility that MreB and aPBPs associate. It is important to note that neither MreB patch rotational movement, nor aPBP activity, depend on each other (van Teeffelen *et al*., 2011; Cho *et al*., 2016); but whether diffusive MreB molecules functionally interact with aPBPs is not known. Further work is thus required to investigate the spatial and functional relationship between MreB (or at least a sub-population of it) and aPBPs.

How does Rod-mediated cell wall synthesis apparently drive its own motion, while aPBPs, in principle mediating the exact same reactions, are more diffusive? One possibility is that RodA's TG activity drives directional movement, and that its interaction with short, dynamic MreB arcs essentially reinforces this movement, while the aPBPs move along a similar trajectory for a short time, but produce shorter PG chains and diffuse away when the TG reaction is terminated (e.g., through interaction with a putative chain termination factor, or due to an intrinsic capability to produce shorter PG chains). It is noteworthy that MreB was shown to interact with cytoplasmic cell wall precursor synthesis proteins whose localization changed during MreB depletion (Divakaruni *et al*., 2007; White *et al*., 2010; Favini-Stabile *et al*., 2013; Rueff *et al*., 2014), suggesting that MreB might coordinate the availability of precursors to generate a local pool of lipid II to support the activity of the Rod complex. This might be beneficial if RodA's TG activity intrinsically generates longer PG strands than the aPBPs (generating shorter chains may enable the aPBPs to operate on a more limited local supply of lipid II).

In the light of the existence of two independent cell wall synthesis complexes, an important open question is whether flippase activity is associated with one of the complexes, both, or is completely independent. Current data favor the latter hypothesis: heterologous expression of the *Helicobacter pylori* O-antigen flippase Wzk (Elhenawy *et al*., 2016) or *B. subtilis* Amj (Meeske *et al*., 2015) can functionally replace MurJ in *E. coli*. Amj has no homologs in *E. coli* and is thus unlikely to specifically interact with this organism's cell wall synthesis machinery, suggesting that unguided lipid II flipping may be sufficient to sustain bacterial growth. Furthermore, cell wall incorporation after inhibition of either the Rod complex or aPBPs is not zero, implying residual flippase activity. Thus, it is likely that lipid II flippase activity is not strictly dependent on either complex.

FtsZ and the divisome: FtsZ treadmilling drives PG synthesis at the septum

FtsZ, a homolog of tubulin, is essential for cell division in many bacteria. Cytoplasmic FtsZ molecules polymerize at the inner face of the cytoplasmic membrane as a dynamic ring of FtsZ filaments of varying lengths (Michie and Lowe, 2006). This so-called 'Z-ring' and various accessory factors anchor the assembly of a dynamic, spatio-temporally ordered multiprotein complex called the divisome. The divisome contains what are generally assumed to be structural proteins, but also proteins involved in cell wall synthesis (PBPs) and turnover, like amidases and lytic transglycosylases (Egan and Vollmer, 2013). Ultimately, the FtsZ-guided divisome serves the function of facilitating cytokinesis, membrane constriction, synthesis of new cell wall material and finally daughter cell separation. Purified FtsZ is sufficient to initiate constriction of elongated liposomes (Osawa *et al*., 2008, 2009), suggesting that FtsZ itself generates the forces for cell division, powered by GTP hydrolysis (de Boer *et al*., 1992; RayChaudhuri and Park, 1992). This was later challenged by results from experiments showing that constriction does not initiate in the absence of cell wall synthesis (Daley *et al*., 2016). Beyond these observations, the role of FtsZ outside of its anchor function remained largely mysterious.

Several recent studies have addressed this issue with newly available super-resolution techniques. In two parallel studies, Bisson-Filho *et al*. and Yang *et al*. used single molecule tracking and super-resolution microscopy combined with targeted perturbations of division processes to assess the role of FtsZ filaments in the division process in *B. subtilis* and *E. coli* (Bisson-Filho *et al*., 2017; Yang *et al*., 2017). Both groups found that short FtsZ filaments display a rotational, inward movement that coincides with the deposition of new cell wall material. Strikingly, and in contrast to MreB, FtsZ movement was independent of cell wall synthesis and driven by treadmilling, which depended on its GTPase activity. It thus appears that FtsZ generates its own motion, and induces cell wall synthesis during the constriction process. This observation could provide an explanation for previously inconsistent data: while FtsZ treadmilling by itself probably generates enough force to initiate membrane constriction, it is the reinforcement of these constrictions via guided traces of PG material that enables the completion of outer membrane constriction and cytokinesis. This more active, cytoskeleton-driven process of movement (as opposed to MreB's passive motion) might thus be necessary to apply the forces needed for cell division.

An emerging model of PG synthesis for rod-shaped bacteria

One of the most influential unified models of cell wall growth coordination was put forth in a seminal review paper by Höltje (Holtje, 1998). Asking how PG lytic and synthetic processes might be coordinated without compromising cell wall structural integrity, the author proposed that bacteria synthesize a precursor of three parallel, crosslinked PG strands, which would substitute for a single strand concomitantly removed by the coordinated activity of PG hydrolases; he termed this the '3-for-1' model. Höltje assumed that the parallel strands were generated before breaking any bonds in the PG meshwork (to ensure structural integrity), or a 'make before break' mode of sacculus expansion. This model has remained an important conceptual framework, but has not been re-evaluated in the light of subsequent new observations.

We will attempt here to integrate new data on the mechanisms of PG biogenesis with previous observations into an updated model of cell wall biogenesis (Fig. 2). Perhaps one of the most striking recent realizations is that the Rod complex and aPBPs are spatially distinct, yet their activities are interdependent. A possible model is that one cell wall synthesis complex creates a template structure for the other, consistent with what has been suggested by Wientjes *et al.* (Wientjes and Nanninga, 1991) and Cho *et al.* (2016). We speculate that template generation is accomplished by the circumferentially moving population of the Rod complex, as steady, circumferential motion would be conducive to providing a regular template structure. In contrast, the aPBPs exhibit a diffusive motion interspersed with prolonged local persistence (Cho *et al.*, 2016; Lee *et al.*, 2016), which could suggest that their role is to diffuse freely until they recognize a PG trace or gap (e.g., one generated by the Rod complex) and then add new material to the template. Inhibition of a bPBP (PBP2) in *E. coli* results in the generation of PG fragments that are not incorporated into the PG meshwork but rather are rapidly degraded and recycled (Uehara and Park, 2008; Cho *et al.*, 2014), suggesting that in Rod-mediated PG synthesis, PBP2 provides the first point of attachment for nascent PG after (or while) it emerges from RodA-mediated transglycosylation. This first point of attachment would also be an important anchor providing a fulcrum for RodA-driven MreB movement; perhaps this is why inhibition of bPBPs stops MreB motion (Dominguez-Escobar *et al.*, 2011; Garner *et al.*, 2011; van Teeffelen *et al.*, 2011) but allows futile synthesis of PG by RodA (Cho *et al.*, 2014, 2016).

Interestingly, at least in *E. coli*, a bPBP (PBP2) was shown to activate an aPBP (PBP1A) (Banzhaf *et al.*, 2012). The fact that the Rod-associated bPBP stimulates the aPBP's TG activity may suggest that rod-driven PG synthesis starts prior to aPBP-driven PG synthesis. We propose that the aPBPs likely use the Rod-mediated PG template to attach parallel (or possibly antiparallel) PG strands (Fig. 2). In addition to providing lateral directionality of sacculus expansion, this is consistent with the observed existence of crosslinked PG strands containing pentapeptide: enzymatically, the only way to generate PG containing pentapeptides is when nascent PG is crosslinked with another strand of nascent PG (since the terminal D-Ala of the donor strand is lost in the crosslinking reaction). Moreover, pentapeptides are likely rapidly processed by carboxypeptidases associated with cell wall synthesis (Atrih *et al.*, 1999; Santos *et al.*, 2002; Potluri *et al.*, 2010; Moll *et al.*, 2015), but nascent-nascent crosslinks are indeed observed during pulse-chase PG labeling experiments (Burman and Park, 1984). Alternatively, these crosslinked PG strands containing pentapeptide may come from region with presumably less carboxypeptidase activity, for example the septum (Morales Angeles *et al.*, 2017), or from PG generated by several Rod complexes working in parallel, as recently suggested for *B. subtilis* (Billaudeau *et al.*, 2017). It is therefore possible that the Rod template actually consists of a PG 'raft' structure of several strands that are then woven tightly into the cell wall by aPBPs. Why are the aPBPs partially dispensable in *B. subtilis* (dependent on stress-response mediated upregulation of RodA) and not in *E. coli*? Perhaps the Rod complex template itself is enough to mediate sacculus expansion, as long as it is made in sufficient quantity (*B. subtilis* upregulates RodA upon aPBP deletion via the Sigma M cell wall stress sensing pathway) and as long as there is a sufficient stress-bearing 'buffer', that is, a thick cell wall that can partially compensate for localized, inefficient crosslinking.

Our model suggests that the Rod complex might coordinate its cell wall synthesis activity with PG cleavage by endopeptidases, which are required for the insertion of new PG material during cell elongation (Singh *et al.*, 2012; Vollmer, 2012; Dorr *et al.*, 2013) and should therefore immediately precede template attachment. Consistent with this hypothesis, MreB homologs in *B. subtilis* have been shown to direct the activities of elongation-specific endopeptidases LytE and (indirectly) CwlO (Dominguez-Cuevas *et al.*, 2013; Meisner *et al.*, 2013). Further, at least in Gram-negative bacteria, inhibition or depletion of aPBPs (leaving the putative RodA-autolysin complex active) typically leads to a catastrophic loss of the cell wall (Yousif *et al.*, 1985; Satta *et al.*, 1995; Dorr *et al.*, 2014), while defects associated with the Rod complex (which might cause concurrent lack of major autolysin activation) are generally milder and simply

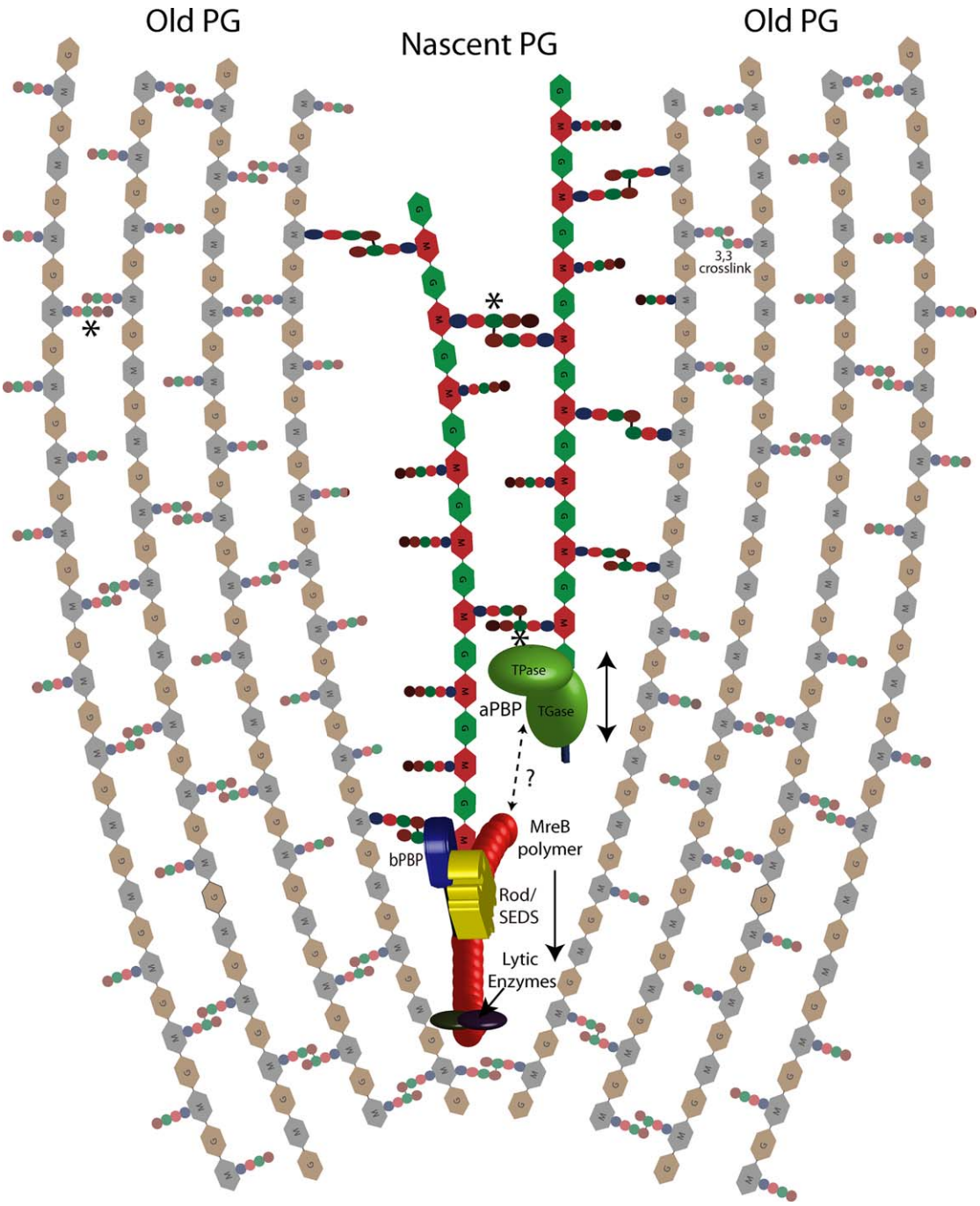

Fig. 2. 'Break before Make' model of peptidoglycan (PG) synthesis complexes.
Rod/SEDS/MreB-associated endopeptidases locally cleave crosslinks in mature PG. RodA generates a PG template, which is attached to the sacculus via bPBPs (only one strand is shown here, note that in principle this could also be a raft structure of multiple parallel strands). The aPBPs then generate additional strands, which are crosslinked with nascent PG on one side and mature PG on the other, ensuring maintenance of structural integrity. Whether the Rod/SEDS/MreB complex interacts with aPBPs remains an open question. Crosslinked pentapeptide (asterisk) is formed when a nascent PG strand containing pentapeptide is crosslinked with another one. PBP-independent 3,3 crosslinks also exist, albeit at low abundance under normal growth conditions.

result in an abrogation of cell elongation with loss of cellular integrity only after prolonged exposure (Tybring and Melchior, 1975; Iwai *et al*., 2002). Measurements of the incorporation of new cell wall material in the presence of antibiotics previously showed that inhibition of aPBPs resulted in a delayed inhibition of cell wall incorporation, and cessation of cell wall synthesis coincided with the onset of lysis (Wientjes and Nanninga, 1991). These

observations are also consistent with Rod-associated cell wall cleavage and template generation, which would proceed even in the absence of aPBPs until a 'tipping point' is reached where accumulated damage caused by the lack of subsequent aPBP-mediated crosslinking results in catastrophic failure of structural integrity. Recent results demonstrate that *E. coli* endopeptidase activity increases aPBP-mediated cell wall attachment during inhibition of bPBP2 (Lai *et al*., 2017), suggesting that endopeptidases might, in addition to priming the Rod system, provide Rod-independent starting points (gaps) for PG synthesis by aPBPs.

It remains to be seen what the roles of lytic transglycosylases (LTGs) are in the cell wall biosynthesis process. The typical PG breakdown products of these enzymes are detected during growth and at increased levels after exposure to cell wall synthesis inhibitors; however, it is currently unclear whether removal of a strand (or strands) of mature PG is actually necessary for the insertion of new material. Alternatively, LTG breakdown products could be the result of the removal of the outer cell layer in Gram-positive bacteria, a proofreading capacity (removing erroneously crosslinked and thus potentially unstable cell wall material), as has been suggested previously (Cho *et al*., 2014), or simply the fact that nascent PG is produced in longer chains at first and then trimmed down to the length most appropriate for the current growth condition (Vollmer and Holtje, 2004; Yunck *et al*., 2016).

In summary, we propose a 'break before make' model. Endopeptidases locally cleave crosslinks in mature PG. RodA generates a PG template, which is attached to the sacculus via bPBPs. Since the Rod complex is not expected to perform an entire rotation around the cell (based on short PG chain lengths measured in various bacteria), these PG degradation events are initially localized and overall structural integrity is thus not immediately compromised. The aPBPs then generate additional strands, which are crosslinked with nascent PG on one side and mature PG on the other, ensuring that the local degradation events initiated by the Rod complex do not accumulate with harmful consequences. Previous simulations and observations regarding bPBP2 inactivation have indeed shown that the cell wall synthesis machinery, even in Gram-negative bacteria, is surprisingly well-buffered and can sustain a fairly high amount of degradation before experiencing catastrophic failure (Huang *et al*., 2008; Lee *et al*., 2014).

Important questions remain unanswered in the context of this model; most importantly, how the Rod complex defines start sites for PG synthesis, how aPBPs recognize the putative Rod template and what role the modulators of PBP activity play [such as the outer membrane localized activators in Gram-negative bacteria, (Paradis-Bleau *et al*., 2010; Typas *et al*., 2010)]. After all, more than 50 years after its emergence, bacterial cell wall research still holds surprises, and is expected to continue doing so.

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References

Anderson, J.S., Meadow, P.M., Haskin, M.A., and Strominger, J.L. (1966) Biosynthesis of the peptidoglycan of bacterial cell walls. I. Utilization of uridine diphosphate acetylmuramyl pentapeptide and uridine diphosphate acetylglucosamine for peptidoglycan synthesis by particulate enzymes from *Staphylococcus aureus* and *Micrococcus lysodeikticus*. *Arch Biochem Biophys* **116**: 487–515.

Anderson, J.S., Matsuhashi, M., Haskin, M.A., and Strominger, J.L. (1967) Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers in the reaction sequence. *J Biol Chem* **242**: 3180–3190.

Arbeloa, A., Segal, H., Hugonnet, J.E., Josseaume, N., Dubost, L., Brouard, J.P., et al. (2004) Role of class A penicillin-binding proteins in PBP5-mediated beta-lactam resistance in *Enterococcus faecalis*. *J Bacteriol* **186**: 1221–1228.

Atrih, A., Bacher, G., Allmaier, G., Williamson, M.P., and Foster, S.J. (1999) Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. *J Bacteriol* **181**: 3956–3966.

Banzhaf, M., van den Berg van Saparoea, B., Terrak, M., Fraipont, C., Egan, A., Philippe, J., et al. (2012) Cooperativity of peptidoglycan synthases active in bacterial cell elongation. *Mol Microbiol* **85**: 179–194.

Bernal-Cabas, M., Ayala, J.A., and Raivio, T.L. (2015) The Cpx envelope stress response modifies peptidoglycan cross-linking via the L,D-transpeptidase LdtD and the novel protein YgaU. *J Bacteriol* **197**: 603–614.

Billaudeau, C., Chastanet, A., Yao, Z., Cornilleau, C., Mirouze, N., Fromion, V., *et al*. (2017) Contrasting mechanisms of growth in two model rod-shaped bacteria. *Nat Commun* **8**: 15370.

Bisson-Filho, A.W., Hsu, Y.P., Squyres, G.R., Kuru, E., Wu, F., Jukes, C., *et al*. (2017) Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *Science* **355**: 739–743.

Bramkamp, M. (2010) The putative *Bacillus subtilis* L,D-transpeptidase YciB is a lipoprotein that localizes to the cell poles in a divisome-dependent manner. *Arch Microbiol* **192**: 57–68.

Brown, S., Santa Maria, J.P., Jr., and Walker, S. (2013) Wall teichoic acids of Gram-positive bacteria. *Annu Rev Microbiol* **67**: 313–336.

de Boer, P., Crossley, R., and Rothfield, L. (1992) The essential bacterial cell-division protein FtsZ is a GTPase. *Nature* **359**: 254–256.

Burman, L.G., and Park, J.T. (1984) Molecular model for elongation of the murein sacculus of *Escherichia coli*. *Proc Natl Acad Sci USA* **81**: 1844–1848.

Cava, F., de Pedro, M.A., Lam, H., Davis, B.M., and Waldor, M.K. (2011) Distinct pathways for modification of the bacterial cell wall by non-canonical D-amino acids. *EMBO J* **30**: 3442–3453.

Cho, H., Uehara, T., and Bernhardt, T.G. (2014) Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell* **159**: 1300–1311.

Cho, H., Wivagg, C.N., Kapoor, M., Barry, Z., Rohs, P.D., Suh, H., *et al*. (2016) Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. *Nat Microbiol* **1**: 16172.

Daley, D.O., Skoglund, U., and Soderstrom, B. (2016) FtsZ does not initiate membrane constriction at the onset of division. *Sci Rep* **6**: 33138.

Denome, S.A., Elf, P.K., Henderson, T.A., Nelson, D.E., and Young, K.D. (1999) *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. *J Bacteriol* **181**: 3981–3993.

Desmarais, S.M., De Pedro, M.A., Cava, F., and Huang, K.C. (2013) Peptidoglycan at its peaks: how chromatographic analyses can reveal bacterial cell wall structure and assembly. *Mol Microbiol* **89**: 1–13.

Di Berardino, M., Dijkstra, A., Stuber, D., Keck, W., and Gubler, M. (1996) The monofunctional glycosyltransferase of *Escherichia coli* is a member of a new class of peptidoglycan-synthesising enzymes. *FEBS Lett* **392**: 184–188.

Divakaruni, A.V., Baida, C., White, C.L., and Gober, J.W. (2007) The cell shape proteins MreB and MreC control cell morphogenesis by positioning cell wall synthetic complexes. *Mol Microbiol* **66**: 174–188.

Dominguez-Escobar, J., Chastanet, A., Crevenna, A.H., Fromion, V., Wedlich-Soldner, R., and Carballido-Lopez, R. (2011) Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria. *Science* **333**: 225–228.

Dominguez-Cuevas, P., Porcelli, I., Daniel, R.A., and Errington, J. (2013) Differentiated roles for MreB-actin isologues and autolytic enzymes in *Bacillus subtilis* morphogenesis. *Mol Microbiol* **89**: 1084–1098.

Dorr, T., Cava, F., Lam, H., Davis, B.M., and Waldor, M.K. (2013) Substrate specificity of an elongation-specific peptidoglycan endopeptidase and its implications for cell wall architecture and growth of *Vibrio cholerae*. *Mol Microbiol* **89**: 949–962.

Dorr, T., Lam, H., Alvarez, L., Cava, F., Davis, B.M., and Waldor, M.K. (2014) A novel peptidoglycan binding protein crucial for PBP1A-mediated cell wall biogenesis in *Vibrio cholerae*. *PLoS Genet* **10**: e1004433.

Egan, A.J., and Vollmer, W. (2013) The physiology of bacterial cell division. *Ann NY Acad Sci* **1277**: 8–28.

Egan, A.J., Cleverley, R.M., Peters, K., Lewis, R.J., and Vollmer, W. (2017) Regulation of bacterial cell wall growth. *FEBS J* **284**: 851–867.

Eiamphungporn, W., and Helmann, J.D. (2008) The *Bacillus subtilis* sigma(M) regulon and its contribution to cell envelope stress responses. *Mol Microbiol* **67**: 830–848.

El Ghachi, M., Derbise, A., Bouhss, A., and Mengin-Lecreulx, D. (2005) Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in *Escherichia coli*. *J Biol Chem* **280**: 18689–18695.

Elhenawy, W., Davis, R.M., Fero, J., Salama, N.R., Felman, M.F., and Ruiz, N. (2016) The O-antigen flippase Wzk can substitute for MurJ in peptidoglycan synthesis in *Helicobacter pylori* and *Escherichia coli*. *PLoS One* **11**: e0161587.

Emami, K., Guyet, A., Kawai, Y., Devi, J., Wu, L.J., Allenby, N., *et al*. (2017) RodA as the missing glycosyltransferase in *Bacillus subtilis* and antibiotic discovery for the peptidoglycan polymerase pathway. *Nat Microbiol* **2**: 16253.

van den Ent, F., Amos, L.A., and Lowe, J. (2001) Prokaryotic origin of the actin cytoskeleton. *Nature* **413**: 39–44.

Errington, J. (2015) Bacterial morphogenesis and the enigmatic MreB helix. *Nat Rev Microbiol* **13**: 241–248.

Espaillat, A., Forsmo, O., El Biari, K., Bjork, R., Lemaitre, B., Trygg, J., *et al*. (2016) Chemometric analysis of bacterial peptidoglycan reveals atypical modifications that empower the cell wall against predatory enzymes and fly innate immunity. *J Am Chem Soc* **138**: 9193–9204.

Favini-Stabile, S., Contreras-Martel, C., Thielens, N., and Dessen, A. (2013) MreB and MurG as scaffolds for the cytoplasmic steps of peptidoglycan biosynthesis. *Environ Microbiol* **15**: 3218–3228.

Fay, A., and Dworkin, J. (2009) *Bacillus subtilis* homologs of MviN (MurJ), the putative *Escherichia coli* lipid II flippase, are not essential for growth. *J Bacteriol* **191**: 6020–6028.

Figge, R.M., Divakaruni, A.V., and Gober, J.W. (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. *Mol Microbiol* **51**: 1321–1332.

Gampe, C.M., Tsukamoto, H., Doud, E.H., Walker, S., and Kahne, D. (2013) Tuning the moenomycin pharmacophore to enable discovery of bacterial cell wall synthesis inhibitors. *J Am Chem Soc* **135**: 3776–3779.

Garner, E.C., Bernard, R., Wang, W., Zhuang, X., Rudner, D.Z., and Mitchison, T. (2011) Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in B. *subtilis*. *Science* **333**: 222–225.

Glauner, B., Holtje, J.V., and Schwarz, U. (1988) The composition of the murein of *Escherichia coli*. *J Biol Chem* **263**: 10088–10095.

Goffin, C., and Ghuysen, J.M. (1998) Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. *Microbiol Mol Biol Rev* **62**: 1079–1093.

Guest, R.L., and Raivio, T.L. (2016) Role of the Gram-negative envelope stress response in the presence of antimicrobial agents. *Trends Microbiol* **24**: 377–390.

Hao, H., Dai, M., Wang, Y., Huang, L., and Yuan, Z. (2012) Key genetic elements and regulation systems in methicillin-resistant *Staphylococcus aureus*. *Future Microbiol* **7**: 1315–1329.

Helmann, J.D. (2016) *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Curr Opin Microbiol* **30**: 122–132.

Hernandez, S.B., Cava, F., Pucciarelli, M.G., Garcia-Del Portillo, F., de Pedro, M.A., and Casadesus, J. (2015)

Bile-induced peptidoglycan remodelling in *Salmonella enterica*. *Environ Microbiol* **17**: 1081–1089.
Holtje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**: 181–203.
Huang, K.C., Mukhopadhyay, R., Wen, B., Gitai, Z., and Wingreen, N.S. (2008) Cell shape and cell-wall organization in Gram-negative bacteria. *Proc Natl Acad Sci USA* **105**: 19282–19287.
Hugonnet, J.E., Mengin-Lecreulx, D., Monton, A., den Blaauwen, T., Carbonnelle, E., Veckerle, C., *et al*. (2016) Factors essential for L,D-transpeptidase-mediated peptidoglycan cross-linking and beta-lactam resistance in *Escherichia coli*. *Elife* **5**: e19469.
Ishino, F., W., Park, S., Tomioka, S., Tamaki, I., Takase, K., Kunugita, H., *et al*. (1986) Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and *rodA* protein. *J Biol Chem* **261**: 7024–7031.
Iwai, N., Nagai, K., and Wachi, M. (2002) Novel S-benzylisothiourea compound that induces spherical cells in *Escherichia coli* probably by acting on a rod-shape-determining protein(s) other than penicillin-binding protein 2. *Biosci Biotechnol Biochem* **66**: 2658–2662.
Jones, L.J., Carballido-Lopez, R., and Errington, J. (2001) Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. *Cell* **104**: 913–922.
Kawai, Y., Daniel, R.A., and Errington, J. (2009) Regulation of cell wall morphogenesis in *Bacillus subtilis* by recruitment of PBP1 to the MreB helix. *Mol Microbiol* **71**: 1131–1144.
Kruse, T., Bork-Jensen, J., and Gerdes, K. (2005) The morphogenetic MreBCD proteins of *Escherichia coli* form an essential membrane-bound complex. *Mol Microbiol* **55**: 78–89.
Laddomada, F., Miyachiro, M.M., and Dessen, A. (2016) Structural insights into protein-protein interactions involved in bacterial cell wall biogenesis. *Antibiotics (Basel)* **5**: 14.
Lai, G.C., Cho, H., and Bernhardt, T.G. (2017) The mecillinam resistome reveals a role for peptidoglycan endopeptidases in stimulating cell wall synthesis in *Escherichia coli*. *PLoS Genet* **13**: e1006934.
Lam, H., Oh, D.C., Cava, F., Takacs, C.N., Clardy, J., de Pedro, M.A., and Waldor, M.K. (2009) D-amino acids govern stationary phase cell wall remodeling in bacteria. *Science* **325**: 1552–1555.
Lavollay, M., Arthur, M., Fourgeaud, M., Dubost, L., Marie, A., Veziris, N., *et al*. (2008) The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J Bacteriol* **190**: 4360–4366.
Leclercq, S., Derouaux, A., Olatunji, S., Fraipont, C., Egan, A.J., Vollmer, W., *et al*. (2017) Interplay between Penicillin-binding proteins and SEDS proteins promotes bacterial cell wall synthesis. *Sci Rep* **7**: 43306.
Lee, T.K., Tropini, C., Hsin, J., Desmarais, S.M., Ursell, T.S., Gong, E., *et al*. (2014) A dynamically assembled cell wall synthesis machinery buffers cell growth. *Proc Natl Acad Sci USA* **111**: 4554–4559.
Lee, T.K., Meng, K., Shi, H., and Huang, K.C. (2016) Single-molecule imaging reveals modulation of cell wall synthesis dynamics in live bacterial cells. *Nat Commun* **7**: 13170.
Lovering, A.L., Safadi, S.S., and Strynadka, N.C. (2012) Structural perspective of peptidoglycan biosynthesis and assembly. *Annu Rev Biochem* **81**: 451–478.
Magnet, S., Arbeloa, A., Mainardi, J.L., Hugonnet, J.E., Fourgeaud, M., Dubost, L., *et al*. (2007) Specificity of L,D-transpeptidases from Gram-positive bacteria producing different peptidoglycan chemotypes. *J Biol Chem* **282**: 13151–13159.
Magnet, S., Dubost, L., Marie, A., Arthur, M., and Gutmann, L. (2008) Identification of the L,D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. *J Bacteriol* **190**: 4782–4785.
Mainardi, J.L., Legrand, R., Arthur, M., Schoot, B., van Heijenoort, J., and Gutmann, L. (2000) Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in *Enterococcus faecium*. *J Biol Chem* **275**: 16490–16496.
Mainardi, J.L., Morel, V., Fourgeaud, M., Cremniter, J., Blanot, D., Legrand, R., *et al*. (2002) Balance between two transpeptidation mechanisms determines the expression of beta-lactam resistance in *Enterococcus faecium*. *J Biol Chem* **277**: 35801–35807.
Mainardi, J.L., Fourgeaud, M., Hugonnet, J.E., Dubost, L., Brouard, J.P., Ouazzani, J., *et al*. (2005) A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. *J Biol Chem* **280**: 38146–38152.
Mainardi, J.L., Villet, R., Bugg, T.D., Mayer, C., and Arthur, M. (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. *FEMS Microbiol Rev* **32**: 386–408.
Mascher, T., Hachmann, A.B., and Helmann, J.D. (2007) Regulatory overlap and functional redundancy among *Bacillus subtilis* extracytoplasmic function sigma factors. *J Bacteriol* **189**: 6919–6927.
McDonough, M.A., Anderson, J.W., Silvaggi, N.R., Pratt, R.F., Knox, J.R., and Kelly, J.A. (2002) Structures of two kinetic intermediates reveal species specificity of penicillin-binding proteins. *J Mol Biol* **322**: 111–122.
McPherson, D.C., and Popham, D.L. (2003) Peptidoglycan synthesis in the absence of class A penicillin-binding proteins in *Bacillus subtilis*. *J Bacteriol* **185**: 1423–1431.
Meeske, A.J., Sham, L.T., Kimsey, H., Koo, B.M., Gross, C.A., Bernhardt, T.G., and Rudner, D.Z. (2015) MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **112**: 6437–6442.
Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekalanos, J.J., Kahne, D., *et al*. (2016) SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* **537**: 634–638.
Meisner, J., Montero Llopis, P., Sham, L.T., Garner, E., Bernhardt, T.G., and Rudner, D.Z. (2013) FtsEX is required for CwlO peptidoglycan hydrolase activity during cell wall elongation in *Bacillus subtilis*. *Mol Microbiol* **89**: 1069–1083.
Michie, K.A., and Lowe, J. (2006) Dynamic filaments of the bacterial cytoskeleton. *Annu Rev Biochem* **75**: 467–492.
Mogensen, T.H. (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* **22**: 240–273.

Mohammadi, T., van Dam, V., Sijbrandi, R., Vernet, T., Zapun, A., Bouhss, A., *et al*. (2011) Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *EMBO J* **30**: 1425–1432.

Moll, A., Dorr, T., Alvarez, L., Davis, B.M., Cava, F., and Waldor, M.K. (2015) A D, D-carboxypeptidase is required for *Vibrio cholerae* halotolerance. *Environ Microbiol* **17**: 527–540.

Morales Angeles, D., Liu, Y., Hartman, A.M., Borisova, M., de Sousa Borges, A., de Kok, N., *et al*. (2017) Pentapeptide-rich peptidoglycan at the *Bacillus subtilis* cell-division site. *Mol Microbiol* **104**: 319–333.

Morgenstein, R.M., Bratton, B.P., Nguyen, J.P., Ouzounov, N., Shaevitz, J.W., and Gitai, Z. (2015) RodZ links MreB to cell wall synthesis to mediate MreB rotation and robust morphogenesis. *Proc Natl Acad Sci USA* **112**: 12510–12515.

Moynihan, P.J., Sychantha, D., and Clarke, A.J. (2014) Chemical biology of peptidoglycan acetylation and deacetylation. *Bioorg Chem* **54**: 44–50.

Osawa, M., Anderson, D.E., and Erickson, H.P. (2008) Reconstitution of contractile FtsZ rings in liposomes. *Science* **320**: 792–794.

Osawa, M., Anderson, D.E., and Erickson, H.P. (2009) Curved FtsZ protofilaments generate bending forces on liposome membranes. *EMBO J* **28**: 3476–3484.

Paradis-Bleau, C., Markovski, M., Uehara, T., Lupoli, T.J., Walker, S., Kahne, D.E., and Bernhardt, T.G. (2010) Lipoprotein cofactors located in the outer membrane activate bacterial cell wall polymerases. *Cell* **143**: 1110–1120.

Potluri, L., Karczmarek, A., Verheul, J., Piette, A., Wilkin, J.M., Werth, N., *et al*. (2010) Septal and lateral wall localization of PBP5, the major D,D-carboxypeptidase of *Escherichia coli*, requires substrate recognition and membrane attachment. *Mol Microbiol* **77**: 300–323.

Quintela, J.C., Caparros, M., and de Pedro, M.A. (1995) Variability of peptidoglycan structural parameters in Gram-negative bacteria. *FEMS Microbiol Lett* **125**: 95–100.

RayChaudhuri, D., and Park, J.T. (1992) *Escherichia coli* cell-division gene ftsZ encodes a novel GTP-binding protein. *Nature* **359**: 251–254.

Rebets, Y., Lupoli, T., Qiao, Y., Schirner, K., Villet, R., Hooper, D., *et al*. (2014) Moenomycin resistance mutations in *Staphylococcus aureus* reduce peptidoglycan chain length and cause aberrant cell division. *ACS Chem Biol* **9**: 459–467.

Reed, P., Veiga, H., Jorge, A.M., Terrak, M., and Pinho, M.G. (2011) Monofunctional transglycosylases are not essential for *Staphylococcus aureus* cell wall synthesis. *J Bacteriol* **193**: 2549–2556.

Reichmann, N.T., and Grundling, A. (2011) Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. *FEMS Microbiol Lett* **319**: 97–105.

Rice, L.B., Carias, L.L., Rudin, S., Hutton, R., Marshall, S., Hassan, M., *et al*. (2009) Role of class A penicillin-binding proteins in the expression of beta-lactam resistance in *Enterococcus faecium*. *J Bacteriol* **191**: 3649–3656.

Rueff, A.S., Chastanet, A., Dominguez-Escobar, J., Yao, Z., Yates, J., Prejean, M.V., *et al*. (2014) An early cytoplasmic step of peptidoglycan synthesis is associated to MreB in *Bacillus subtilis*. *Mol Microbiol* **91**: 348–362.

Ruiz, N. (2008) Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proc Natl Acad Sci USA* **105**: 15553–15557.

Sanders, A.N., and Pavelka, M.S. (2013) Phenotypic analysis of *Escherichia coli* mutants lacking L,D-transpeptidases. *Microbiology* **159**: 1842–1852.

Santos, J.M., Lobo, M., Matos, A.P., De Pedro, M.A., and Arraiano, C.M. (2002) The gene *bolA* regulates *dacA* (PBP5), dacC (PBP6) and *ampC* (AmpC), promoting normal morphology in *Escherichia coli*. *Mol Microbiol* **45**: 1729–1740.

Satta, G., Cornaglia, G., Mazzariol, A., Golini, G., Valisena, S., and Fontana, R. (1995) Target for bacteriostatic and bactericidal activities of beta-lactam antibiotics against *Escherichia coli* resides in different penicillin-binding proteins. *Antimicrob Agents Chemother* **39**: 812–818.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev* **32**: 234–258.

Scheffers, D.J., and Tol, M.B. (2015) LipidII: just another brick in the wall? *PLoS Pathog* **11**: e1005213.

Sham, L.T., Butler, E.K., Lebar, M.D., Kahne, D., Bernhardt, T.G., and Ruiz, N. (2014) Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science* **345**: 220–222.

Shih, Y.L., Le, T., and Rothfield, L. (2003) Division site selection in *Escherichia coli* involves dynamic redistribution of Min proteins within coiled structures that extend between the two cell poles. *Proc Natl Acad Sci USA* **100**: 7865–7870.

Siegel, S.D., Liu, J., and Ton-That, H. (2016) Biogenesis of the Gram-positive bacterial cell envelope. *Curr Opin Microbiol* **34**: 31–37.

Singh, S.K., SaiSree, L., Amrutha, R.N., and Reddy, M. (2012) Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of *Escherichia coli* K12. *Mol Microbiol* **86**: 1036–1051.

Strahl, H., Burmann, F., and Hamoen, L.W. (2014) The actin homologue MreB organizes the bacterial cell membrane. *Nat Commun* **5**: 3442.

Strominger, J.L., Park, J.T., and Thompson, R.E. (1959) Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. *J Biol Chem* **234**: 3263–3268.

Strominger, J.L., Blumberg, P.M., Suginaka, H., Umbreit, J., and Wickus, G.G. (1971) How penicillin kills bacteria: progress and problems. *Proc R Soc Lond B Biol Sci* **179**: 369–383.

van Teeffelen, S., Wang, S., Furchtgott, L., Huang, K.C., Wingreen, N.S., Shaevitz, J.W., and Gitai, Z. (2011) The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. *Proc Natl Acad Sci USA* **108**: 15822–15827.

Tybring, L., and Melchior, N.H. (1975) Mecillinam (FL 1060), a 6beta-amidinopenicillanic acid derivative: bactericidal action and synergy in vitro. *Antimicrob Agents Chemother* **8**: 271–276.

Typas, A., Banzhaf, M., van den Berg van Saparoea, B., Verheul, J., Biboy, J., Nichols, R.J., *et al*. (2010)

Regulation of peptidoglycan synthesis by outer-membrane proteins. *Cell* **143**: 1097–1109.
Uehara, T., and Park, J.T. (2008) Growth of *Escherichia coli*: significance of peptidoglycan degradation during elongation and septation. *J Bacteriol* **190**: 3914–3922.
Ursell, T.S., Nguyen, J., Monds, R.D., Colavin, A., Billings, G., Ouzounov, N., *et al*. (2014) Rod-like bacterial shape is maintained by feedback between cell curvature and cytoskeletal localization. *Proc Natl Acad Sci USA* **111**: E1025–E1034.
Vollmer, W. (2012) Bacterial growth does require peptidoglycan hydrolases. *Mol Microbiol* **86**: 1031–1035.
Vollmer, W., and Holtje, J.V. (2004) The architecture of the murein (peptidoglycan) in Gram-negative bacteria: vertical scaffold or horizontal layer(s)? *J Bacteriol* **186**: 5978–5987.
Welzel, P. (2007) A long research story culminates in the first total synthesis of moenomycin A. *Angew Chem Int Ed Engl* **46**: 4825–4829.
White, C.L., Kitich, A., and Gober, J.W. (2010) Positioning cell wall synthetic complexes by the bacterial morphogenetic proteins MreB and MreD. *Mol Microbiol* **76**: 616–633.
Wientjes, F.B., and Nanninga, N. (1991) On the role of the high molecular weight penicillin-binding proteins in the cell cycle of *Escherichia coli*. *Res Microbiol* **142**: 333–344.
Yang, X., Lyu, Z., Miguel, A., McQuillen, R., Huang, K.C., and Xiao, J. (2017) GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. *Science* **355**: 744–747.
Yousif, S.Y., Broome-Smith, J.K., and Spratt, B.G. (1985) Lysis of *Escherichia coli* by beta-lactam antibiotics: deletion analysis of the role of penicillin-binding proteins 1A and 1B. *J Gen Microbiol* **131**: 2839–2845.
Yunck, R., Cho, H., and Bernhardt, T.G. (2016) Identification of MltG as a potential terminase for peptidoglycan polymerization in bacteria. *Mol Microbiol* **99**: 700–718.
Zhao, H., Sun, Y., Peters, J.M., Gross, C.A., Garner, E.C., and Helmann, J.D. (2016) Depletion of undecaprenyl pyrophosphate phosphatases disrupts cell envelope biogenesis in *Bacillus subtilis*. *J Bacteriol* **198**: 2925–2935.